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GUIDANCE

Guidance on information requirements and chemical safety assessment

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

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

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Appendix R7-1: Recommendations for nanomaterials

1. INTRODUCTION TO APPROACHES TAKEN FOR APPENDICES CONCERNING INFORMATION REQUIREMENTS

The three appendices concerning information requirements (appendices to R7a, R7b and R7c) have been developed in order to provide advice to registrants for use when preparing registration dossiers for nanomaterials[1]. The content of the appendices implements the advice provided by the REACH Implementation Project on Nanomaterials 2 (RIP-oN2) on specific aspects of information requirements concerning materials in nano form.

The final report of the RIP-oN2 project contains a large amount of information including within its scope applicability of the methods, research gaps etc. This appendix implements only the agreed outputs (i.e. the recommendations for guidance update on which there was consensus).

In the appendices only guidance on the endpoints for which a recommendation has been made in the RIP-oN2 report is included. In the absence of any specific recommendation, either because the endpoint is not relevant for nanomaterials (e.g. flash point or surface tension), or the guidance already provided is considered to be equally applicable to nanomaterials or because more research is needed before developing advice, no additional guidance for the endpoint has been included in this appendix.

Note that new parameters or endpoints (such as ventilation rate, or gill pathologies) have been proposed only when these were explicitly recommended to be included as guidance updates in RIP-oN2.

For further information (e.g. recommended further research & development or reasoning for the advice provided for guidance updates, the reader can refer to the final report of RIP-oN2. (http://ec.europa.eu/environment/chemicals/nanotech/index.htm).

[1] See Recommendation on the definition of nanomaterial adopted by the European Commission

2. RECOMMENDATIONS FOR PHYSICO-CHEMICAL PROPERTIES ARISING FROM RIP-oN 2 for NANOMATERIALS

2.1 General remarks

2.1.1 Sample preparation

Sample preparation is widely recognised as one of the most critical steps towards successful characterisation and subsequent (eco)toxicological testing of nanomaterials, in which there are many variables to consider when designing a method for preparation. Common issues regarding sample preparation include storage and stability of the test material; the chemical composition of the test media; characterisation of stock dispersions, and; characterisation of samples (prepared from stock dispersions) prior to administration/testing (OECD, 2010). Preliminary guidance on sample preparation for the physico-chemical characterisation of nanomaterials, covering properties including particle size distribution, shape, specific surface area, octanol-water partition coefficients, degree of agglomeration and dispersion behaviour, is available (OECD, 2010). ISO 14887:2000 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. 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. 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) and support the characterisation of nanoparticles in inhalation exposure chambers (ISO 10808:2010).

An important component of sample preparation is the need to have "reliable" sampling, such that 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 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.

Also in relation to sample preparation, it is necessary to be aware that aggregates and agglomerates of nanomaterials can form in solution, powder and aerosol forms, and their presence is influenced by a number of factors including the method of synthesis, storage, handling and environmental conditions. An agglomerate is defined as a collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components. An aggregate is a particle comprising of strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components (ISO 27687:2008).

In addition, it is known that the observations and interpretation of toxicity, and fate and behaviour, as a result of exposure to agglomerates may or may not be associated with the primary particle's characteristics. 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 (OECD, 2009). OECD (2009) suggests for nanomaterials with a non-zero width of the distribution that the degree of agglomeration should be characterised. Other measurands include the average agglomeration number (AAN), which is derived from the ratio of the

volume based median particle size to the average equivalent spherical volume derived from BET gas adsorption.

In addition to aggregation and agglomeration, the behaviour of particles in solution 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, temporally stable phase (a suspension down to the molecular level) 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 (OECD, 2010).

A dispersion is a suspension of discrete insoluble particles in a fluid, which may falsely have the visible appearance of a solution (i.e. the product of the conversion of a solid substance to liquid form by mixture with a solvent). A dispersion of an insoluble material may elicit a different response from that anticipated from the classical molecular or elemental toxicity expected from the chemical composition. 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 environment. 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 determined primarily by the environment of the nanoparticles. In solution, 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. 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.

If a nanomaterial is soluble in 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 bulk (non-nanoscale) solubilised substances. If, however, the nanomaterial under investigation is insoluble or sparingly soluble in biological or environmental media, then it will likely be presented to the test system in a particle form.

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 experiment (OECD, 2010).

Other important considerations to take into account during sample preparation include the influence of contaminants and impurities on (eco)toxicological test results. Adverse effects on a number of species used in PNEC derivations for nanomaterials have been attributed to particle impurities (e.g. Cheng et al., 2007: Brayner et al., 2006).

Of particular concern is the influence of endotoxin on certain testing results. Endotoxin

(lipopolysaccaride) is a constituent of the outer cell wall of Gramnegative bacteria and as such is found ubiquitously within the environment. Endotoxin however can 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). As such the establishment of the presence or level of endotoxin in a test sample is useful as a preliminary undertaking during the preparation of a sample for toxicological testing. International standards are available for the testing of nanomaterials (ISO 29701:2010) although issues regarding endotoxin contamination are not necessarily nano-specific and are equally relevant for other particles or aqueous substances undergoing toxicological evaluation.

In order to eliminate potential confounding of 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, consistent with the substance identification requirement

2.1.2 Physico-chemical properties

With regard to nanomaterials characterisation, it is important to note that different techniques will suit different sample forms (e.g. aerosol, suspensions etc.) and, in many cases, no individual technique can satisfy the need for a meaningful characterisation of nanomaterials (Stone et al., 2009; Tran et al., 2008). Multiple techniques should therefore be used where possible in order to formulate an appropriate understanding of the nanomaterial's properties, and the optimum set of required techniques should be selected and justified based on the specific nanomaterial type and form under investigation. The need for multi-method characterisation and material-specific selection of techniques applies across a range of nanomaterial properties and would facilitate the gathering of data on multiple metrics.

Table R.7.1-5 Summary of use of physico-chemical properties in Section R.7.1.1.6, gives an overview of every endpoint and the impact of it on other physicochemical tests, on toxicology, ecotoxicology and risk assessment. Regarding granulometry and the impact on toxicology, it should be noted (in addition to the information already in the table) that:

- Knowledge of high aspect ratio particles and specific surface area may inform interpretation of some toxicity test results.

- Particle shape is an important parameter in the characterisation of nanoparticles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media.

2.1.3 Evaluation of available information

Comparison of the experimentally determined physico-chemical property with a suitable reference material and a scientifically justified QSAR prediction is often, if not always, recommended to provide reassurance that the experimentally derived value is acceptable and has not been influenced by the presence of impurities in the product. A number of particle based reference materials are available from commercial sources and National or Community Measurement Standard Bureaus e.g. NPL, IRMM, NIST

2.2 Specific advice for endpoints

2.2.1 Water solubility

Water solubility is covered in Section R.7.1.7. 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. For nanomaterials, 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. Further information on these issues is provided in Section 2.1.2. on Sample Preparation. It should also be ensured that no undissolved material contributes to what is being measured.

Additionally, it should be taken into consideration when following the workflow shown in Figure R.7.1-5 (in the parent guidance – i.e. "The three properties, solubility, hydrolytic stability and acid dissociation constant are inter-related. It is not possible to measure any of these without some knowledge of the other two"), that in the case of nanomaterials the preliminary test assessing solubility might need to be performed by instrumental means rather than visual, as shown in the Figure R.7-1.1 below.

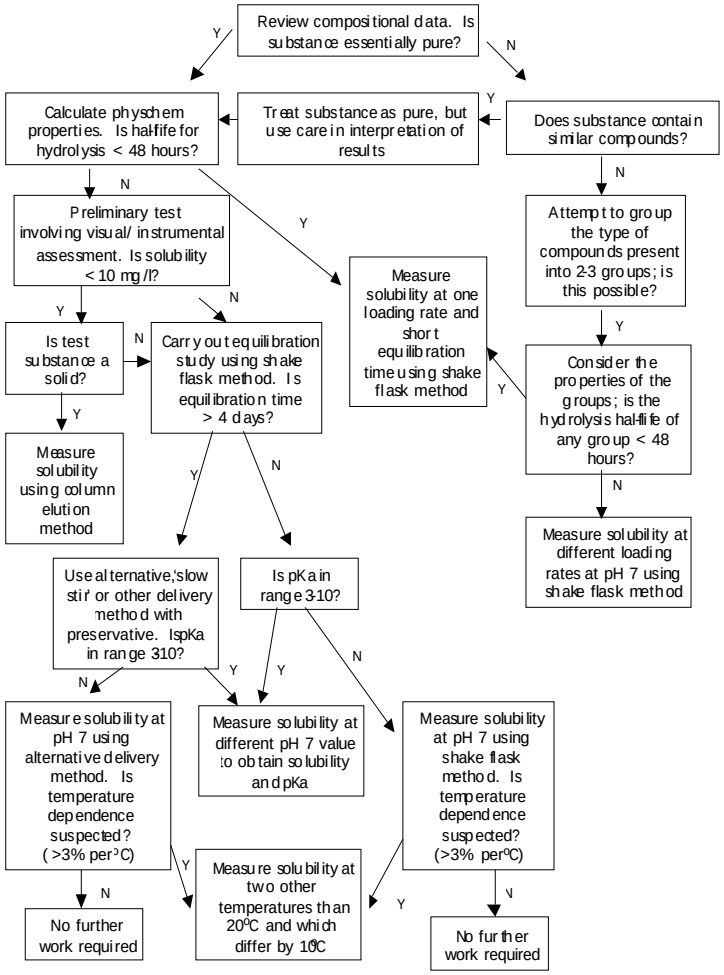


Figure R. 7-1.1: Testing strategy for solubility, hydrolysis and pKa

2.2.2 Partition coefficient n-octanol / water

Section R.7.1.8.3.includes information regarding the experimental data on partition coefficient n-octanol/water including testing methods. In this respect it is important to note that, following a review of the applicability of test guidelines to nanomaterials, OECD concluded that test guidelines (TG) 107, 117 and 123 might be applicable under some circumstances or to some classes of manufactured nanomaterials, although further work is required to determine this and modify the TGs, if it is considered necessary (OECD, 2009). Results might be impacted upon by the presence of a colloidal suspension, which could be present if the manufactured nanomaterial does not completely dissolve (OECD, 2009).

Additionally, in the same section, when treating "Difficult to test substances", it should be noted that for nanomaterials, 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 two distinct phenomena and it is important to differentiate between them. Further information on these issues is provided in Section 2.1.2. on Sample Preparation

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". 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" (HSE, 2000)

- "Stationary source emissions – Determination of mass concentration particulate matter (dust) at low concentrations – manual gravimetric method" (BS ISO 12141:2002)

- "Stationary source emissions – Manual determination of mass concentration of particulate matter" (BS ISO 9096:2003)

- "Ambient air quality – Standard gravimetric measurement method for the determination of the PM2.5 mass fraction of suspended particulate matter" (BS ISO 14907:2005)

- "Workplace atmospheres – Ultrafine, nanoparticle and non-structured aerosols – Inhalation exposure characterization and assessment" (ISO/TR 27628:2007)

- "Nanotechnologies – Health and safety practices in occupational settings relevant to nanotechnologies" (ISO/TR 12885:2008)

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 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 R7-1.1 to R7-1.4). 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 (OECD, 2009), 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 and ISO/TR 12885:2008.

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 R7-1.1 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 (1997): 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	Dry powders/granulates Size range: 2-200 microns	MMAD cannot be determined

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.		
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 (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	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

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. ISO/13322-1:2004 and ISO/13322-2:2006 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. ISO/13322-1:2004 and ISO/13322-2:2006 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; ISO 13318-2:2007; ISO 13318-3:2004) 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. 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	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.

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.		
Ultrasonic spectroscopy (ISO/20998-1:2006) 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. Measurement results may vary with different vol%.	Particles in colloids, dispersions and emulsions Size range: 10 nm - 3 mm	Attenuation spectrum, from which the particle size distribution based on mass/number 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)	Particles in powder and suspension form	Average particle size for a

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.	Size range: 1-300 nm	sample, estimated by mathematical adaption of a diffractogram
X-ray diffraction (XRD) (BS EN 13925-1, BS EN 13925-2 and BS EN 13925-3) 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; ISO/13321:1996; ASTM E2490 – 09) 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 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.	Particles or droplets dispersed in liquids Size range: 1 - 1000 nm	Size distribution based on mass/number. Average particle size and polydispersity index (dimensionless; measure of broadness of the size

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 (Lövestam et al., 2010).		distribution).

Table R7-1.2 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 (Ma-Hock et al., 2007). 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 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 provides an informative description.		
Rotating drum method (prEN 15051-2) 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 (Schneider & Jensen, 2008).	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 (prEN 15051-3) 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 (Schneider & Jensen, 2008).	Dry powders/granulates/friable products Size range: 0.5-10,000 µm	MMAD can be determined via an appropriate coupled analytical technique.

Table R7.-1.3. 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 R7-1.4 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; ISO 10808:2010; ISO 28439:2011) 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. 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	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.

two methods (Ma-Hock et al., 2007). 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 the equations do not give unambiguous results in the case of polydisperse aerosol size distributions. ISO/TR 27628:2007 provides an informative description of this method.	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.
Optical Particle Counter (OPC) OPC is a widely used method for detecting and counting aerosolised particles, and operates across a	Particles in an aerosol Size range: 0.3 – 17 µm	Particle number concentration

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 (Ma-Hock et al., 2007). ISO/TR 27628:2007 provides an informative description of this method.		
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 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.	Particles in an aerosol Size range: 0.06 - 45 µm	Particle size/size distribution*, from which mass median diameter can be calculated, with knowledge of the density of the particles

Using the methods listed in Tables R7-1.1 to R7-1.4, 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. 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 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 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" (CEN, 2006).

materials. 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 standard is currently under revision (draft of European standard available) and the final publication is expected for 2013. The standard has been divided in 3 parts (a general part and one part for each method).

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 (e.g. Heitbrink et al., 1992). 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* (Upton et al., 1990), 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 R7-1.2 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 R.7-1.4 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 R7-1-3.

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:

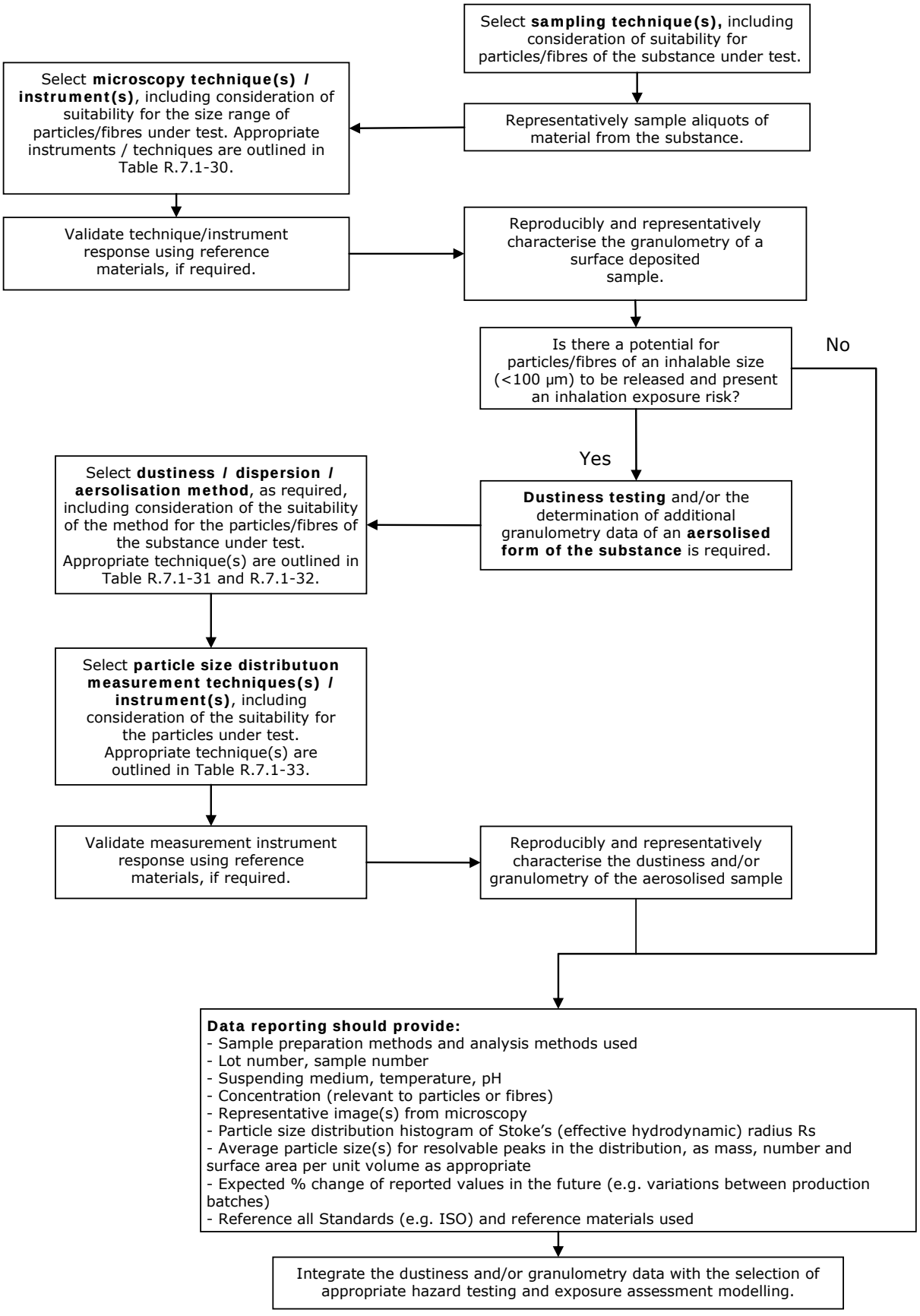


Figure R7-1.2 ITS for granulometry

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 OECD, 2009).

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 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 (Barrett, 1980).

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) 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.

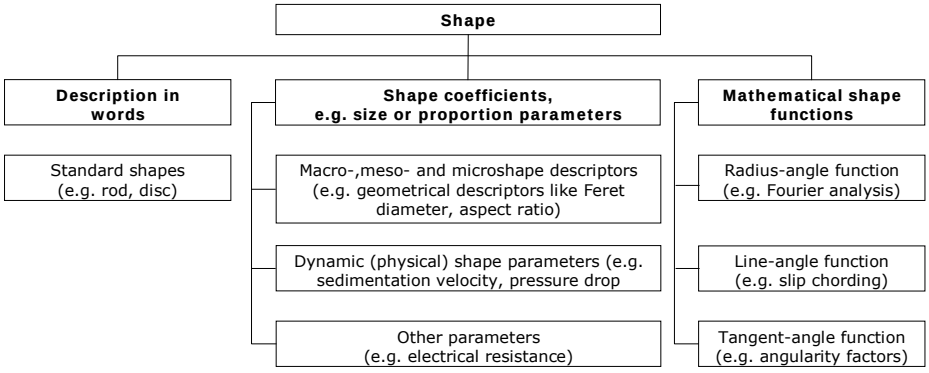


Figure R7-1.3 Classification of some methods for shape description (adapted from ISO 9276-6:2008)

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 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 R7-1.5). 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 R7-1.5 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	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

available in ISO/TR 27628:2007. ISO 13322-1:2004 and ISO 13322-2:2006 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 a 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 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. ISO 13322-1:2004 and ISO 13322-2:2006 provide general guidance for measurement description and its validation when determining particle size by static	Particles in solid, powder and suspension form. Size range: < 0.01–10 µm. Particularly suitable for the particle size range of 10 nm – µm	Image, providing opportunity to determine macro-, meso- and 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.	Particles in air or dispersed in a liquid Size range: 1nm – 8 µm	Image, providing opportunity to determine macro-, meso- and microdescriptors of shape

ISO TR/27628:2007 provides an informative description of this method. **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 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.	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.	Image, providing opportunity to determine macro-, meso- and microdescriptors of shape

Using the methods listed in Table R7-1.5, 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 R7-1.6) and proportion (Table R7-1.7) descriptors of macroshape, mesoshape descriptors (Table R7-1.8), combination of shape descriptors (Table R7-1.9) and roughness descriptors (which represent microshape properties) (Table R7-1.10) are available (ISO 9276-6:2008). Fractal dimensions are necessary to distinguish between mesoshape (concavity) and microshape (descriptors).

Table R7-1.6 Geometric macroshape descriptors (reproduced from ISO 9276-6:2008)

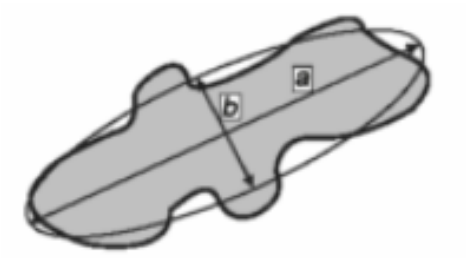
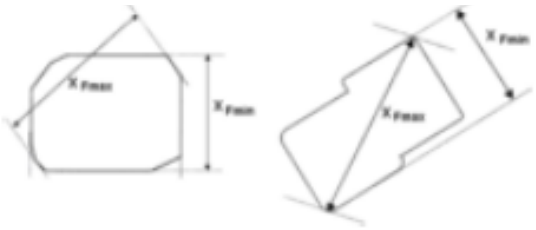
Lengendre ellipse of inertia 	An ellipse with its centre at the particle's centroid and with the same geometrical moments, up to the second order, as the original particle area The major and minor axes are given by x_{Lmax} and x_{Lmin} respectively Robust measurements.
Feret diameters x_{Fmax} and x_{Fmin} 	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: $A = x_E \cdot x_{LG} \qquad P = 2(x_E + x_{LG})$

Table R7-1.7 Geometric Proportion macroshape descriptors (reproduced from ISO 9276-6:2008)

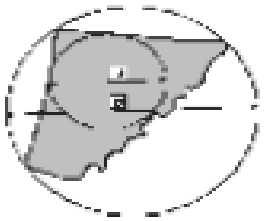
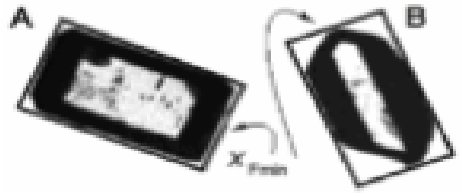
Ellipse ratio	Ellipse ratio = x_{Lmin}/x_{Lmax} where x_{Lmin} and x_{Lmax} are the lengths of the axes of the Legendre ellipse (Also used: elliptical shape factor) More robust paramneter than aspect ratio
Aspect ratio	For not very elongated particles: Aspect ratio = x_{Fmin}/x_{Fmax}
Elongation	For 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 R7-1.8 Mesoshape descriptors (reproduced from ISO 9276-6:2008)

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: $\bar{x}_F = \frac{1}{\pi \int^{x} x_F(\alpha) d\alpha}$
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 R7-1.9 Combination of shape descriptors (reproduced from ISO 9276-6:2008)

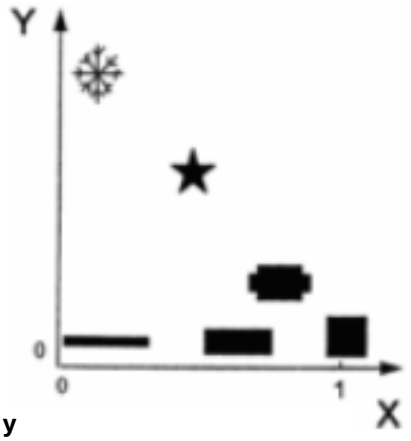
Concavity/robustness ratio Ω_3	Secondary mesoshape descriptor:
 **Key** X robustness Ω_1 Y largest concavity index Ω_2	$$\Omega_3 = \frac{\Omega_2}{\Omega_1} = \frac{\omega_2}{\omega_1}$$

Table R7-1.10 Roughness descriptor (reproduced from ISO 9276-6:2008)

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, 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).

R.7.1.19.4 Conclusions on shape

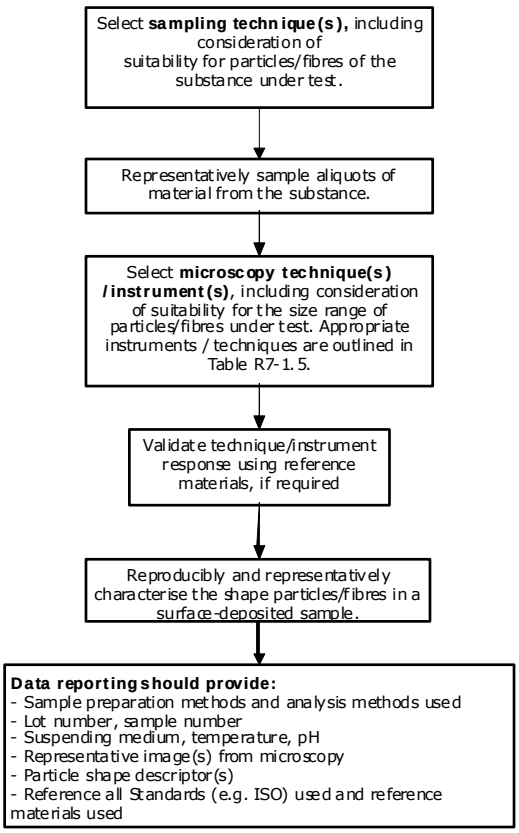
Shape is an important parameter in the characterisation of particles, with contextual value to the assessment of deposition, adsorption kinetics, and hazard assessment in biological media. Three corresponding levels of shape can be distinguished: macroshape, mesoshape and microshape. The shape of particles 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 R7-1.4) presents an integrated testing strategy 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 (SCENIHR, 2009). 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) (OECD, 2009).

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 (SCENIHR, 2010; Kreyling et al., 2010).

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. Duffin et al., 2002, Duffin et al., 2007, Stoeger et al., 2006; Oberdörster et al., 2005). In addition, it has been observed in several nanotoxicity studies that effects correlate with surface area (e.g. Brown et al., 2001; Donaldson et al., 1998; Oberdorster et al., 1992; Tran et al., 2000) 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 (OECD, 2009).

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 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 R.7.1-Y) (Brunauer et al., 1938). 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 R7-1.11 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; ISO 18757:2005) 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 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 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. (OECD, 2010). 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 (OECD, 2009). 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

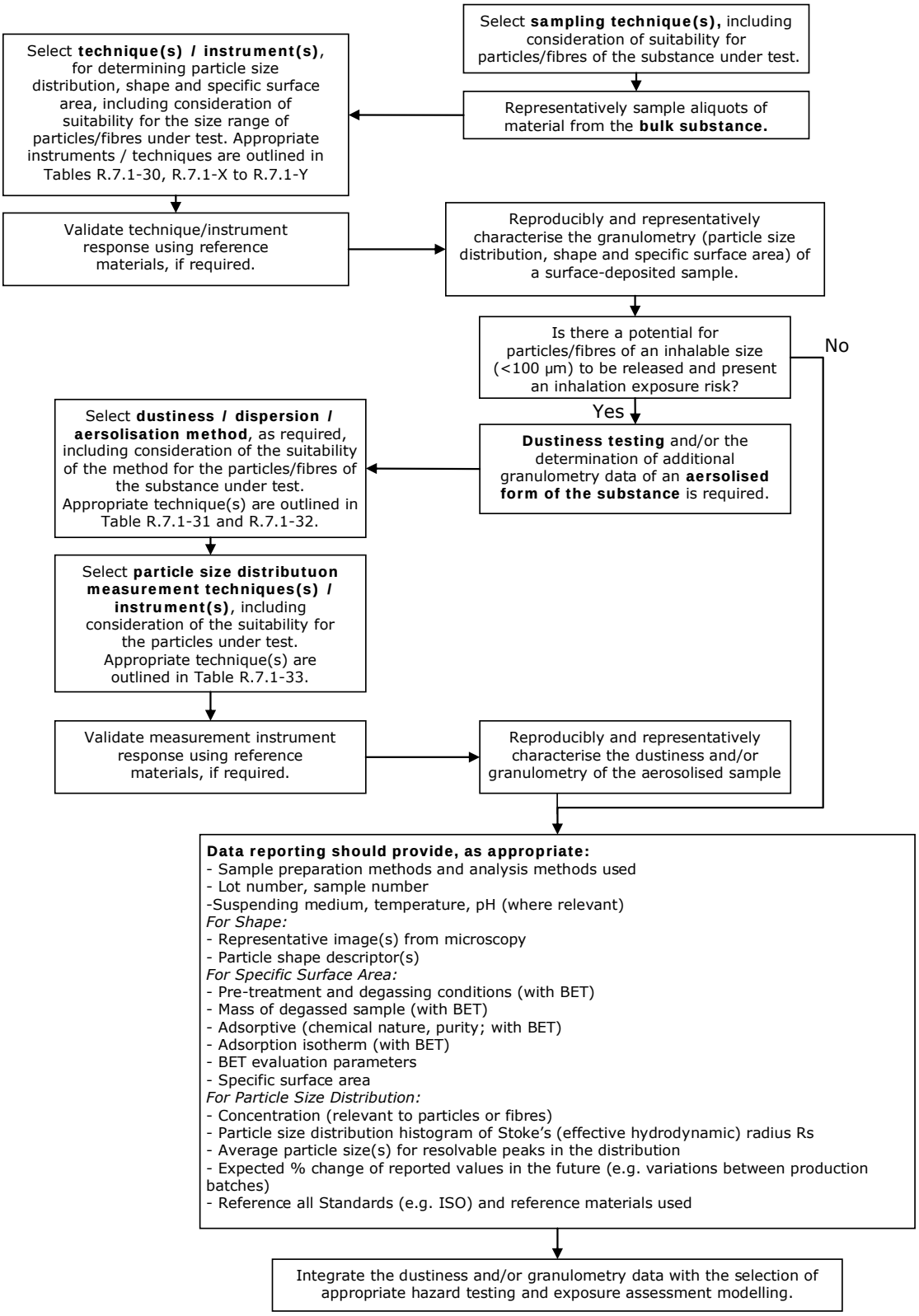


Figure R7-1.5. Joint ITS for particle size distribution, surface area and shape

2.2.4 Adsorption / desorption

The methods for determining this endpoint are shown in Table R.7.1-33 Methods for the measurement of adsorption.

With regard to nanomaterials the distribution coefficient Kd has to be based on actual testing using one of the methods for the measurement of adsorption outlined in Table 7.1-33 since estimations of Kd derived from the organic carbon-water partition coefficient (Koc) and the octanol-water partition coefficient (Kow) have no or questionable merit when it comes to nanomaterials.

3. RECOMMENDATIONS FOR TOXICOLOGICAL INFORMATION REQUIREMENTS FROM RIP-oN 2 for NANOMATERIALS

3.1 General advisory notes

These advisory notes do not propose a protocol but aim to provide useful advice and references to relevant resources.

Please note that Chapter R7c includes a section on toxicokinetics (Section R.7.12. Guidance on toxicokinetics) information about toxicokinetics and nanomaterials can be found in the Appendix to R7c.

3.1.1 Advisory note on the consideration of rat lung overload within inhalation toxicity assessment

The term 'lung overload' or 'particle overload' as it is also known, is a phenomenon associated with exposure to poorly soluble, low toxicity (PSLT) particles and occurs when a threshold dose of particles is achieved within the lung. During chronic exposure to PSLT particles, the lung burden of particles increases until a steady state or equilibrium is achieved between deposition and clearance of particles (Miller 2000) as shown by the A, B and C traces in Figure R7-1.6. Below the lung overload threshold, particles are cleared via normal mechanisms at a normal clearance rate, generating little or no appreciable response.

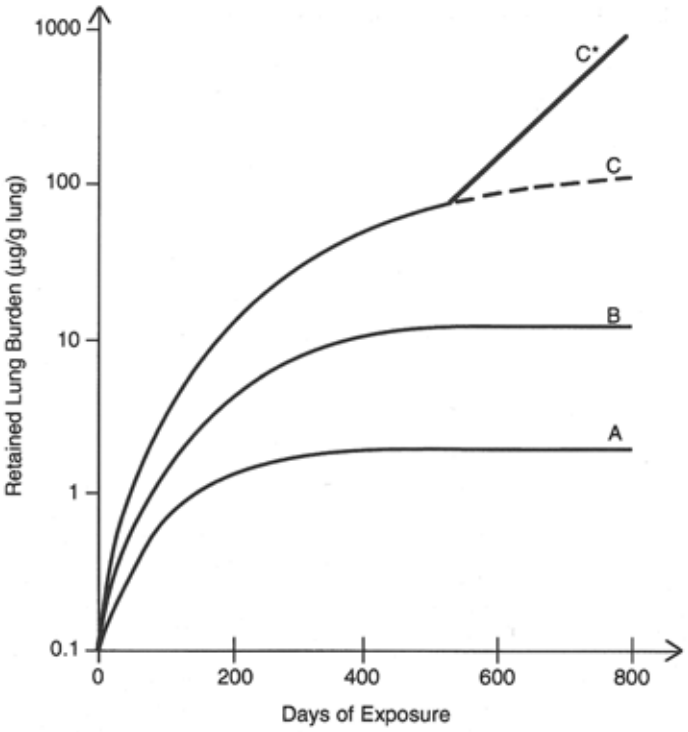


Figure R7-1.6: Schematic representation of the relationship between retained lung burden and length of exposure leading to the phenomenon of lung overload. Curves A, B, and C are associated with progressively increasing exposure concentrations. If the exposure level is sufficiently high and the length of exposure sufficiently long, alveolar macrophage-mediated

clearance of particle can be overwhelmed. When this occurs, retained lung burden increases linearly with further exposure (curve C*). Reproduced from Miller (2000).

However, once the threshold has been reached, the clearance mechanisms of the lung become overloaded which 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. This is shown in the C* trace of Figure R7-1.7 whereby at the point of threshold, particle retention occurs exponentially rather than an equilibrium being established (as demonstrated by the dashed line).

The result of this rapid net increase in particle accumulation is lung inflammation, cessation of alveolar-mediated clearance and an increase in accumulation of particle laden macrophages within the lung alveoli. The continued build up of particles leads to a higher rate of transfer to lymph nodes and accumulation of particles in the lung interstitia. Persistent inhalatory exposure leads to chronic inflammation which in turn is likely to lead to fibrosis, alveolar cell proliferation (hyperplasia), the conversion of cells to cell types not normal associated with the specific lung location (metaplasia). The final result may be local tumour formation (neoplasia) as shown in Figure R7-1.7 (Mauderly 1996; Miller 2000; Oberdorster 1996). This occurs only at high particle inhalatory exposure and is known as the overload phenomenon.

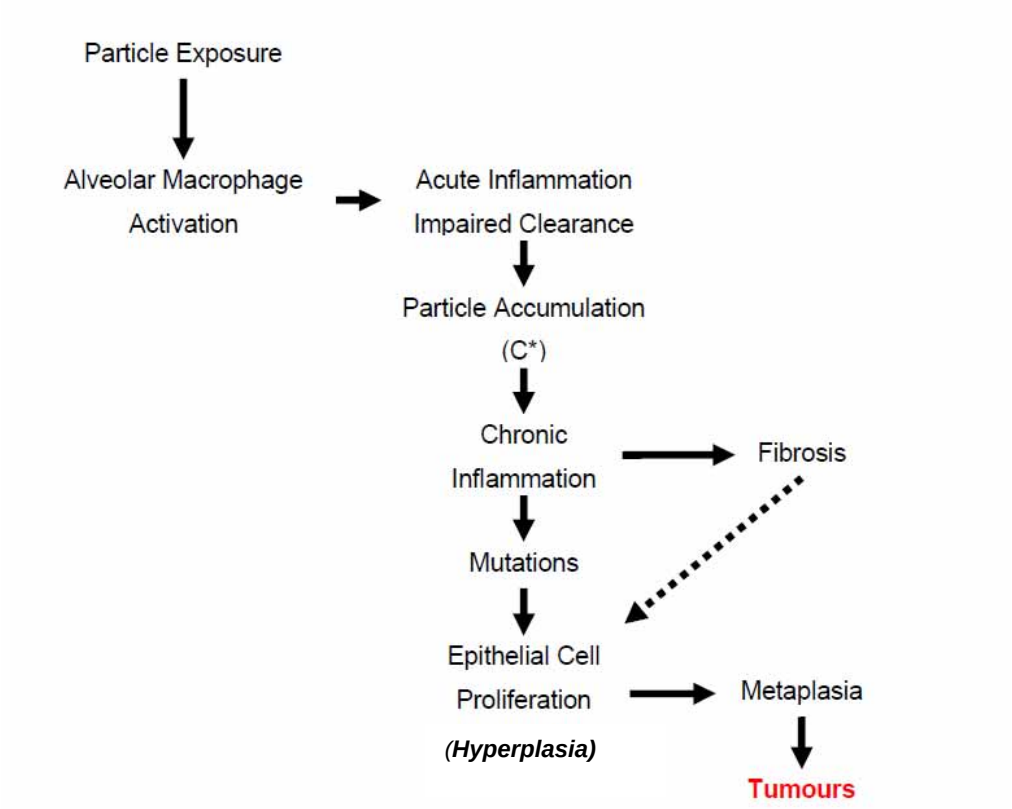


Figure R7-1.7: Suggested pathogenic sequence of effects of chronically inhaled particles in rats. Adapted from Oberdörster (1996).

The driving force behind this cascade of effects is thought to be the particle load rather than an intrinsic property of the particles themselves. The situation of overload is most commonly associated with repeated inhalation exposure to particles but it can also occur after single or repeated instillation of particles into the lung (due to high deposition fraction as a result of direct instillation) or possibly as a result of a single massive inhalation exposure (Mauderly 1996). As such since this phenomenon occurs at high level of inhalatory exposure, it is often

argued that the observed effects are a product of the experimental condition and not necessarily a true reflection on the intrinsic toxic potential of the particles to cause inflammation, fibrosis and cancer. Indeed this also raises the question of particular sensitivity to lung overload between different species (e.g. between different experimental species or between an experimental species such as rats and humans). In a comparative study assessing the long-term pulmonary response of rats mice and hamsters to inhalation of pigmentary grade titanium dioxide, the authors found species differences. Lung burden was shown to be lower in hamsters at concentrations which caused overload in rats and mice. Also the inflammatory and pathological responses were less severe in mice than rats and diminished with time irrespective of the similar lung burdens (Bermudez et al. 2002).

It should however be noted that this is only the case for PSLT particles. Exposure to highly reactive or toxic particles may cause inflammation, fibrosis and cancer at non-overload conditions due to intrinsic properties of the particles themselves. Inflammation, fibrosis and cancer in rats arising from high exposure to PSLT particles could be a result of the exposure conditions (overload) rather than a result of an intrinsic particle property.

The question of which dose metric best describes the association between deposited dose in the lung, overload conditions and subsequent pathogenic effects is particularly pertinent. There have been several suggested metrics with the first being particle volume as suggested by Morrow et al. (1988). Morrow hypothesised that overload begins when 6 percent of the macrophage volume is filled with particles and total cessation of AM-mediated clearance occurs when 60 percent of the macrophage volume is filled. Such a driver of lung overload has also been more recently suggested for carbon nanotubes (Pauluhn 2010). However, two further metrics have been discussed as important in driving lung overload. The first is surface area and there are several studies which suggest that, as metric, particle surface area correlates well with induced pathogenic events (Elder et al 2005; Borm et al. (2004)). In a study by Tran et al. (2000), 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, the authors suggested that based on the shape of the statistical relationship for lung response to particles, the presence of a threshold at approximately 200-300 cm2 of lung burden was indicated. In relation to surface area as a driving metric, due to their known high level of surface area, the potential for overload effects may be increased with those nanomaterials which exhibit a high biologically accessible surface area.

The third suggested metric is that of mass. Whilst some studies indicate mass as a less sensitive indicator of lung overload (Warheit et al. 1997) there is a study showing an improved relationship between the mass of three forms of PSLT particles, and the generation of inflammation due to lung overload.

The generation of overload conditions may be seen as a point of weakness within a study design and hinder accurate risk assessment due to the suggested differences in species susceptibility introducing further uncertainty. Indeed in a retrospective analysis by Valberg et al. (2009) they analysed studies considering the lifetime tumour occurrence in rats after repeated dose short term intratracheal instillation of 19 different PSLT particles. Including other drawbacks within the studies (such as the lack of low-dose studies) the authors pointed towards significant issues with study design that resulted in lung overload in the test subjects. They argued that the response of rats to PSLT particle lung overload is stereotyped and unique to that species and pointed towards human exposure to demonstrate this. Specifically workers historically exposed to potentially lung-overloading burdens of inhaled dust (e.g., coal workers, underground miners using diesel equipment) do not exhibit an established lung-cancer excess despite the potential for lung overload. As such in rats, when the lung-overload threshold is exceeded, rats develop lung tumours from ongoing inflammation as opposed to particle-specific toxicity, whilst humans do not (Valberg et al. 2009).

Based on this evidence the authors suggested that the reported results for PSLT particles were

not a reliable basis for predicting human lung cancer risk. Such a criticism could be placed on all studies of PSLT particles which may generate overload conditions due to dosing regimes or exposure levels.

The interpretation of data obtained after high doses of PSLT particles should be approached with caution and appropriate discussion should be given to the mechanistic driver behind any pathogenic effects detected. The reason for this is to establish the relevance to humans and if alteration of the default assessment factors is warranted or appropriate in the derivation of exposure limits.

For further information, review articles covering this subject include Miller (2000) which provides an excellent in-depth discussion of particle deposition, clearance and lung overload; Borm et al. (2004) which discusses the importance of overload in the context of risk assessment.

3.1.2 Advisory note on the consideration of assay inhibition / enhancement (interference)

Various nanomaterials have on occasion been found to interfere with several commonly used assays utilised 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, nanoparticles may bind to assay components including the substrates (such as CNT with the reagent in MTT assays; Belyanskaya *et al*. 2007) or the biomarker being measured, (such as LDH and cytokine proteins, see for example Davoren *et al*. 2007).

A summarised list of potential sources of interferences with commonly used assays has been developed by Kroll et al. (2009) and reproduced within Table R7-1.12.

Table R7-1.12: Nanoparticle interference with cytotoxicity assays (reproduced from Kroll et al., 2009)

Cytotoxicity assay	Detection principle	NP interference	Altered readout	Particle / Reference
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/	Fluorimetric detection of phosphatidylserine exposure (apoptosis marker)	Ca2+-depletion	Reduced indication of apoptosis	
Propidium iodide	Propidium iodide staining of DNA (necrosis marker)	Dye adsorption	Reduced indication of necrosis	Carbon nanoparticles
Neutral red	Colorimetric	Dye	Reduced	Carbon

	detection of intact lysosomes	adsorption	indication of cell viability	nanoparticles
Caspase	Fluorimetric detection of Caspase-3 activity (apoptosis marker)	Inhibition of Caspase-3	Reduced indication of oxidative stress	Carbon nanoparticles
Stress response				
DCF	Fluorimetric detection of ROS production	Fluorescence quenching	Reduced indication of oxidative stress	Carbon nanoparticles
Inflammatory response				
ELISA	Colorimetric detection of cytokine secretion	Cytokine adsorption	Reduced indication of cytokine concentration	Carbon nanoparticles Metal oxide nanoparticles

It should be noted that this list is not exhaustive and proper testing should be performed where possible as a matter of course to check for inhibition or enhancement of test results.

Within certain standard methodologies such as ISO/FDS 29701 (Nanotechnologies - endotoxin tests on nanomaterial samples for in vitro systems), the method requires the use of sample 'spikes' (addition of a known sample control to the test sample) to test for inhibition or enhancement of the spiked control. This is calculated by assessing the returned value against the expected value which should be a cumulative value of the spike and sample.

Any alteration to this may indicate inhibition (return of a value less than expected) or enhancement (return of a value greater than expected) of the assay. The use of sample spikes is encouraged as it allows a simple yet effective method of investigating potential assay interference and would give greater confidence in derived results. This is especially important due to the uncertainty that surrounds the effect of nanomaterials on the performance of routinely used assays.

The use of such methods to investigate possible inhibition or enhancement of results should be carried out wherever possible irrespective of standard method requirement; however this may not always be possible. In many of the studies reported it is not possible to ascertain whether the assays were adequately controlled to assess for interference. Thus, as a general precaution, it is advisable to use more than one assay to assess the endpoint or effect in question, as advised by Landsiedel et al. (2009) for establishing genotoxicity. The potential for inhibition or enhancement of the test result may impact on numerous test methods. In certain cases, the potential for assay interference has been identified for some nanomaterials, for example carbon nanotubes are suggested to interfere with the MTT assay (Wörle-Knirsch et al.

2006) and as such may cause issues with tests such as OECD TG 431/EU B.40 Human Skin Model tests (EPISKIN™, EpiDerm™) due to their use of the MTT assay. However knowledge on nanomaterial assay interference is incomplete and so precautions to ensure the validity of an assay, such as the 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 to validate obtained results.

3.1.3 Advisory note on the consideration of bacterial assay interference

Assessment of substances with regard to genotoxicity is generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation, clastogenicity and aneuploidy.

One such test, the bacterial reverse mutation (Ames) test (OECD TG 471/EU B.12/13: Bacterial reverse mutation test (in vitro)), uses aminoacid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (Ames et al., 1975; Maron et al,. 1983; Gatehouse et al., 1994). The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid (histidine). The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain (OECD TG471, 1997). A positive test indicates that the test substance might act as a mutagen, or hold carcinogenic potential (as cancer is often linked to DNA damage).

Generally, the major drawback of the Ames test is that it is difficult to translate prokaryotic data for eukaryotic genotoxicity testing, and the test is known to generate false positive results (Khandoudi et al., 2009). Indeed, 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 (Eastmond et al., 2009). In relation to nanomaterials, a recent review of the applicability of genotoxicity tests to NM questioned whether the Ames test was accurately representative of NM genotoxicity (Landsiedel et al., 2009). The Landsiedel study reported that of those studies reviewed, results were predominantly negative (5/6 studies). The group speculated that it is likely that some NMs are not able to cross the bacterial wall, whilst others kill the test organism as they are bactericidal.

Based on this evidence, it is advisable that any 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.

3.2 Specific advice for endpoints

3.2.1 Skin and eye irritation / corrosion and respiratory irritation

The test method(s) described in the guidance are considered applicable to nanomaterials. However, regarding the use non-testing data; i.e. Sections 7.2.3.1, 7.2.4.1 (on non human data), Appendixes R.7.2-2 and R.7.2-3 (on QSARs and expert systems) and Figures R.7.2-2 and R.7.2-3 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this. the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.2 Skin and respiratory sensitisation

The test method(s) described in the guidance are considered as applicable to nanomaterials as they are to other substances. However, regarding the use non-testing data; i.e. Sections R7.3.3.1, R7.3.4.1 and R7.3.5.1 (on non human data), and Figure R.7.3-1 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very

limited at this time. In addition to this, the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.3 Acute Toxicity

Regarding the use non-testing data; i.e. Sections R7.4.3.1, R7.4.4.1 (on non human data), and R7.4.5.1(on classification and labelling) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such in silico models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by case basis only.

Additionally, when considering the testing strategy for acute toxicity (Section R.7.4.6.3), with respect to new data generation it should be noted that the route of exposure to be used for acute toxicity evaluation depends on the nature of the substance (e.g. gas or not, molecular weight, log Kow, solid with inhalable particle size (e.g. nanomaterials)) and should reflect the most likely route of human exposure. Consequently the ITS for acute toxicity endpoint (Figure R.7.4-1) has to consider not only to consider if the substance is gaseous or not, but also if the substance is inhalable.

3.2.4 Repeated dose toxicity

Regarding the use non-testing data; i.e. Sections R7.5.3.1, R7.5.4.1 (on non human data), and R7.5.6.2 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

Additionally, when considering the testing strategy for repeated dose toxicity (Section 7.5.6) it should be noted that:

- When performing an inhalation test for PSLT particles the issues surrounding lung overload should be considered

- As inhalation may be the most likely route for nano(particles) exposure, further modification of the OECD method TG 422 may be required with full justification.

3.2.5 Reproductive and development toxicity

Regarding the use non-testing data; i.e. Section R7.6.4.1 (on non human data), and R7.6.6.2 (on integrated testing strategy) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

3.2.6 Mutagenicity and Carcinogenicity

Regarding the use non-testing data; i.e. Sections R7.7.3.1, R7.7.4.1 R7.7.10.1 R7.7.11.1 (on

non human data), R7.7.6.2 (on ITS on mutagenicity) and R.7.7.13 and Figure R.7.7-2 (on ITS on carcinogenicity) it is necessary to take into account that the use of non-testing data such as read-across, grouping or (Q)SAR approaches in addressing data gaps for nanomaterials is very limited at this time. In addition to this the use of such *in silico* models for nanomaterials has also yet to be established or accepted. Therefore the use of non-testing approaches for nanomaterials in deriving an assessment of hazard for humans must be scientifically justified and applied strictly on a case-by-case basis only.

The guidance gives a list of methods for *in vitro* testing for mutagenicity in Table R.7.7-2, the list includes the *in vitro* gene mutation study, as specified in Annex VII of REACH (See Section 7.7.6.3). In this respect, it should be noted that solid particles, including some nanomaterials, may not penetrate the cell wall of bacteria and as such this assay may not allow a robust evaluation of (nano)particle mutagenicity as discussed in the bacterial mutagenicity advisory note (See Section 3.1.3.). Therefore, the bacterial mutation assay should not be used as a single 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.

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