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Guidance on information requirements and chemical safety assessment

Appendix R7-1 for nanomaterials applicable to Chapter R7a Endpoint specific guidance

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Appendix R7-1 for nanomaterials applicable to Chapter R7a - Endpoint specific guidance

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Version	Changes	Date
Version 1	First edition	April 2012
Version 2	• Update of section 2.1.1 on sample preparation, to provide specific indications on the parameters for characterisation, pre-requisites and preparation for any nanomaterial. • Update of section 2.2.1 on water solubility, to include alternative guidelines that could be used for this endpoint and to flag the non-applicability of insolubility as a waiver for other endpoints • Update of section 2.2.2 on partition coefficient n-octanol/water, to strengthen the message that guidelines recommended for this endpoint for non-nanomaterials are not applicable for nanomaterials and recommend other parameters that could be considered instead. • Update of section 2.2.4 on adsorption/desorption, to clarify that the methods recommended in the parent guidance for this endpoint are not applicable for nanomaterials and recommend other parameters that could be considered instead. • New advisory note on testing and sampling strategy and sample preparation for human health endpoints (section 3.1.1); • Reorganisation of the (general) advice regarding non-testing methods in section 3.1.1 instead of under each specific endpoint to avoid repetition • Update of advisory notes on consideration on lung overload (section 3.2.1.1); • Update of the section on repeated dose toxicity (section 3.2.1); • Update of the section on mutagenicity (section 3.2.2)	May 2017

PREFACE

Three appendices concerning information requirements (appendices to IR&CSA Guidance Chapters R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing REACH registration dossiers that cover "nanoforms"[1].

The advice provided in this document focuses on specific recommendations for testing materials that are nanomaterials[2]. Part of the advice provided is not strictly nano-specific and may for instance also be applicable to other particulate materials (e.g. relevance of dissolution rate). However, when such advice has been included, it is because it is considered that the issue covered is especially relevant for nanomaterials and should be part of the nano-specific guidance.

In the absence of availability of any suitable specific provision (either because the endpoint is not relevant for nanomaterials, because the guidance already provided is considered to be equally applicable to nanomaterials as to non-nanomaterials, or because more research is needed before developing advice) no additional guidance for the endpoint has been included in this appendix.

This appendix intends to provide advice specific to nanomaterials and does not preclude the applicability of the general principles given in Chapter R.7a (i.e. the parent guidance). Moreover, when no advice has been given in this appendix for a specific endpoint the advice provided in the parent Guidance should be followed.

Please note that this document (and its parent guidance) provides specific guidance on meeting the information requirements set out in Annexes VI to XI to the REACH Regulation.

General information for meeting the information requirements such as collection and evaluation of available information, and adaptation of information requirements is available in Chapter R.2 to R.5 of Guidance on IR&CSA).

Moreover, when considering the use of data already available *Appendix R.6-1: for nanomaterials applicable to the Guidance on QSARs and Grouping* of *Chemicals* [1] may be useful as it provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance.

1 Please see *How to prepare registration dossiers that cover nanoforms: best practices* [153]

2 See Recommendation on the definition of nanomaterial adopted by the European Commission

Table of Contents

Table of Tables

Table of Figures

2 RECOMMENDATIONS FOR PHYSICO-CHEMICAL PROPERTIES

2.1 General remarks

2.1.1 Sample preparation

The following section focuses on preparation of the sample, thus it is implied that a choice of testing material(s) has already been made and that they are representative of the registered substance and/or the relevant nanoforms.

Sample preparation is widely recognised as one of the most critical steps towards successful characterisation and subsequent testing of nanomaterials. There are many variables to consider when designing a method for sample preparation. Common issues to be considered regarding sample preparation include storage, colloidal and chemical stability of the tested nanomaterial, the chemical composition of the test media, characterisation of stock dispersions and characterisation of samples (prepared from stock dispersions) prior to administration/ testing [2].

The hazards posed by all possible forms of the substance covered by a registration, including nanoforms, must be addressed by the toxicological and ecotoxicological information provided in the registration dossier. In order to show that the test material(s) chosen are appropriate to represent the substance and/or the nano(forms) being assessed, some information should be reported in the endpoint study record under the test material information field in IUCLID. The following parameters should be provided :

- Chemical composition (as described in the ECHA *Guidance for identification and naming of substances under REACH and CLP*)
- Size (as a minimum the D50, but particle size distribution is recommended)
- Shape and aspect ratio
- Surface chemistry

Moreover, appendix R6-1 *for nanomaterials applicable to the guidance on QSARs and Grouping of Chemicals* [1] provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform) of the same substance. The Guidance gives some (additional) parameters that may be required to be able to assess whether the available hazard data are applicable for different nanoforms of a substance. The registrant may wish to consider characterising the test material taking into account such parameters, in order to be able to follow the above-mentioned guidance. For example, the dissolution rate, surface chemistry and dispersability have been reported as a founding base for the grouping of the nanomaterials [1].

Besides all these parameters, ISO 14887:2007 [3] outlines procedures for the preparation of good dispersions from various powder/liquid combinations for particle size analysis of substances in general. Suggested dispersion procedures for a range of nanomaterials are also emerging in the scientific literature e.g. in [4] and [5].

However, such procedures should be carefully examined to determine if they are adequate for the test material under consideration and modifications may be required for different materials. For example with regard to inhalation toxicity testing, standards are available that outline procedures for the generation of metal nanoparticles using the evaporation/condensation method (ISO 10801:2010 [5]) and support the characterisation of

nanoparticles in inhalation exposure chambers [5].

An important component of sample preparation is "reliable" sampling. In reliable sampling the test aliquot used for measurement represents the physical and chemical characteristics of the entire sample. The characterisation of particle properties like size, form and specific surface area requires very careful sampling and sample splitting practices to be followed. ISO 14488:2007 [3] specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level and is of particular relevance to the measurement of particle size, size distribution and surface area.

In order to eliminate potential errors in the interpretation of results due to particle contaminants/impurities, data from the characterisation of the test material including its purity and, if technically feasible, quantities of identified contaminants and impurities should be considered prior to the start of a study, consistently with the substance identification requirement.

Also in relation to sample preparation, it is necessary to be aware that aggregates and agglomerates of nanomaterials can form in the dispersion, powder and aerosol forms, and that their presence is influenced by a number of factors including the method of synthesis, storage, handling and environmental conditions. "Aggregate" means a (larger) particle comprised of strongly bound or fused particles. "Agglomerate" means a collection of weakly bound particles. (EC Recommendation on the definition of nanomaterial).

The state of agglomeration or aggregation is recognised as an important parameter influencing the interpretation of characterisation and testing of nanomaterials ("as received", "as used", "as dosed / as exposed") and should therefore be considered during sample preparation. A number of measurands have been proposed for assessing agglomeration or aggregation state, including the effective cross-section, determined by measuring aerodynamic/light scattering properties or by electron microscopy ([6], [7]).

Draft test guidelines and a guidance document on agglomeration behaviour and dissolution rate of nanomaterials in aquatic media are under development within the OECD and would allow characterisation and quantification of agglomeration behaviour (see section 2.2.2).

In addition to aggregation and agglomeration, the behaviour of particles in liquid media presents some additional important aspects and challenges to recognise. In particular, it can be difficult to distinguish between when a nanomaterial is *dispersed* and when it is *dissolved* due to its small particle size. It is important to recognise that solubility and dispersibility are two distinct phenomena. Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) such that a single, homogeneous, stable phase results, and is relevant to solids, liquids and gases. Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). Historically, the term "dissolved" meant the component of a liquid sample that had passed through a 0.45µm (or similar) filter. However, as (colloidal) dispersions of nanoparticles might also pass through such filters, it is recommended that use of the term "dissolved" should be restricted to the formation of true solutions, and where both liquid and particulates are present the term "dispersed" should be used ([2], [8]).

By applying a combination of ultracentrifugation and ultrafiltration techniques it is however possible to measure the amount of truly soluble fraction (see [4] and [9]).

A dispersion is a suspension of a heterogeneous mixture of nanomaterials comprising a liquid and a finely dispersed solid material, which may falsely have the visible appearance of a solution. Dispersion stability is an important parameter to assess in the context of sample preparation. The dispersion of particles is determined by intermolecular forces involving particle-particle interactions as well as those between the particles and their surrounding

matrix. Due to attractive forces (e.g. van der Waals interactions) particles tend to agglomerate unless stabilised by surface charge or steric effects. As a result, the state of dispersion is dynamic and changes with time to potential dissolution and/or higher agglomeration. Dispersion is determined by interactions between the properties of the nanoparticles and properties of the surrounding matrix. In liquid media, slight modifications in pH, ionic strength and concentrations of molecular constituents can significantly alter the dispersion of particles. For aerosolised powders, the situation can be even more complex as the concentration and diffusion characteristics of the aerosol can cause the state of dispersion to change over time.

The state of dispersion is typically assessed using comparative particle size measurements and requires a reliable method of measuring the baseline particle size distribution of the material. By comparing changes in particle size distribution, a qualitative assessment or proxy measure of the state of dispersion can be made. As an example of measurement methods applicable for spherical particles: Zeta potential measurement, combined with Dynamic Light Scattering (DLS), also enables the stability of nanoparticle dispersions to be monitored and a qualitative understanding of the agglomeration process to be achieved. Other methods such as particle tracking analysis can also be used when applicable for the tested substance [10].

2.1.1.1 General considerations for (Eco)-Toxicological testing

In order to start with relevant sample preparation the Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials OECD No. 36 ENV/JM/MONO(2012)40 should be considered. Further guidance on sample preparation may be found in Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines OECD No. 40 ENV/JM/MONO(2014)1 [8], ENV/JM/MONO(2014)1/ADD1 [11], and [12] reflecting the outcome of the discussion of the OECD's work on nanosafety during the Testing Programme of Manufactured Nanomaterials [13].

For example, the following aspects are considered important in sample preparation:

- Characterization of the physicochemical properties of nanomaterials (e.g. particle size distribution, shape, specific surface area, composition, impurities, and surface chemistry) and the state present in the test medium (degree of agglomeration/sedimentation).
- Nanomaterials test item preparation and dispersion (including stability) should take into account the characteristics of the test media [4]. Due to their particular nature in the (eco)toxicological test media, the physico-chemical properties of the nanomaterials as well as the potential (eco)toxicological effects are highly influenced by the interactions with the bio-physicochemical surroundings in these media. Thus, testing should be carried out with accompanying analytics to monitor the exposure concentration. For nanomaterials the use of only chemical analysis is not sufficient, as further explained in the discussion later on dose metrics.
- Sample preparation also needs to be controlled, consistent, relevant, reliable and robust, as the testing stages may include e.g. the use of powder and/or dispersion depending on the end-point, and the test item may have undergone a multi-stage process of preparation.
- Selected sample preparation procedure (and controls, if applied) should be justified and sufficiently reported in the robust study summary.
- Since the most appropriate dose metrics may not be known, the use of other dose metrics than mass-based, such as surface area and particle counts, are to be provided in addition to the mass metrics, when available. These measurements will increase the ability to interconvert doses from mass to particle counts and/or to surface area and are considered as essential while diminishing the uncertainty related to the conversion when the metrics are used independently and consequently reducing the amount of testing required.

If a nanomaterial is soluble and has a high dissolution rate (see section 2.2.1) in relevant biological or environmental media, then it is likely to be presented to the test system in its molecular or ionic form and can therefore be expected to elicit the same response as non-nanoscale solubilised substances e.g. the salts of metallic substances used as positive reference versus the metal ionic form stemming from the nanomaterial. If, however, the nanomaterial under investigation is insoluble or sparingly soluble in biological or environmental media, then it will probably be presented to the test system in a particle form. In this case, the advice provided in *Appendices for nanomaterials applicable to Chapters R.7a (this document), r R.7b and R.7c* would apply.

In addition, nanoparticles may interact with the liquid phase components, partially or totally yielding soluble or dispersed transformation products (as well as some solubilised nanomaterial itself) that may influence the overall toxicity and fate processes. This possibility needs to be taken into account when selecting the media and procedures as well as in the assessment of the result of any test ([2], [14]).

Other important considerations to take into account during sample preparation include the influence of contaminants (including biological contaminants) and impurities on (eco)toxicological test results. For example, metallic impurities such as Co and Ni catalysts used in the production process of the nanoparticles were shown to inhibit hatching in zebrafish embryos (e.g. [15]).

Also of particular concern for nanomaterials is the influence of endotoxin on certain test results. Endotoxin (lipopolysaccaride) is a constituent of the outer cell wall of gram-negative bacteria and as such is found ubiquitously within the environment. Endotoxin can however generate a range of toxic effects either at the whole organism level causing responses such as fever, 'endotoxin shock' and death, or at the cellular level via the triggering of inflammatory cascades leading to the secretion of pro-inflammatory mediators.

Due to the potent response endotoxin can generate in biological assays, toxicity testing of a contaminated test sample may lead to a confounding of results (including a potential false positive). Therefore the establishment of the presence or level of endotoxin in a test sample is an important preliminary undertaking during the preparation of a sample for toxicological testing. Endotoxin can be measured using in vitro methods such as the macrophage activation test, which has been validated by European Committee on Validation of Alternative Test Methods and proposed as a reliable method for determining the pyrogenicity of engineered, research-grade nanomaterials [16]. International standards are available for the testing of nanomaterials [17]. Although issues regarding contamination are not nano-specific, the increased relative surface area of nanophase systems compared to other particles means that the possible amounts of adsorbed endotoxin (e.g. grams adsorbed endotoxin per gram of material) are significant [18].

The existence of false negatives has also to be accounted for, for instance in cases where exposure of the organism is underestimated (e.g. Ames test, insoluble particles etc.). Due to differences in fate and behaviour between nanomaterials and traditional chemicals in different test environments, a testing strategy/decision tree approach on dispersion, dissolution, dispersion stability and aggregation recommended in OECD No. 40 [8] may be considered for nanomaterials. This approach takes into account e.g. the effects of pH, DOM, NOM/proteins, and ionic strength and should be accounted for until specific test guidelines and guidance documents developed by OECD are made publicly available (see 2.1.1). Considerations and measurement of dissolution rate and dispersion stability in the media will not only help to find the appropriate testing strategy and test conditions, but will also help in the interpretation of the results. This information would also be useful for nanomaterial grouping and read-across [1].

2.2 Specific advice for endpoints

2.2.1 Water solubility

Water solubility is covered in Section R.7.1.7 of the parent guidance. In the case of nanomaterials it is necessary to take into account that water solubility has the potential to increase for materials in the nano-size range due to their decreasing particle size and it may also be affected by their shape and surface coating. For nanomaterials, the dissolution rate and degree of dispersion also play an important role in mobility of the substance. However, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved due to its small particle size. It is important to recognise that solubility and dispersibility are different and distinct phenomena, with different implications on testing and characterisation, and it is important to differentiate between them. This situation is not unique to nanomaterials, and indeed the parent guidance already highlights that "measurement of the solubility of sparingly soluble compounds requires extreme care to generate saturated solutions of the material without the introduction of dispersed material". However, this problem may be further amplified in the case of sparingly soluble nanomaterials. Further information on these issues is provided in section 2.1.1 on Sample Preparation. It should also be ensured that no undissolved material contributes to what is being measured as being dissolved material.

The OECD has examined the applicability of its test guidelines for nanomaterials and OECD publications have stated that OECD TG 105 [19] (Water solubility) is not always appropriate for testing of nanomaterials [11].

This is the case when the substance in question has low water solubility, and where the possibility of generating a dispersion also exists. Measurement of water solubility using the OECD TG 105 guideline may still be of value for nanomaterials that are water soluble and have a high and fast dissolution rate.

2.2.1.1 Other guidelines and protocols for solubility

Measurement of the rate and extent of dissolution, as supporting information and/or as an alternative method when OECD TG 105 is not applicable, is highly recommended as dissolution rate in relevant biological and environmental media is relevant given that this affects the bioavailability of substances in the (biological) environment (OECD No. 62 [20]). For instance, data on dissolution rate may be useful in determining what type of testing is required for aquatic toxicity testing (see for instance section 1.2.1 in *Appendix R.7-1 to Chapter R.7b*). OECD 29 [21] allows to test dissolution and transformation for test duration varying between 1 up to 28 days with a usual duration of 7 days being applied. When choosing the testing material for this endpoint, it should be noted that testing the smallest particle size (as recommended by the guideline) may not be adequate in the case of nanomaterials.

OECD 62 [20] mainly focuses on dissolution rate and the setting of qualitative thresholds of high or > 70 % of dissolution into another form, moderate between 10 and 70 %, low >1 and below 10 % and negligible < 1%, all estimated for a test duration of 7 days.
The OECD 29 testing protocol on transformation/dissolution [21] provides advice on how to determine the rate and extent to which metals and sparingly soluble metal compounds can produce soluble available ionic and other metal bearing species in aqueous media. The measurement of dissolution rate and the qualitative thresholds developed further in OECD No. 62 provide further advice on how to apply the transformation/dissolution protocol on metallic nanomaterials. Furthermore, there are two additional test guidelines for determining the dissolution rate of nanomaterials under development within OECD that will be applicable instead of the OECD 29 once they are available:

- Test Guideline for the Dissolution Rate of Nanomaterials in the Aquatic Environment,
- Guidance Document on Agglomeration and Dissolution behaviour of Nanomaterials in Aquatic Media.

Measurements of agglomeration and aggregation can also be useful together with the dissolution rate using the guidance documents above once available.

2.2.1.2 (In)solubility as a waiver

In the parent Guidance Section R.7.1.7.1 it is noted that water insolubility is used as a regulatory trigger for waiving certain physicochemical and ecotoxicological endpoints. However for nanomaterials insolubility alone is not relevant as a justification for test waiving. The high insolubility of a nanomaterial does not necessarily indicate that toxicity is unlikely. Exposure cannot be excluded, as even an insoluble nanomaterial may be bioavailable to the test organisms due to nano-specific properties e.g. size and dispersibility. Furthermore, test guidelines not appropriate for highly insoluble substances may be applicable for nanomaterials with specific adaptation.

Taking into account the nano-specific properties and constraints in assessing the solubility of nanomaterials by currently available standard methods such as OECD TG 105 (Water solubility), waiving the information requirement based on high insolubility should always be accompanied with robust technical and scientific justification.
For instance, further information on dissolution, agglomeration and sedimentation could be used as a part of the weight of evidence to justify an alternative testing strategy (e.g. including a sediment toxicity test).

2.2.2 Partition coefficient n-octanol/water

Section R.7.1.8.3 of the parent guidance, includes information regarding experimental data on n-octanol/water partition coefficient including testing methods. The n-octanol/water partition coefficient (K_{ow}) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of the largely immiscible solvents n-octanol and water. In a two-phase system, nanoparticles behave differently from organic molecules. The fate of nanoparticles may not be predicted by equilibrium partitioning ([22], [23]) as nanoparticles cannot reach thermodynamic equilibrium by distributing between two phases, water and n-octanol, due to their particulate nature. Therefore, OECD TGs recommended in the parent ECHA Guidance for partition coefficient n-octanol/water, i.e. OECD TG 107, OECD TG 117 and OECD TG 123, are in most cases not applicable to nanoparticles ([6], [8], [12]). Results might be impacted upon by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve ([2], [8]).

Nevertheless, if it is shown that the nanomaterial is quickly and highly dissolved, and the presence of particles can be excluded the parent guidance will apply. Taking into account the above, measurement of n-octanol/water partition coefficient may still be of value for organic nanomaterials that are water soluble and have a high dissolution rate (see section 2.2.1).

The use of n-octanol/water partition coefficient (K_{ow}) might lead to erroneous interpretation of the environmental fate or bioconcentration [22]. Taking into account the nano-specific properties and constraints in assessing the n-octanol/water partition coefficient (K_{ow}) of the nanomaterials by currently available standard methods, waiving the information requirement based on n-octanol/water partition coefficient should always be accompanied by a robust technical and scientific justification on the applicability of the used test method (e.g. nanomaterial being water soluble or have a high and fast dissolution rate).

With respect to parent Guidance section 7.1.8.3, "Difficult to test substances", it should be noted that due to the small particle size of nanomaterials, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved. It is important to recognise

that solubility and dispersibility are two distinct phenomena and it is important to differentiate between them. Further information on these issues is provided in section 2.1.1 on Sample Preparation.

2.2.2.1 Other guidelines and protocols for K_{ow}

Regarding nanomaterials, currently there are no proper standard methods for fate descriptors to predict the behaviour and transport of nanomaterials in the environment and biological media as alternative to n-octanol/water partition coefficient ([22], [23]). There are, however, properties other than equilibrium partitioning that may be used to predict fate and transport of the nanomaterials in the environment and organisms. Agglomeration, aggregation, deposition and attachment are considered to be informative properties to predict behaviour of the nanoparticles ([22], [24], [25], [26]). Alternative fate descriptors for nanoparticles are further discussed in section 2.2.4 on adsorption/desorption.

In the OECD, there is ongoing activity on development of the following test guidelines for determining the agglomeration behaviour of nanomaterials:

- Test Guideline for Agglomeration Behaviour of Nanomaterials in different Aquatic Media [27]
- Guidance Document for Agglomeration behaviour and Dissolution rate of Nanomaterials in Aquatic Media.

Assessment of agglomeration of nanomaterials is to be conducted in accordance with the OECD test guideline when it is available[3].

Other non-testing methods can also be considered in case K_{ow} measurement is not applicable. A list of and details on the models and specific parameters under development is available in Appendix 1.

[3] The draft is available at: http://www.oecd.org/env/ehs/testing/test-guidelines-for-comments-section3-degradation-and-accumulation.htm

2.2.3 Granulometry

2.2.3.1 General considerations on the advice given by RIP-oN 2

Granulometry is, as expected, the central issue for nanomaterials. For that reason it is the endpoint requiring most recommendations to cover nanomaterials. The need for modifications starts already with the definition of what is considered to be covered by the term "granulometry".

Regarding this issue, the RIP-oN2 report offers two alternatives:

- Granulometry refers only to particle size distribution
- Granulometry includes shape and surface area in addition to particle size distribution

The RIP-oN report offers different alternatives, but the advice is, in essence, the same: shape and surface area are parameters that need to be taken into account (for instance because of the impact on toxicology), so either they are considered together with the granulometry or proposed to be new endpoints.

For the purpose of structuring the granulometry section within this appendix it has been considered to be clearer and more helpful to the reader to restrict the scope of text concerning granulometry to consider only particle size distribution and to add two additional sections for discussion of shape and surface area.

As the sections for discussion of shape and surface area are completely new, the original guidance structure has been maintained and they appear in this appendix numbered as if they were new sections in the body of the document (Sections R.7.1.19 and R.7.1.20).

Finally a new Section 2.2.3.3 has been added showing a joint integrated sampling strategy for the three parameters (particle size distribution, shape and surface area)

2.2.3.2 Recommendations for granulometry (as particle size distribution)

The potential release of particles into the workplace or environment is an important consideration in the design and operation of many industrial processes and safe handling of substances. Release of particles may present a safety hazard and may cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles, allowing risks to be evaluated, controlled and minimised. Measurement of the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges. It is worth noting that the particle size distribution and the behaviour of the airborne fraction may be different to those determined for the powdered substance.

Particle size is a fundamental attribute of disperse materials. When a group of particles are of differing sizes, they may be described by a particle size distribution. Granulometry can be defined as the determination of particle size distribution. When a group of particles are of differing sizes, they may then be described by a Particle Size Distribution.

Section R.7.1.14, quotes the European standard EN 481 "Workplace Atmospheres – size fraction definitions for measurement of airborne particles" [28]. The standard provides definitions of the inhalable, thoracic and respirable size fractions, and target specifications (conventions) for sampling instruments to measure these fractions. In addition to that document, the following recommended documents provide background information and sampling guidelines, representing the current state-of-the-art, to effectively characterise and monitor exposures in the workplace:

- Method for Determination of Hazardous Substances MDHS 14/3 "General methods for sampling and gravimetric analysis of respirable and inhalable dust" [29]

- "Stationary source emissions – Determination of mass concentration particulate matter (dust) at low concentrations – manual gravimetric method" [30]
- "Stationary source emissions – Manual determination of mass concentration of particulate matter" [31]
- "Ambient air quality – Standard gravimetric measurement method for the determination of the PM2.5 mass fraction of suspended particulate matter" [32]
- "Workplace atmospheres – Ultrafine, nanoparticle and non-structured aerosols – Inhalation exposure characterization and assessment" [33]
- "Nanotechnologies – Health and safety practices in occupational settings relevant to nanotechnologies" [34]

The latter two reports (which are the only two of the list above that are specific to nanomaterials) are also relevant when referring to the measuring of the appropriate fractions.

As it was foreseeable, Section 7.1.14.2 (Available information on granulometry) dealing with test methods for granulometry, is the one needing the most adaptation. For that reason we are reproducing here the text of Section R.7.1.14.2 in its entirety as proposed to be modified by the RIP-oN.

R.7.1.14.2 Available information on granulometry

Testing data on granulometry

The characterisation of particles requires very careful sampling and sample fractionation practises to be followed. ISO 14488:2007 [35] specifies methods for obtaining a test aliquot from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative with a defined confidence level. Further information is available in Section 2.1.1 of this appendix on Sample Preparation.

Many methods are available for particle size measurements, but none of them is applicable to the entire size range (see Tables 1 to 4). Multiple techniques should be used where possible in order to formulate a complete understanding of the particle properties, and the optimum set of required techniques should be selected based on the specific substance and form under investigation. Methods for determining particle size distribution are designed to provide information on the transportation and sedimentation of insoluble particles in water and air. The OECD test guideline applicable to measuring the particle size distribution is OECD TG 110. It is important to note that Method A of OECD TG 110 (sedimentation, or centrifugation) is not considered applicable to nanomaterials [36] as it is useful only in the range 2 μm < Rs < 100 μm. However, alternative standardised equipment (e.g. centrifugal sedimentation) can be used in accordance with this method. Method B of OECD TG 110 (electron microscopy) requires a necessary but minor deviation in the data reporting for nanomaterials (i.e. particles/fibres of less than 5 microns in length and less than 100 nm in diameter). Details of methods capable of measuring nanoparticle size distributions are provided in ISO/TR 27628:2007 [33] and ISO/TR 12885:2008 [34].

These methods are generally applicable and frequently in use. They are used to calculate the effective hydrodynamic radius of both fibrous and non-fibrous particulates without prior inspection indirectly from other measurements of particle size and density. If applied properly, they represent an estimate of the aerodynamic property and mass fractions present and as such can indicate whether or not respirable particles may be present. They are applicable to water insoluble (i.e. water solubility < 10^{-6} g/l) substances and cover the range 5nm-100 μm

In the case of materials which can form fibres; which is initially confirmed using light microscopic examination to determine the approximate nature of the particles (e.g. plates, needles, etc.), an additional set of measurements is recommended to help identify the potential health hazards arising from inhalation or ingestion. This is comparatively specialised, infrequently required and involves specialised microscopic examination (e.g. TEM, SEM). A fibre is a water insoluble particle with an aspect ratio (length/diameter > 3) and diameter <

100 µm.

Image analysis of particle size can be used to determine the aspect ratios of fibrous particles. Image analysis generates data by capturing direct images of each particle. This provides users with the ultimate sensitivity and resolution as subtle differences in particle size can be accurately characterised. Images of each individual particle are also recorded, providing a further visual verification of the data and also enabling detection of important phenomena such as agglomeration, breakage and foreign particles. A range of industries (e.g. pharmaceuticals, biotechnology, abrasives, ceramics, polymers, explosives and toners) are increasingly using image analysis systems in order to characterise their products.

Table 1: Methods to determine particle size distribution of the material as it is

Method and details	Material and size range	Data type
Optical microscopic examination It is preferable to prepare samples directly in order not to influence shape and size of the particles. This method determines distribution of particles of respirable and inhalable size and does not refer to airborne dust or dispersed or nebulised particles. Optical microscopy can be used to examine likelihood of fibres present by comparing similarities to known fibrous or fibre releasing substances or other data. Extreme care required during sample preparation to avoid fibre breaking and clumping. Care should also be taken to avoid contamination by airborne fibres. Samples might be prepared by: (a) producing suspensions in water by gentle hand agitation or vortex mixing or (b) transfer of dry material onto copper tape either directly or by spraying of the dry fibres by use of atomiser or pipette. Length and diameter distributions should be measured independently at least twice and at least 70 fibres counted. No two values in a given histogram interval should differ by > 50% or 3 fibres, whichever is larger. The presence of long thin fibres would indicate a need for further, more precise measurements. This method might be suitable to determine the distribution of fibres of respirable and inhalable size.	Particles of all kinds, including fibres Size range: 0.2–5000 µm. Fibre diameters as small as 0.2 µm and as large as 100 µm and lengths as small as 5 µm and as large as 300 µm	Particle size/size distribution, from which mass median aerodynamic diameter (MMAD) can be calculated with knowledge of the particle density. Fibre number as defined by WHO [37] Aspect ratio > 3:1, fibre length > 5 microns
Sieving Sieving using wire-mesh sieves and perforated sheet metal sieves is not suitable to determine the distribution of particles of respirable and inhalable size since their range is only 100-10,000 microns. Micro mesh sieves (range 5-100 micron) may give better results. However, since these sieves are generally operated in combination with mechanical or ultrasonic vibration, modification of median size and form may result. Sieving not suitable to determine distribution of particles of respirable size, but might be suitable to determine particles of inhalable size.	Dry powders/granulates Size range: 100–10,000 microns (wire mesh/metal sieves) and 5-100 (micromesh)	MMAD cannot be determined

Sedimentation (gravitational settling) Method is based on gravitational settling of particles in liquid and the effective hydrodynamic radius is determined. Effective hydrodynamic radius distribution should be measured 3x with no two values differing by >20%. Requires sufficient numbers of radius intervals be used to resolve the radius distribution curve. Binary or ternary mixtures of latex spheres (2-100 microns) are recommended as calibration material. Method might be suitable to determine the distribution of particles of respirable and inhalable size.	Dry powders/granulates Size range: 2-200 microns	MMAD cannot be determined
Electrical Sensing Zone (e.g. Coulter) method Samples are suspended in an electrolytic solution. As the particle is drawn through an aperture, the change in conductance gives a measure of particle size. The important parameter is the settling velocity in the liquid phase, which depends on both density and diameter. Particles having a density of several g/cm^3 can be determined. Applicable to particles that are complete electrical isolators in the fluid. Difference in density between particles and fluid must not be too large. Method might be suitable to determine the distribution of particles of respirable and inhalable size	Dry powders/granulates (non-conducting) Size range: 1-1000 microns	MMAD cannot be determined
Phase Doppler Anemometry Expensive technique. Particle size distribution can be measured either in air or in liquid. The method presupposes that the particles are spherical with known refractive index. Method might be suitable to determine the distribution of particles of respirable and inhalable size	Dry powders/granulates Size range: 0.5-80 microns (in air); 0.5-1000 microns (in liquid)	MMAD cannot be determined
Transmission Electron Microscopy (TEM) TEM can be used for samples collected from the air or prepared in suspension on a TEM grid, including those from separation and sampling instruments. Powder preparation is very easy and fast for this method. TEM enables qualitative assessment of size and form of particles, and differentiation between agglomerates and primary particles. Quantitative determination of size distribution of primary particles is achievable in cases where agglomeration is not significant. TEM has a very high local resolution (nm) and is capable of imaging lattice planes and individual rows of atoms with resolution better than 0.2 nm. Additions to TEM can provide further information e.g. Scanning Transmission Electron Microscopy	Particles in solid, powder and suspension form. Size range: < 0.1 – 10 µm. Particularly suitable for the particle size range of 1 - 500 nm.	Particle size/size distribution, from which number/mass median diameter can be calculated with knowledge of the particle density

(STEM), High-Resolution TEM (HRTEM) or in-situ measurements using Environmental TEM, which offers the potential for dispersed samples to be characterised. However, TEM is a highly work-intensive method and requires manual preparation of samples. Dispersions need to be diluted (to ca. 1%) or prepared into work-intensive cryo-sections. Drying samples under vacuum for analysis may alter the size and shape of the particles being characterised. An extremely small area of the sample is analysed, which might not be representative enough. The comparatively small share of evaluated particles (ca. 1,000) results in limited statistical precision. Only a two-dimensional projection of particles is visible and can be evaluated; and the interpretation of pictures is difficult. Picture analysis is impossible if agglomeration is significant. Contours of particles may not be clearly resolved in some samples. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects. Further informative information on this method is available in ISO/TR 27628:2007 [33]. ISO/13322-1:2004 [38] and ISO/13322-2:2006 [39] provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.		
Scanning Electron Microscopy (SEM) SEM can be used for samples collected from the air or prepared in suspension on an SEM grid, including those from separation and sampling instruments. Sample preparation is easier than for TEM, and only a small quantity of sample needed. Testing possible with undiluted dispersions and emulsions. SEM enables non-destructive testing of samples, and provides an image of the sample structure with very precise size determination at high local resolution. This method can be used *in-situ* as Environmental SEM. A representative sample of the material must be used. Where samples are not electrically conducting, plasma sputter-coating the surface-adhered particles with a layer of a conducting material is often required. This process may modify the sample being characterised. Only a small section of the sample is pictured and imaging is limited to surface features. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects. Further informative information on this method is available in ISO/TR 27628:2007 [33]. ISO/13322-1:2004 [38] and ISO/13322-2:2006 [39] provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.	Particles in solid, powder and suspension form. Size range: < 0.01 – 10 µm. Particularly suitable for the particle size range of 10 nm – 1 µm.	Particle size/size distribution, from Which number/mass median diameter can be calculated with knowledge of the particle density

Centrifugal Sedimentation (ISO 13318-1:2001 [40]**; ISO 13318-2:2007** [41]**; ISO 13318-3:2004** [42]**)** Measures the particle size distribution of particulate materials dispersed in a liquid by fractionation. Centrifugal sedimentation methods are based on the rate of settling, under a centrifugal field, of particles in a liquid. The relationship between settling velocity and particle size reduces to the Stokes equation at low Reynolds numbers. Thus, the calculation of particle size using this method is dependent on Stokes law. This technique can be used to supply data in accordance with Method A of OECD TG 110 [36]. When using optical turbidity detection, the measuring range depends on the density of the material, the viscosity of the medium and the number of revolutions of the centrifuge. High absolute precision of particle size through calibration with a particle standard, and high resolution compared with other methods. A small quantity of sample is sufficient. This method involves fewer artefacts and possible errors than integral methods (e.g. light scattering), which measure all fractions together without separation. However, the measuring concentration is very low and therefore significant dilution is necessary. The potential for agglomeration must be considered, and the suspension / emulsion must be stable for analysis. A sedimentation liquid suitable for the sample must be determined, in which a density gradient can be established for measuring. The measuring time for samples with small particles is long. For evaluation, the density and optical constants of particles must be known. Evaluation of a fine fraction in a wide distribution can be critical. When using x-ray detection, the measuring range depends on the density of material. Implementation and evaluation is simple, without the need for calibration, gradients, Mie correction or optical information. A high resolution of distribution spectra is possible, and only a small quantity of sample is required. This method provides good statistics, with 10^{10} particles assessed in one measuring activity. However, dilution to ~ 5% necessary and, for evaluation, the density of particles must be known.	Particulate materials dispersed in a liquid Size range: 0.1 to 5 µm	Settling velocity (m s-1), from which particle size can be calculated based on Stokes law.
Ultrasonic spectroscopy (ISO/20998-1:2006 [43]**)** Allows determination of the size distribution of one or more material phases dispersed in a liquid. Measurements can be made for concentrations of the dispersed phase ranging from 0.1- 50% by volume. Enables dynamic changes in the size distribution to be monitored, including agglomeration or flocculation in a concentrated system. However, this method is air- and temperature-sensitive. Parameter adjustment is complex.	Particles in colloids, dispersions and emulsions Size range: 10 nm - 3 mm	Attenuation spectrum, from which the particle size distribution based on mass/number

Measurement results may vary with different vol%.		can be extracted via a model (which may be empirical or based on first principles)
Small Angle X-ray Scattering (SAXS) (ISO/TS 13762:2001 [44]**)** Allows determination of the particle size distribution of ultra-fine powders and suspensions. The requirement for particle dispersion of the sample is not as strict as for other methods. SAXS cannot distinguish pores from particles and therefore cannot be used for powders consisting of porous particles. This method assumes that particles are isotropic and spherically shaped, and thus has limited applicability to powders containing particles whose morphology is far from spherical e.g. non-spherical nano-objects such as carbon nanotubes. In addition, due to the need for a concentrated sample, an interference effect between particles may arise.	Particles in powder and suspension form Size range: 1-300 nm	Average particle size for a sample, estimated by mathematical adaption of a diffractogram
X-ray diffraction (XRD) (EN 13925-1 [45]**, EN 13925-2** [46] **and EN 13925-3** [47]**)** XRD estimates the average particle size by mathematical adaptation of a simulated diffractogram to real measurement. Enables crystallinity to be quantified with high statistical relevance, and avoids the need for representative sampling. Crystal structures of existing phases and equipment- and sample-specific parameters must be known. It is important to note that particle size does not equal crystallite size. Other factors can also influence the peak width, such as microstrain, lattice defects and temperature factors. Larger crystalline samples (>1mg) are required for analysis.	Single crystal or polycrystalline materials Crystallite size range: ~1-100 nm	Average particle size for a sample, estimated by mathematical adaptation of a diffractogram.
Dynamic Light Scattering (DLS)/Photon Correlation Spectroscopy (PCS) (ISO/22412:2008 [48]**; ISO/13321:1996** [49]**; ASTM E2490 – 09** [50]**)** Enables rapid and simple estimation of an average particle size and measurement of the broadness of the size distribution of sub micrometre-sized particles or droplets dispersed in liquids. For	Particles or droplets dispersed in liquids	Size distribution based on mass/number. Average particle

nanoparticles in suspension, DLS/PCS is one of the most commonly employed techniques providing *in situ* characterisation of size and size distribution and is often applied with zeta potential measurements to provide an indication of the particle suspension stability with respect to time and medium. Only a small quantity of sample is needed, and in the particle size range < 100 nm, no refractive indices are necessary. DLS/PCS is of particular benefit to toxicity assessment as it measures size in solutions that more accurately resemble the exposure conditions. An extension of this technique for high concentration opaque suspensions is Photon Cross Correlation Spectroscopy (PCCS), which provides particle size and stability of nanoparticle suspensions. However, extensive sample dilution is necessary. This method is of limited use when particles are difficult to maintain in a dispersed state or when particles of > 2 µm in size are present. This method is temperature sensitive and only enables low resolution. Optical parameters must be known for data analysis, and this method is not suitable for particles with different optical properties. It is noted that Dynamic Light Scattering (DLS) does not provide a full particle size distribution. DLS measures fluctuations in the intensity of scattered light caused by Brownian motion, from which the hydrodynamic diameter is calculated, enabling estimation of the particle size distribution. Thus, even though DLS does not measure particle size distribution directly, this method provides a good background for the estimation of the full particle size distribution. The method also provides a number (the 'polydispersity index') indicating the polydispersity of the particle population. There are several software routines that facilitate the calculation of a particle size distribution from DLS data, but the adequacy and the comparability of these routines needs to be further evaluated [51].	Size range: 1 - 1000 nm	size and polydispersity index (dimensionless; measure of broadness of the size distribution).

Table 2: Methods to generate/sample airborne dispersed or nebulised particles

Method and details	Material and size range	MMAD
Cascade impaction Cascade impactors can be used to obtain the size distribution of an aerosol (i.e.. in this context a dust cloud). Air samples are drawn through a device which consists of several stages on which particles are deposited on glass or glass fibre. Particles will impact on a certain stage depending on their size. The cut off size can be calculated from the jet velocities at each stage by weighing each stage before and after sampling and the MMAD derived from these calculations. A well established technique to measure the distribution of particles of respirable or inhalable size. However, cascade impaction may fail to describe the dimension of high aspect ratio nanoparticles when they no longer follow aerodynamic rules [52]. Conventional cascade impactors will have size selective stages limited to the capture of particles greater than ~250 nm. This is a sampling method and also requires aerosolisation. ISO/TR 27628:2007 [33] provides an informative description.	Particles in an aerosol Size range: 0.1-20 µm and 0.5-80 µm	MMAD can be determined via an appropriate coupled analytical technique.
Low Pressure Impactor (ELPI) ELPI is a type of cascade impactor that combines inertial collection with electrical particle detection to provide near-real-time aerosol size distributions for particles larger than 7 nm in diameter. Aerosol particles are charged in a unipolar ion charger before being sampled by a cascade impactor. The upper size limit of the instrument is 10 µm, but in practice reliable data can be obtained only up to about 2.5 µm due to significant losses at larger particle sizes. Collected aerosol particles are available for offline analysis, but this is also a limitation as it does not provide a direct measurement. It does however enable a range of off-line analytical methods to be used with samples, including electron microscopy and chemical speciation. ELPI has useful application in relation to exposure estimation. Data from the lowest stage have relatively large uncertainty due to losses and uncertainties of the true size channel width.	Particles in an aerosol Size range: 7 nm – 10 µm	MMAD can be determined via an appropriate coupled analytical technique or by calculation.

ISO/TR 27628:2007 [33] provides an informative description.		
Rotating drum method (EN 15051-2) [53] This method is based on size selective sampling of an airborne dust cloud produced by the repeated lifting and dropping of a material in a rotating drum. Air drawn through the drum passes through a specially designed outlet and a 3-stage fractionating system consisting of two porous polyurethane foams and a membrane filter. The mass of dust collected on each collection stage is determined gravimetrically to give a direct measure of the biologically relevant size fractions. This method simulates a wide range of material handling processes in industry and determines the biologically relevant size functions of a material in the airborne state. Full size distributions can be obtained by analysing the contents on the dust collection stages. This method is suitable to determine the distribution of particles of respirable or inhalable size. Rotating drum dustiness tests are usually performed as three replicate tests and need quite large amounts of test material, typically 300–600 g. It has been highlighted that such large amounts of test material may not be practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material [54].	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.

Continuous drop method (EN 15051-3) [55] This method is based on the size selective sampling of an airborne dust cloud produced by the continuous single dropping of material in a slow vertical air current. The dust released by dropping material is conducted by the airflow to a sampling section where it is separated into the inhalable and respirable fractions. This method is suitable to determine the distribution of particles of respirable or inhalable size. The continuous single-drop method requires a total amount of 500 g for the required five single test runs. It has been highlighted that such large amounts of test material may not be practical if very toxic and/or costly materials are to be tested and there is a need for test systems that can be operated under controlled atmospheric environments using much smaller amounts of material [54].	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.

Table 3: Methods that measure inhalable fractions only or that give no detailed distributions

Method and details	Material and size range	Data type
Elutriation Particles are drawn out on a column at varying velocity. The velocity is used to calculate particle size and the weight of the remaining sample at a particular velocity is used to calculate the distribution. The method is limited to particles >15 microns. The method is not suitable to determine the distribution of particles of respirable size, but might be suitable to determine the distribution of particles of inhalable size	Dry powders/granulates Size range: 15-115 microns	MMAD cannot be determined
Air jet sieve Air is aspirated through a weighted sample on a fine sieve and the weight loss measured. The method is capable of estimation of the non-floatable fraction of the material under investigation. Aggregation of the particles will result in unreliable values. In addition, since the lower detection limit is only 10 micron, this method is not suitable to determine the distribution of particles of respirable size. The method is not suitable to determine the distribution of particles of respirable size, but might be suitable to determine the distribution of particles of inhalable size.	Particles of all kind Size range: 10-10,000 microns	MMAD cannot be determined
Cyclons		

The use of a cyclone is a simple approach to determining whether respirable and/or inhalable particles are present in the test atmospheres by constructing the cyclone cut off points at 4.25 and 100 microns. By measuring the weight of particles which pass through the cyclone it can be decided whether more sophisticated methods have to be applied to determine the size distribution of the particles smaller than 10 micron. This method is suitable to determine the fraction of particles of respirable and inhalable size.	Particles of all kind Size range: 0.1-200 microns	MMAD cannot be determined

Table 4: Methods of measuring airborne dispersed or nebulised particles

Method and details	Material and size range	Data type
Scanning Mobility Particle Sizer (SMPS) (ISO 15900:2009 [56]**; ISO 10808:2010** [5]**; ISO 28439:2011** [57]**)** SMPS operates by charging particles and fractionating them based on their mobility when passing between electrodes. This method combines a Differential Mobility Analyser (DMA) and an Optical Particle Counter (OPC). SMPS detects and counts nanoparticles, and enables measurement of the particle size distribution and count median diameter of nano aerosols, up to 10^8 particles /cm³. This method also allows evaluation of nanoparticle surface area, mass dose, composition and dispersion to support effective analysis of inhalation toxicity testing results. SMPS also has useful application in relation to exposure estimation. Measurement with SMPS is the only currently available method that meets all of the following requirements in the size range below 100 nm: i) measurement of particle size distribution during particle exposures in a continuous manner with time resolution appropriate to check stability of particle size distribution and concentration; ii) measurement range of particle sizes and concentrations covers those of the nanoparticle aerosols exposed to the test system during the toxicity test; iii) particle size and concentration measurements are sufficiently accurate for nanoparticle toxicity testing and can be validated by ways such as calibration against appropriate reference standards; iv) resolution of particle sizing is sufficiently accurate to allow conversion from number-weighted distribution to surface area-weighted or volume-weighted distribution.	Particles in an aerosol Size range: ~3 – 800 nm -115 microns	Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles.

However, SMPS is relatively slow and requires a scanning approach to measure different size intervals in series. This method is restricted to ambient temperatures below 35 °C (due to evaporation of butanol in the CPC) and requires aerosolisation of the sample. SMPS cannot distinguish between agglomerates and primary particles. For non-spherical particles (e.g. HARN), estimation of diameter and mass concentration by SMPS can result in significant error. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods [52]. It is important to know the stability of the source, since rapid changes of the size distribution, particle concentration, or both, can affect measurement of the size distribution. This is relevant to consider for nanomaterials, which have a high tendency to agglomerate in the atmosphere		
Fast Mobility Particle Sizer (FMPS) FMPS enables determination of the size distribution of sub-micrometer aerosol particles, up to 10^7 particles / cm^3 (depending on particle size). Measurements can be made with a time resolution of one second or less, enabling visualization of particle size distributions in real time. However, FMPS is typically less sensitive than the SMPS at low particle concentrations.	Particles in an aerosol Size range: ~5 - 560 nm	Size distribution based on number counted (number count per size interval). From the distribution, MMAD can be calculated, with knowledge of the density of the particles
Diffusion batteries The operation of diffusion batteries is based on the Brownian motion of the aerosol particles. Depositional losses through diffusion are a function of particle diameter. By measuring diffusion based deposition rates through systems with varying geometries, it is possible to determine particle size distribution. The deposition systems are usually placed together in series to form a diffusion battery. The diffusion battery can be designed for determination of particle sizes as low as 2 nm depending upon instrument setup. This method has useful application in relation to exposure estimation. The primary property measured is the diffusion coefficient of the particles and this has to be converted to particle diameter. The instrument needs to be operated with a particle counter (typically a continuous flow Condensation Particle Counter) in order to determine the number concentration before and after each diffusion stage. Inversion of the raw data to real size distribution is complex and the solutions of	Particles in an aerosol Size range: 0.005 – 0.1 µm	Particle number in intervals according to diffusion diameter, from which the median diffusion diameter can be determined with knowledge of the density of the particles.

the equations do not give unambiguous results in the case of polydisperse aerosol size distributions. ISO/TR 27628:2007 [33] provides an informative description of this method.		
Optical Particle Counter (OPC) OPC is a widely used method for detecting and counting aerosolised particles, and operates across a wide temperature range (0 – 120 °C). Enables agglomerates/aggregates of primary particles to be measured and counted. OPC has useful application in relation to exposure estimation. However, OPC is insensitive to particles smaller than approximately 100-300 nm in diameter and provides insufficient coverage of potential primary particle. Assembling data of measurements from SPMS and OPC to provide a whole picture of particle size distribution is not appropriate, due to the different principles employed by the two methods [52]. ISO/TR 27628:2007 [33] provides an informative description of this method.	Particles in an aerosol Size range: 0.3 – 17 µm	Particle number concentration
Laser scattering/diffraction In general, the scattering of the incident light gives distinct pattern which are measured by a detector. This technique is particle property dependent – i.e. material has unique scattering and diffraction properties which are also particle size dependent. It is important to calibrate the instrument with similar material (of the same size range as the material to be measured). Laser scattering techniques are suitable for geometric particles, viz spheres, cubes and monocrystals. Particle size will be established optically. The MMAD can be calculated by means of a calculation correction. The method is suitable to determine the distribution of particles of respirable and inhalable size. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.	Particles of all kind Size range: 0.06-100 µm	Particle size/size distribution*, from which mass median diameter can be calculated, with knowledge of the density of the particles.
Light scattering aerosol spectrometer (LSAS) LSAS is a type of light scattering instrument, applicable for measuring the size, number concentration and number/size distribution of particles suspended in a gas. LSAS can be used for the determination of the particle size distribution and particle number concentration at relatively high concentrations of up to 10^{11} particles/m^3. The large measurement range of LSAS may result in high uncertainty in	Particles in an aerosol Size range: 0.06 - 45 µm	Particle size/size distribution*, from which mass median diameter can be

nanoscale measurements. Measurements may be dependent on the reflectivity of particles. Laser diffraction assumes a spherical particle shape. Test products should therefore have no extreme aspect ratios, with a restriction of 1:3 for non-spherical particles. This method has limited applicability really suitable in the sub-100 nm range. In the range below several microns, results strongly depend on optical constants of particles.		calculated, with knowledge of the density of the particles

Using the methods listed in Tables 1 to 4, the following information should be presented (as appropriate):

- Sample preparation methods and analysis methods used
- Lot number, sample number
- Suspending medium, temperature, pH
- Concentration (relevant to particles or fibres)
- Representative image(s) from microscopy
- Particle size distribution histogram from the applied measurement technique
- Average particle size(s) for resolvable peaks in the distribution, as mass number and surface area per unit volume as appropriate
- Expected % change of reported values in the future (e.g. variations between production batches)
- Reference all Standards (e.g. ISO) and reference materials used.

Rules for the graphical representation of particle size analysis data in histograms, density distributions and cumulative distributions are specified in ISO 9276 1:1998 [58]. It also establishes a standard nomenclature to be followed to obtain the distributions mentioned above from the measured data. In a graphical representation of particle size analysis data, the independent variable, i.e. the physical property chosen to characterise the size of the particles, is plotted on the abscissa (x-axis). The dependent variable, which characterises the measure and type of quantity (e.g. number, mass) is plotted on the ordinate (y-axis). ISO 9276-2:2001 [59] provides the relevant equations for the calculation of average particle sizes or average particle diameters and moments from a given particle size distribution. It is assumed that the size distribution is available as a histogram. It is nevertheless also possible to apply the same mathematical treatment if the particle size distribution is represented by an analytical function. It is furthermore assumed in ISO 9276-2:2001 [59] that the particle size of a particle of any other shape may also be represented by the diameter of an equivalent sphere, e.g. a sphere having the same volume as the particle concerned.

It is advantageous to have accurate information about the propensity of materials to produce particulate aerosol (including the *dustiness* of the material). No single method of dustiness testing is likely to represent and reproduce the various types of processing and handling used in industry. The measurement of dustiness depends on the test apparatus used, the properties of the dust and various environmental variables. The measurand of dustiness is the ratio of the inhalable dust produced by the dustiness test procedure, in milligrams, to the test mass of material used for the test, in kilograms. There are a number of methods for measuring the dustiness of bulk (non-nanoscale) materials, based on the biologically relevant aerosol fractions defined in EN 481. Two methods (the rotating drum method and the continuous drop method) are detailed in EN 15051 "Workplace atmospheres – Measurement of the dustiness of bulk materials – Requirements and reference test methods" [60].

Dustiness is a relative term (derived from the amount of dust emitted during a standard test procedure). This is dependent on the method chosen, the condition and properties of the tested bulk material, and various environmental variables in which the tests are carried out. Thus, the two methods in EN 15051 may provide different results (the methods are intended to simulate handling processes).

The particle size distribution of a dust cloud may be different from the powder source. Studies on dust generation by free falling powders have demonstrated that the manner in which the powder is handled may be as important as the dust generating capacity of the material, in terms of the resulting exposure. Falling height has an important influence on dust generation and release for more than one reason. The higher the impact, the more dissemination of dust there is. Moreover, the greater the falling height, the greater flow of entrained air, which

favours dust dissemination. This shows the importance of process design and adequate work practices.

There have been many interesting studies on material flow which demonstrate that the influence of the various factors is not so obvious. For example, it is sometimes erroneously assumed that a powdered material with a larger proportion of coarse particles offers less dust hazard; however, a higher proportion of coarse particles in the material may actually increase dustiness due to a *decrease in the cohesion of the material as the proportion of coarse particles increases* [61], and also due to the agitation of the fine particles as there are more collisions with large particles. The higher the impact between particles, the more dissemination of dust there is.

The aerosolisation/sampling methods in Table 2 are used in the determination of the distribution of respirable particles and (to a lesser extent) the distribution of inhalable particles. These methods generate aerosol test atmospheres and require coupled particle detection instrumentation.

The particle detection methods in Table 4 can be used to characterise the distribution of aerosolised particles. These methods are preferred since they measure particles in the air and as such the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD), but are subject to limitations. All particle size instrumentation have ranges of particle size limited by the principle of operation. Therefore more than one type of instrument is often used with overlapping size ranges. Often depending on the material, these size distributions may not match exactly, because different measuring principles deliver different equivalent diameters. Moreover, the lower sizes of 1nm to 3 nm cannot be accurately measured in aerosol measurement instrumentation because of diffusion losses in tubes or at the inlet of the instruments. Depending on the number based particle size distribution the particle number concentration will be determined too low and particle counters with different valid lower size limit will give different particle number concentrations. Aerosolisation of substances for particle size distribution characterisation also results in a degree of artificiality if the engineering set-up introduces an upper limit on the aerosol size as a result of the operational conditions (e.g. flow rate and exit orifice). The upper size limit can be predicted using Stoke's equation. Other methods that measure inhalable fractions only or that give no detailed distributions are detailed in Table 3.

Published data on granulometry

Particle size measurements have been published in the peer-reviewed literature. No electronic databases that are specific to particle size data could be found at the time of publication.

(End of R.7.1.14.2)

Regarding the evaluation of available information on granulometry (Section R.7.1.14.3), it is advised to perform particle size characterisation not only of the material under investigation but also of the airborne dust where appropriate. It as also important to remember that the original particle size distribution is highly dependant of the industrial processing methods used and care should be taken to ensure that the measurement and assessment activity considers any changes to the particle size distribution by subsequent environmental or human transformations.

When considering the uncertainty on granulometry it has to be noted that aerosolisation of substances for particle size distribution characterisation also results in a degree of artificiality if the engineering set-up introduces an upper limit on the aerosol size as a result of the operational conditions (e.g. flow rate and exit orifice). The upper size limit can be predicted using Stoke's equation.

For reaching conclusion on granulometry (See Section R.7.1.14.4) it has to be taken into account that the potential release of particles into the workplace or environment is an important consideration in the design and operation of many industrial processes and safe

handling of substances. Release of particles may present a safety hazard and could cause adverse health effects to humans and affect the environment. It is therefore important to obtain data about the propensity of substances to be released as particles or fibres, allowing risks to be evaluated, controlled and minimised. Measurement of the release of particles from powdered substances has similarities to the conventional measurement of the dustiness of a powder, but with significant differences in the methods and instrumentations suited to different particle size ranges.

In addition, the particle size distribution is needed to inform the decision regarding which route of administration is most appropriate for the acute toxicity and repeat dose toxicity animal studies. A number of methods are provided for determining the particle size fractions which are then used to assess the possible health effects resulting from inhalation of airborne particles in the workplace. A number of methods covering different ranges of particle sizes are available though none of them is applicable to the entire size range. Multiple techniques should be used where possible in order to formulate a complete understanding of the particle properties, and the optimum set of required techniques should be selected based on the specific substance and form under investigation.

Finally, taking the previous recommendations into consideration the integrated testing strategy (ITS) for granulometry would be as shown in the workflow:

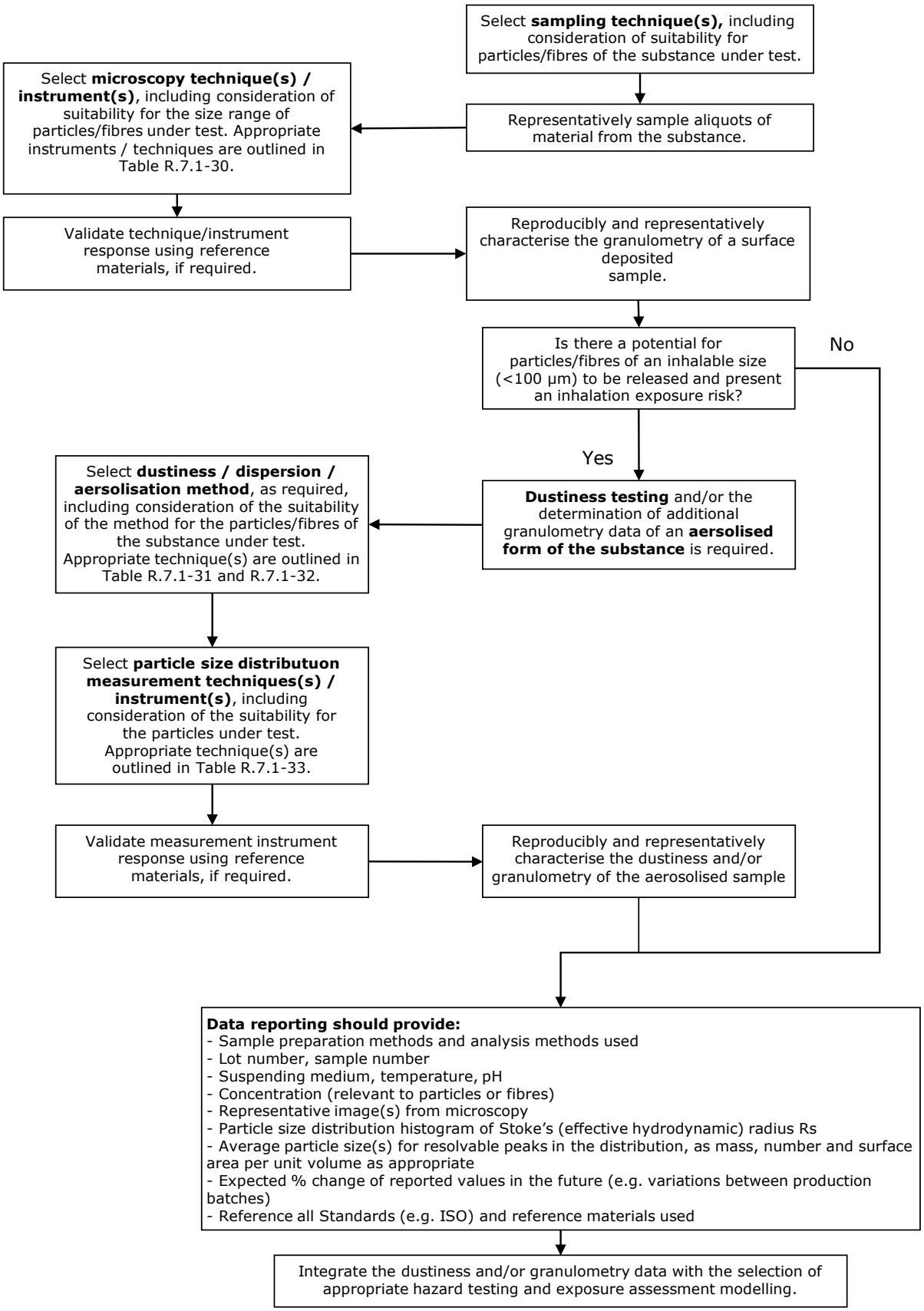


Figure 1: ITS for granulometry

2.2.3.3 Recommendations for shape

R.7.1.19 SHAPE

Solid particulates/granulates with identical composition can have a variety of well- or ill-defined shapes, including spheres, rods, tubes, fibres and plates, which may have different physical, chemical, and biological properties. Shapes are determined by the way in which the entities are bound together and particles will assume the shape that minimises free energy and is kinetically achievable under given environmental conditions. Particle shape is an important parameter in the characterisation of some nanoparticles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Knowledge of high aspect ratio particles may inform interpretation of some toxicity test results.

Definition of shape

Shape is a qualitative or, at best, semi-quantitative geometrical description or dimension-less term(s) of the extremities of the particle or collections of particles, their agglomerates or aggregates, that make up the material under investigation (adapted from [6]).

Particles may have readily definable shapes such as spheres, rods, or defined crystal morphologies. More often, particle shape is much more variable and 'shape factors' such as sphericity, circularity, aspect ratio, convexity and fractal dimension are needed to characterise shape.

ISO 9276-6:2008 [62] specifies rules and nomenclature for the description and quantitative representation of particle shape and morphology. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape.

Macrodescription is a description of the overall form of a particle in terms of the geometrical proportions of the particle. In general, simple geometrical descriptors calculated from the size measurements made on the particle silhouette are used. Low-order Fourier descriptors can also be regarded as macrodescriptors.

Mesodescription provides information about details of the particle shape and/or surface structure that are in a size range not much smaller than the particle proportions, like Barrett's roundness and concavity [63].

The following mesodescriptors can be defined:

a) morphological mathematical descriptors, computing robustness and largest concavity index;

b) a concavity tree, providing general insight into the organisation of concavities and their complexity;

c) angularity descriptors, determining those parts of the boundary that are active in the abrasion process:

i. an angularity factor, selecting the apices on corners which are coincident with the convex hull because it is these points that will make contact with the surface of another particle,

ii. a quadratic spike parameter, taking into account those spikes that are outside a circle, of area equal to that of the particle, centred over the particle centroid,

iii. slip chording, generating information on the number of cutting edges and their sharpness in the facet signature waveform;

d) fractal dimension, providing data on the overall structural complexity by consideration of a larger measurement step;

e) Fourier descriptors, of higher order than macrodescriptors, specifying the smaller-scale components of morphology;

f) bending energy, measuring the overall complexity of contour lines.

Microdescription determines the roughness of shape boundaries using two of the descriptors mentioned above:

- fractal dimension, measured using a measurement step smaller than that

used for structural description;

- higher-order Fourier descriptors/coefficients for surface-textural analysis.

R.7.1.19.1 Information requirements on shape

The study does not need to be conducted if the substance is marketed or used in a non-solid or non-granular form. Shape determination requires information on water solubility. Fibre length and diameter distribution require information on the fibrous nature of the product and on stability of the fibrous shape under electron microscope conditions.

The summary should include a microscopy image of the particle and a qualitative or semi-quantitative geometrical description of the extremities of the particle and/or collections of particles, agglomerates or aggregates that make up the material under investigation. Size-independent macro-, micro- and meso-shape descriptors (examples are ratios of extensions in different directions; unit [meter/meter] such as aspect ratio or fractal dimension are available (ISO 9276-6:2008 [62]) and should be used wherever possible. A combination of terms and/or measurands may be needed to describe shape; this is essential to circumvent the challenges already foreseeable where materials are capable of concurrently exhibiting multiple shapes in a sample which may present different hazard potentials. Information should also be included on the temperature at which measurements were made, purity of the sample used, physical state, method used and reference substance used (if any).

The level of inspection used in a method is a very practical criterion for the classification of the method, since many methods provide shape information at different size levels. Another convenient way of classifying methods is to differentiate between those which derive shape descriptors from particle images and those which derive shape descriptors from physical properties:

a) Calculation of geometrical descriptor/shape factors:

Geometrical shape factors are ratios between two different geometrical properties, such properties usually being some measure of the proportions of the image of the whole particle or some measure of the proportions of an ideal geometrical body that envelops, or forms an envelope around, the particle. These results are macroshape descriptors similar to an aspect ratio.

b) Calculation of dynamic shape factors from physical equivalent diameters:

These shape factors are similar to geometrical shape factors except that at least one physical property is considered in the comparison. Usually, the results are expressed as the ratio of equivalent diameters, e.g. Stokes sedimentation velocity to volume-equivalent diameter x_{Stokes}/x_V.

c) Morphological analysis:

Morphological analysis descriptors give mean values of particle shape that are not much smaller than the proportions of the whole particle. A typical example is concavity analysis.

d) Analysis of the contour line (shape boundary):

Multiple operations on the grey-level pixel image of a particle can produce a set of shape descriptors which can be correlated with the topology or surface texture of the particle.

e) Analysis of the physical spectra:

Multiple operations on, or the mathematical treatment of, the physical spectra of a single particle can extract the shape of information as a set of descriptors. Such a procedure has been described for shape analysis by azimuthal light scattering and diffraction spectroscopy.

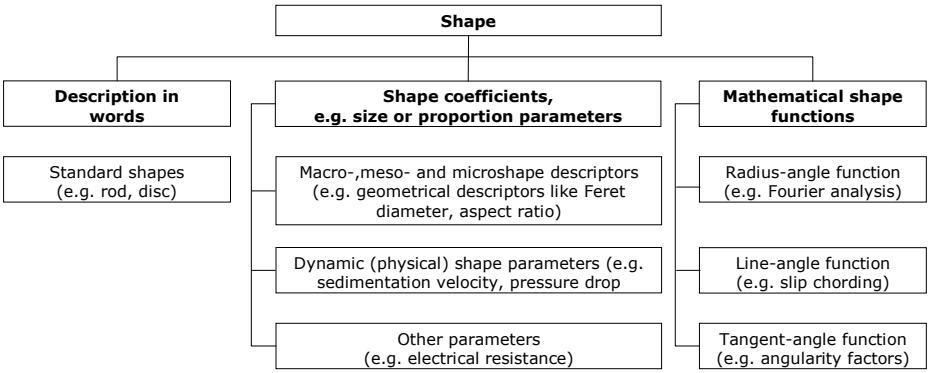


Figure 2: Classification of some methods for shape description (adapted from ISO 9276-6:2008 [62])

In the context of hazard assessment of nanomaterials, there are three forms in which properties should be characterised: "as produced", "as dosed / as exposed", and at the point(s) of interaction within the organism (which are sometimes collectively referred to as "as tested", but this and the equally un-specific term *in situ* require some further description of the context). "As dosed / as exposed" should reflect as much as possible the state of the substance to which humans and /or environment are exposed. The latter (at the point of interaction with the organism) is the most challenging measurement, because invasive techniques usually cannot be used without compromising the integrity of the organism and possibly invalidating the test, but acknowledged to be of more interest to advancing mechanistic toxicology rather than to regulatory toxicology. Although potentially confounded by issues of artefacts, insufficient statistical reliability, and difficulties in measurement and interpretation, an indirect way of assessing this form is through post-exposure evaluation, examining the shape distribution (i.e. a description of the proportion of particles with particular shapes in a sample) of particles in cells, tissues, organs or the environmental compartment after exposure.

R.7.1.19.2 Available information on shape

Testing data on shape

The characterisation of particle properties requires very careful sampling and sample splitting practices to be followed. ISO 14488:2007 [35] specifies methods for obtaining a test sub-sample from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative of the whole sample with a defined confidence level. Further information is available in Section 2.1.1 on Sample Preparation

A number of different methods for the qualitative or semi-quantitative description of particle shape and morphology are available (

Table 5). The shape of particles is usually determined by electron microscopy (e.g. TEM, SEM), which includes many qualitative and semi-quantitative techniques to investigate the morphology (size and shape) and also the aggregation state.

The choice of an appropriate shape description method depends on the measurement technique available and the particle system under examination (in particular its size range). Methods based on mathematical operations on contour lines (e.g. fractal dimension analysis or

Fourier analysis) require a relatively high resolution of particle images. This may be obtained by using a scanning electron or light microscope. Apart from such factors, the results of shape analysis may also be significantly affected by sample preparation (e.g. by the sample size and its representativeness of the whole sample) by particle orientation in 2D-analysis.

Table 5: Methods for the qualitative or semi-quantitative description of particle shape and morphology

Method and details	Material and size range	Data type
Transmission Electron Microscopy (TEM) TEM can be used for samples collected from the air or prepared in suspension on a TEM grid, including those from separation and sampling instruments. Powder preparation is very easy and fast for this method. Enables qualitative assessment of size and shape of particles, and differentiation between agglomerates and primary particles. TEM has a very high local resolution (nm) and is capable of imaging lattice planes and individual rows of atoms with resolution better than 0.2 nm. Additions to TEM can provide further information e.g. Scanning Transmission Electron Microscopy (STEM), High-Resolution TEM (HRTEM) or in-situ measurements using Environmental TEM, which offers the potential for dispersed samples to be characterised. However, TEM is a highly work-intensive method and requires manual preparation of samples. Dispersions need to be diluted (to ca. 1%) or prepared into work-intensive cryosections. Drying samples under vacuum for analysis may alter the size and shape of the particles being characterised. An extremely small area of the sample is analysed, which might not be representative enough. The comparatively small share of evaluated particles (ca. 1,000) results in limited statistical precision. Only a two-dimensional projection of particles is visible and can be evaluated; and the interpretation of pictures is difficult. Picture analysis is impossible if agglomeration is significant. Contours of particles may not be clearly resolved in some samples. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects. Further informative information on this method is available in ISO/TR 27628:2007 [33]. ISO 13322-1:2004 [38] and ISO 13322-2:2006 [39] provide general guidance for measurement description and its validation when determining particle size by static and dynamic image analysis, respectively.	Particles in solid, powder and suspension form. Size range: < 0.1 – 10 µm. Particularly suitable for the particle size range of 1 - 500 nm.	Image, providing opportunity to determine macro-,meso- and microdescriptors of shape
Scanning Electron Microscopy (SEM) SEM can be used for samples collected from the air or prepared in suspension on a SEM grid, including those from separation and sampling instruments. Sample	Particles in solid, powder and suspension form. Size range: < 0.01–10 µm.	Image, providing opportunity to determine macro-, meso- and

preparation is easier than for TEM, and only a small quantity of sample needed. Testing possible with undiluted dispersions and emulsions. SEM enables non-destructive testing of samples, and provides an image of the sample structure with very precise determination of size and shape at high local resolution. This method can be used in-situ as Environmental SEM. A representative sample of the material must be used. Where samples are not electrically conducting, plasma sputter-coating the surface-adhered particles with a layer of a conducting material is often required. This process may modify the sample being characterised. Only a small section of the sample is pictured and imaging is limited to surface features. The quality of the images to be analysed is of critical importance, and care must be taken to avoid bias introduced by orientation effects. Further informative information on this method is available in ISO/TR 27628:2007 [33]. ISO 13322-1:2004 [38] and ISO 13322-2:2006 [39] provide general guidance for measurement description and its validation when determining particle size by static	Particularly suitable for the particle size range of 10 nm – µm	microdescriptors of shape
Scanning Probe Microscopy (SPM) SPM includes both atomic force microscopy and scanning tunnelling microscopy (STM), which are all based, with some minor modifications, on a scanning probe (called the tip), which is moved across a substrate where particles have been deposited. SPM techniques allow individual nanoparticles and aggregates to be profiled in three dimensions from which shape can be studied. This is an advantage over SEM and TEM, which can measure only two dimensions. Air samples or liquid dispersions can be assessed, including those from separation and sampling instruments. SPM images give directly the three-dimensional morphology of complex samples such as carbon nanotubes, and can resolve simultaneously both their atomic structure and the electronic density. SPM enables rapid sample analysis under ambient conditions, and requires minimal sample preparation. For analysis, the sample must disperse onto and adhere to a substrate. The roughness of the substrate must be less than the size of the particles being measured to avoid a lack of clarity regarding image interpretation. Although SPM can resolve horizontal and vertical details to fractions of a nanometre, it is unable to deal with large changes in vertical profile occurring over a few nanometres. ISO TR/27628:2007 [33] provides an informative description of this method.	Particles in air or dispersed in a liquid Size range: 1nm – 8 µm	Image, providing opportunity to determine macro-, meso- and microdescriptors of shape
Optical microscopic examination It is preferable to prepare samples directly in order not to influence shape and size of the particles. This method provides images for the characterisation of the shape and distribution of samples of respirable	Particles of all kinds, including fibres. Size range: 0.2–	Image, providing opportunity to determine macro-, meso- and microdescriptors of shape

and inhalable particles and does not refer to airborne dust or dispersed or nebulised particles. Optical microscopy can be used to examine likelihood of fibres present by comparing similarities to known fibrous or fibre releasing substances or other data. Extreme care required during sample preparation to avoid fibre breaking and clumping. Care should also be taken to avoid contamination by airborne fibres. Samples might be prepared by (a) producing suspensions in water by gentle hand agitation or vortex mixing or (b) transfer of dry material onto copper tape either directly or by spraying of the dry fibres by use of atomiser or pipette. Length and diameter distributions should be measured independently at least twice and at least 70 fibres counted. No two values in a given histogram interval should differ by > 50% or 3 fibres, whichever is larger. The presence of long thin fibres would indicate a need for further, more precise measurements.	5000 µm. Fibre diameters as small as 0.2 µm and as large as 100 µm and lengths as small as 5 µm and as large as 300 µm.	

Using the methods listed in

Table 5, the following information should be

presented:

- Sample preparation methods and analysis methods used
- Lot number, sample number
- Suspending medium, temperature, pH
- Representative image(s) from microscopy
- Shape descriptor(s)
- Reference to all Standards (e.g. ISO) used and reference materials used

Published data on shape

No electronic databases that are specific to particle shape data could be found at

the time of publication. Software used with commercial instruments characterising shape by image analysis often contain libraries of reference shapes to categorise the particles under test.

R.7.1.19.3 Evaluation of available information on shape

Experimental data on shape

Shape is very often not a specific physico-chemical property of a substance. The original shape is highly dependent on the industrial processing methods used and can also be affected by subsequent environmental or human transformations. In that respect any published data on shape will only be pertinent to that particular sample or process.

Macroshape descriptors represent the geometrical proportions of particles. Most of them are ratios of descriptors of different geometrical properties. Geometrical (Table 6) and proportion (Table 7) descriptors of macroshape, mesoshape descriptors (Table 8), combination of shape descriptors (Table 9) and roughness descriptors (which represent microshape properties) (Table 10) are available (ISO 9276-6:2008 [62]). Fractal dimensions are necessary to distinguish between mesoshape (concavity) and microshape (descriptors).

Table 6: Geometric macroshape descriptors (reproduced from ISO 9276-6:2008 [62])

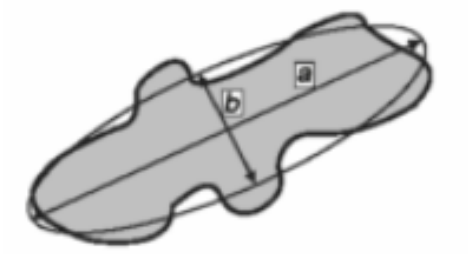
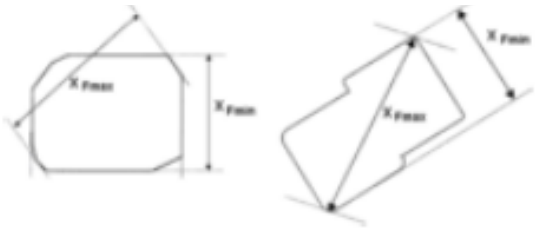
Lengendre ellipse of inertia 	An ellipse with its centre at the particle's centroid and with the same geometrical moments, up to the second order, as the original particle area The major and minor axes are given by x_{Lmax} and x_{Lmin} respectively Robust measurements.
Feret diameters x_{Fmax} and x_{Fmin} 	Distances between parallel tangents Maximum diameter x_{Fmax} corresponds to the "length" of the particle Minimum diameter x_{Fmin} corresponds to the "breadth" of the particle
Length x_{LF}	Feret diameter perpendicular to the minimum Feet diameter
Geodesic length x_{LG}, thickness x_E 	Better approximations for very long and concave particles, such as fibres Robust method determining xLG as an approximation for geodesic length and $x_{E,}$ using the following equations for an area and perimeter-equivalent rectangle: $A = x_E \cdot x_{LG}$ $P = 2(x_E + x_{LG})$

Table 7: Geometric Proportion macroshape descriptors (reproduced from ISO 9276-6:2008 [62])

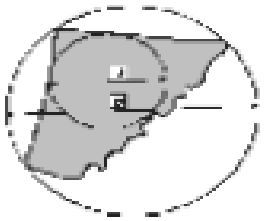
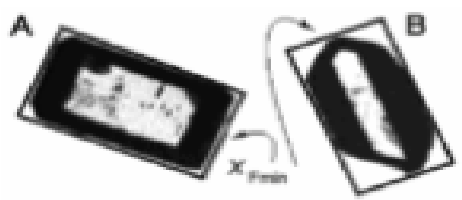
Ellipse ratio	Ellipse ratio = x_{Lmin}/x_{Lmax} where x_{Lmin} and x_{Lmax} are the lengths of the axes of the Legendre ellipse (Also used: elliptical shape factor) More robust paramneter than aspect ratio
Aspect ratio	For not very elongated particles: Aspect ratio = x_{Fmin}/x_{Fmax}
Elongation	For very elongated particles such as fibres: Elongation = x_E/x_{LG} (Also used: eccentricity)
Straightness	For very elongated particles (reciprocal of curl): Straightness = x_{Fmax}/x_{LG}
Irregularity (modification ratio) 	Relationship between the diameter of the maximum inscribed circle d_{imax} and that of the minimum circumscribed circle d_{cmin}: Irregularity = d_{imax}/d_{cmin} Also used: modification ratio)
Compactness	Degree to which the particle (or its projection area) is similar to a circle, considering the overall form of the particle: Compactness = $\frac{\sqrt{(4A/\pi)}}{x_{F\max}}$ Roundness R_n is also used, but is less robust: $R_n = 4A/\pi x_{F\max}^2$
Extent	Extent = $\frac{A}{x_{F\max} - x_{F\min}}$ (Also used: bulkiness)
Box ratio 	Ratio for the Feret Box area to the projected area: Box ratio = A/A_{box} $A_{box} - x_{F\min}.x_{LF}$ Very sensitive to orientation

Table 8: Mesoshape descriptors (reproduced from ISO 9276-6:2008 [62])

Wadell's sphericity ψ	$\psi = (x_v / x_s) = \pi \cdot x_v^2 / s$
Circularity *C*	Degree to which the particle or its projection area) is similar to a circle, considering the smoothness of the perimeter: $C = \sqrt{\frac{4\pi A}{p^2}} + \frac{x_A}{x_P}$ (Term under square root sign is called from the factor, FF)
Solidity	Measure of the overall concavity of a particle: Solidity=A/A_c Where A_c is the area of the convex hull (envelope) bounding the particle Global surface concavity index (CI) and concavity are also used: $CI = \frac{A_c - A}{A}$ $Concavity = \frac{A_c - A}{A_c}$
Convexity	Convexity=P_c/P Where P_c is the length of the perimeter of the convexity hull (envelope) bounding the particle
Average concavity	$\psi_{FP} = \frac{\bar{x}_F}{x_P}$ Where the angle-average Feret diameter $\bar{x}_F$ is given by: $\bar{x}_F = \frac{1}{\pi \int_x^x x_F(\alpha) d\alpha}$
Particle robustness Ω_1  Object	$\Omega_1 = \frac{2\omega_1}{\sqrt{A}}$ Where ω_1 is the number of erosions necessary to make the silhouette disappear completely
Largest concavity index Ω_2  Object Convex hull Complement B to convex hull	$\Omega_2 = \frac{2\omega_2}{\sqrt{A}}$ Where ω_2 is the number of erosions necessary to make the residual silhouette, set with respect to the convex hull of area A_C disappear completely

Table 9: Combination of shape descriptors (reproduced from ISO 9276-6:2008 [62])

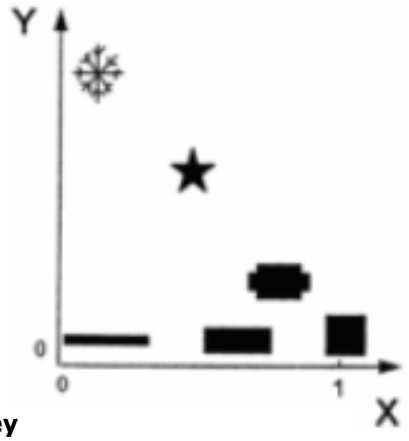
Concavity/robustness ratio Ω_3	Secondary mesoshape descriptor:
 **Key** X robustness Ω_1 Y largest concavity index Ω_2	$\Omega_3 = \frac{\Omega_2}{\Omega_1} = \frac{\omega_2}{\omega_1}$

Table 10: Roughness descriptor (reproduced from ISO 9276-6:2008 [62])

Fractal dimension D_F	
	The relationship between the length of the perimeter $P(\lambda)$ and the length λ of the steps is linear on a log-log plot, known as a Richardson plot The data are first normalized by dividing by the maximum Feret diameter The upper limit for the step size is giving by: $\lambda = 0{,}3x_{F\max}$ The equation of the straight line is: $\log P(\lambda) = (1 - D_F)\log \lambda + \log b$

Non-Experimental data on shape

At present, there are no QSPR/QSAR tools available for accurately predicting particle shape. Therefore the property will need to be experimentally determined.

Remaining uncertainty on shape

It is useful to distinguish between aggregates and agglomerates. While an aggregate may be considered to be permanent in most situations, agglomerates may break up under certain circumstances. As small particles often form agglomerates, sample pre-treatment (e.g. the addition of dispersing agents, agitation or low-level ultrasonic treatment) may be required

before the shape can be determined. However, great care must be taken to avoid changing the shape or size of the particle during sample preparation and the influence of any dispersant on testing results.

A combination of terms and/or measurands may be needed to describe shape; this is essential to circumvent the challenges already foreseeable where materials are capable of concurrently exhibiting multiple shapes in a sample which may present different hazard potentials.

Problems associated with image analysis are manifold and errors can be introduced in the generation of shape descriptors. These errors can exist at many levels, but most of them are fundamentally different from those observed in the more traditional techniques used for the characterisation of dispersed matter. Such shape descriptor errors are usually introduced by the protocols necessary to perform calculations on any given image (ISO 13322-1:2004 [38], Annex D). The common sources of errors which occur when performing image analysis and in the comparison of image analysis protocols include image resolution, binarization and algorithms for calculating shape descriptors (ISO 9276-6:2006 [62]).

R.7.1.19.4 Conclusions on shape

Shape is an important parameter in the characterisation of particles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape. The shape of particles is usually determined by electron microscopy (e.g. TEM, SEM), which includes many qualitative and semi-quantitative techniques to investigate the morphology (size and shape) and also the aggregation state.

Concluding on C&L and Chemical Safety Assessment

Shape is not used as a classification and labelling criterion. However, it can be used in the chemical safety assessment in considering risks associated with the substance.

R7.1.19.5 Integrated testing strategy (ITS) for shape

The following schematic diagram (Figure 3) presents an integrated testing strategy for shape.

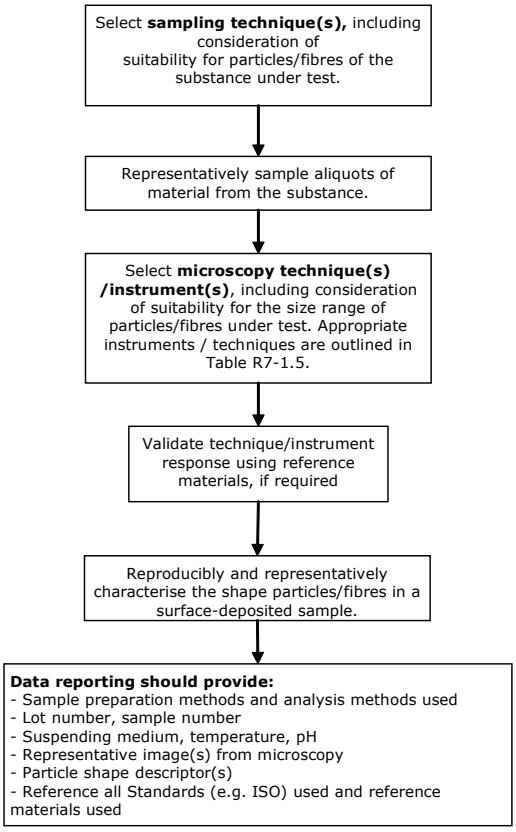


Figure 3: ITS for shape

2.2.3.4 Recommendations for surface area

R.7.1.20 SURFACE AREA

For particle-based substances, the surface plays an important role in influencing the physical and chemical interactions. As chemical reactions take place at surfaces, a sample of material with a high specific surface area to volume ratio can be expected to have a higher reactivity than a sample of the same material with a low specific surface area to volume ratio.

Surface area is an important parameter in the characterisation of nanoparticles, with emerging evidence of quantitative value as a dose metric or descriptor for hazard assessment. The total surface area should not be confused with the specific surface area where smaller particles have a larger specific surface area independent of whether they are present as primary, agglomerated or aggregated particles. For nanoscale materials, the reduction in size is accompanied by an inherent increase in the surface-to-volume ratio.

The specific surface area will dictate the surface charge in cases where nanomaterials are surface functionalised. This in turn has direct consequences on (a) nanomaterial interaction (i.e., agglomeration) with other naturally occurring particulate matter (i.e. contaminant vectors); (b) route of exposure as a function of surface ligand-biological interface (i.e. bioaccumulation pathway, bioavailability); and (c) mechanisms of toxicity (e.g. dose response curves normalized for surface area may indicate different results compared to results presented on a per mass basis) [6].

The volume specific surface area (VSSA) is determined from the entire particulate powder material including the whole size range distribution, with all external and/or internal surfaces. It characterises the entire particulate surface area per volume of a solid and/or powder material. The VSSA can be used to distinguish dry solid nanostructured material from non-nanostructured material based on its integral material surface area per material volume ([64], [65]).

The toxicity of some nanoparticles has been demonstrated in a number of studies to be related to their small size and therefore high surface area (e.g. [66], [67], [68], [69]). In addition, it has been observed in several nanotoxicity studies that effects correlate with surface area (e.g. [70], [71], [72], [73]) to a greater extent than mass as a dose metric. Other studies have demonstrated that the mass or volume may be a better descriptor in some cases. No scientific consensus has been reached at this stage regarding whether a single metric will be appropriate or possible given the complexity of different toxicological profiles and physico-chemical characteristics.

Definitions of surface area

Surface area is defined as the area of the exposed surface of a single particle, or more generally, the area of the exposed surface of a certain amount of a material [6].

Surface area as an extensive quantity depends on the amount of the material, and therefore a better comparable characteristic is the ratio of the surface area to the mass of a certain amount of a material. This is the so called specific surface area which is an intensive quantity and thus independent of the amount of the material. The volume specific surface area (VSSA) of a material is an ensemble measurement, only valid for the entire material as analysed; if a fraction/subset of the material (e.g. fractionated by size) is analysed, this subset will have a different VSSA which may be above or below the VSSA of the initial entire material.

Specific surface area = surface area of a material divided by its mass

[SI unit: m^2/kg].

Volume specific surface area = density multiplied by the specific surface area

[SI unit: m^2/cm^3].

R.7.1.20.1 Information requirements on surface area

The study does not need to be conducted if the substance is marketed or used in a non-solid or non-granular form. Specific surface area requires information on water insolubility. Fibre length and diameter distributions require information on the fibrous nature of the product and on stability of the fibrous shape under electron microscope conditions.

The summary should include a determination of the specific surface area [m^2/kg] and (where appropriate) the calculated volume specific surface area [m^2/cm^3] of the material under investigation, the temperature and conditions at which measurements were made, purity of the sample used, physical state, method used and reference substance used (if any).

R.7.1.20.2 Available information on surface area

Testing data on surface area

The characterisation of particle properties requires very careful sampling and sample splitting practices to be followed. ISO 14488:2007 [35] specifies methods for obtaining a test sample from a defined sample of particulate material (powder, paste, suspension or dust) that can be considered to be representative of the whole sample with a defined confidence level. Further information is available in Section 2.1.1 of this appendix on Sample Preparation.

By far the most common technique for measurement of the surface area of particles is by gas absorption measurements using Brunauer, Emmet and Teller (BET) adsorption isotherm theory (Table 11) [74]. This is a high vacuum method and requires a clean, dry sample of the nanomaterial. Nitrogen is the most common adsorbate, although many other gases such as argon, carbon dioxide, or krypton are also used. The BET technique involves measuring the amount of adsorbate released on vaporisation. The BET surface represents the surface area that is freely accessible to gases. The primary particle diameter (assumed as equivalent sphere diameter) is subsequently calculated from already available specific surface area and density of particles. Although this method provides measurement of two parameters simultaneously, i.e. size as well as surface area, the drawback of this procedure is in the assumption of a monodispersed spherical system which reports only an average size and does not provide the size distribution or a surface area distribution.

Emerging techniques for measuring particle surface area of nanoparticles in dispersion are being commercialised but are not yet standardised, such as the NMR analysis system for specific surface area determination of nano dispersions. This technique is based on the fact that liquid in contact with or “bound” to the surface of a particle behaves differently from that of the “free” liquid. Bound liquid molecules undergo restricted motion while free liquid can move unrestricted. The NMR relaxation time of liquid “bound” to the particle surface is much shorter than that of “free” liquid, the difference can be several orders of magnitude. In most situations there is a rapid exchange between liquid molecules on the surface and in the rest of the fluid, and an average relaxation time can be measured; this is then a direct measure of the amount of available particle surface area.

Table 11: Brunauer, Emmet and Teller (BET) method for determination of surface area

Method and details	Material and size range	Data type
BET method (ISO 9277:2010 [75]**; ISO 18757:2005** [76]**)** Enables determination of the total specific external and internal surface area of by measuring the amount of physically absorbed gas. Commonly applied to determine the surface area of nanomaterials. Allows an assessment of the agglomeration state of powders. Method assumes a mono-dispersed spherical system and provides a measurement of the surface area of a dry particle, which is not necessarily representative of the surface area of the particle when dispersed in the exposure medium. In order to ensure proper working conditions and correct data evaluation, the apparatus performance should be monitored periodically using a surface-area reference material. The BET method cannot reliably be applied to solids which absorb the measuring gas. ISO 9277:2010 [75] is applicable to adsorption isotherms of type II [disperse, nonporous or macroporous solids] and type IV [mesoporous solids, pore diameter between 2-50 nm]. ISO 18757:2005 [76] is applicable for determination of the total specific external and internal surface area of disperse or porous [pore diameter > 2 nm] fine ceramic materials.	Disperse or porous solids (e.g. powders)	Specific surface area (m^2/kg)

When reporting results from using the BET method, the following information should be presented:

- sample preparation methods and analysis methods used
- lot number, sample number
- pre-treatment and degassing conditions, e.g. degassing in a vacuum or in inert gas flow, temperature and duration of degassing;
- mass of degassed sample;
- adsorptive (chemical nature, purity);
- adsorption isotherm (na, plotted against relative pressure, p/p0), measurement temperature;
- evaluation parameters: multipoint or single-point determination, BET plot or range of linearity, monolayer amount, BET parameter C, molecular cross-sectional area used;
- specific surface area;
- references for all Standards (e.g. ISO) and reference materials used.

Published data on surface area

No electronic databases that are specific to particle surface area data could be

found at the time of publication.

R.7.1.20.3 Evaluation of available information on surface area

Experimental data on surface area

Surface area is not a specific physico-chemical property of a substance. Any published data on surface area will only be pertinent to that particular sample or process.

Non-Experimental data on surface area

At present, there are no QSPR/QSAR tools available for accurately predicting the surface area of nanomaterials. Therefore the property will need to be experimentally determined.

Remaining uncertainty on surface area

In many cases specific surface area measurements are derived quantities that depend on the nature of the probe molecule [77]. In the case of porous materials, it is often useful to distinguish between external and internal surface. The external surface is usually regarded as the envelope surrounding the discrete particles or agglomerates, but is difficult to define precisely because solid surfaces are rarely smooth on an atomic scale. The external surface include all the prominences and also the surface of those cracks which are wider than they are deep; the internal surface comprises the walls of all cracks, pores and cavities which are deeper than they are wide and which are accessible to a test gas (the adsorptive). In practice, the demarcation depends on the methods of assessment and the nature of the pore size distribution; hence accessibility of pores depends on the size and shape of gas molecules, the area of, and the volume enclosed by, the internal surface as determined by gas adsorption will depend on the adsorptive molecules (molecular sieve effect).

Not all particulate materials are amenable to a meaningful VSSA determination, for example where the specific surface area of substances with complex structural assemblies where the internal components are intrinsically not measurable.

R.7.1.20.4 Conclusions on surface area

For particle-based substances, the surface plays an important role in influencing the physical and chemical interactions. Surface area is an important parameter in the characterisation of nanoparticles in particular, with emerging evidence of quantitative value as a dose metric / descriptor for hazard assessment. The surface area will dictate the surface charge in cases where nanomaterials are surface functionalised, with direct consequences on nanomaterial interaction (i.e. agglomeration) with other naturally occurring particulate, route of exposure as a function of surface ligand-biological interface and mechanisms of toxicity [6]. By far the most common technique for measurement of the surface area of particles is by gas absorption measurements using Brunauer, Emmet and Teller

(BET) adsorption isotherm theory.

Concluding on C&L and Chemical Safety Assessment

Surface area is not used as a classification and labelling criterion. However, it can be used in the chemical safety assessment in considering risks associated with the substance.

R.7.1.20.5 Integrated testing strategy (ITS) for surface area

The tiered approach to testing (Section R.7.1.14) combined with the choice of an appropriate test method and implemented in conjunction with the ITS for granulometry (R.7.1.14.4) represents an integrated testing strategy for specific surface area.

2.2.3.3 Joint Integrated strategy for particle size distribution, surface area and shape

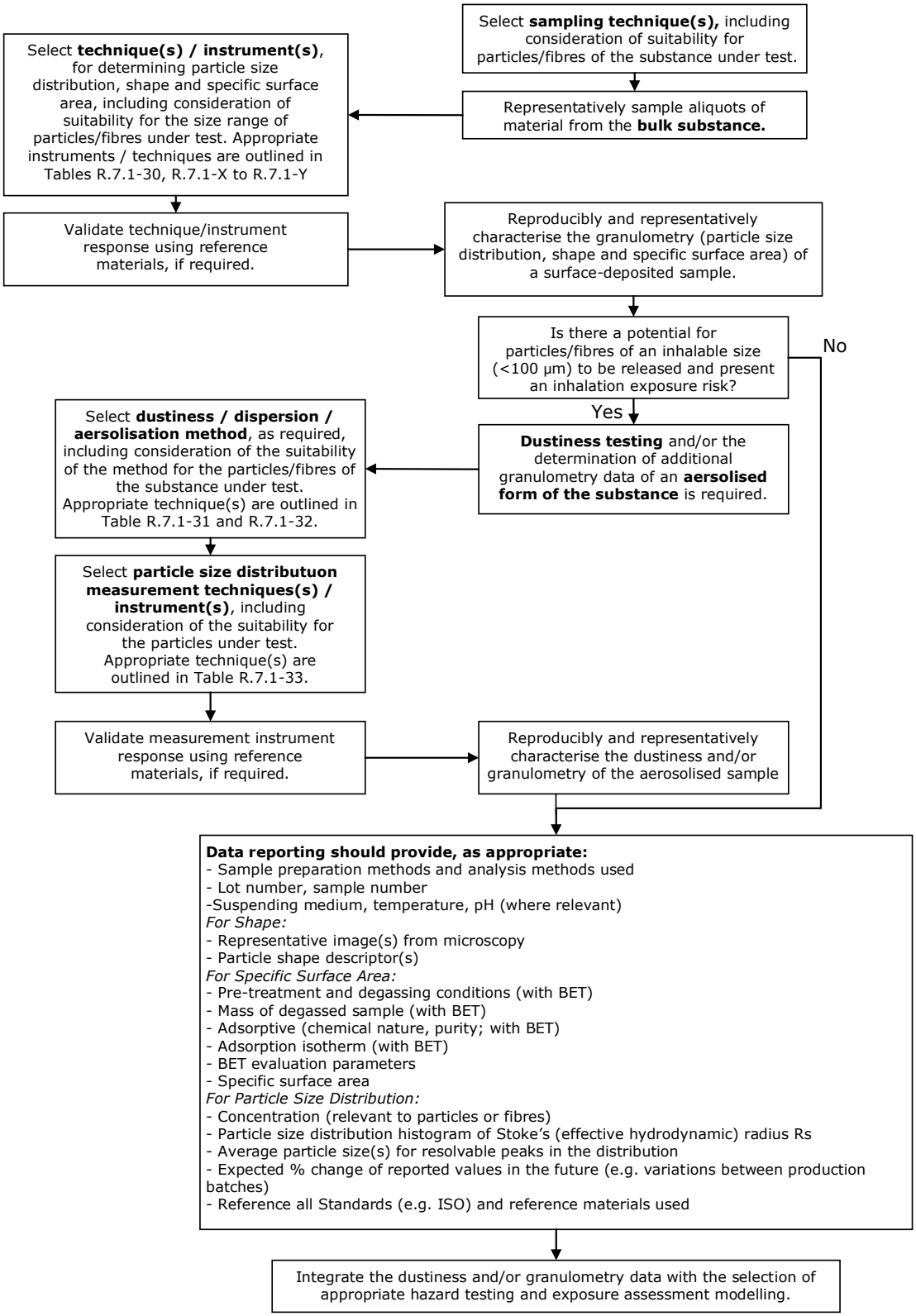


Figure 4: Joint ITS for particle size distribution, surface area and shape

2.2.4 Adsorption/desorption

In the parent guidance, the methods for determining this endpoint are shown in Table R.7.1-14 "Methods for the measurement of adsorption". Adsorption/desorption measurements are used in fate modelling to indicate which compartment in the environment will be exposed the most or might need to be considered in hazard and risk assessment. These measurements help to determine in which environmental compartment (e.g. soil, sediment or water) the substance is most likely to end up and whether it is likely to be mobile or immobile in the environment. For instance, high adsorption to soil would show that both soil and sediment are highly relevant environmental compartments to be considered in hazard assessment.

Adsorption[4] is temporary (reversible) or permanent bonding between the substance and a surface. With regard to nanomaterials, the distribution coefficient between solid phase and a liquid phase K_d may have to be based on actual testing since estimations of K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}) might not applicable when it comes to nanomaterials. K_d measurement is also based on the assumption of thermodynamic equilibrium between liquid and solid phase. Equilibrium partitioning does not apply to undissolved nanoparticles ([12], [22], [23];) as described in section 2.2.2 "Partitioning coefficient n-octanol/water". Hence, nanoparticles do not always form solutions, but instead may form colloidal dispersions, which are multiphase systems and thermodynamically unstable. Thus, nanoparticle dispersions can be kinetically stable for a long period of time (typically through electrostatic or steric stabilization) but they will never reach thermodynamic equilibrium and consequently cannot be equilibrated with an additional phase [23], [78].

Therefore, nanoparticles strive to reduce their surface energy by attaching to each other. This attachment can be:

- homoagglomeration/aggregation between the particles of the same nanomaterial, or,
- heteroagglomeration/aggregation with other particles or with e.g. organic matter, or
- to the interface between phases (deposition or attachment).

Because of our inability to accurately quantify the physicochemical forces contributing to particle attachment, this step is typically described by an empirical parameter termed the particle attachment efficiency (α) that needs to be determined in agglomeration (hetero-agglomeration) or deposition experiments [22], [78].

OECD TG 106 Adsorption – Desorption Using Batch Equilibrium Method is not applicable to nanomaterials because it is currently not possible to differentiate between adsorbed or aggregated/agglomerated nanoparticles settled during the centrifugation step, and a new TG needs to be developed ([8], [79]). However, if it is shown that a nanomaterial is quickly and highly dissolved, it can be assessed in the same way as traditional chemicals and the parent guidance will apply.

It is necessary to take into account the nanoparticle specific properties and constraints in assessing the adsorption/desorption of nanoparticles by currently available methods, based on K_d derived from the organic carbon-water partition coefficient (K_{oc}) and the octanol-water partition coefficient (K_{ow}), such as OECD TG 106. Consequently, waiving the information requirement based on low adsorption/desorption should always be accompanied with a robust technical and scientific justification of the applicability of the used test method.

2.2.4.1 Other guidelines and protocols for K_{oc} or K_d

OECD TG 312 Leaching in Soil Columns [80] allows study of the mobility and leaching of the

[4]Please note that distribution/partitioning does not equal adsorption, and neither does sorption, which consists of aDsorption and aBsorption phenomena.

test substance into deeper soil layers or ground water. Using OECD TG 312, K_d values can be derived from column leaching studies and these are considered generally applicable for nanomaterials.

Alternative approaches and measurements to describe adsorption/desorption of nanoparticles based on the determination of retention of nanomaterials in soils by screening techniques [23] or K_d and other equations based on colloidal suspensions or particles not reaching thermodynamic equilibrium have been discussed.

Other parameters than K_d or log K_{oc} could be considered for nanoparticles such as (hetero)agglomeration, aggregation, particle attachment and removal. Agglomeration behaviour has been identified as an important parameter affecting the environmental behaviour of nanomaterials. The agglomeration parameter depends on the physicochemical characteristics of the nanomaterial itself, the physicochemical characteristics of the suspension medium, suspension preparation, concentration of the nanomaterial and concentration of other substances and particles in the suspension. The agglomeration behaviour is controlled by kinetics (energy barriers) rather than thermodynamic equilibrium. Therefore information on the agglomeration and aggregation behaviour of nanomaterials is recommended to be generated before their further testing.

- The Draft OECD TG on Agglomeration Behaviour of Nanomaterials in Different aquatic Media [27] is available at http://www.oecd.org/env/ehs/testing/test-guidelines-for-comments-section3-degradation-and-accumulation.htm.

Determination of sorption is critical to assessing amounts of nanomaterials released to surface waters, and to soils and sediments ([81], [82], [83]; [84]). Particle attachment and removal from wastewater can be used as another alternative approach to predict sorption of nanomaterials.

- For example OECD TG 303A "Aerobic Sewage Treatment Simulation Test" may be used as an indirect measurement to predict sorption of nanomaterials into sludge by determining the distribution of the nanomaterials between sludge and effluent.

These alternative approaches are still under development and further validation is needed. When they are available they will be recommended as a means to provide suitable alternative information on the sorption and agglomeration/aggregation of nanomaterials. Pre-assessment of dissolution rate and agglomeration behaviour of nanomaterials is needed before proceeding with any alternative measurement of their attachment or deposition ([8], [12], [85]).

Other non-testing methods can also be considered in case the K_{oc} and K_d measurement are not valid. A list of available models to predict alternative fate descriptors for nanomaterials is available in Appendix 1.

3 RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS for NANOMATERIALS

3.1 General advisory notes

3.1.1 General advisory note on testing and sampling strategy and sample preparation for human health endpoints

These advisory notes do not propose a protocol but, instead, aim to provide useful advice with regard to specific aspects that are particularly important for nanomaterials testing, and references to relevant resources. For a testing material identified by its physico-chemical characterization as being a nanomaterial, the testing strategy is dependent on its solubility and dissolution potential in relevant biological fluids and testing media. Figure 5 below shows a decision tree that can be used to determine whether nanospecific advice should be used, or, due to the conclusions on the nanomaterial's properties, the advice provided by the parent guidance can be followed instead

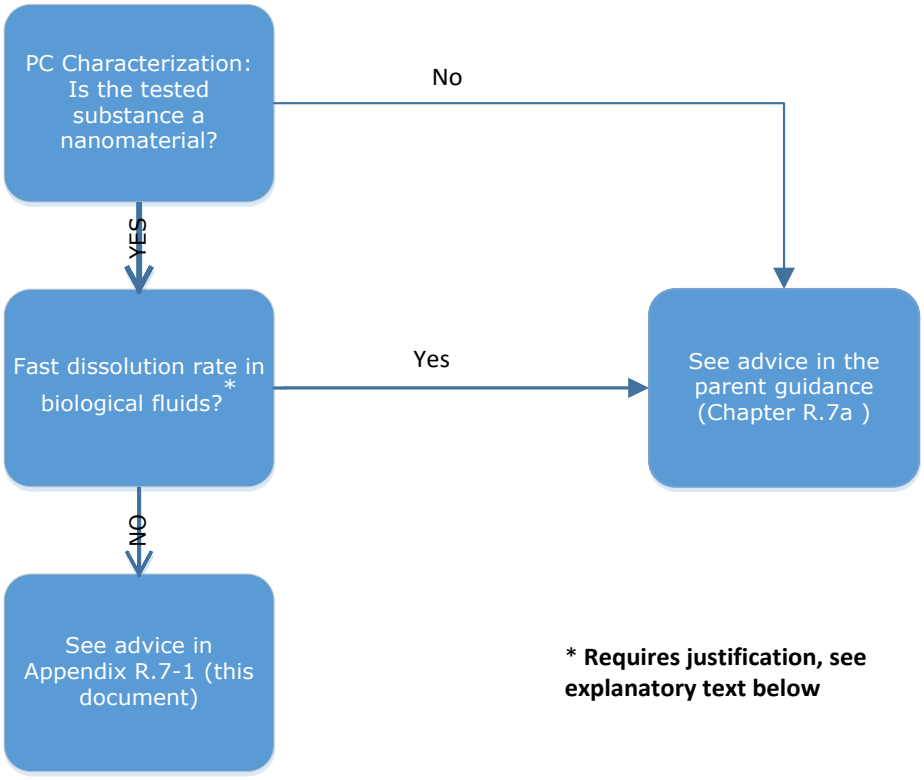


Figure 5: Decision tree for nanomaterials testing for human health endpoints

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has stated that many nanomaterials will have considerable solubility and that for "*these materials the interaction with living systems remains close enough to the bulk chemical agent to justify the use of well-established toxicological testing procedures and approaches*" [86]. The latest approaches for the risk assessment of nanomaterials recommend a similar strategy in which the dissolution rate and equilibrium in water is a primary key element [87]. Water solubility may give a first indication on a nanomaterial (non)biopersistence [88]. For example, as an initial pragmatic approach to assess the biopersistence of nanomaterials in the context of risk assessment in occupational settings, BAuA [89] proposed that nanomaterials with a water

solubility above 100 mg/l could be considered as soluble[5] (and thus not biopersistent). The water-soluble nanomaterials are generally not biopersistent. Nevertheless, different biological media may influence both the kinetics of dissolution and the saturation concentration [90]. In addition, some water insoluble nanomaterials may be non-biopersistent in biological fluids and this can be assessed from data on the dissolution rate. A nanomaterial's dissolution is a time-dependent process (depending on the rate of solubilisation and the surface area) and is directly related to a nanomaterial's *in vitro* or in vivo biopersistence that decreases with increasing dissolution rate [88]. Although no exact cut-off value has been proposed for dissolution rate, it needs to be very fast (i.e. close to instantly dissolved) [87].The determination of the dissolution rate provides an insight on how a certain particle may interact with its biological environment [91].

Consequently, for the nanomaterials for which there is evidence of fast dissolution rate in relevant biological fluids and testing media the advice provided in the parent guidance applies [92].

For the nanomaterials that do not have fast dissolution rate in relevant biological fluids and testing media, further guidance is given in this document.

3.1.1.1 Test material characterization and reporting[7]

Prior to toxicological testing, the sample characterization and preparation including special considerations on dispersion and dosimetry, should be performed, as advised in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials (ENV/JM/MONO(2012)40), and as specified in Section 2.1.1 of this Appendix. Additional useful information can be found in the report of the OECD expert meeting on the physical chemical properties of manufactured nanomaterials and test guidelines (ENV/JM/MONO(2014)15). A harmonized preparation of the teste sample will enable the comparison of the data and their further use. Information on the characterisation of test material serves multiple purposes:

a) enables linking to the identity (in this case also of the nanoform being covered in the dossier) and therefore supports data relevance,
b) facilitates interpretation of test results
c) provides general information on the material's properties 'as test sample' to support handling/storage and repeatability/reproducibility of results, and
d) may facilitate the use of toxicological data for grouping of the nanoforms of a substance or justifying read-across between nanoforms, and between nanoforms and the bulk form (For further information see *Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping* [1]).

Section 2.1.1 and 2.2 of this Appendix explain in detail the importance of these physico chemical parameters for toxicological testing and also gives information on how these parameters can be determined.

The chemical composition, the physicochemical properties, and the interaction of the nanomaterials with biological systems influence its potential hazard. The hazards posed by all possible forms of the substance covered by a registration, including nanoforms, must be addressed by the toxicological and ecotoxicological information provided in the registration dossier. In order to show that the test material(s) chosen are appropriate to represent the substance and/or the nano(form(s)) being assessed, some information should be reported in the endpoint study record under the test material information field in IUCLID. The following parameters should be provided:

[5] Please note this value is only used as an indication for (non) biopersistence and should not be used as a threshold for solubility/insolubility in other contexts (such as triggering a waiver for insolubility for environmental endpoints)

- Chemical composition (as described in the ECHA *Guidance for identification and naming of substances under REACH and CLP*);
- Size (as a minimum the D_{50}, but particle size distribution is recommended);
- Shape and aspect ratio;
- Surface chemistry.

Moreover, *Appendix R6-1 for nanomaterials applicable to the guidance on QSARs and Grouping of Chemicals* [1] provides an approach on how to justify the use of hazard data between nanoforms (and the non-nanoform(s)) of the same substance. The Guidance details some (additional) parameters that may be required to be able to assess whether the available hazard data are applicable for different nanoforms of a substance. The registrant may wish to consider taking into account such parameters when characterising the test material, in order to be able to follow the above-mentioned guidance. For example, the dissolution rate, surface chemistry and dispersability have been reported as a founding basis for the grouping of the nanomaterials ([1], [93]).

3.1.1.2 Biological Sampling[7]

Currently there are no OECD test guidelines specifically adapted for nanomaterials testing for human health endpoints[6]. However, this document aims to give some supplementary recommendations on specific aspects that, although not entirely nanomaterial specific (e.g. lung overload), are particularly important for nanomaterial testing.

The biological samples to be collected in the *in vivo* toxicological studies are specified in the relevant test guidelines. However, if there is an indication that the nanomaterials would be distributed in other tissues not listed in the OECD TGs, then the collection of these additional tissues is recommended.

It is advised to keep the samples to allow the performance of later analysis (e.g. storage by chemical or physical tissue fixation for microscopy [94], freezing for burden analysis ([95], [96].

3.1.1.3 Use of Non-Animal Testing Approaches [7]

Article 25 of the REACH regulation specifies that testing on vertebrate animals should be conducted only as a last resort, i.e. only when all other avenues have been exhausted. Therefore, there is an obligation to look at existing data and data from non-animal methods of hazard assessment before considering any new tests using vertebrates. Registrants are advised to keep informed on ongoing developments and validation efforts of the OECD and the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM), as well as on the regulatory acceptance of new methods by ECHA [97]. Implementation of non-animal approaches for nanomaterials requires the prior consideration of all available information, including context-specific nanomaterial characterisation, which is a critical requirement for grouping and read-across and quantitative structure–activity relationships (QSARs). In addition, relevant and reproducible *in vitro* systems may be used. Adverse Outcome Pathways (AOPs) specific to nanomaterials are under development at the OECD and offer new approaches to integrated assessment.

Regarding the use of non-testing data for nanomaterials, it is necessary to take into account

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[6] The update of OECD TG 412 and TG 413 to cover nanomaterials testing is currently under preparation. The drafts (when publicly available) may already provide some guidelines for testing nanomaterials.

[7] This advice is applicable for all endpoints relevant for human health, i.e. not only to those having a nanospecific entry in this document.

that:

- The use of *in silico* models (e.g. QSARs) for nanomaterials has also yet to be established. Thus, the use of these models for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied on a case-by-case basis only. However, in any case results from non-testing methods can be useful information in the context of weight of evidence or can provide essential information for the planning of an animal test. A range of *in silico* models, such as those used to determine nanomaterial kinetics, QSARs and physiologically based pharmacokinetic (PBPK) models have been developed for nanomaterials ([98], [99], [100] [101], [102] [103].

- The use of grouping and read-across approaches is another step to consider before performing animal testing. In this respect, it is advised to consider the ECHA guidance *Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping of the Chemicals* [1] when data on other (nano)forms[8] of the same substance are available. Regarding read-across and/or grouping between (nano)forms of different substances the advice provided in the ECHA Guidance Chapter R.6 on QSARs and Grouping of the Chemicals [104] and its nanospecific appendix [1] may be considered.

3.1.1.4 *In vitro* studies

In accordance with Article 13(1) of the REACH regulation, "*Information on intrinsic properties of substances may be generated by means other than tests, provided that the conditions set out in Annex XI are met*". The information from *in vitro* tests should always be considered before performing an animal test.

It has been shown that many *in vitro* assays (e.g. [105], [106], [107]) are applicable to nanomaterials when the nano-specific parameters are considered, and can be effectively used as part of a weight of evidence approach [2], [108], [109]. REACH Annex XI includes provisions for the acceptance of data from *in vitro* studies.

According to OECD 43, [110] for *in vitro* testing the "Characterisation of the materials should be undertaken in the cell culture medium used both at the beginning of treatment and, where methodologies exist, after treatment. The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the *in vivo* system".

3.1.2 Advisory note on the consideration of assay interference

Various nanomaterials have on occasion been found to interfere with several commonly used assays used to determine their cellular or toxic effects. For example, some nanomaterials may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanomaterials may bind to assay components including the substrates (e.g. CNT with the reagent in MTT 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide assays; [111]) or the biomarker being measured, (e.g. lactate dehydrogenase (LDH) and cytokine proteins; see for example [112]). Please note that other factors such as coatings or impurities may also have an influence on the assay.

A summary list of potential sources of interference with commonly used assays has been developed by Kroll *et al.* [113] and is reproduced in the table below.

[8] The term (nano)form intends to cover nanoforms and non-nanoforms of the substance

Table 12: Potential sources of interference with commonly used assays

Cytotoxicity assay	Detection principle	Nanoparticle interference	Altered readout	Particle type
Cell viability				
MTT	Colorimetric detection of mitochondrial activity	Adsorption of substrate	Reduced indication of cell viability	Carbon nanoparticles
LDH	Colorimetric detection of LDH release	Inhibition of LDH	Reduced indication of necrosis	Trace metal-containing nanoparticles
Annexin V/ Propidium iodide	Fluorimetric detection of phosphatidylserine exposure (apoptosis marker) Propidium iodide staining of DNA (necrosis marker)	Ca^{2+}-depletion Dye adsorption	Reduced indication of apoptosis Reduced indication of necrosis	Carbon nanoparticles
Neutral red	Colorimetric detection of intact lysosomes	Dye adsorption	Reduced indication of cell viability	Carbon nanoparticles
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of oxidative stress	Carbon nanoparticles
Stress response				
Dichlorofluorescein (DCF) ()	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				

ELISA(enzyme-linked immunosorbent assay)	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

It should be noted that the above list is not exhaustive and the potential for inhibition or enhancement of test results should always be investigated. The agglomeration, dispersion and/or dose may influence the outcome of the test.

Within some standard methodologies, the method requires the use of spiked sample (addition of a known reference/control sample) to test for inhibition or enhancement of the spiked control. This is evaluated by assessing the measured value against the expected value, which should be a cumulative value of the spike and of the sample.

Assay interference should always be investigated wherever possible, irrespective of standard method requirement; however, this may not always be possible. Furthermore, for many of the studies reported, it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, if other methods for assessing interference are not available, as a general precaution, it is advisable to use more than one assay to assess the studied endpoint or effect, as for example advised by Landsiedel *et al*. [114] for the genotoxicity endpoint. The potential for inhibition or enhancement of the test result may impact numerous test methods.The potential for assay interference has been identified for some nanomaterials in certain cases, for example carbon nanotubes are suspected to interfere with the MTT assay [115] and this may cause issues with tests such as OECD TG 431/EU B.40 bis Human Skin Model tests (EPISKIN™, EpiDerm™) which use the MTT assay. However, knowledge on nanomaterial assay interference is incomplete and so precautions to ensure the validity of an assay, such as the above-mentioned use of control spikes could be used.

Due to the potential for interference resulting in misleading results in numerous assays, utmost care should be taken in testing for such interference.

3.2 Specific advice for individual endpoints

3.2.1 Repeated dose toxicity

As highlighted in the general testing strategy for nanomaterials in Figure 5, for nanomaterials that do not have a fast dissolution rate in relevant biological fluids and testing media, further guidance for testing is provided in this document. Poorly soluble particles (PSPs) are part of this category.

For PSPs, the rat lung burden is an important issue to consider in the toxicological outcome and therefore a special chapter within this section (3.2.1.1) is included to addres this. For fibre-like particles, in addition to the overload of macrophages, frustrated phagocytosis has also been proposed as playing a role in their toxicity [116].

When considering the nanomaterials testing strategy for repeated dose toxicity (Section 7.5.6) it should be noted that:

- Especially for workers (and in some cases for consumers (e.g. in case of sprayable products)) inhalation may be the most likely route of exposure to nano(particles), nano aerosols and dustHence, the repeated dose toxicity studies are recommended to be performed via inhalation, unless there is convincing information (e.g. uses, dissolution rate, etc.) that justifies another route. Any modification of the protocols described in OECD TG 412 and 413 ([117] and [118]) should be justified;

- When dose range finding studies or repeated dose studies are performed, for PSPs, it is recommended to collect additional toxicokinetic data as described in *Appendix R7-2 for nanomaterials applicable to Chapter R7c Endpoint specific guidance*). In addition, to

make full use of the test, if there is a particular concern it is recommended to address it within the study design (e.g. accumulation, specific organ toxicity, etc.).

- When performing an inhalation test for PSPs the possibility for lung overload should be considered. The data on lung burden and clearance may be useful arguments in the context of read-across.

- To monitor the fate and effects of PSPs in the body it is recommended to collect the samples at several time points and/or from different organs. Data from range-finding studies, if proven robust, could be used to determine the appropriate sampling times). It is not intended to advise here on the use of extra animals for the additional analyses. However, it is important to find a balance between performing additional analyses and indication of toxicity

- Since the lower respiratory tract (i.e., the alveoli) is the primary site of deposition (depending on agglomerate size) and retention for inhaled nanoparticles, bronchoalveolar lavage (BAL) analysis is a useful technique to predict and quantitatively estimate pulmonary inflammation and damage (for further information on BAL parameters please see OECD TGs 412 and 413 [117] [118]). BAL analysis allows dose-response and time-course changes of alveolar injury to be suitably investigated. Therefore, for nanomaterials testing, it is highly recommended to include BAL analysis (further details are given in Section R.7.5 (repeated dose toxicity) of *Chapter R7.a of the Guidance on IR&CSA* (Endpoint specific guidance) [92].

- It is strongly advised to use more than one different dose-describing metric and to include the mass metric. The choice of method(s) selected should be justified as described in Section 3.2.1.1.1.

3.2.1.1 Advisory note on the consideration of lung burden within inhalation toxicity assessment

This section describes the concept of rat lung burden of PSPs and the associated effects, the differences between species and the extrapolation of the results to humans, relevant dose metrics and suggested thresholds. Care should be taken when interpreting lung burden in the context of human risk assessment. Lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans unless it can be clearly substantiated otherwise. When designing a new study, the doses to be used in repeated dose inhalation studies should not exceed the maximum tolerated dose. OECD TGs 412 [117] and 413 [118] provide advice on dosages to be used. This includes the provision that the highest dose should elicit unequivocal toxicity without causing undue stress to animals or affecting their longevity.

Results from inhalation studies in rats have shown that the PSP can induce serious adverse pulmonary effects if inhaled in high concentrations due to material accumulation, as lung clearance mechanisms are not able to remove materials at the same time or at a higher rate as the dose is delivered. This condition named "lung overload", occurs when the retained particle burden in the lung exceeds a certain threshold [119].
The term 'lung overload', is a phenomenon associated with exposure to PSP and occurs when a threshold level of particles is reached within the lung. During prolonged exposure of rats to PSP, the lung burden of particles increases until equilibrium is reached between deposition and clearance of particles [120] as shown by the curves A, B and C in Figure 6. This equilibrium can be reached very fast or may take up to many days. Below the lung overload threshold, particles are cleared via normal mechanisms at a constant clearance rate, in general generating little or only a minor or reversible response (exposure concentrations in curves A and B).

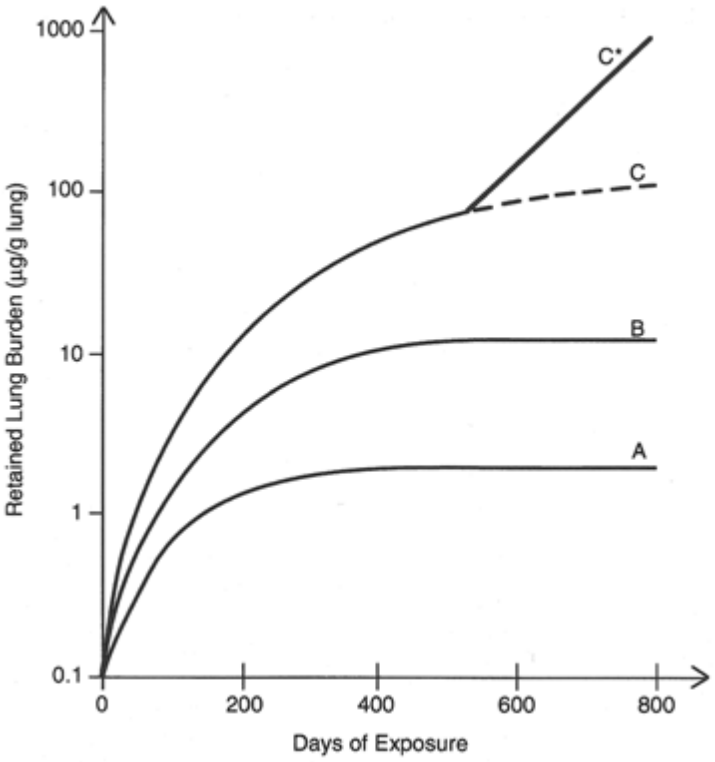

Figure 6: Schematic representation of the relationship between retained lung burden and duration of exposure leading to the phenomenon of lung overload. Curves A, B, and C are associated with progressively increasing exposure doses. If the exposure level is sufficiently high and the duration of exposure sufficiently long, alveolar macrophage-mediated clearance of particles can be overwhelmed. When this occurs, the retained lung burden increases linearly with further exposure (curve C*). Reproduced from [120].

Once the threshold has been reached, the clearance mechanisms of the lung become overloaded. This is typified by a progressive reduction of particle clearance from the deep lung, reflecting a breakdown in alveolar macrophage (AM)-mediated dust removal due to the loss of AM mobility [119]. This is shown in the C* curve of Figure 6 whereby at the point of threshold, particle retention occurs linearly rather than an equilibrium being established (as demonstrated by the dashed line).

The result of this net increase in particle accumulation is lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages and/or free (non-phagocyted) particles within the lung alveoli. The potential progression of inflammatory reactions toward a granulomatous type in rats was found to depend on the exposure duration and the level of the particle (surface) burden in the lung [121] as well as of the volumetric load [122].

The situation of lung burden is most commonly associated with repeated inhalation exposure of rats to PSP but it can also occur after single or repeated instillation of PSP into the lung (due to a high deposition fraction as a result of direct instillation) or possibly as a result of a single massive inhalation exposure [123]. Since this phenomenon occurs at relatively high exposure levels of respirable PSPs it is often argued that the observed adverse effects are a product of the lung burden caused by experimental conditions and not always a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer. Exposure to highly reactive or toxic particles may cause inflammation, fibrosis and cancer at lower exposure levels (non-overload conditions) due to intrinsic properties of the particles themselves.

In the studies performed with PSP the measurement of changes in lung burden over post-exposure time(s) provides essential information on lung clearance and allows clarification of the deposited vs the exposed particle amount. Different imaging techniques may also be used for a semi-quantitative assessment of the PSPs in the tissue [124]. The assessment of the dissolution potential as an indicator for biopersistence can also be done using *in vitro* systems [91].

Information on clearance and biopersistance is important in the context of read-across and weight of evidence.

The rat is currently considered the most sensitive species for inhalation toxicity testing for nanoparticles. However, as it can be difficult to interpret the findings of overload of alveolar macrophages in rat studies, a better understanding of the rat lung burden and its relevance to humans is needed. Several studies have assessed the responses to lung overload in different species, and the relevance of this data for humans. For instance, in a comparative study assessing the long-term pulmonary response of rats, mice and hamsters to inhalation of ultrafine grade titanium dioxide [96], the same air concentrations caused overload effects in rats and mice but not in hamsters. Also, the inflammatory and pathological responses were less severe in mice than in rats and they diminished with time irrespective of the similar lung burdens ([96], [125]). However, in relation to the relevance of animal data for humans, other studies have pointed out that the lung responses to high lung burdens of PSP of low toxicity can be qualitatively similar in rats and humans [126]. Based on experience with exposure of coal miners, a specific interstitial particle sequestration compartment is hypothesed [127]. Borm *et al* [122] discuss whether this mechanism could explain why humans, in contrast to rats, seem not to have an increased risk of lung cancer under lung overload conditions [122]. Nevertheless, there seems to be some conditional evidence for particle overload associated with impaired clearance in coal miners [122].

Therefore, the use of existing data, obtained after exposure to high doses of PSP, cannot automatically be dismissed as irrelevant in the context of risk assessment and the interpretation of such data should be approached with caution. In the caseof adverse effects observed in animals under overload conditions, the relevance for humans has to be assumed *a priori*; any claimed non-relevance for humans must be supported by data.

For further information, there are several review articles covering the subject of lung overload such as Miller [120], who provided an in-depth discussion of particle deposition, clearance and lung overload. Borm *et al* [128] discussed the importance of overload in the context of risk assessment whereas in an editorial of Borm et al, [122] the state of the art concerning lung particle overload concepts is summarized. These reviews also present different views on how to assess lung overload and how to interpret the data and emphasize the fact that the topic is still under debate.

In conclusion, lung effects observed in animals exposed to PSP by inhalation should be considered relevant for humans, unless it can be clearly substantiated otherwise.

3.2.1.1.1 Metrics

The question of which dose metric best describes the association between deposited dose in the lung, and subsequent inflammation and impaired clearance function is particularly relevant. There have been several suggested metrics but volumetric load of AM and surface area appear to be the most relevant [122] in interpreting lung overload-related as well as other adverse effects and in establishing limit concentrations. Morrow *et al*. [119] hypothesised that overload begins when the particulate volume exceeds approximately 60 μm^3/AM (which produces a 6% increase in the average alveolar macrophage volume) and that total cessation of AM-mediated clearance occurs when the particulate volume exceeds 600 μm^3/AM (producing a 60% increase in the average alveolar macrophage volume). Extending the Morrow concept, Pauluhn ([129], [130]) modelled a generic particle volume threshold for agglomerated PSP.
Oberdoerster et al. [131] suggested that the particle surface area better correlates the

overload with retarded clearance. Several studies suggest that, particle surface area correlates well with induced pathogenic events in lung ([128], [132], [73]). In a study by Tran *et al*. [73] data from a series of chronic inhalation experiments on rats with two poorly soluble dusts (titanium dioxide and barium sulphate) was analysed. The results indicated that when lung burden was expressed as particle surface area, there was a clear relationship with the level of inflammation and translocation to the lymph nodes. Most usefully, based on the shape of the statistical relationship for lung response to particles, the authors suggested the presence of a threshold at approximately 200–300 cm^2 of lung burden for "low-toxicity dusts" in rats.

Whilst some studies indicate mass as a less sensitive indicator of lung overload [133], the mass concentration is still important because there is already a large body of data and research on the exposure to and toxicity of particles using the mass-based metric. Therefore, for the sake of comparison(s), the mass concentration should always be reported.

Other studies ([134], [135]) found that the particle number or the number of functional groups in the surface of nanoparticles ([136], [137]) was the best dose metric.

The most relevant dose metric seems to vary depending on the specific nanoparticle in question. Particle volume, surface areas, mass, particle number as well as number of functional groups should be reported in order to establish the dose metric that best describes the association between deposited dose in the lung, overload conditions and the subsequent pathogenic effects and in order to establish the dose metric most relevant for risk assessment.

It is therefore vital to fully characterise test materials, so that the measured response can be retrospectively correlated with multiple-dose metrics, without the need for repeat testing. In general, the more metrics are reported the better.

In conclusion, it is strongly advised to use more than one different dose-describing metric and to justify the choice of the selected methods.

3.2.1.1.2 Overview of the recommendations for lung burden

- Data from existing studies performed with high doses of PSPs showing adverse effects cannot automatically be dismissed as irrelevant for humans
- When planning/performing, new studies, the use of excessively high doses should be avoided (in order not to exceed the maximum tolerated dose)
- Lung burden data may provide useful information on the pulmonary (retained) dose as well as on clearance behaviour and may support the read-across and weight of evidence approaches
- The most relevant metric should be used and mass metric should always be included. It is strongly recommended to use more than one metric.

3.2.2 Mutagenicity and Carcinogenicity

3.2.2.1 Advisory note on the consideration of bacterial assay interference

Genotoxicity assessment generally relies on a combination of *in vivo/in vitro* effect and indicator tests to assess effects for three major endpoints concerning genetic damage: i.e. gene mutation, clastogenicity and aneuploidy. It is now clear, from the results of international collaborative studies and the large databases that are currently available for the assays evaluated, that no single assay can detect all genotoxic substances [138].

The bacterial reverse mutation (Ames) test (OECD TG 471 [139]/EU B.12/13: Bacterial reverse mutation test (*in vitro*)) detects point mutations in *Salmonella typhimurium* and *Escherichia*

coli ([140], [141]; [142]). In relation to nanomaterials, a review of the applicability of genotoxicity tests to nanomaterials questioned whether the Ames test was accurately representative of nanomaterial genotoxicity [114]. The Landsiedel study [114] reported that of those studies reviewed, results were predominantly negative (5/6 studies). The group speculated that it is likely that some nanomaterials are not able to cross the bacterial wall, whilst others kill the test organism as they are bactericidal. According to OECD 43 [110], *'The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials*'. Likewise, Doak *et al.* [143] concluded that "although the Ames test is a reliable genotoxicity screen for the analysis of chemicals, it does not appear to be suitable for the assessment of nanomaterials".

Based on this, it is advisable that any negative data harvested from such bacterial mutation tests should be followed up with other assays after the initial screening, perhaps via implementation of a battery of standardised genotoxicity testing methods covering an as wide as possible variety of potential genotoxic mechanisms. In addition to the use of other assays, determination of cellular uptake by appropriate methods will help in the interpretation of *in vitro* genotoxicity assays.

3.2.2.2 General considerations for Mutagenicity and Carcinogenicity

The parent guidance Section 7.7 provides the general testing strategy for mutagenicity and carcinogenicity. The advice provided in the parent guidance should be followed together with the recommendations given in this section.

The guidance gives a list of methods for *in vitro* testing for mutagenicity in Table R.7.7-2, and the list includes the *in vitro* gene mutation study, as specified in Annex VII of REACH (See Section 7.7.6.3). The bacterial mutation assay should not be used as the only test for (nano)particle mutagenicity, but instead be used in conjunction with a range of mammalian cell gene mutation tests to reduce the potential for confounded results due to interference with a test method. Measurement of cellular uptake by appropriate methods is highly advised for bacterial as well as for mammalian cell genotoxicity/mutagenicity tests. Moreover, the use of metabolic activation system (S9) in *in vitro* studies can affect the outcome of the tests: like for any other tested chemical, S9 can induce the formation of mutagenic metabolites (in case the nanomaterial can be metabolised); also, the addition of proteins (contained in S9) can modify the cellular uptake of nanomaterials ([144], [143] and [145])

During the OECD/WPMN expert meeting on the Genotoxicity of Manufactured Nanomaterials in Ottawa, Canada in November 2013 [110], several consensus statements were agreed and found useful to investigate the genetic toxicity testing of nanomaterials. Several of these recommendations are also supported in other scientific literature (e.g. see reviews by Magdolenova *et al*. [144], Pfuhler *et al*. [145], Doak *et al*. [143]):

1. "*The use of the Ames test (TG 471* [139]*) is not a recommended test method for the investigation of the genotoxicity of nanomaterials"* [110]

   According to the recent discussions, it is advised to perform another *in vitro* mutagenicity study in mammalian cells, such as the gene mutation test on mammalian cell (OECD TG 476 [146] or 490 [147]) that is required according to 8.4.3. However, an *in vitro* gene mutation study in bacteria is a data requirement for Annex VII 8.4.1 with potentially important regulatory consequences (e.g. follow-up in vivo testing). Therefore, a negative outcome in the Ames test should be considered valid only if there is proof of bacterial wall penetration and on absence of bactericidal activity by the nanomaterial.

2. "*Measures of cytotoxicity based on cell proliferation that are described in the test guidelines are appropriate for determining the top concentration to be applied for in vitro tests of nanomaterials. It is appropriate in some cases to consider wider concentration spacing than the standard √10 in order to ensure that any potential concentration-response relationship is well characterized, and at concentrations not associated with cytotoxicity*." [110]

3. "*The extent of cellular uptake is a critical factor to consider when interpreting test results. In some circumstances, a lack of uptake in a mammalian cell may indicate a low intrinsic hazard from a direct genotoxicity perspective*". [110]

   The importance of cell uptake was also pointed out by the EU Nanogenotox project (http://www.nanogenotox.eu/files/PDF/nanogenotox_web.pdf). Several parameters (e.g. *inter alia* agglomeration and protein coating) can influence cell uptake.

4. "*The test guidelines program should consider modification of the in vitro micronucleus assay to recommend, where cytochalasin B is used, its addition using a post-treatment or delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the nanomaterial in the absence of cytochalasin B*". [110]

   According to Annex VIII 8.4.2 of REACH, a micronucleus test (OECD TG 487 [148]) or a chromosomal aberration test (OECD 473 [149]) is required. The EU Nanogenotox project showed that the "Guideline for the testing of chemicals *in vitro* mammalian cell micronucleus test (OECD TG 487) is applicable for nanomaterials but may need some adaptation in order to provide predictive results in vivo" [110] (http://www.nanogenotox.eu/files/PDF/nanogenotox_web.pdf). A project on the adaptation of the *in vitro* mammalian cell micronucleus assay (TG 487 [148]) for nanomaterials testing was approved in 2015 in the OECD WPMN rolling work plan (Project 4.95: Guidance Document on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials). The study focuses on physico-chemical characterisation of nanomaterials and protocol modifications (selection of cell type with respect to uptake mechanisms, use of cytochalasin B, timing of exposure to nanomaterials, specification of controls, dose ranges and dose metrics).

5. *"Prior to conducting an in vivo genotoxicity study, there is a need to conduct some toxicokinetic investigations to determine if the nanomaterial reaches the target tissue, where the target tissue is not the site of contact. In the absence of data to the contrary, the test is not applicable for detecting primary genotoxicity if the nanomaterial does not reach the target tissue."* [110]

6. In the absence of toxicokinetic information demonstrating systemic availability and/or exposure of target tissue(s), it is recommended to investigate the genotoxic effects in the site of contact tissue(s). "*There are insufficient data to recommend one route of administration over another. The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s).*" [110]

   Currently inhalation is considered the most likely route for human exposure to nanomaterials - at least for workers - (See R.7.a, Section R.7.5.6). The selected route of administration should be justified (and the issue of exposure of target tissues should be addressed).

Appendix 1. Models for fate for nanomaterials

There is on-going research and development of modelling tools to assess the fate of nanomaterials. The list of methods given in Table 13 below is not exhaustive and includes methods based on attachment affinity and dissolution rate of nanomaterials. Further Information on these methods that may be used to predict fate and transport of nanomaterials in the environment and organisms can be found at for instance at [150].
Further information on the models and their validation status can be found in the referenced publications for each model.

Table 13: Overview of some models for fate for nanomaterials

Model	Overview	Output	Link to the model tools	References
SimpleBox4nano (SB4N): Classical multimedia mass balance modelling system	The model expresses engineered nanoparticles (ENP) transport and concentrations in the environmental compartments (air, water, soil, etc.) accounting processes such as aggregation, attachment, and dissolution. The model solves simultaneous mass balance equations.	The output is mass concentrations of ENPs as free dispersive species, heteroaggregates with natural colloids, and larger natural particles in each compartment in time and at steady state.	http://www.rivm.nl/simplebox	[151]
NanoDUFLOW: Spatiotemporally explicit hydrological model	Feedbacks between local flow conditions and engineered nanoparticles (ENPs) fate processes, such as homo- and heteroaggregation, resuspension and sedimentation, are modelled.	The outputs are the concentrations of all ENP forms and aggregates in water and sediment in space and time, and retention.	DUFLOW Modelling Studio (v3.8.7) software package with a set of specific processes defined by the user via the NanoDUFLOW submodel.	[152]
Steady-state distribution model	Multimedia model was developed using nanospecific process descriptions such as homo- and heteroaggregation, dissolution and sedimentation to estimate the steady-state distribution	The output is nanoparticle / mass concentrations in water and sediment, and its distance from the source.	As a first case study in Praetorius *et al.*, [26] a river model was used.	[26]

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