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JRC SCIENCE FOR POLICY REPORT

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Front page image: graphic elaboration of a SEM image, plate-like shapes, Au "nanocoins" on polymer substrate (SEM imahe by A. Valsesia, Joint Research Centre, © European Commission)

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# Contents

# Executive Summary

This document aims to support the implementation of the European Commission's Recommendation on the definition of nanomaterials (2011/696/EU). It is a follow-up of a previous JRC Science for Policy Report on concepts and terms used in the definition. The present report addresses identification of nanomaterials by measurements and discusses options and points to consider when assessing whether a particulate material is a nanomaterial or not according to the definition of nanomaterials.

The primary criterion against which particulate materials should be assessed is the median of the number-based distribution of the external dimensions of the particles. The particles to be measured are the constituent particles of the material, regardless whether these particles appear separate from one another or are parts of aggregates or agglomerates. Reliable assessment of particle size measurement results requires a detailed and unambiguous specification of the quantity that is intended to be measured (the measurand), including at least the physical principle of the measurement technique, the applied sample preparation protocol and the data analysis procedure. The chosen measurand must allow a reliable classification of a material according to the EC nanomaterial definition. Results for particle size should always be reported along with the size range which was covered by the measurement and which must be sufficiently large to allow reliable conclusions on the particle size distribution of the analysed material.

The main steps in the nanomaterial identification process by measurements are collecting information on the material, acquiring knowledge of the measurement method(s), matching method(s) and material, sample preparation, measurement/analysis and decision (nanomaterial / no nanomaterial). Each of these steps must be carried out with great care including good measurement practices.

All particle size analysis techniques require that samples are prepared in a certain way (e.g. by dispersion) before they can be analysed experimentally. Therefore appropriate sample preparation procedures, and in particular validated dispersion protocols, are pivotal in the process of making unbiased particle size measurements. To be acceptable such procedures must be effective, efficient and reproducible and must not compromise the integrity of the particle size distribution of the original material.

A variety of screening and confirmatory techniques is available to analyse particle size distributions. Screening techniques do not measure directly the number-based distribution of the external particle dimensions, but they are fast and inexpensive and still allow to positively identify a material as a nanomaterial. Confirmatory techniques

are usually more costly and time-intensive, but may provide a more reliable classification allowing to resolve doubts (or disputes). They are also used when screening methods do not allow classification. All these techniques have their specific technical capabilities (measurement principle, size range, sensitivity, etc.) and suitability for certain materials. For a correct classification whether a material is a nanomaterial or not according to the European Commission's definition, a thorough knowledge of the measurement method to be applied is therefore indispensable to correctly interpret the outcome of a measurement and to understand whether a specific technique is fit for the purpose.

The volume specific surface area (VSSA) can serve as a proxy to identify nanomaterials, provided that certain requirements are fulfilled. This can facilitate the classification of a material according to the nanomaterial definition. However, results for the number-based size distribution take precedence over an assessment based on VSSA.

Reliable measurement results can be obtained if a reference measurement system is implemented. Such a system, which is typically based on documented and validated methods and reference materials, will provide confidence in the result and the final assessment. Documentary standards, validated methods and reference materials are already available for some nanomaterials, and more can be expected in the future. Best practices should be applied when reference measurement systems are not available. This report provides examples and practical options for consideration, including a flowchart that can assist users with relevant technical knowledge in the identification of nanomaterials.

# 1 Introduction

In 2011, the European Commission (EC) adopted the Recommendation (2011/696/EU) on the definition of nanomaterial [1], in the following called the 'EC NM definition':

*'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.*

*In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.*

The EC NM definition further specifies:

*By derogation […], fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.*

*[…]'particle', 'agglomerate' and 'aggregate' are defined as follows:*

*(a) 'particle' means a minute piece of matter with defined physical boundaries;*

*(b) 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;*

*(c) 'aggregate' means a particle comprising of strongly bound or fused particles.*

*Where technically feasible and requested in specific legislation, compliance with the definition […] may be determined on the basis of the specific surface area by volume. A material should be considered as falling under the definition […] where the specific surface area by volume of the material is greater than 60 m²/cm³. However, a material which, based on its number size distribution, is a nanomaterial should be considered as complying with the definition […] even if the material has a specific surface area lower than 60 m²/cm³.*

The EC NM definition was developed to provide a common basis for regulatory purposes across all areas of European Union (EU) policy. Since its publication, regulatory provisions were adopted in the EU jurisdiction which explicitly address nanomaterials and contain regulatory definitions of the term 'nanomaterial'. The latter were derived from the EC NM definition, adopting it either as a whole or in its core parts, for example in:

- the Biocidal Products Regulation (EU) No 528/2012 [2];
- the Medical Devices Regulation (EU) 2017/745 [3];
- the annexes of the Chemicals Regulation REACH (EC) No 1907/2006, which were amended in 2018 [4].

Furthermore, the EC NM definition, or core parts thereof, is already used in some Member States (e.g. Belgium, Denmark, France, Sweden) and in Norway to define a nanomaterial or (in France) a substance at the nanoscale for the purposes of national registration and notification schemes. The EC NM definition is also referred to in EFSA's guidance on risk assessment of the application of nanoscience and nanotechnologies in the European food and feed chain [5]. EFSA's Scientific Committee advises to take it (and any future reviews) into consideration when assessing the safety of materials consisting of particles.

It is therefore appropriate to support the implementation of the EC NM definition by (i) clarification of its key concepts and terms and (ii) practical guidance on how to identify nanomaterials through measurements. These needs were also voiced by numerous stakeholders in their responses to a survey carried out by the European Commission's Joint Research Centre (JRC) [6,7,8].

A JRC Science for Policy Report, which focusses on key concepts and general and specific terms and explains them in the context of the EC NM definition, was published in February 2019 [9]. The present report gives specific advice on how to identify materials based on the criteria laid down in the EC NM definition, mainly through analytical approaches. It focusses on how to meet the technical challenges that arise when attempting to reliably identify a material as nanomaterial according to the EC NM definition.

These two reports are intended to facilitate the practical implementation of the EC NM definition across all relevant legislative areas. The considerations aim at a harmonised and coherent implementation of the EC NM definition in any specific regulatory context at Community and national levels, they are not specific to any particular piece of legislation. Such horizontal considerations may be complemented in the future by additional sectoral guidance documents, e.g. targeted advice for groups of materials relevant for sector-specific legislation.

Classification of a material as a nanomaterial requires that at least 50 % of the particles have one or more external dimensions ('size') between 1 nm and 100 nm. A descriptor commonly used for evaluating particle size distributions is the $x_{50,0}$ value, also called the median. For a number-based particle size distribution it is the size at which 50 % of the analysed particles of a sample have an external dimension of less than this value and the other 50 % of the particles in that sample are larger than that size. If the median is 100 nm or less, then the material is a 'nanomaterial' according to the EC NM definition. The median, as well as other particle number fractions, are often derived from the cumulative particle size distribution. Particle size and its distribution can be determined by a variety of techniques, each having its region of applicability in terms of materials and measurement capability. The experimental determinations of the particle size, and the particle number fraction, are essential parts in the assessment of a material against the criteria in the EC NM definition. Therefore this report starts with providing general considerations on particle size and what to measure, including external dimensions, the particle size range that should be covered, the median and constituent particles.

Section three is dedicated to reference measurement systems, including relevant existing documentary standards, reference materials and representative test materials, and good measurement practice in the context of nanomaterial identification.

The next section addresses sample preparation and its importance for a successful and reliable analysis of the particle size distribution in any material. Despite the technical progress made in recent years, a universally applicable standard operating procedure to prepare an arbitrary sample for size measurement of its constituent particles is not available. Each material requires specific treatment, but there are certain general and essential aspects that need to be taken into account when preparing samples for testing. A reliable result of the particle size distribution cannot be obtained without suitable sample preparation.

Section five explains the concept of screening and confirmatory methods and reviews specific measurement techniques and their output in relation to the requirements of the EC NM definition. It also includes information on what to specifically consider when choosing a measurement procedure to determine a particle size distribution (PSD).

The volume specific surface area (VSSA) is a material property that can serve as proxy for particle size and hence may be used to identify nanomaterials. In practice, certain conditions must be fulfilled so that both the size and the VSSA based criteria can allow identification of nanomaterials in a consistent manner. Section six therefore discusses the requirements that need to be fulfilled for including the VSSA in a measurement strategy to identify nanomaterials.

The next section discusses the approach to identify nanomaterials from a practical viewpoint, i.e., the main steps that should be considered in the process of identifying nanomaterials. This includes collection of information on the material, knowledge of the measurement techniques, the selection of appropriate measurement techniques, sample preparation and the evaluation of experimental data, and how these steps can be integrated into a logical flowchart. This section also points out several tools that were specifically developed to facilitate the identification of nanomaterials, such as software for image analysis and an e-tool that guides the user through a flowchart.

The final section summarises the core points of the report and provides and concluding remarks.

Abbreviations and non-exhaustive lists of relevant documentary standards are provided in the Annex.

This report mainly focusses on the specific practical challenges that may be encountered when attempting to assess a material against the criteria of the EC NM definition. The scope of this report does not include technical details on specific measurement methods or quality assurance, as those can be found in the literature. Neither does this report cover methods to measure exposure to nanomaterials or the detection and quantification of nanomaterials in products as it is more appropriate to discuss these challenges in the practical implementation of specific regulations. The terms 'method' (generic description of a logical organisation of operations used in a measurement) and 'technique' (a way that a method is realised or implemented) are used interchangeably in this report.

# 2 Particle size

## 2.1 External dimension

A particle size threshold is the principal criterion to be used for classifying particulate materials against the EC NM definition. Hence, application of the definition and implementation of the Recommendation relies strongly on the availability of reliable particle size measurement results. For a discussion of the term (particle) 'size', or external dimension, please see section 2.6 of Ref [9].

The size of simple regularly shaped particles, such as spheres or cubes, can be readily described by a single size parameter such as a characteristic linear dimension of the particle. In contrast, the constituent particles of real-world products can have very different morphologies (Figure 2.1) and their size can be described by many characteristic dimensions, or size parameters, depending upon the orientation of the particles.

Different size parameters may not be comparable with each other, and, moreover, results obtained for a specific parameter may significantly differ when obtained with different measurement techniques. Indeed, most particle size analysis techniques apply different physical measurement principles and they employ data analysis algorithms which often make implicit assumptions about specific properties such as particle shape, density and refractive index. This means that most instruments, or techniques, do not directly measure the fundamental particle size characteristic but they measure other properties that are correlated with the particle dimensions [10]. Taking also into consideration the influential impact of different sample preparation strategies (see Section 4), the property 'particle size' is method- (or operationally) defined. The results obtained for method-defined particle size measurands are often expressed as 'equivalent sphere diameters' (or radii). The equivalent sphere diameter is the computed diameter of a virtual sphere that creates the same signal response in the size measurement process as the particle in question. A correct interpretation and comparison of such results is only possible if the measurand – that is, the 'quantity intended to be measured' – is precisely specified [11,12].

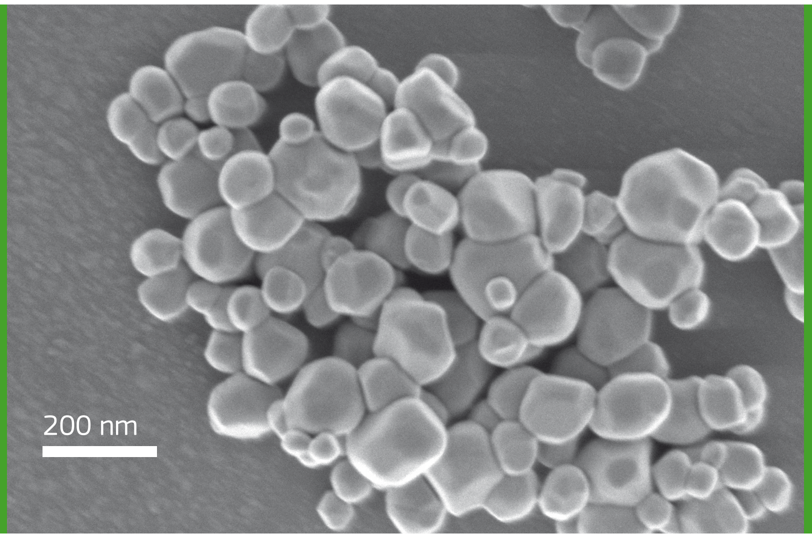


**FIGURE 2.1**

SEM micrograph of titanium dioxide nanoparticles

(Joint Research Centre, © European Commission).

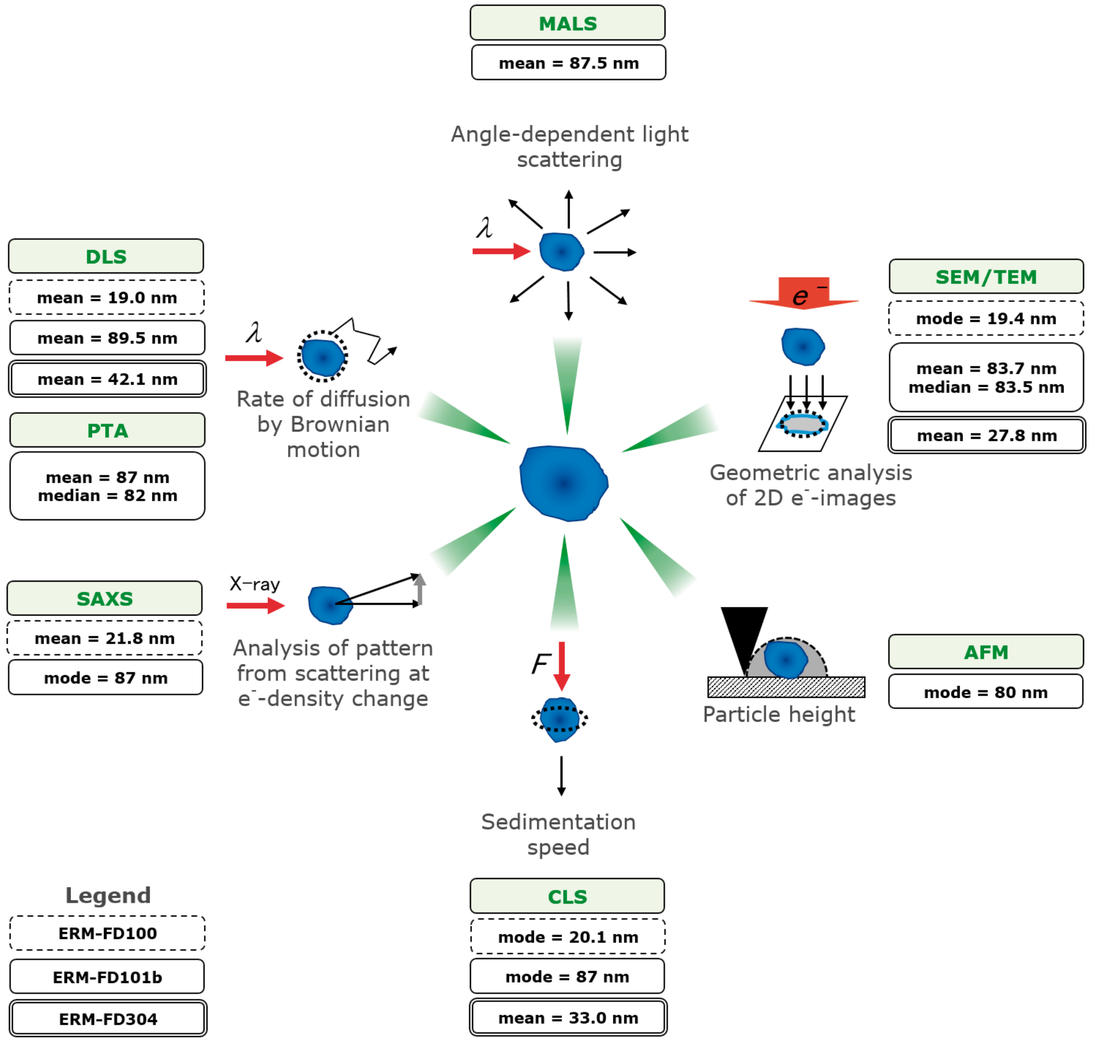


**FIGURE 2.2**

Equivalent sphere diameters of monodisperse near-spherical nominally 20 nm and 80 nm silica nanoparticles (dashed and single-lined boxes) and slightly polydisperse near-spherical nominally 40 nm silica nanoparticles (double-lined boxes) corresponding to method-defined measurands as measured with different selected techniques indicated in the green boxes (data source [13, 14, 15]: certified reference materials ERM®-FD100, ERM®-FD101b and ERM®-FD304).
Abbreviations are explained in the Annex.

As shown in Figure 2.2, for monodisperse populations of (near-) spherical particles (e.g. ERM-FD100 and ERM-FD101b), size results obtained with different measurement techniques and for distinctly different method-defined measurands agree fairly well, thus demonstrating the merits of the equivalent diameter sphere concept for this type of materials. When the particle population becomes more polydisperse, measurements become more challenging because the underlying physical measurement principles are usually only valid for idealised sample systems. As can be seen from the particle size results assigned to the slightly more polydisperse ERM-FD304, the equivalent sphere diameter concept starts losing its meaning, which results in more pronounced differences amongst different measurands. During the characterisation study of ERM-FD304 most laboratories participating in the interlaboratory comparison study were unable to reliably analyse the particle size using electron microscopy. Hence, the assigned size value is only indicative rather than certified.

Eventually, the equivalent sphere diameter can differ widely from the real external dimensions when the size of particles with distinctly non-spherical shapes such as rods and platelets is measured (Figure 2.3).

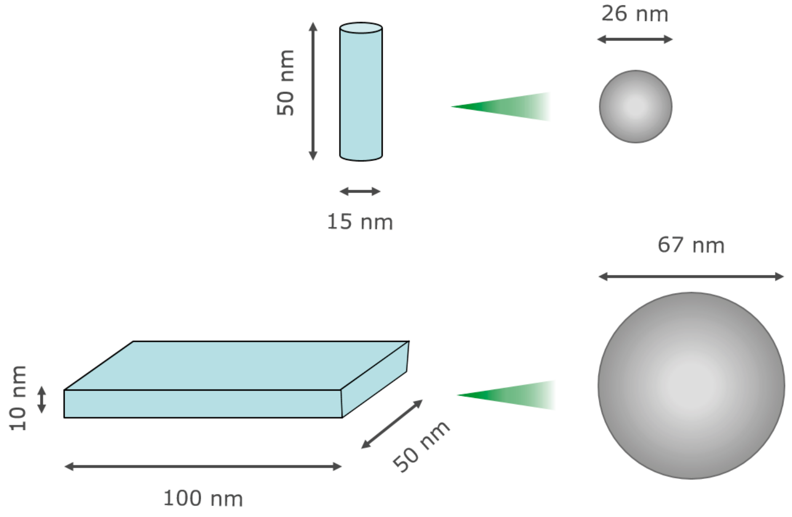


**FIGURE 2.3**

Rod- and platelet-like particles and their corresponding volume equivalent sphere diameters illustrating the weakness of the equivalent sphere diameter concept for particles with significantly different dimensions.

The inherent risk of employing the sphere equivalent diameter concept in a naive way, i.e. without prior knowledge of the shape of the particles, is further illustrated for a titanium dioxide nanorod material (Figure 2.4). Here, the area-equivalent circular diameter (ECD), which is a common descriptor for analysing the size of particles imaged by microscopy-based techniques, clearly overestimates the smallest dimensions (e.g. minimum Feret diameter, $x_{\text{Fmin}}$, maximum inscribed circular diameter) and, at the same time, underestimates the largest dimensions (e.g. maximum Feret diameter, $x_{\text{Fmax}}$) of elongated particles. In particular in the context of classifying materials according the EC NM definition, where the minimum external dimension can be a decisive factor, one can easily run the risk of false negative classifications, i.e. not classifying a material as a nanomaterial when it is one.

Any geometric descriptor [16] (minor axis, major axis, $x_{\text{Fmin}}$, $x_{\text{Fmax}}$, $x_{\text{Fmean}}$, etc.) that results in a median size value below 100 nm is sufficient to show that the particle has at least one external dimension in the nanoscale. Since irregular particles exhibit multiple external dimensions, the EC NM definition specifies that at least one of these dimensions shall be in the range of 1 nm to 100 nm, even if the particles' other dimensions are outside that range. Then such a particle will be counted for the fraction of particles having one or more external dimensions between 1 nm and 100 nm. In a previous JRC report [9], both the minimum Feret and the maximum inscribed circular descriptors are suggested for the implementation of the EC NM definition as they allow direct assessment of the particles' minimum external dimension. However, these size parameters must be chosen prudently and in line with the purpose of the EC NM definition as erroneous results can otherwise be obtained for particles of complex shapes (e.g. bent rods, particles with concave elements) [9].

As emphasised before, the measurand definition is a critical step of any measurement as it gives a description of 'what is (intended to be) measured'. This should, however, not be confused with the question 'what to measure'. The latter is a distinctly different issue as it refers to a very specific and defined quantity that needs to be measured, for example, in response to a customer request. For example, to demonstrate to public authorities that a particulate material intended to be placed on the market conforms to the regulatory requirements, the applicant should, along with the reported results, provide adequate evidence that the method-defined measurand complies with the required specifications.

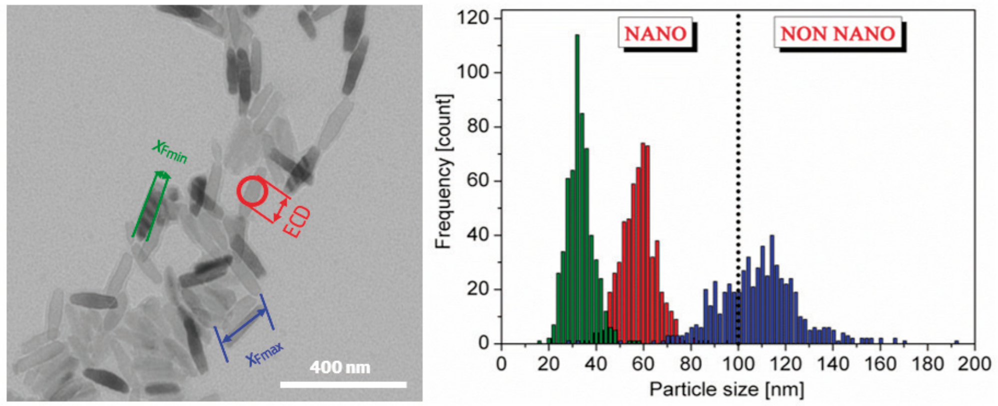
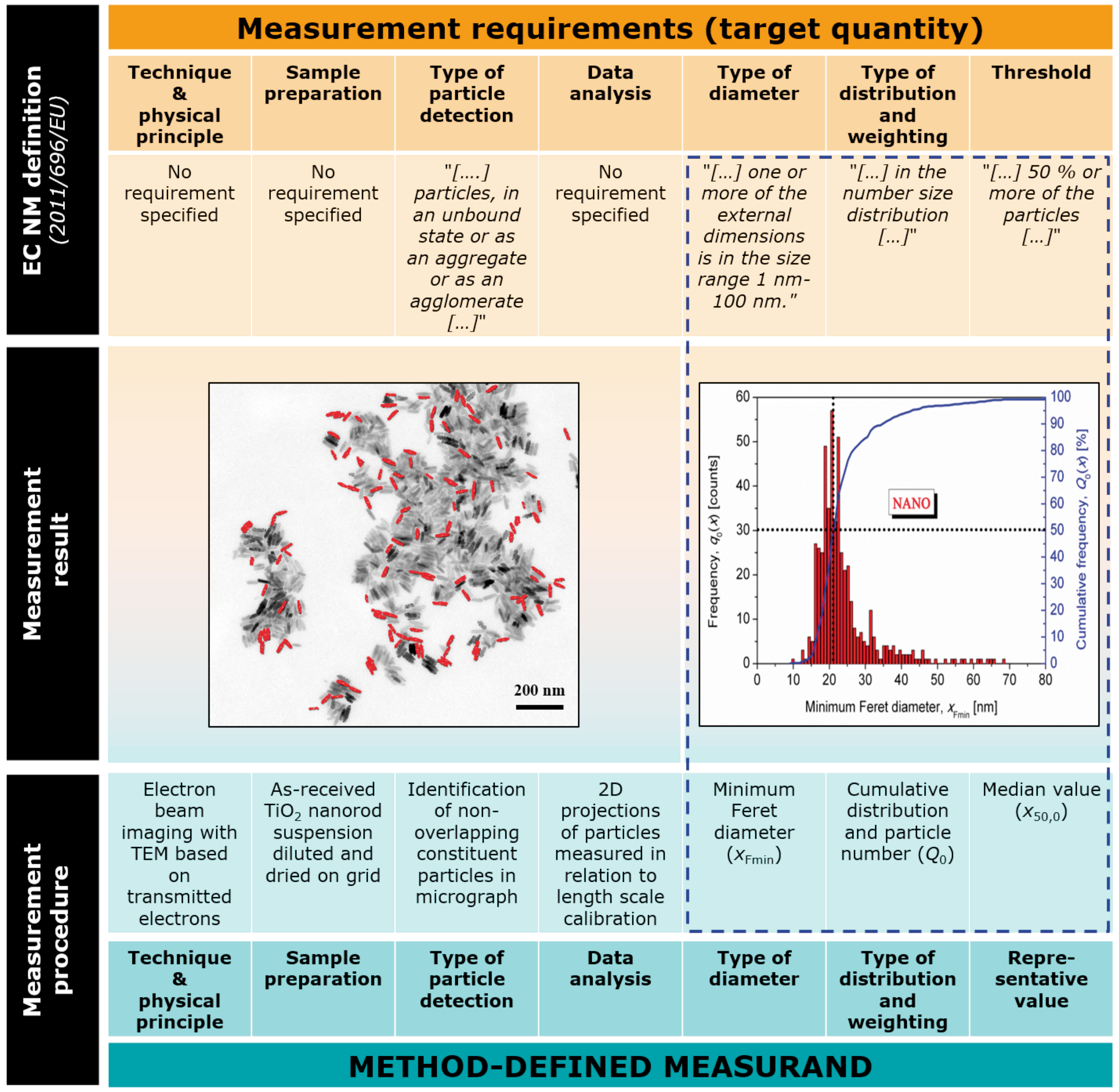


**FIGURE 2.4**

TEM micrograph of titanium dioxide nanorods with simplified representation of different particle size measurands, and corresponding number size distributions for $x_{\text{Fmin}}$ (green), $x_{\text{Fmax}}$ (blue) and ECD (red)

(TEM image, Joint Research Centre, © European Commission).

A systematic approach for classifying particulate materials according to the EC NM definition is presented in Figure 2.5 and Figure 2.6. The top part of the figures summarises the particle size-based measurement requirements (target quantity) and corresponding quantitative criteria, as defined by the different elements of the EC NM definition. The bottom section describes the method-defined measurand of a transmission electron microscopy (TEM) and particle tracking analysis (PTA) based measurement procedure (or method), respectively. In the presented approach, the particle size measurement procedure is split up into seven distinct stages [12], with each stage corresponding to a specific part (i.e. measurement technique, sample preparation, particle detection, data analysis, type of diameter or descriptor, type of distribution and weighting, representative value) of the procedure. If each stage is carefully and fully described, then the combined information will give an unambiguous definition of the property which is intended to be measured by the measurement procedure. In these



**Measurement requirements (target quantity)**

| | Technique & physical principle | Sample preparation | Type of particle detection | Data analysis | Type of diameter | Type of distribution and weighting | Threshold |
|---|---|---|---|---|---|---|---|
| **EC NM definition** *(2011/696/EU)* | No requirement specified | No requirement specified | *"[….] particles, in an unbound state or as an aggregate or as an agglomerate […]"* | No requirement specified | *"[…] one or more of the external dimensions is in the size range 1 nm-100 nm."* | *"[…] in the number size distribution […]"* | *"[…] 50 % or more of the particles […]"* |
| **Measurement result** | | | | | | | |
| **Measurement procedure** | Electron beam imaging with TEM based on transmitted electrons | As-received $TiO_2$ nanorod suspension diluted and dried on grid | Identification of non-overlapping constituent particles in micrograph | 2D projections of particles measured in relation to length scale calibration | Minimum Feret diameter ($x_{Fmin}$) | Cumulative distribution and particle number ($Q_0$) | Median value ($x_{50,0}$) |
| | **Technique & physical principle** | **Sample preparation** | **Type of particle detection** | **Data analysis** | **Type of diameter** | **Type of distribution and weighting** | **Representative value** |

**METHOD-DEFINED MEASURAND**

**FIGURE 2.5**

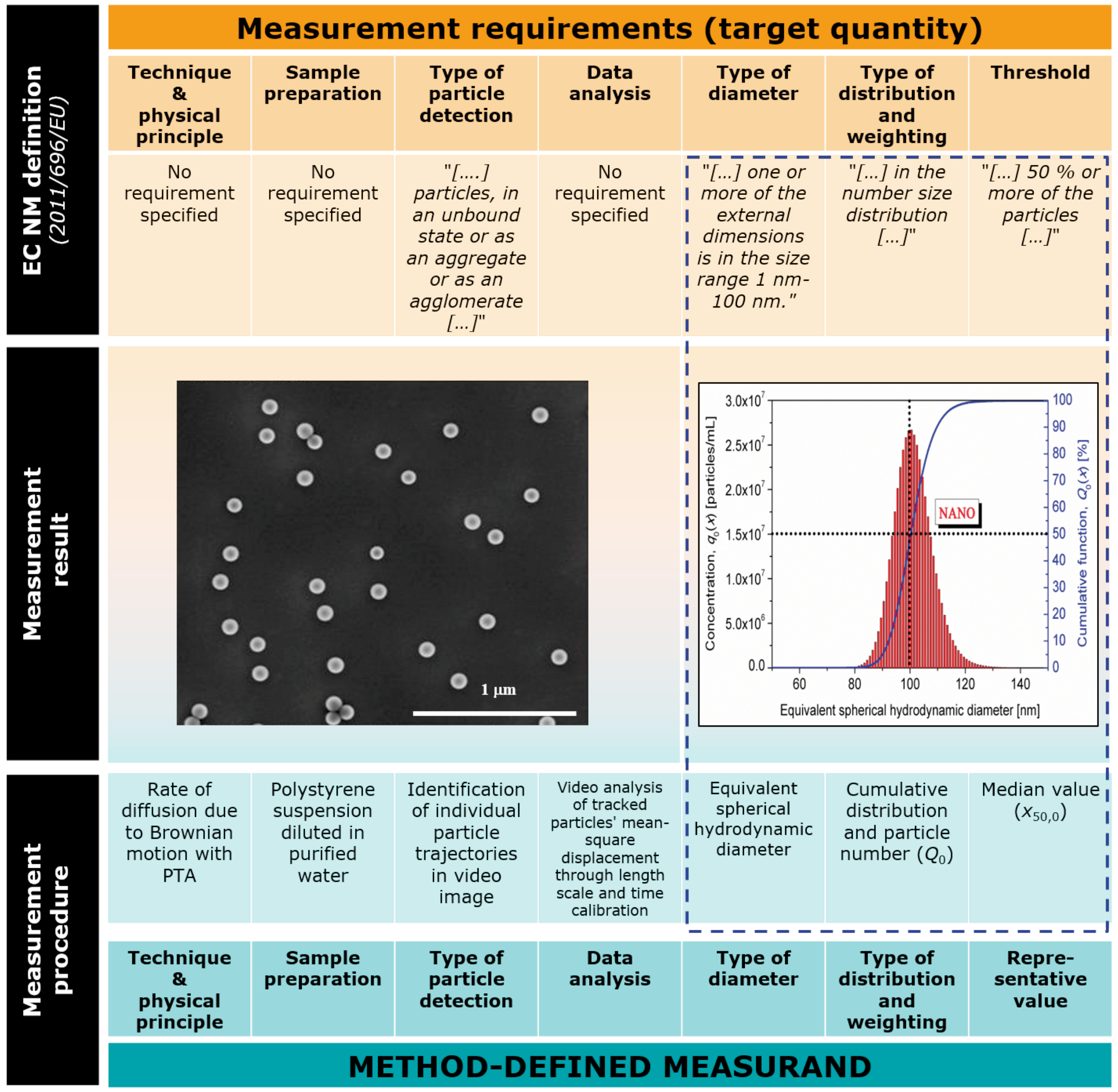
Classification of a titanium dioxide nanorod material according to the EC NM definition.

(TEM image, Joint Research Centre, © European Commission)

examples, the minimum external dimension (i.e. width) of titanium dioxide nanorods and the sphere equivalent diameter of polystyrene latex particles are measured with TEM and PTA, respectively. In both examples, the titanium dioxide and polystyrene latex materials are classified as ‘nanomaterial’.

Compared to the method-defined measurand, the measurement requirements outlined in the EC NM definition are less specific as they neither prescribe the measurement technique to be employed, nor do they specify requirements regarding sample preparation and data analysis. Such partial specification gives, on the one hand, a certain degree of flexibility as it allows the applicant to develop and validate her/his own measurement procedure around the regulatory requirements. On the other hand, it is specific enough to avoid that techniques are selected solely on an easy-to-use, or availability, basis, rather than on a fit-for-purpose principle.

To assist laboratory managers in selecting the most appropriate particle size measurement technique for a given particulate material, we further elucidate the key elements of the EC NM definition in the following sections. More information on measurement procedures, or methods, which are suitable for screening and confirmation purposes of particulate materials, can be found in Section 5.



**Measurement requirements (target quantity)**

| | Technique & physical principle | Sample preparation | Type of particle detection | Data analysis | Type of diameter | Type of distribution and weighting | Threshold |
|---|---|---|---|---|---|---|---|
| **EC NM definition** *(2011/696/EU)* | No requirement specified | No requirement specified | *"[….] particles, in an unbound state or as an aggregate or as an agglomerate […]"* | No requirement specified | *"[…] one or more of the external dimensions is in the size range 1 nm-100 nm."* | *"[…] in the number size distribution […]"* | *"[…] 50 % or more of the particles […]"* |
| **Measurement result** | | | | | | | |
| **Measurement procedure** | Rate of diffusion due to Brownian motion with PTA | Polystyrene suspension diluted in purified water | Identification of individual particle trajectories in video image | Video analysis of tracked particles' mean-square displacement through length scale and time calibration | Equivalent spherical hydrodynamic diameter | Cumulative distribution and particle number ($Q_0$) | Median value ($x_{50,0}$) |
| | **Technique & physical principle** | **Sample preparation** | **Type of particle detection** | **Data analysis** | **Type of diameter** | **Type of distribution and weighting** | **Representative value** |

**METHOD-DEFINED MEASURAND**

**FIGURE 2.6**

Classification of a polystyrene latex nanoparticle material according to the EC NM definition.

(SEM image, Joint Research Centre, © European Commission)

## External dimensions of particles and considerations on measurand definitions

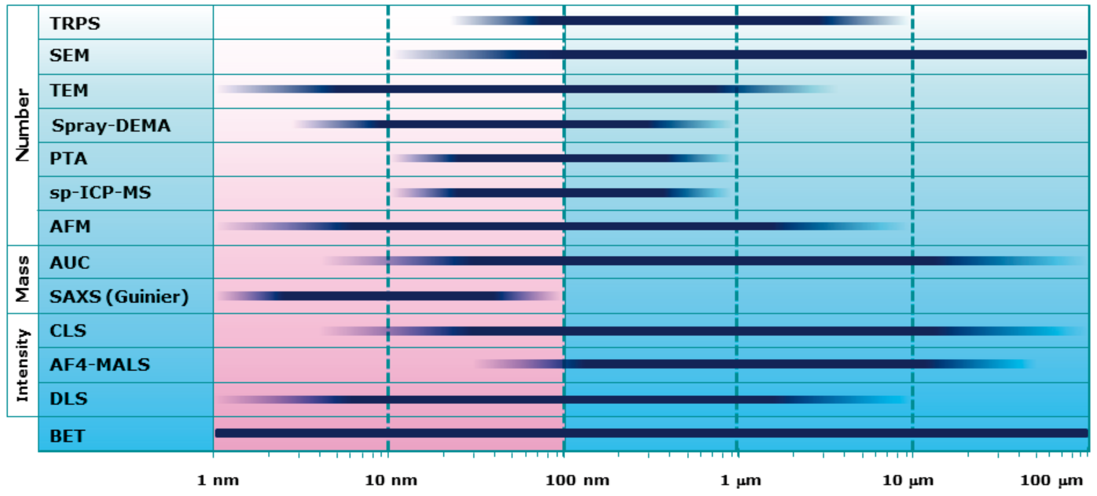
- Many particle size measurement techniques produce equivalent sphere diameters and tend to overestimate the minimum external dimensions of the particles.
- ‘Particle size’ is method-defined and a reliable assessment requires a detailed specification of the measurand, including at least the physical principle of the measurement technique and the data analysis procedure.
- The chosen measurand must allow a reliable classification according to the EC NM definition.

## 2.2 Particle size range of measurement techniques

A broad range of measurement techniques is available for measuring different types of particle size parameters, including equivalent sphere diameters and size parameters related to the particles' external dimensions. One of the main 'intuitive' criteria for choosing the most appropriate technique for analysing a specific material is, amongst others [17], the technique's particle size (working) range.

As shown in Figure 2.7, each technique has a specific working range in which it can reliably measure the size of particles. In rare cases, the lower and upper limits can be well-defined, for example, due to instrumental specifications such as the mesh size of sieves. However, for the majority of the techniques, both the lower and upper limits, which typically coincide with the measurement procedure's limits of quantification (LOQ), cannot be easily defined as they are strongly affected by the type and quality (e.g. concentration, optical properties, density, width of the distribution) of the material under investigation [17, 18]. For example, the lower LOQ of a measurement procedure based on DLS may be even lower than 10 nm when analysing strong light scatterers such as gold nanoparticles, but may only be 15 nm for silica nanoparticles as these scatter the light only weakly. On the other hand, the upper LOQ depends on the density (and size) of the particles, i.e. DLS results are only valid for particles that are colloidally stable during the time of the measurement. Also, DLS can analyse particles of 20 nm in diameter and particles of 2000 nm in diameter. However, due to the underlying light scattering physics, DLS cannot easily analyse a mixture of particles of 20 nm and 2000 nm in diameter because the intensity of the light scattered by the large particles may completely override the measurement signal from the smaller particles, thus underestimating the finer fraction in favour of coarser fractions. Similar material-dependent limitations are seen for most techniques whose measurement principles are based on the physics of light scattering or sedimentation. The working range of microscopy-based techniques such as Scanning Electron Microscopy (SEM) and TEM is considerably less affected by material- and size-dependent parameters. However, if the variation of particle size in a given sample is very large (i.e. polydisperse materials), then the working range can only be extended by combining the results of images taken at different magnifications [19]. The use of measurement techniques such as Small-Angle X-ray Scattering (SAXS) whose working range does typically not extend beyond 100 nm can produce unreliable results if particles bigger than 100 nm in diameter are measured. The DLS and TEM examples show that the particle size ranges typically claimed by instrument manufacturers in technical specifications must not be confused with the useful or effective working range of a measurement procedure.

In recent years, the measurement performance and the level of quality assurance for common particle size measurement techniques have been improved through the joint efforts of researchers and technical experts in academia, government laboratories and industry working actively together in numerous collaborative (e.g. NanoLyse, NanoChOp, NanoDefine, QualityNano, MetVes) and standardisation projects. Despite the technological advances, techniques are still unable to measure across the entire size range relevant for the EC NM definition, that is, from 1 nm well into the micrometre region.



TRPS: tunable resistive pulse sensing
SEM: scanning electron microscopy
TEM: transmission electron microscopy
Spray-DEMA: differential electrical mobility analysis on sprayed suspensions
PTA: particle tracking analysis
sp-ICP-MS: single-particle inductively coupled plasma mass spectrometry
AUC: analytical ultracentrifugation
SAXS: small-angle X-ray scattering
CLS: centrifugal liquid sedimentation
AF4-MALS: asymmetrical-flow field flow fractionation multi-angle light scattering
DLS: dynamic light scattering
BET: Brunauer–Emmett–Teller

**FIGURE 2.7**

Theoretical particle size working ranges of selected techniques along with their measurement signal weighting regime (i.e. light intensity or extinction, particle mass, particle number). The particle size ranges are indicative only and exact specifications may vary from one instrument to another. Note that the effective particle size ranges can be significantly smaller depending on the type of material being analysed.

Therefore, the statement from 2012 that no single technique alone can cover, in a single measurement and for all materials, the complete size range from 1 nm to well above 100 nm, as it would be required for a universal assessment according to the EC NM definition, is still valid today [20].

In the absence of techniques that allow for such universal assessment, lab managers should select the measurement technique that fits the PSD of a given material best. Although the determination of the material's effective PSD, and its associated characteristic parameters, is the ultimate objective of the analytical framework in question, based on preliminary knowledge, such as specifications from material synthesis and material provenance information, one will often be able to cautiously estimate the width of the PSD. If no prior information on the material is available, it is highly recommended to make particle size range-resolved measurements using different techniques of overlapping working ranges. Based on the estimated PSD and taking into account material compatibility issues, a suitable technique for screening and/or confirmatory purposes (see Section 5) can be selected. Ideally, the estimated average particle size of the material should be near the middle of the technique's particle size working range as this will reduce the risk of false negative or false positive material classification. Despite careful selection of a suitable technique, one can never completely rule out the possibility of not detecting significant number fractions of particles, especially beyond the lower and/or upper LOQ of the applied method, due to the limited working range, or small dynamic range.

In particular for materials whose PSDs span more than one order of magnitude, measurements may need to be conducted using more than one technique, or using different levels of magnification (in case of microscopy), to ensure a representative sampling and analysis of the material's entire PSD. As a consequence, to assess whether a material is a nanomaterial or not, multiple datasets and/or partial PSDs from different methods need to be combined. Currently, this process of combining partial PSDs to form a new distribution remains an important knowledge gap in the field of particle size analysis, as validated procedures are still lacking. The impact on the overall measurement uncertainty when combining PSDs must be carefully quantified by the analyst.

In any case, for a meaningful assessment of the results, the particle size measurement results must be reported along with the size range in which the size was measured.

### Particle size ranges of techniques versus method working ranges

- The effective working range of a method can be significantly smaller than the theoretical particle size range claimed by the instrument manufacturer.
- The upper and lower limits of the working range depend on the type and quality of the material.
- Prior knowledge of the material can facilitate in selecting a suitable technique.
- Combining partial PSDs is a challenging process that significantly contributes to the overall measurement uncertainty.
- Particle size results should always be reported along with the size range in which the size was measured.

## 2.3 Median of the number based distribution

The basic principle of the EC NM definition is, in short, to classify a particulate material by the fraction of its constituent particles in the size range of 1 nm to 100 nm, reported on the basis of the particle number-based particle size distribution, or the number size distribution. In the definition the particle number fraction threshold for classifying a material as a nanomaterial has been set to 50 % (median), or to a lower threshold if warranted by specific concerns regarding health, safety, environment or competitiveness. Results of particle size measurements can be represented in different ways depending on specific applications or customer demands [21,22]. In the context of implementing the EC NM definition, both the particle size value at a specific relative fraction as well as the relative fraction corresponding to a given particle size value can be determined from a cumulative distribution function, i.e. with the particle size values plotted on the horizontal axis and the relative fractions or percentiles plotted on the vertical axis.

Figure 2.8 shows the cumulative distribution function for a Pigment Yellow 83 representative test material IRMM-386 (European Commission, Joint Research Centre, Geel, Belgium) as obtained by disc-type centrifugal liquid sedimentation (CLS) after dispersing in an aqueous solution. By finding the 50th percentile value along the vertical axis, then moving horizontally to the cumulative distribution function, and finally moving vertically down, one finds a median particle size value of 155 nm (solid red arrows). By applying the same strategy in the reversed direction (dotted black arrows), one can find that 32% of the particles have a Stokes diameter of 100 nm and smaller. While the latter approach is not specifically addressed in the EC NM definition, it may be relevant for compliance to future sectoral legislation.

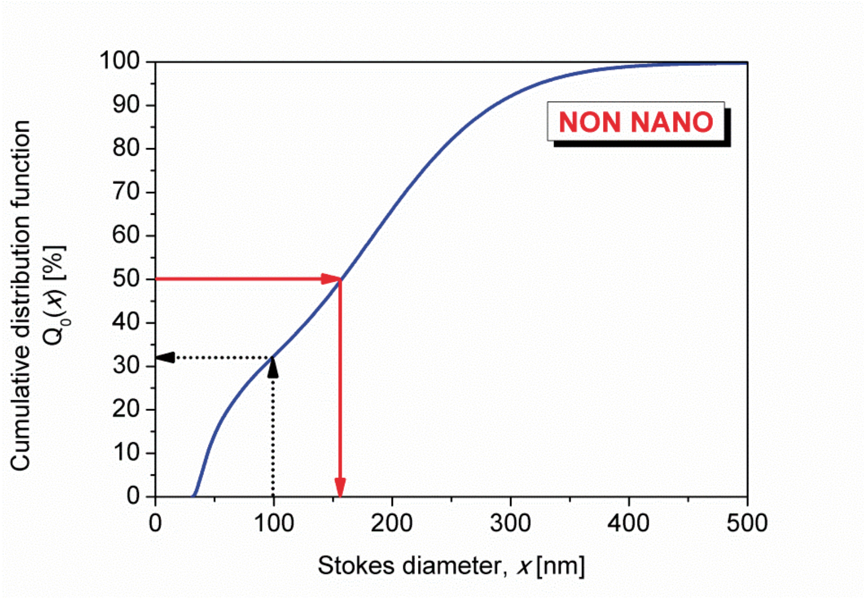


**FIGURE 2.8**

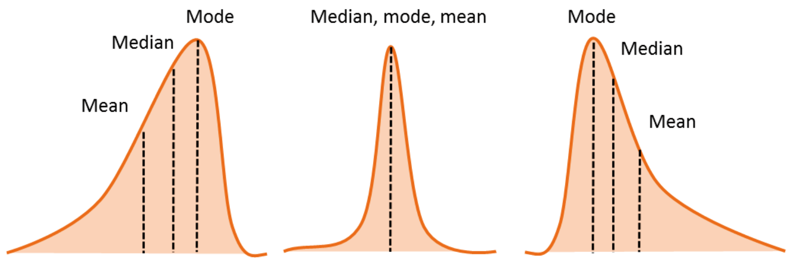
Cumulative number size distribution of Pigment Yellow 83 by disc-type CLS; red and black arrows indicate the determination of the median particle size value and the particle fraction using a threshold of 100 nm, respectively (data source: representative test material IRMM-386).

As shown in the elaborated example, the median value is a measure of the central tendency that divides the entire distribution into two equal parts, with one half representing particles with a size below 155 nm and the other half representing particles with a size above 155 nm. Compared to other measures of central tendency, such as the arithmetic mean, the median value is less affected by outliers and skewed data (Figure 2.9), and in addition, it remains meaningful in case the distribution consists of different distinct populations (e.g. multimodal distribution). The median is the formal criterion that indicates whether a material is a nanomaterial or not. Only for highly monodisperse or symmetric PSDs, the typical central values (e.g. mean, mode and median) will be very similar, and under those conditions, the mode and mean may be used as reliable estimate of the median.

Together with the working range and material compatibility (see Section 2.2), also the ability to directly determine sum or cumulative distribution functions is an important aspect when choosing a suitable measurement technique [17]. Such sum functions can be directly generated from counting techniques, such as electron microscopy, PTA and cuvette-type CLS. Other techniques such as DLS and disc-type CLS yield histograms or density distributions which need to be converted to cumulative distributions in order to easily retrieve the median size value or the fraction of particles up to a certain size threshold.

**FIGURE 2.9**

Simplified representation of measures of central tendency and their relationship for three distributional shapes: negative skew (left), normal or symmetric (centre) and positive skew (right).



## The median value of the particle size distribution

- The median value, which divides a distribution into two equal parts, can be determined from the cumulative number size distribution.
- The median value allows classification of a material according to the EC NM definition.

## 2.4 Constituent particles

### 2.4.1 What are constituent particles

The definition of ‘nanomaterial’ is primarily based on the external dimensions of the constituent particles of the material, regardless whether these particles appear separate from one another or are parts of aggregates or agglomerates. The definition continues to define ‘agglomerates’ as weakly bound particles and ‘aggregates’ as strongly bound or fused particles. In practice, there is a continuum of binding strengths with an upper range of the strengths that also hold the atoms or ions of an individual particle together. In principle, agglomerates may be distinguished from aggregates based on the way the constituent particles are bound together and their relative surface areas (cf. ISO/TS 80004-2). However, there is no commonly agreed quantitative criterion how to distinguish agglomerates from aggregates.

To get a better idea of the nature of constituent particles, it is useful to review how particles are actually generated. Particles can be generated by milling of larger entities or by growing from gases/solutions/plasmas.

In the latter case, illustrated in Figure 2.10, a seed particle (which can be an impurity or a random molecule) grows in size by the addition of other atoms or molecules, resulting in amorphous, monocrystalline or polycrystalline particles, or amorphous particles. If the number of particles per volume is low enough, this growth continues unimpeded and a collection of individual particles emerges. At higher particle number concentrations, particles may touch each other and stick together to form agglomerates bound by weak bonds like electrostatic or van der Waals forces. These weak bonds may be strengthened by e.g. sintering processes or by accretion of material, resulting in aggregates of strongly bound or fused particles. Also aggregates may clump together, forming agglomerates of aggregates, which in turn consist of constituent particles in the meaning of the definition.

When creating particles by grinding or milling, the resulting particles are always in close contact with other particles and adhere to one another. The outcome of a milling process therefore often is a collection of agglomerates.

Apart from being created during the production of particles, agglomerates can also be formed while preparing a material for analysis, e.g. concentrating a

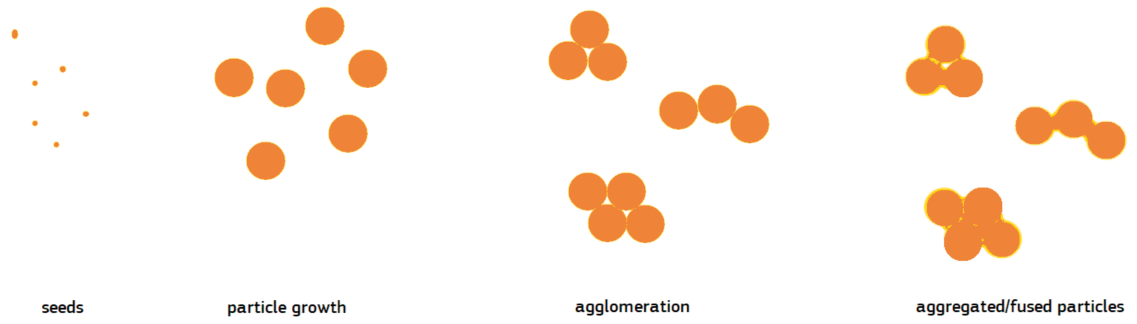


**FIGURE 2.10**

Stages of particle growth from the first seed particle until the formation of aggregated/fused particles. Note that not all particle growth results in agglomerated or fused particles.

suspension, evaporating the liquid of a suspension for preparation of analysis by electron microscopy, adding electrolytes or a different liquid to a suspension, changing the pH of a suspension.

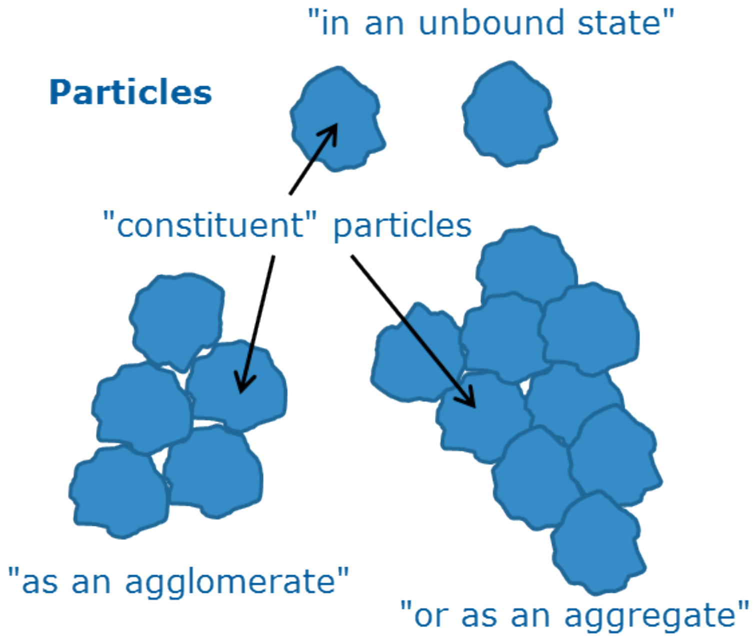
Constituent particles are all particles that once were individual particles but appear as parts of aggregates or agglomerates as well as particles that appear separate from one another. This excludes the possible seed particle and individual crystals of polycrystalline particles, but includes the particles that make up agglomerates and the particles that are combined to form aggregates (Figure 2.11). The situation is more complex with particles in which a core of one substance is completely covered by a shell of another substance (core-shell particles). Such particles clearly are not aggregates/agglomerates and in this case the outer edge of the shell forms the relevant boundary of the particle. The determination of accurate external dimensions can be challenging particuarly for particles with a metallic core and an organic shell. In these cases, the organic shell has very different properties from the metallic core (e.g. density, contrast in an electron microscope), which can lead to inaccurate results. For an illustration of such problems see Figure 2.12. It should also be noted that in case of potential ambiguities, such as complex or multicomponent composition, specific legislation or case-by-case decisions may provide clarity for the identification of constituent particles.

To summarise, the relevant particles for the definition of 'nanomaterials' are the constituent particles in an unbound state or as parts of aggregates and agglomerates.

The figures below show examples of unbound particles, agglomerates/aggregates and core-shell particles to illustrate the difference between these.

**FIGURE 2.11**

The concept of particles in an unbound state, constituent particles of an agglomerate (bottom left) and of an aggregate (bottom right). Note that identifying the boundaries between the constituent particles in an aggregate may be challenging.



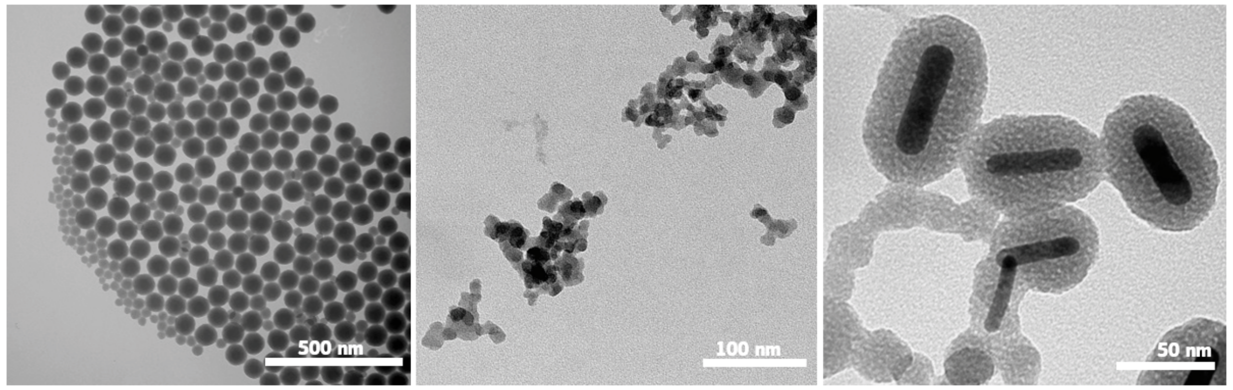


**FIGURE 2.12**

TEM images.
Left: individual spherical SiO2 particles.
Centre: aggregated/agglomerated $SiO_2$ particles.
Right: Core-shell particles consisting of Au nanorods (dark) and $SiO_2$ coating (light), next to smaller $SiO_2$ particles without an Au core.

(Joint Research Centre, © European Commission)

## 2.4.2 Identification of constituent particles

There are two ways to identify constituent particles of agglomerates and aggregates, namely trying to split an ensemble into the constituent particles or trying to identify the constituent particles within an ensemble.

### *2.4.2.1 Splitting a material into constituent particles*

Disintegration, commonly called dispersion, is the process of splitting ensembles into constituent particles. (see Section 4). In principle, a material can be dispersed in a liquid (forming a suspension) or a gas (forming an aerosol). For nanomaterials, suspension in liquids is more common.

Sufficient energy has to be introduced to overcome the binding energy between the constituent particles in order to separate the constituent particles of an agglomerate. In practice, two sources of energy are widely used, either alone or in combination:

- <u>Binding energy between a liquid and a particle:</u> When adding a liquid to a powder, the liquid molecules weakly bind to the surface of the particle if the particles can be wetted by the liquid. This reduces the free surface energy of the system. The forces between liquid molecules and particles can already break weak bonds between particles, hence breaking up weakly bound agglomerates. Specific substances that bind stronger to the particles than the liquid can be added. These 'dispersing aids' (e.g. surfactants) reduce the free surface energy even more and hence can break up stronger bound agglomerates. Liquids of different properties (polarity, pH, with or without dispersing aids) can be tried in order to maximise the breaking up of agglomerates.
- <u>Ultrasound:</u> The use of ultrasonic energy is widespread. Sonication with ultrasound creates miniscule bubbles, which implode and give rise to local temperature peaks and very fast micro-jets of fluid. These micro-jects exert mechanical stress in the area of the previous bubble, which rips agglomerates apart.

Increasing the energy input by ultrasound will increase the dispersing power of the approach until a limit is reached that is specific for each material. Increasing the energy above this limit will not lead to better de-agglomeration, but only to a higher contamination of the sample: it has been shown that sonication not only breaks up agglomerates, but also knocks particles off the ultrasonic probes (when used), thus generating nano- and microparticles that do not belong to the test material [23] and can lead to a wrong classification of a material as nanomaterial.

After dispersion, particles may re-agglomerate so a stabilisation of the dispersion is necessary.

Regardless of the dispersion method, one usually cannot break up strongly bound aggregates. As noted above, the binding forces in strongly bound aggregates can approach the binding forces in an individual particle, hence raising the sonication energy too much will result into breaking the constituent particles themselves into smaller particles, which can lead to an incorrect classification of a material as nanomaterial. If the dispersion is successful, deagglomeration takes place, agglomerates break up and their constituents (aggregates or constituent particles) are dispersed in liquid or gas.

Even if a material is not fully broken up into its constituent particles, this may be sufficient to classify a material correctly as nanomaterial: If at a certain stage of dispersion more than 50% of the analysed particles are smaller than 100 nm, then a material fulfils the definition of 'nanomaterial'. Further dispersion (if possible) can only result in even more particles having external dimensions below 100 nm, hence confirming the classification of nanomaterial. A rather questionable (but often used) approach is to assume that for not fully deagglomerated particles the particle size distribution inside the agglomerates/aggregates is the same as in the unbound particles. In this approach a decision is therefore made on the basis of the deagglomerated particles alone, even if a large (or even the major) part of the material is still present as agglomerates.

### 2.4.2.2 *Determining the external dimensions of constituent particles within an aggregate or agglomerate*

There is only one technique that has the potential to determine the dimensions of constituent particles in aggregates and agglomerates, namely electron microscopy (EM). The technique is discussed in more detail elsewhere (see Section 5.2.1 and refs. [24] and [25]), this section is limited to a general discussion. For recommendations on how to find out whether a sample contains agglomerates/aggregates please see Section 4.

EM offers the possibility to view the morphology of particles. This often allows a good assessment of what constitutes an individual particle and what is an agglomerate or aggregate of various particles. The limitations of the method are twofold:

- As any image analysis method, EM requires images of a high quality to make reliable assessments. Samples consisting of light elements (especially carbon but also silicon) do not have a very good contrast and hence lead to a poor image quality. This is not a major problem as long as the particles are well-dispersed and come from a suspension without other substances. However, the image quality decreases significantly for particles in a matrix (e.g. foodstuff) or particles on top of each other, which makes identification of constituent particles in such samples very difficult. This is shown in Figure 2.13, where the EM images of same particles are shown as derived from aqueous suspensions and as embedded in a thin polymer film.
- Automatic image analysis is only possible for fairly well-dispersed, not strongly agglomerated/aggregated materials (ISO 13322-1 'Image analysis' limits the automatic analysis to non-touching particles). Although most image analysis software contains algorithms to separate touching particles, operator intervention is still needed to assess the size of constituent particles in dense agglomerates and aggregates. As the external dimensions of several hundreds to thousands of particles need to be determined, EM is slow and expensive for particles which require significant operator input, but fast and cost-effective for materials which lend themselves to automated image analysis. Also the surrounding medium may cause problems for automated image analysis, as shown in Figure 2.13.

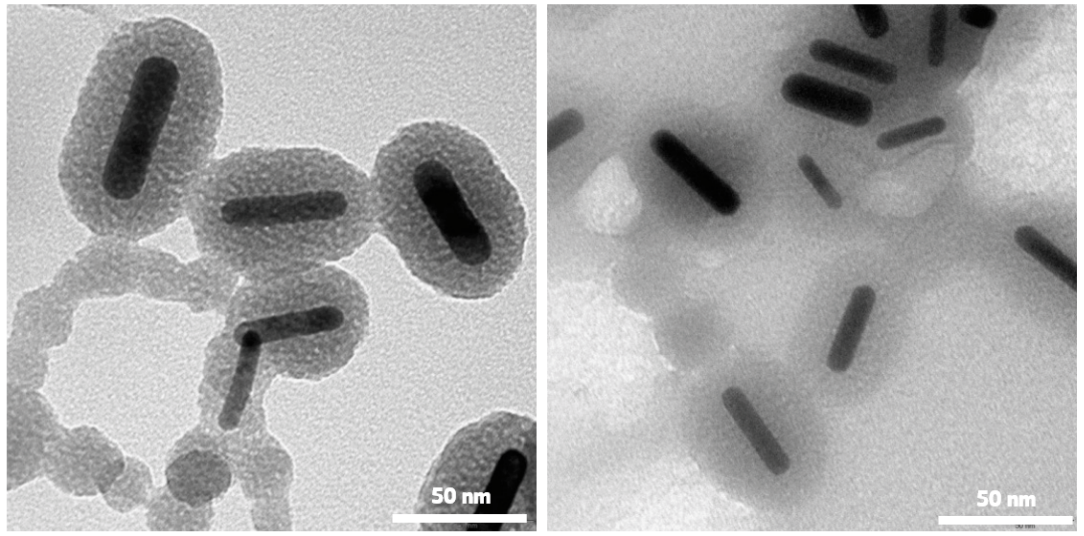


**FIGURE 2.13**

TEM images of core-shell particles of Au nanorods (dark) with a $SiO_2$ coating (light).
Left: Particles without medium. Right: The same particles in a thin layer of polyvinylpyrrolidone. The poor contrast between the $SiO_2$ part of the particle and the surrounding medium can hamper automated image processing and analysis.

(Joint Research Centre, © European Commission)

## Constituent particles, agglomerates and aggregates

- Particles in materials can exist as unbound particles, but occur more often as constituent particles of agglomerates and aggregates.
- Agglomerates can be broken up into constituent particles; aggregates usually cannot.
- Incomplete dispersion does not necessarily impede correct classification of a material as nanomaterial. However, classification of a material as not being a nanomaterial requires either the complete dispersion of a material into its constituent particles or measurement of the dimensions of constituent particles within aggregates/agglomerates.
- Electron microscopy can be used to determine the dimensions of constituent particles within aggregates/agglomerates. This requires high-quality images and considerable time for evaluation.

# 3 Reference measurement system

## 3.1 Harmonised methods

### 3.1.1 International standards

Reliable measurements require documented and validated methods. The methods need to be documented to ensure that the experiments can be reproduced. Not only do the methods have to be documented, they also need to be validated, meaning that the performance of a method has been demonstrated for the intended purpose and the reliability of the results has been assessed. In principle, each laboratory can develop its own methods and validate them. However, international and national standardisation bodies have emerged in which groups of experts develop such methods, thus eliminating the need for each laboratory to develop their own and ensuring (inter)national agreement on the used method(s). This activity is particularly important for method-defined properties like the equivalent spherical diameters of nanoparticles: application of standard methods is a fundamental requirement for comparable measurement results and consistent implementation of legislation. In the field of nanotechnology, important standardisation bodies are ISO (International Organization for Standardization) and ASTM (ASTM International, formerly known as American Society for Testing and Materials) as well as CEN (European Committee for Standardization). CEN and ISO have concluded an agreement to avoid duplication of work. Regardless of the organisation developing the documentary standards the two categories of main interest here are:

- standards that describe the design and use of a specific instrument or technique, the measurement principle and the fundamentals of signal and data analysis algorithms. Examples are ISO 22412 “dynamic light scattering” [26] or ISO 17867 “small-angle X-ray scattering” [27];
- standards that describe an often complex set of operations for measuring specific properties of specific (types of) materials, including sampling, sample preparation, specific material treatment, reporting. Examples are ISO/TS 11888 “Characterization of multiwall carbon nanotubes – Mesoscopic shape factors” [28] or ISO/TS 10867 “Characterization of single-wall carbon nanotubes using near infrared photoluminescence spectroscopy” [29].

Application of documentary standards means following a detailed sequence of operations according to written specifications. As with any other activity, theoretically knowing how to perform an action does not necessarily mean that one is also able to perform this action in practice. Therefore, laboratories need to demonstrate their competence to carry out measurements according to a certain standard method and also demonstrate that they can apply the standard method correctly [30]. This validation is performed using reference materials (see Section 3.2).

### 3.1.2 OECD Test Guidelines

OECD (Organisation for Economic Co-operation and Development) – similar to ISO and CEN providing documentary standards – provides detailed descriptions of methods used for the assessment of the safety of chemicals: the OECD Test Guidelines (TGs) for the testing of chemicals. The OECD TGs are a collection of the most relevant internationally agreed testing methods used as standards by governments, industry and independent laboratories to assess the safety of chemicals. They are primarily used in regulatory safety testing and subsequent notification and registration of chemicals [31].

The data obtained by following the TGs are recognised by all OECD member countries and other adherents to the OECD agreement of Mutual Acceptance of Data (MAD). This agreement establishes that data generated in the testing of chemicals in an OECD member country (or other State adhering to the agreement) in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice shall be accepted in other member countries for purposes of assessment and other uses relating to the protection of man and the environment [32]. However, interpretation of the results remains the respective jurisdiction's own prerogative.

A number of OECD TGs relevant to the size measurement of nanoparticles are currently under development, e. g. a new TG on Determination of the (Volume) Specific Surface Area (VSSA) of Manufactured Nanomaterials, a new TG on particle size and size distribution of manufactured nanomaterials (to complement the OECD TG110 on Particle Size Distribution/Fibre Length and Diameter Distribution).

In the European Union the Test Methods Regulation [33] lays down test methods that shall be used for testing of chemicals under EU legislation. However, other international test methods can also be used for testing if recognised by the Commission or the responsible Agency as being appropriate. Most of the tests in the Test Method Regulation  are equivalent to the OECD Test Guidelines, while other derive from the United Nation's Manual of Tests and Criteria from the United Nations Committee of Experts on the Transport of Dangerous Goods [34]. Regarding nanomaterials, currently no method relevant for the assessment of the particle size and/or size distribution of nanomaterials is included in the test methods regulation.

## 3.2 Good measurement practice

Many measurement results are obtained with the purpose to support a decision in a legal context. This means that these results must be sufficiently robust to stand up to scrutiny when challenged, for instance, by the general public or in court. Such measurement results must be obtained in a well-designed measurement/analytical quality assurance system that provides confidence and credibility in the reliability of the laboratory's measurement results. Good measurement systems are based on several pillars: facilities, equipment, personnel, procedures and standards. These pillars must be understood, modelled, managed and measured to assure that the laboratory's measurement results meet the required level of quality. In the case of measurements at the nanoscale, the same principles of quality assurance also apply.

Facilities and equipment must be suitable for the work in question and must be properly maintained and calibrated. Staff must dispose over the necessary resources and must be trained appropriately. Adequate measurement procedures must be selected that have been checked (validated) for their potential to yield correct results. Proper selection and handling of Certified Reference Materials (CRMs) ensures the correctness of calibrations and ultimately of the measurement results. More information about these generic requirements can be found in ISO/IEC 17025 [30].

The definition of the measurand is fundamental to assess quantitative data about nanomaterials. The background is elaborated in section 2. There has to be consistency between equipment, procedures and reported measurement results. In order to allow comparable results between laboratories, metrological traceability of their results is of utmost importance. In the nanoscale, the measurand is often depending on the combination of a measurement technique and a corresponding procedure. As described in section 2.1, particle size can be determined using different physical measurement principles that may provide intrinsically different results. It is important that the identity of the measurand is stated unambiguously to allow reliable comparison of the results with other data, as this is otherwise not possible.

A similar issue is what defines the procedural workflow that provides a measurement result in the end. Different terminology exists to describe quantitative analysis, such as 'measurement procedure' in the VIM (International vocabulary for metrology) [11] or 'method' in ISO/IEC 17025 [30] and many laboratories would refer to it as their standard operating procedure (SOP). Often, the measurement technique is incorrectly described as 'the method'. For instance, TEM is not a method, but a measurement technique. If a nanomaterial is embedded in a specific matrix (e.g. in food or cosmetics), the method is a combination of a sample preparation protocol, the measurement technique (e.g. TEM, DLS) and a specific type of nanomaterial. Different materials may often require different sample preparation conditions that can have an impact on the determined particle size and particle concentration. Similarly, it is not appropriate to analyse previously unstudied materials with a method that was developed for other types of samples under the assumption that the method gives correct results in the same way. For most fields in analytical sciences, a method needs to be developed and also validated for a specific sample type and this is also the case for nanomaterials. In the measurement procedure, many aspects such as sample selection, sample preparation, sample transfer, calibration, defined measurement conditions and data evaluation have to be specified and documented in detail.

The ultimate test and proof that a method is fit for its intended purpose is its validation, which confirms that the method under consideration has measurement capabilities consistent with what the application requires. Good method validations are not *ad hoc* exercises, but examine a candidate method against previously defined parameters and performance criteria. These criteria are based on the performance that a method has to achieve to be fit for purpose. Another requirement for method validation is the application of conditions that reflect the situation in the later analysis (routine conditions). The prime objective of a validation study is not to achieve results under a *best case* scenario, but to reflect the performance that can be expected during the future routine use of the method.

Whereas many guidelines exist for method validation in chemical analysis, the analyst may encounter new challenges for nanoparticle measurements. In principle, the same method performance parameters as for other analytical sectors are applicable, such as (but not limited to): *linearity, working range, selectivity, limit of detection/quantification, trueness, repeatability, intermediate precision, and robustness.* These parameters can be assessed in a single laboratory; the parameter *reproducibility* is assessed in an interlaboratory validation study.

In some cases, assessment of certain parameters might be difficult or not applicable. For example, some particle size analysis techniques, such as DLS or cuvette-type CLS, do not require calibration of the signal response with reference particles. For the determination of the detection and quantification limits, two different cases can be generally distinguished: firstly, the mass fraction or mass concentration of the particles and secondly, the minimum and maximum particle size diameters that mark the lower and upper end of the working range. For the investigation of selectivity, potentially interfering particles and matrix constituents could be already identified during method development and optimisation [35]. The validation parameter *precision* should be considered at three levels: *repeatability, intermediate precision and reproducibility*. Appropriate guidance is given in ISO 5725-3 [36]. The validation parameter *trueness* is assessed by estimating the magnitude of the experimental bias and by determining if it is statistically significant. Bias is typically determined by using CRMs or making use of data coming from an interlaboratory comparison, or comparing the new method with a reference method (listing in the order of preference). If these options are not available, spiked samples for recovery determination can be used for particle concentration analysis. It is preferable that more than one particle size or concentration level is checked. The validation parameter *robustness* is assessed through small, but deliberate changes to the method variables where the effect on the method performance is studied. The method variables selected shall be based on the observations made during method development and shall represent the *critical* method variables that have most influence on the method performance or method result.

The use of fit for purpose methods in daily practice requires regular, systematic performance monitoring. A basic requirement is the use of appropriate standards for calibration. Two essential metrological concepts have to be considered in the selection of a calibrant: *metrological traceability and measurement uncertainty.* If the calibrant is appropriately selected, it links the measurement results with the respective SI unit (such as metre or kilogram) through a chain of unbroken comparisons. Care has to be taken that the definition of the measurand of the calibrant matches the quantity to be measured.

The provision of a quantitative indication of the accuracy of a measurement result is named the measurement uncertainty and is essential for the comparison of measurement results, either among themselves or with reference values given in a specification or documentary standard. An estimate for the measurement uncertainty can be obtained in a dedicated study, from results of a method validation, by modelling the measurement procedure, or from quality control data over an extended period of time (control charts, proficiency testing schemes) [37].

The best way to demonstrate the competence of a laboratory is through accreditation. It is a third party, impartial and independent process to assess that an organisation is competent to perform specific tests, inspections, measurements and certifications. In the case of the measurement system for nanomaterials, this would involve the accreditation of calibration and testing activities (ISO/IEC 17025) [30] and proficiency testing (ISO/IEC 17043) [38]. As nanotechnology is an emerging technology, there are currently still rather few organisations that hold an accreditation for one of the mentioned activities. At European level, there is no centralised database to search for accredited laboratories; however the overarching organisation *European co-operation for Accreditation* provides a website [39] with links to all European national accreditation bodies. Individual laboratories can be found on the respective websites of these national accreditation bodies.

## 3.3 Quality assurance tools

Apart from other quality assurance monitoring tools such as performance checks of measuring and testing equipment, the use of control charts, analysis of blinded samples, and testing of retained samples, the best option to check the continuous performance of a laboratory and its methods is achieved by making use of appropriate CRMs. They provide a means to confirm the metrological traceability of a measurement result and are the best way to assess the trueness of a measurement result. In practice, there is still a lack of CRMs for nanoparticle analysis, especially of matrix CRMs. In these cases, laboratories should consider to use in-house quality control samples. Guidance for their preparation is given in [40]. In general, many analytical laboratories have problems not only to find reference materials, but also other types of test materials for other purposes. For example, representative test materials [41] can be used for method development or precision determination in method validations. In the case of demonstrated sufficient homogeneity, they can also serve as test samples in collaborative trials. Apart from the development and distribution of CRMs [42] for properties in the nanoscale, the JRC also hosts a repository [43] of representative industrial nanomaterials.

Regarding the availability and use of reference materials, it is important to clarify the terminology. The term *reference material (RM)* can be used in two ways. The superordinate *reference material* describes both non-certified and certified reference materials. The respective subordinate terms were developed by the ISO committee on Reference Materials (ISO/REMCO) for generic use across all scientific disciplines. ISO Guide 30 [44] provides the terminology for it:

*Reference Material (RM): material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.*

*Certified Reference Material (CRM): reference material (RM) characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability.*

This means that the term *reference material* can be used for describing the overarching, generic concept and/or for describing non-certified materials of proven homogeneity and stability. CRMs have a much higher added value for the analyst as they carry not only certified values based on a thorough characterisation of the material, but they also provide an estimate of the uncertainty of the certified values and a link to a metrological reference point.

Consequently, the applications of reference materials are different. Non-certified RMs can be used for method development and method validation. However, measurements that need a *trueness* component, such as measurements for calibration or quality control, have to be checked with *certified RMs.* Only CRMs can contribute this quantitative component, including the metrological traceability that can link a laboratory result to a common reference, resulting in comparability of data.

The selection of the right CRM is important in order to benefit most of the use of the CRM. Great care has to be taken to choose a CRM whose certified properties match exactly the measurand, i.e. the quantity intended to be measured (see also Section 3.2 on good measurement practice).

Most RMs certified for properties in the nanoscale cover particle size values of monodisperse, spherical particles. While these materials are very useful for calibration of and performance checks of measurement equipment and methods, they are less suitable for quality control checks when measurements of irregular-shaped, polydisperse nanomaterials are involved or when particles in complex matrices need to be analysed. Hence, there is a clear need for CRMs that represent polydisperse, irregularly shaped nanomaterials, especially when embedded in (simple and complex) matrices. However, the development and production of these CRMs still need considerable efforts. The certification of such materials is also depending on the availability of appropriate, validated methods.

Additionally, as for all kinds of other CRMs, one single nanomaterial CRM might not be sufficient to cover the whole working range of a method or the range that needs to be covered for a correct implementation of the EC NM definition. Thus, several CRMs might be necessary to cover an appropriate measurement range.

As a part of the European project NanoDefine (www.nanodefine.eu), a review of existing calibration and reference materials was made [45]. Certified reference materials from different suppliers are available for various properties: silica, gold, silver and polystyrene particles are mostly used for the determination of particle sizes; titanium dioxide and alumina materials are available for the measurement of the specific surface area. The review concludes that only a small number of CRMs are available.

There are some databases that list reference materials, such as the COMAR database hosted by BAM (www.comar.bam.de) that includes also materials certified for different physical properties. The database *Nanoscale Reference Materials* (www.nano-refmat.bam.de) is complementary to COMAR by listing materials that are relevant in the nanoscale range. It includes CRMs, but also non-certified reference materials for particle size and other nanoscale dimensions, such as surface flatness, porosity, height or depth.

Additional to reference materials, proficiency testing (PT) schemes serve as another pillar in quality assurance. For a laboratory participation in PT schemes is a way to determine the laboratory precision and the correctness of its results. In addition to the information on how the laboratory results match an assigned or consensus value (z-score), also information about the dispersion of the laboratory's results in comparison with the other laboratories can be obtained (zeta-score). At the moment, PTs or other interlaboratory comparisons are mostly organised as part of research projects. For instance, the Technical Work Area 34 [46] *Nanoparticle Populations* of the Versailles Project on Advanced Materials and Standards (VAMAS) is occasionally organising interlaboratory comparisons in the frame of specific projects. With increasing progress of analytical capabilities and the need for (regulatory) compliance, hopefully commercial PT providers will also come into play.

The EC NM definition asks for certain specifications to decide whether a material is a nanomaterial or not. As discussed earlier in this report, a series of techniques can be used for screening purposes, but only a limited number of techniques are fit to confirm if the median of a number-based PSD is smaller than 100 nm. This means that only a subsection of techniques and methods are suitable to answer the relevant question. Likewise, the availability of tools for quality assurance (validated methods, CRMs, PT) is still rather limited, but with more progress in development of these tools, the situation will improve over time.

## Reference measurement system

- Documentary standards from international standardisation bodies exist that describe the application of various methods.
- The OECD provides Test Guidelines that are internationally agreed testing methods used as standards by governments, industry and independent laboratories to test and assess the safety of chemicals in a regulatory context. While standards and test guidelines eliminate the need for each laboratory to develop their own method, laboratories still need to demonstrate correct application of the method.
- Although non-certified reference materials (RMs) are useful for method development and validation, only certified reference materials (CRMs) can provide a check of the trueness of quantitative results. There is a strong need for more reference materials certified for properties in the nanoscale.
- Important tools to assure the quality of measurements of nanomaterials are the use of validated methods, certified reference materials and participation in proficiency testing.

# 4 Sample preparation

Many analytical instruments, including those commonly used for particle size analysis, require a dedicated process to make the original test material amenable for analysis. Depending on the instrument and the type of material to be analysed, the sample transformation process, further referred to as ‘sample preparation’, can significantly vary from a simple procedure such as dilution to a very complex multi-step protocol. Elaborated dispersion protocols are often required for powdered materials as most techniques, such as DLS, CLS, PTA, etc., cannot handle dry powders. Even direct techniques based on imaging (e.g. SEM, TEM) significantly benefit from having samples available as stable dispersions because measuring the size of particles deposited onto a specimen substrate works best for monolayers of individual particles and when the particles are not too strongly agglomerated.

As illustrated in Figure 4.1, sample preparation is an essential part of almost every analytical measurement procedure or method. It may often seem trivial at first glance, but its consequences and impact on the final measurement result can be considerable. Due to the many different physicochemical properties of materials and various instrumental requirements, a universal sample preparation protocol that fits all – or even a standardised protocol that fits a group of materials with similar properties – is not available. The main challenge is thus to select, or develop, for each material/instrument combination a sample preparation strategy that converts a representative portion (taken from the original material) into a form suitable for analysis. It is critical that the applied sample preparation process does not compromise the integrity and quality of the sample so that the obtained results are reproducible and representative for the original material. Although specific quality assurance measures can be taken to underpin the reliability of the final measurement results (see Section 3), one can never be completely sure that the results obtained on a modified or converted sample are unbiased. Therefore, laboratory managers should give full details of the applied sample preparation routines as these will help regulators (or applicants) to make appropriate decisions.

The following sections aim to provide a brief summary of different sample preparation steps that may be considered when developing a sample preparation protocol for a specific particle size analysis application. These steps are mainly, but not exclusively, relevant for dispersing powders in a liquid. Due to the versatility and distinct properties of materials and instruments we can not give detailed instructions, or advice, on which sample preparation steps to be used. However, analysts and laboratory managers may find valuable inspiration from specific and/or generic protocols available in literature [47].

The effective stability (resistance to re-agglomeration and/or selective sedimentation) of a prepared sample for the time between sample preparation and the end of the measurement should be verified.

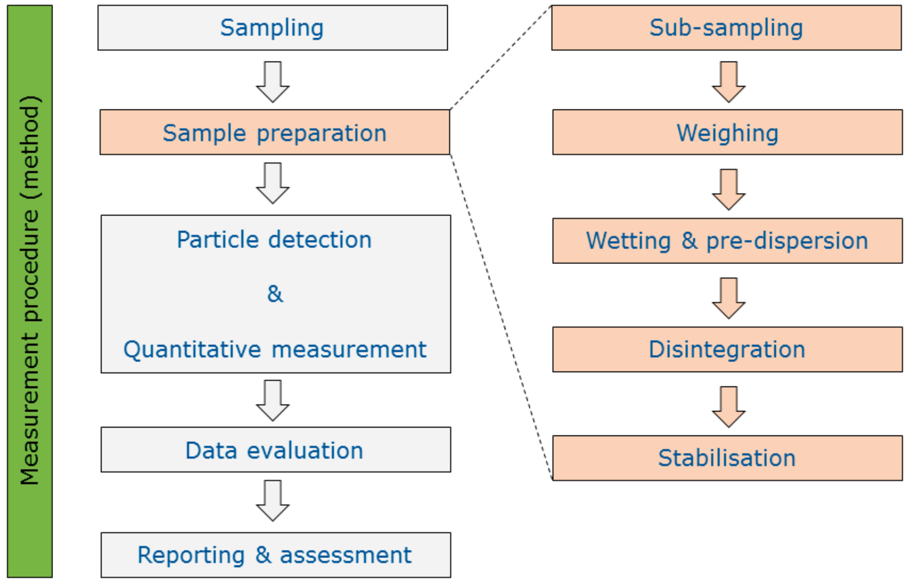


**FIGURE 4.1**

Traditional workflow of a generic (particle size) measurement procedure (left chain) and different possible sample preparation steps (right chain).

## 4.1 Sampling and subsampling

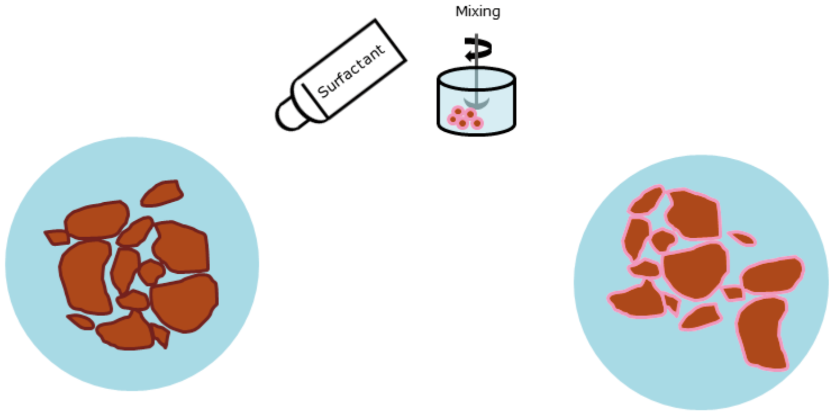
Sampling and sub-sampling are the first physical steps in any analytical measurement procedure. During these steps, one applies a strategy that helps deciding where and how to retrieve one or more portions from a bulk material. As the results obtained on the test samples, prepared from the retrieved portions, are used to infer conclusions about (the particle size distribution of) the original material, and ultimately to make possible decisions in a regulatory context, it is of utmost importance that the sampled test portions are fully representative for the bulk material. As explained in Section 3, the main elements (i.e. sample preparation, measurement, data analysis) of the measurement procedure can be embraced by dedicated quality assurance measures allowing the analyst to make assessments in a quantitative and metrologically underpinned manner. These quality assurance measures, however, do not cover the sampling step. Therefore, the assessment made on the analysed samples cannot be easily extrapolated to the (bulk) material from which the samples have been retrieved. This is particularly true for powder materials that consist of heterogeneous discrete particles and which can be sensitive to segregation or de-mixing. To ensure the representativeness of the samples, one should apply a sampling plan that is based on internationally recognised procedures (e.g. ISO 14488 [48]).

## 4.2 Wetting and pre-dispersion

The first stage in producing a colloidally stable dispersion from dry particles requires the displacement of the solid-air (moisture) interface with a solid-liquid interface (Figure 4.2). The ease with which this can be achieved can vary depending on the particular combination of particle and dispersant media. In many cases, such as hydrophilic particles and aqueous solutions, mixing will occur spontaneously with mechanical stirring being sufficient to place the solid particles in intimate contact with the dispersant liquid. In other cases the powder itself may be intrinsically hydrophilic but through the production process or during storage it may accumulate via atmospheric contamination a thin contaminant layer of hydrophobic material. In such cases contacting the particles with a small amount of a pure or diluted polar solvent such as methanol or ethanol prior to mixing with aqueous media may be sufficient to ensure an adequate wetting behaviour. Should this fail to give satisfactory results, it may be necessary to use a specific wetting agent or surfactant before adequate pre-dispersion can be achieved prior to the de-agglomeration.

**FIGURE 4.2**

Simplified representation of a dispersion process consisting of particle wetting and mechanical mixing. Left: before mixing, right: after mixing. The mixing ensures that the agglomerated constituent particles are convered by surfactant as indicated by the pink border around each particle.



## 4.3 Disagglomeration

The next step in the process is the breaking up of the agglomerates (if present) by the input of mechanical mixing and/or ultrasonication energy (Figure 4.3).

The principle of ultrasonication is based on using high-frequency sound waves to break apart particle agglomerates by cavitation. In the laboratory, ultrasonication may be applied using a variety of techniques of which the most common are bath sonication and immersion probe sonication. The simplest method, bath-type sonicator (indirect sonication), is easy to use and is available in most laboratories. As the ultrasonic waves need to traverse firstly the bath liquid and then the wall of the sample container before reaching the actual sample suspension, the energy reaching the sample is much lower than when using an ultrasonic probe directly immersed in the sample suspension. The main disadvantage of direct sonication is the physical contact between the probe and the suspension, which can result in chemical or particulate contamination of the sample (see Figure 4.4) due to unavoidable erosion of the probe head. To minimise the risk of this problem, care should be taken to ensure that the sonicator and probe combination are always used according to the instructions provided by the manufacturer and that regular inspections of the probe tip be made to detect the onset of wear damage.

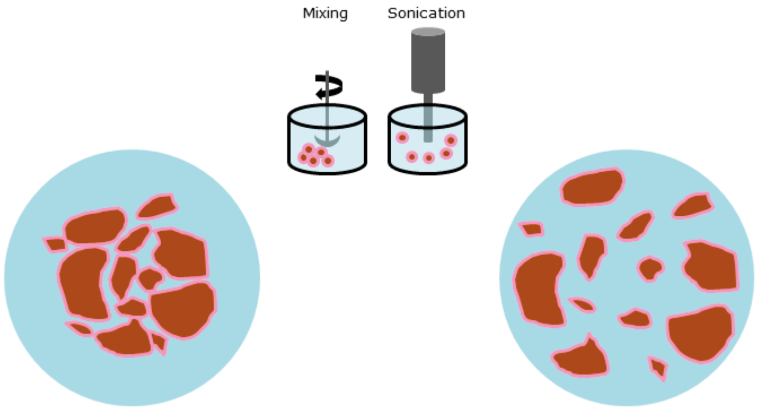


**FIGURE 4.3**

Simplified representation of breaking up agglomerates by mechanical mixing and ultrasonic energy.
Left: before sonication,
right: after sonication.

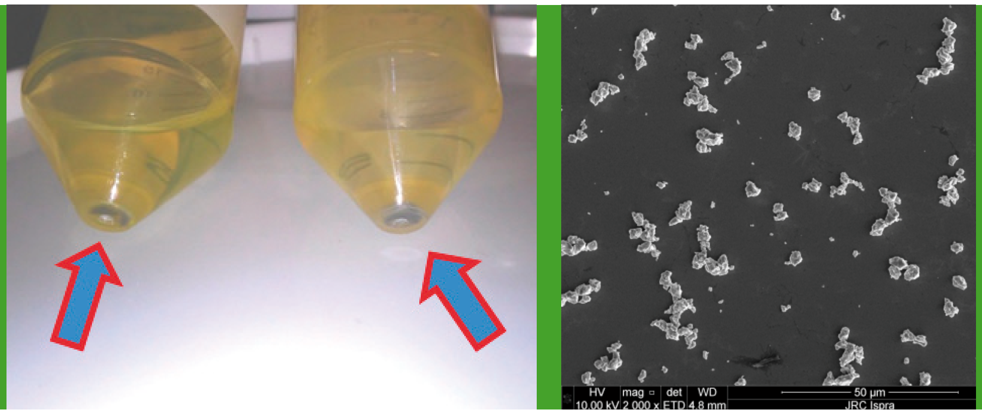
In addition to the immersion probe sonication method there are a number of less commonly available methods which can achieve similar results but by using indirect sonication and thus presenting a reduced risk of sample contamination. For relatively small sample volumes (up to 2 mL) vial sonication is a viable alternative to probe sonication. In this method the samples to be treated are contained in small plastic/glass vials which are held directly in contact with a metal block which is, in turn, in contact with the ultrasonic transducer. An alternative to this is the cup horn sonicator which functions as a high intensity ultrasonic water bath into which multiple samples in sealed tubes can be immersed for treatment. Both of these approaches can produce ultrasonic power densities which are similar to that of probe sonicator but with the advantage that the samples are only in contact with their container, effectively eliminating the risk of any particulate contamination which can occur with immersion probes. An additional advantage is that the use of sealed vials eliminates any potential hazard from aerosol generation.

An important aspect in sample preparation is to know and to report the effective amount of power that is delivered to a sample. Although many different sonicator and probe combinations are commercially available, the nominal power output either cited by manufacturers or displayed on the sonicator device is a measure of the electric energy that is delivered to the convertor (to which the probe is connected to). The displayed power is not a reliable indicator for the actual power absorbed by any specific sample. Various factors such as the probe size and shape, sample volume, particle concentration, probe immersion depth, vessel shape, liquid temperature and viscosity cause that a significant amount of input power is attenuated and dissipated. It is therefore recommended that the power density of the sonication set-up is determined. Bath sonicators have a typical maximum power density of about 2 W $cm^{-2}$ while much greater maximum power densities (i.e. up to several hundred of W $cm^{-2}$) can be achieved by probe/vial/cup-horn sonicators. A suitable protocol for estimating the effective acoustic power output from generic probe-type sonicators when operating at a variety of instrument settings has been presented by Taurozzi et al. [49].

From measurements based on this type of protocol it has been seen that the absorbed power depends not only on the power setting of the instrument (amplitude and cycle time) but also is strongly influenced by the diameter of the probe's radiating face – in practice there is an approximately linear relationship between acoustic energy output to the sample and the probe diameter. In the case of probe sonication the sample volume is an important factor in determining the quality of the final dispersion and it is relevant to consider the volume specific energy density (W s $mL^{-1}$) and sonication time rather than total energy absorbed. The energy required to disagglomerate particles can vary strongly with the specific material but as an indication, effective power output values of (1-2) W s $mL^{-1}$ and treatment times of 10-30 minutes are suitable starting points for many inorganic materials. Similar values are applicable also for the use of vial sonication where sample volumes are likely to be fixed at approximately 1.5 mL or 2 mL.

**FIGURE 4.4**

Left: Organic pigment samples after probe sonication showing particulate contamination (residue) from the probe sonicator.
Right: SEM image of metallic residue produced by probe sonicator (© NanoDefine).



## 4.4 Stabilisation

When powder agglomerates have been broken up into smaller agglomerates or constituent particles and dispersed in a liquid, these will inevitably undergo random movements due to Brownian motion and liquid convection. This leads to collisions between particles which will bring them again into sufficiently close contact as to allow re-agglomeration of the particles due to van der Waals (vdW) interactions. To reduce or avoid this destabilisation of the dispersion, steps must be taken to ensure that colliding particles cannot come sufficiently close as to allow the onset of vdW attraction. The main ways to keep particles apart and thus maintain a stable colloidal dispersion are electrostatic stabilisation and steric stabilisation (Figure 4.5).

**FIGURE 4.5**

Simplified representation of electrostatic and steric stabilisation of a suspension.

### 4.4.1 Electrostatic stabilisation

Many types of nanoparticles dispersed in polar liquids spontaneously develop a degree of surface charge relative to their surrounding dispersion media. The surface charge may be intrinsic to the interface of the particle and polar solvent or the result of adsorbing charged molecules on the particle surface. Provided that all the particles exhibit the same polarity of charge and the magnitude of the charge is sufficient, then mutual repulsion between particles can keep the particles separated so minimising the possibility of re-agglomeration. Zeta potential measurements can be useful to check whether a dispersion is colloidally stable for a sufficiently long period of time. An absolute value of the zeta potential $\zeta$ larger than 25 mV ($|\zeta| > 25$ mV) is an indication for colloidal stability. This effect, electrostatic stabilisation, is often the simplest route to implement since it relies on the intrinsic properties of the particle surface or the use of small molecules to modify the particle surface. In comparison to when using a long-chain polymeric stabiliser, electrostatic stabilisation does not have a significant influence on the hydrodynamic diameter of the particles. In cases where the particles in dispersion do not naturally have a sufficient level of charge, adjustments to pH or chemisorption of a suitably charged molecule, may be able to increase surface charge sufficiently to stabilise the dispersion.

The major disadvantage of electrostatic stabilisation is that the effectiveness of stabilisation of the particles in solution depends on the nature of the surrounding solvent (aqueous or polar organic) and in particular on the pH, ionic strength and the presence of multivalent ions.

### 4.4.2 Steric stabilisation

Steric stabilisation of colloidal particles is achieved by attaching (grafting or chemisorption) macromolecules to the surfaces of the particles. When the coated particles collide the protruding polymer chains interact generating a repulsive force which prevents particles from becoming sufficiently close as to allow the onset of agglomeration by vdW attraction. The use of such steric stabilisation has a number of advantages over electrostatic stabilisation including:

- relative insensitivity to the presence of electrolytes giving efficacy in both polar and non-polar dispersion media;
- maximum concentration of solids;
- reversibility of flocculation.

The number of possible surfactants and stabilisers which can be used in preparing colloidal dispersions for analysis is very large and whenever possible selection should be based on prior knowledge of the materials being examined. Unfortunately, no optimal, universally applicable stabiliser can be proposed but studies in nanotoxicology have shown that certain natural polymers can offer possible solutions to stabilizing a wide range of nanoparticle materials. Examples of such natural polymers include bovine serum albumin, plant derived polyphenol/polysaccharides or other natural organic matter. Such polymers, in addition to their wide applicability, offer the advantages of being readily available, and biocompatible. The main disadvantage is that their ability to disperse and stabilise may not be as effective as that of a correctly selected conventional chemical surfactant.

## 4.5 Verifying the stability of dispersions

In order to check the effective stability (resistance to re-agglomeration) of dispersed solutions, periodic verifications should be made of the apparent particle size distribution from stock dispersions using at least one technique which is sensitive to changes in particle size and quantity. The absolute accuracy of the measurement is not critical provided it is reproducible and in this respect a prime candidate (measures both size and quantity) for such verification would be Centrifugal Liquid Sedimentation - assuming the expected particle size range and density are compatible with the lower size limit of the CLS instrument being used. CLS can be used to very sensitively reveal any change in size distribution. Reagglomeration changes the particle size distribution and often leads to sedimentation. However, certain dispersions may change over time due to gravitational sedimentation only (without re-agglomeration) and this can be easily reversed. It is therefore recommended that before verifying size distributions with CLS or other appropriate alternative the sample dispersion should be re-homogenised by a short period (few minutes) of bath sonication.

## 4.6 Verifying the effectiveness of dispersion protocols

For the implementation of the EC NM definition, a dispersion protocol can be considered effective if it yields samples which mainly consist of non-agglomerated/non-aggregated particles. To monitor the effectiveness of a protocol, one requires analytical methods which can reliably distinguish constituent particles from agglomerates and aggregates. Suitable methods are those based on electron microscopy (EM) techniques (e.g. SEM, TEM). Where such methods are available and suitably prepared samples (specimens) are possible the effectiveness of a dispersion protocol maybe evaluated by direct imaging to determine the proportion of free (non-touching) particles compared to aggregates and agglomerates. As EM methods require dry samples, care must be taken to optimise the EM specimen preparation to minimise re-agglomeration of particulates during specimen drying [50,51,52]. As mentioned earlier, the use of protocols involving powders directly transferred onto a stub (without bringing them first in dispersion) is not recommended.

In the case where it is not possible to verify agglomeration and aggregation state with EM it is recommended that efforts be made to empirically verify that the protocol has been optimised to produce the minimum mean particle size possible with the facilities available for dispersion. To do this the dispersion steps should be applied systematically using different sonication times and fixed power settings with the mean particle size being measured by methods such as CLS or DLS. The most suitable sonication time and power should be chosen as the time when further incremental increases in sonication time and/or in power do not result in significant further changes in mean size. It should be noted that sonication of many materials produces a stable minimum size beyond which further sonication is ineffective while other materials may reach a minimum after which the mean size begins to increase due to undesirable fusion of small particles into larger aggregates.

## 4.7 Summary

Many of the common particle size analysis techniques need, or benefit from having, samples which are available as stable suspensions. Many materials consist of dried powders which need to be dispersed in compatible liquids before they can be analysed with such analytical instruments. Thus, sample preparation, and in particular validated dispersion protocols, are pivotal in the process of making unbiased particle size measurements. To be acceptable it is necessary that such procedures are effective, efficient and reproducible and that they do not compromise the integrity of the PSD of the original material.

In developing dispersion protocols it has been found that only few agglomerated powdered materials can be adequately dispersed by means of low energy mixing (e.g. stirring/shaking/vortexing) or by the use of low intensity ultrasound sonication (e.g. sonication bath). Instead, it is usually necessary to apply high intensity sonication instruments (e.g. vial or probe sonication) which have a nominal power output of at least 100 W. The use of sonication, although often effective in breaking up agglomerates, does introduce a important variable in the measurement process as a wide variety of sonication instruments with different nominal output powers, probe types and dimensions and efficiency exists. As the intensity of sonication is critical to the dispersion process it is highly recommended that the effective power density of a sonicator is determined calorimetrically to allow easy implementation of the dispersion protocol in other laboratories using different types of sonication devices. As an indication of appropriate sonication conditions for a range of common inorganic nanomaterials, acoustic power densities in the range of 1 W s $mL^{-1}$ to 2 W s $mL^{-1}$ applied for time periods of 10-30 minutes have been found to be a reasonable starting point for the development of dispersion protocols.

The use of immersion sonication probes should be done with caution as metallic particles can be released due to wear of the probe in some cases already after only a few hours of use of a new probe or probe head. It is advisable that if a laboratory requires to regularly produce nanoparticle dispersions consideration should be given to using a vial- or possibly a cup-sonicator system to avoid the risk of contamination from probe degradation.

In addition to optimising the input of energy to de-agglomerate the solid materials, it may be necessary to take additional steps to ensure that the dispersed particulates resist re-agglomeration for a period of time which is compatible with the measurement technique being used. In the case of aqueous dispersions this may be achieved by natural electrostatic stabilisation, possibly assisted by appropriate modification of the pH, but more commonly it may be necessary to add an appropriate surfactant. The choice of a suitable surfactant will depend on the specific material being tested and in particular prior knowledge about properties such as its hydrophobicity, charge and chemical functionalisation may help in selecting an effective stabiliser. In cases where such information is unknown the use of selected natural polymers such as bovine serum albumin or humic acid may provide a broadly applicable but not necessarily optimal solution for water-based dispersions.

## Sample preparation

- Sample preparation and validated dispersion protocols are pivotal in the process of making unbiased particle size measurements.
- Such procedures should be effective, efficient and reproducible and must not change constituent particle size distribution of the original material.
- Stabilisation of a dispersion and the effectiveness of dispersion protocols should be carefully verified, in terms of disagglomeration/re-agglomeration and of homogeneous distribution/sedimentation.

# 5 Measurement techniques

This section provides brief descriptions about specific measurement techniques that can be used for the determination of the particle size distribution of a material. The section describes those techniques that are considered most applicable to the majority of materials, but it does not aim for completeness. New techniques, and techniques under development, are not mentioned in this section though they may even be preferable or more cost-efficient than the techniques included in this report. In any case, techniques need to be selected bearing in mind both the properties of the material to be investigated and the measurement performance characteristics of the available techniques. An extensive discussion can be found in ref [24 and 25]. Important issues to consider when selecting a technique for a specific sample are:

- **Number of particles investigated by the technique:** A reliable experimental determination of the median, or any other characteristic parameter, of a distribution requires a sufficiently large number of data points (or particles). Some techniques (e.g. DLS, CLS, SAXS) probe ensembles of particles whereby often thousands of particles are measured simultaneously and their composite signal is used to extract a PSD. The obvious advantages of these techniques are the high measurement speed and the robustness of the extracted distributions. The disadvantages are loss of information on individual particles and that results are usually signal intensity-based instead of number-based, as required by the EC NM definition. Other techniques (e.g. PTA, EM) investigate particles individually and construct the particle size distribution from these particle-by-particle based measurement results. The main advantage of these counting techniques is that the derived distributions are intrinsically number-based. However, here, the main point of concern is the minimum number of particles to be counted. Sometimes, it is claimed that at least hundreds or even thousands of particles need to be analysed in order to reliably model the PSD. Instead, the actual minimum number of particles to be analysed strongly depends on the material under investigation (i.e. narrow or broad PSD) and the measurement uncertainty that is required for a reliable final assessment [53]. For example, it has been experimentally demonstrated that for a relatively monodisperse population of titanium dioxide nanorods, as few as 100 particles can be sufficient to obtain robust PSDs [54].
- **Measurements of suspensions or dry particles:** Some techniques measure particles in suspension, others measure in their dry state. While it is of course possible to convert a dry powder into a suspension and (to a lesser degree) a suspension into a dried powder, these steps may change the particles and definitely the aggregation/agglomeration state and may change the apparent particle size (e.g. particles may aggregate/agglomerate, the surface of the particles may be transformed or lost etc.). See also Section 4 for more information.
- **Type of samples that can be measured:** Some techniques can measure a large variety of particle types, whereas others are limited to particles of a certain density, refractive index or chemical composition. With regard to chemical composition, there are some

- techniques that are not suitable for organic particles. Several other techniques are less reliable for particles consisting of light elements (silicon, boron etc.).
- **Ability to deal with aggregates or agglomerates:** Some techniques will measure the size of aggregates and agglomerates rather than the size of the constituent particles, whereas other techniques have the ability to determine the external dimensions of particles within aggregates and agglomerates.
- **Size resolution and size range:** Techniques differ in size resolution (being able to distinguish, e.g. a 60 nm diameter particle from a 70 nm particle) and size range (minimum and maximum size of particles that can be measured). With respect to the size range, nearly all techniques are limited to roughly one order of magnitude for any single measurement. This means that in a given measurement for instance particles from 5 nm to 50 nm or from 50 nm to 500 nm in diameter can be measured, but not from 5 nm to 500 nm.
- **Availability of documentary standards:** Documentary standards harmonise the application of techniques and thus lead to a better reliability and comparability of measurement results. Lack of documentary standards does not render a method unsuitable, but requires more efforts for method validation by the laboratory. Full references to the documentary standards mentioned in this section can be found in the Annex of this report.

There is no technique that combines only advantageous features. Depending on the material, a compromise between required accuracy, cost and sample throughput must be found.

Measurement techniques and methods presented in this section are grouped into two categories. This grouping is done by the reliability of the classification allowed by the method:

**Screening methods:** include measurement techniques that are fast and often inexpensive but still allow for many materials a positive identification as nanomaterial.

**Confirmatory methods:** include measurement techniques that are usually more cost- and time-intensive. They are used when screening methods do not allow classification, as well as in cases of doubt or dispute for which they normally provide a more reliable classification.

## 5.1 Techniques used in screening methods

The interplay between sample preparation, identification of constituent particles and the accurate determination of the external dimensions of these constituent particles requires complex and often time- and resource-intensive measurement techniques. However, of interest is often not the exact number-based distribution as such, but a decision whether a material needs to be classified as nanomaterial or not.

For example for classification as 'nanomaterial' only, it is irrelevant whether the median diameter is 60 nm or 90 nm as in both cases the material in question will be classified as nanomaterial. Similarly, it does not matter whether 20% or 30% of particles have external dimensions between 1 nm and 100 nm, as in both cases the material will be classified as not a nanomaterial.

Likewise, it is acceptable for screening methods to measure something different from the number-based distribution of the external dimension of the constituent particles, as long as there is a reliable correlation with this metric and the external dimension which allow reliable classification as nanomaterial or not a nanomaterial.

For example, mass-based distributions will underestimate the contribution of smaller particles. Therefore, if 50% or more of the mass-based distribution consists of particles smaller than 100 nm in diameter, then the fraction of the number-based distribution will be larger. Therefore, such a method could reliably identify a material as nanomaterial, but it cannot prove that a material is not a nanomaterial. The same reasoning holds true for any other commonly used metric to describe particle size distributions, be they based on particle volume or intensity of scattered light.

Fast and comparatively economic methods may help to screen a large number of materials (hence the term 'screening methods') for falling under the definition. Ideally, a screening method should allow a reliable decision for the majority of materials. Materials for which no decision is possible based on the screening methods must be further investigated using more complex methods. Therefore, screening methods should have the following properties:

- Enable for many materials a reliable decision whether they fall under the EC NM definition.

  Note that current screening methods can only identify nanomaterials without a doubt; their use for classification of materials as not being nanomaterials may not be possible.
- If a decision can be taken, it should be reliable and without false negative assessments, i.e. without classifying a material incorrectly as not a nanomaterial.
- It should be clear when the method or technique is unsuitable for such a reliable decision so as to continue further testing.
- It should be fast and economically feasible to realise the desired cost savings.

The main purpose of screening methods is to implement the EC NM definition in an economically affordable way, i.e. to minimise costs. This can only be achieved if a significant part of materials do not have to be tested again by a confirmatory method, as otherwise the cost of the screening methods is simply added to the cost of the confirmatory method. This section lists possible measurement techniques and describes their scope and limitations. The description of the individual techniques will be kept brief – more detailed descriptions and information concerning measurement principles are given, for example, in Ref [20, 25] and the performance of various techniques was also summarised in Ref [24].

Techniques used for screening purposes do not determine the external diameters directly, but rather measure other material properties and relate these to the dimensions of ideal particles (usually spheres). Descriptive electron microscopy imaging allows a quick check whether the assumptions inherent in the various techniques are approximately met and hence greatly improves the reliability of classification. Contrary to particle size determinations by electron microscopy, descriptive electron microscopy images can be made fast and inexpensively.

The EFSA guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain requires electron microscopy to be used as one technique to characterise the particle size of engineered nanomaterials [5].

A factor encountered most often is the fact that a certain technique cannot distinguish between individual (constituent) particles and agglomerates/aggregates, which would often result in a false negative classification as ‘not a nanomaterial’.

### 5.1.1 Dynamic light scattering (DLS)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. All materials (organic, inorganic) can be measured. As for analysis of any suspension, reliable results depend on obtaining stable suspensions. Re-agglomeration or sedimentation of large particles/agglomerates/aggregates makes results invalid.

Particle sizes that can be measured: 1 nm to 10 µm with the best results above 10 nm. Larger particles give a much stronger signal than smaller particles, so this size range refers to materials with a relative narrow size distribution (about one order of magnitude). For more polydisperse samples, the fraction representing the smaller particles will be under-represented.

**Measurement principle:**

DLS measures the fluctuation of scattered light caused by Brownian motions. From this, it calculates the speed of diffusion of entities from their random movement. These entities can be individual particles, but also aggregates or agglomerates. It does this by measuring many entities simultaneously. The measurement result after having applied specific data evaluation algorithms is the hydrodynamic diameter of equivalent spheres, i.e. the diameter of spheres that would diffuse as fast as the ensembles in the sample.

DLS results are scattered light intensity-weighted, which gives much more weight to larger particles than to smaller ones. The number-based median diameter will always be smaller than the intensity-based diameter given by the instrument.

*Note: Instruments often include the function of converting the intensity-weighted results into a number-weighted one, but this conversion is not reliable and is explicitly deprecated in the respective ISO standard [26].*

**Documentary standards available:** ISO 22412

**Main advantages and disadvantages:**

- ☺ Possibility to measure particles smaller than 10 nm in diameter
- ☺ Fast and robust when using the best established evaluation algorithm
- ☹ Does not yield external dimensions but equivalent hydrodynamic diameters
- ☹ Measures the size of aggregates/agglomerates and not their constituent particles
- ☹ Does not provide number-based distributions at a reasonable measurement uncertainty
- ☹ Low particle size resolution

**Relation of the result to the parts of the definition:**

External dimensions: DLS does not measure the actual external dimensions of the particle, but it determines the hydrodynamic size of an equivalent sphere. The smallest external dimension is usually smaller than this diameter.

Constituent particles: DLS measures the size of ensembles moving together, i.e. aggregates and agglomerates are regarded as one particle. This means that the size of the constituent particles can never be larger than the size of the entities measured by DLS. If the sample contains aggregates and/or agglomerates, the size of the constituent particles is considerably smaller than the size determined by DLS.

Number-based diameters: DLS gives results in intensity-weighted diameters, which is always larger than the number-based diameter.

**Possible outcome:**

All metrics determined by DLS are always larger (often significantly larger) than the number-based median of the external dimensions of the constituent particles. Inversely, the median of the number-based distribution of the external dimension may be (and usually is) smaller, but is never larger than the result given by DLS. This means that if the intensity-based average equivalent diameter measured by DLS is below 100 nm, the material is certainly a nanomaterial.

If DLS gives an average diameter above 100 nm, further measurements by other techniques are needed as the median diameter of the number-based distribution may be considerably smaller.

### 5.1.2 Centrifugal liquid sedimentation (CLS) / analytical ultracentrifugation (AUC)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. All materials (organic, inorganic) can be measured. The effective density of particles must be different from the density of the dispersing liquid. Most instruments require that the particle density is higher than the density of water, which can make the measurement of organic particles difficult (note that some instruments also allow measurement of particles with a lower density than the surrounding liquid).

The effective density of all particles must be uniform and known.

As for analysis of any suspension, reliable results depend on obtaining stable suspensions from which the test sample is taken. Re-agglomeration of large particles/agglomerates/aggregates makes results invalid as does sedimentation prior to measurement.

Particle sizes that can be measured: Below 5 nm (if high density material) to 100 µm. Particularly for disc- and cuvette-type analytical centrifuges (i.e. CLS) measurement of particles with diameters smaller than 5 nm are usually problematic due to the very long sedimentation time.

**Measurement principle:**

The CLS/AUC technique uses the fact that large particles sediment faster than small particles of the same density. The particles are sedimenting in a centrifugal field and the sedimentation time is measured. Using the known or assumed effective particle density and the known centrifugal force, the Stokes diameter, i.e. the diameter of spheres that would sediment at the same velocity is calculated.

*Note: Instruments often include the function of converting the intensity-weighted results into a number-weighted one, but this conversion is only reliable for simple particle systems.*

The centrifugation principle is applied by two closely related groups of techniques namely analytical centrifugation (CLS) with a cuvette or disc and analytical ultracentrifugation (AUC). The difference between the two is that AUC uses a much higher rotational speed. In addition, the only commercial producer of AUC also uses a different type of detector than those used by analytical centrifugation equipment.

**Documentary standards available:** ISO 13318-1, ISO 13318-2 and ISO 13318-3

**Main advantages and disadvantages:**

- ☺ Possibility to measure particles smaller than 10 nm
- ☺ Good size resolution
- ☺ For AUC only: Applicable to broad size distributions from 5 nm to 100 µm
- ☹ Does not yield external dimensions but equivalent diameters
- ☹ Measures the size of aggregates/agglomerates rather than constituent particles
- ☹ Does not provide number-based distributions at a reasonable measurement uncertainty

**Relation of the result to the parts of the definition:**

External dimensions: CLS/AUC does not measure actual external dimensions but gives the diameter of equivalent spheres, i.e. spheres that sediment as fast as the particles measured.

Constituent particles: CLS/AUC measures the equivalent diameters of sedimenting ensembles, i.e. aggregates and agglomerates are regarded as one particle. This means that the size of the constituent particles can never be larger and is usually smaller than the size of the entities measured by CLS/AUC.

Number-based diameters: The results of CLS/AUC are light extinction (or intensity) or mass-based (depending on the detector), which gives larger weight to larger particles.

**Possible outcome:**

The metrics determined by CLS/AUC are larger than the number-based diameter of the external dimensions of the constituent particles. This means that if the mass-based average equivalent diameter measured by CLS/AUC is below 100 nm, the material is certainly a nanomaterial.

If CLS/AUC gives an average diameter above 100 nm, further measurements by other techniques are needed as the median diameter of the number-based distribution may be considerably smaller.

### 5.1.3 Tunable resistive pulse sensing (TRPS)

**Applicable to:**

Particles in suspension, size range: 40 nm to 10 µm. The suspension must be compatible with an electrolyte (i.e. in aqueous suspension or in a solvent miscible with aqueous solutions).

**Measurement principle:**

TRPS utilises a particle's movement through a size-changeable pore. Particles must be suspended in an electrolyte, as the ionic current is measured. When a particle passes through the pore, it creates a reduction in the ionic current flow, which is registered as a blockade (resistive pulse signal). Its magnitude is proportional to the particle's volume. If the size of the pore is precisely controlled and calibrated, for example with a particle size standard, then the size and concentration can be measured simultaneously on a particle-by-particle basis.

**Documentary standards available:** none

**Main advantages and disadvantages:**

- ☺ Yields a number-based size distribution of spheres of equivalent volume
- ☺ Gives a number concentration (particles/mL)
- ☺ Good size resolution
- ☹ Size range limited to particles larger than 40 nm
- ☹ Does not measure external dimensions directly, but gives the size of spheres of equivalent volume
- ☹ Cannot distinguish between aggregates/agglomerates and constituent particles

**Relation of the result to the parts of the definition:**

External dimensions: TRPS measures the particle volume, hence gives the diameter of spheres of equivalent volume. This diameter is larger than the smallest external diameter of a particle.

Constituent particles: TRPS measures the volume of ensembles that move together, so aggregates/agglomerates cannot be distinguished from constituent particles. Hence the diameter obtained by TRPS can never be smaller than the diameter of the constituent particle.

Number-based diameters: It is directly achieved from the TRPS measurements.

**Possible outcome:**

The fact that aggregates and agglomerates are seen as individual particles biases the results towards higher values. Similarly, the diameter of a sphere of equivalent volume is larger than the smallest external dimension of the particle. Furthermore, most particles < 40 nm in diameter are not detected. This means that if TRPS yields a median diameter of less than 100 nm, the material is a nanomaterial. No reliable conclusion can be drawn for materials for which TRPS yields a median diameter larger than 100 nm. In this case, further measurements with confirmatory methods are needed.

### 5.1.4 Particle tracking analysis (PTA)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. Most materials (organic, inorganic) can be measured.

As for analysis of any suspension, reliable results depend on obtaining stable suspensions. Re-agglomeration or sedimentation of large particles/agglomerates/aggregates makes results invalid.

Particle sizes that can be measured: 10 nm to 1 µm. The ideal size range for the technique is between 100 nm and 1 µm, whereas particles below 30 nm are often not measurable, unless they scatter light strongly. The stated size range also refers to materials with a narrow size distribution. For materials with a broad size distribution (larger than one order of magnitude), smaller particles can be very hard to detect.

**Measurement principle:**

PTA uses a microscope to take videos of entities (individual particles, agglomerates, aggregates) that move under Brownian motion. If the magnification is known, the speed of each entity can directly be calculated and converted into a particle's translational diffusion coefficient. Subsequently, the diameter of a sphere that would diffuse with the same speed is calculated. Larger particles are better visible than smaller particles so the technique may miss some or all small particles.

PTA results are number-weighted.

**Documentary standards available:** ISO 19430

**Main advantages and disadvantages:**

- ☺ Yields number-based size distributions
- ☺ Among the screening methods with a fairly good size resolution
- ☹ Does not yield external dimensions but equivalent diameters
- ☹ Measures the size of aggregates/agglomerates rather than constituent particles
- ☹ Cannot easily deal with broad size distributions as the fraction of small particles can be underestimated

**Relation of the result to the parts of the definition:**

External dimensions: PTA does not measure the external dimensions of the particle, but it determines the hydrodynamic diameter of an equivalent sphere, i.e. the diameter of spheres that diffuses with the same speed.

Constituent particles: PTA measures the size of ensembles moving together, i.e. aggregates and agglomerates are regarded as one particle. This means that the size of the constituent particles can never be larger and is usually smaller than the size of the entities measured by PTA.

Number-based diameters: PTA measures number-based diameters. Due to the better visibility of larger particles, smaller particles might be under-represented, hence shifting the measurement results to larger diameters.

**Possible outcome:**

The metrics determined by PTA are larger than the number-based diameter of the external dimensions of the constituent particles. This means that if the number-based median equivalent diameter measured by PTA is below 100 nm, the material is certainly a nanomaterial.

If PTA gives a median diameter above 100 nm, further measurements by confirmatory techniques are needed.

### 5.1.5 Single particle inductively coupled plasma mass spectrometry (sp-ICP-MS)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. The technique is limited to inorganic particles/particles containing a high fraction of elements heavier than neon (Ne) and of known composition.

As for analysis of any suspension, reliable results depend on obtaining stable suspensions. Re-agglomeration or sedimentation of large particles/agglomerates/aggregates makes results invalid.

Particle sizes that can be measured: 10 nm to 1 µm. The actual lower size limit depends strongly on the material. Gold particles as small as 10 nm can be measured, whereas the lower size limit for silica particles is 50 nm [55] . Note that gold particles are the easiest type of particles to measure for sp-ICP-MS (best repeatability, smallest size possible, ease of calibration). This is why most publications show the result on gold nanoparticles, which gives an overoptimistic impression of the applicability of the method.

**Measurement principle:**

ICP-MS is a well-established technique for the determination of elements in which a liquid is introduced into a plasma. This plasma vaporises and ionises the individual atoms which are subsequently measured by a mass spectrometer. In single particle ICP-MS, the suspensions are highly diluted to ensure that particles enter the plasma one after the other. Each particle thus results in a distinct cloud of ions and this allows measuring the number of atoms in each particle. From the number of atoms in each particle, the mass of each particle is calculated and from the mass, assuming a specific geometry (usually spherical), an external dimension of a particle of the same mass is calculated.

From the ensemble of individual particles, the number-based equivalent diameter is calculated.

Knowledge of the chemical composition of a particle is crucial, as not all elements can be determined by ICP-MS. Elements that cannot be measured (e.g. the oxygen in silica) are added based on the known composition. The SPC "single-particle calculation tool" software [56] allows the determination of the number-based size distribution of nanoparticles in a sample from sp-ICP-MS analysis and can be used with any type/brand of ICP-MS to evaluate single particle ICP-MS data.

**Documentary standards available:** ISO/TS 19590:2017

**Main advantages and disadvantages:**

- ☺ Yields number-based size distributions
- ☺ Good size resolution
- ☺ Specific for chosen elements, hence insensitive to the presence of other, chemically different particles in the sample
- ☹ Does not yield external dimensions but equivalent diameters
- ☹ Measures the size of aggregates/agglomerates rather than constituent particles

- ☹ Does not count small particles ("small" depends on the material and can mean smaller than 10 nm, but also smaller than 50 nm)
- ☹ Cannot be applied to all materials

**Relation of the result to the parts of the definition:**

External dimensions: sp-ICP-MS does not measure the actual external dimensions of the particle, but gives the result of a sphere of the same mass.

Constituent particles: sp-ICP-MS measures the mass of entities entering the plasma simultaneously, i.e. aggregates and agglomerates are regarded as one particle. This means that the size of the constituent particles can never be larger and is usually smaller than the size of the entities measured by sp-ICP-MS.

Number-based diameters: sp-ICP-MS measures number-based diameters. Note that for several industrially important materials (silicon dioxide, titanium dioxide), the limit of detection is around 50 nm. This means that a significant number of small particles might be missed for such materials.

**Possible outcome:**

The metrics determined by sp-ICP-MS are larger than the number-based diameter of the external dimensions of the constituent particles. In addition, for many materials particles smaller approximately 50 nm are not detected and therefore not considered in the median. This means that if the number-based median equivalent diameter measured by sp-ICP-MS is below 100 nm, the material is certainly a nanomaterial.

If sp-ICP-MS gives a median diameter above 100 nm, further measurements by other techniques are needed as the diameter may reflect the size of aggregates/agglomerates rather than constituent particles. In addition, for non-spherical particles, external dimensions may be smaller than for spheres.

## 5.1.6 Small-angle X-ray scattering (SAXS)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. SAXS can in principle also measure powders, but the result is the size distribution of the void between the particles, which is not suitable for the purpose of the EC NM definition.

As for analysis of any suspension, reliable results depend on obtaining stable suspensions. Re-agglomeration or sedimentation of large particles/agglomerates/aggregates makes results invalid.

Particle sizes that can be measured: 1 nm to approximately 100 nm. For materials with a very narrow size distribution, significantly larger particles can be measured.

**Measurement principle:**

X-rays are scattered by the electrons of particles and the scattering angle depends on the wavelength of the light and the size of the particles. The scattering curve (scattering intensity versus scattering angle) is fitted. For regular particles (spheres, discs, cylinders), the particle size distribution is obtained; for irregular particles, a mean radius of gyration is calculated from the fit.

**Documentary standards available:** ISO 17867. The current standard only covers monodisperse particles, but a revision covering the determination of particle size distributions is in development. A second standard covering the determination of the specific surface area by SAXS is under development.

**Main advantages and disadvantages:**

- ☺ Can determine the size of constituent particles in aggregates and agglomerates if the particles in the aggregate/agglomerate are separated by an interface with a lower electron density (e.g. gaps, organic shells)
- ☺ Can be employed to determine the volume specific surface area (VSSA)
- ☹ Limited to particles smaller than 100 nm, produces unreliable results if particles bigger than 100 nm in diameter are measured
- ☹ Determination of external diameters requires assumption of the shape of the material; for irregular particles, an equivalent diameter is obtained
- ☹ Measures the size of aggregates/agglomerates rather than constituent particles (unless the constituent particles are separated by an interphase of lower electron density)
- ☹ Does not provide number-based distributions
- ☹ Low particle size resolution

**Relation of the result to the parts of the definition:**

External dimensions: SAXS does not measure the external diameter of the sample. For regular particles (spheres, discs, cylinders), the dimensions of the chosen model are derived. For irregular particles, the result is the radius of gyration, i.e. the radius of a sphere that would have the same moment of inertia as the measured particle.

Constituent particles: A "particle" for SAXS is anything that is not separated by another (dispersing) phase, so aggregates and agglomerates are regarded as single particles. This means that the size of the constituent particles can never be larger and is usually smaller than the size of the entities measured by SAXS.

Number-based diameters: SAXS measures basically scattering-intensity based equivalent diameters. The exact metric, however, depends on the evaluation algorithm used.

**Possible outcome:**

The metrics determined by SAXS are larger than the median of the external dimension of the constituent particles. It is therefore tempting to conclude that if SAXS determines the mean diameter as below 100 nm, the material is a nanomaterial.

However, SAXS is limited to particles smaller than 100 nm (with advanced instrumentation pushing the upper limit to 400 nm) and can produce unreliable results if particles bigger than 100 nm in diameter are measured.

This makes the conclusion more complicated, but one can state:

- if the particle size distribution as determined by SAXS has a maximum below 100 nm, then the material is most likely a nanomaterial. The maximum below 100 nm indicates that the majority of particles have been covered and missing the particles larger than 100 nm will not lead to a different conclusion, unless the material is bimodal with the main mode (in terms of particle numbers) larger than 100 nm.
- if the particle size distribution obtained by SAXS does not have a max

imum below 100 nm, no conclusion can be drawn, as it is unknown how many particles are larger than 100 nm.

### 5.1.7 Asymmetrical-flow field-flow fractionation coupled with multi-angle light scattering (AF4-MALS)

**Applicable to:**

Type of samples: Suspensions; powders that can be suspended in liquids. As for analysis of any suspension, reliable results depend on obtaining stable suspensions.

Particle sizes that can be measured: Basically from 30 nm to 50 µm. The actual lower size limit depends strongly on the material – for various particle types the size limit may differ. The upper limit represents the typical inversion point where the normal or Brownian mode elution changes to the steric or hyperlayer elution mode.

**Measurement principle:**

Asymmetrical-Flow Field-Flow Fractionation (AF4) is a separation technique, where particles are separated according to their hydrodynamic diameters. A suspension of particles flows through a narrow channel while a secondary flow (cross-flow) is applied perpendicular to the main flow direction. The external field forces the particles, which move in a laminar flow, close to the membrane which is attached to the bottom channel wall. In the normal or Brownian mode, the smaller particles diffuse (against the field force) to the centre of the flow so they move faster than bigger particles. This leads to a separation in time, as particles with a smaller hydrodynamic diameter emerge faster at the end of the channel than larger particles. After passing through the AF4 channel, the individual particle fractions are basically monodisperse in nature. Individual fractions can be collected and measured off-line or an on-line detector can be applied. However, numerous factors other than particle diameter can influence the elution time of particles, even leading to cases where larger particles elute earlier than smaller ones [57]. Therefore, method development in AF4 is critical and the applicability of a given setup to not yet tested particles has to be carefully investigated.

One way of obtaining particle size information from AF4 data is by calibrating the retention time response, monitored with monodisperse particles of known size. While this approach typically yields a low LOD, the main disadvantage is that this calibration is strictly only valid for materials of the same chemical nature (i.e. if the retention times are calibrated with polystyrene latex particles, the calibration function is only valid for polystyrene latex particles). The relative amount of each particle fraction is derived from the intensity of the signal.

An alternative method is to use a size specific detector such as multi angle light scattering (MALS), generating the combination AF4-MALS. For particles which are sufficiently small compared to the wavelength of the incident light, particle size information can be determined directly from multi-angle light scattering data using the angular dependency of the scattered light intensity. For each time slice, the radius of gyration, or the root-mean-square diameter, can be calculated. As after separation by AF4 the fractions are near monodisperse, the diameter is more reliable than a diameter determined from a polydisperse sample. The major disadvantage is that it is difficult to derive a PSD of the whole sample from the PSDs at each time point.

Apart from MALS, also other detectors like DLS, ICP-MS and sp-ICP-MS (described above) can be used. Also the use of these detectors benefits from the monodispersity of the sample after passing through the AF4 channel, but also share the problem of constructing an overall PSD of the sample from the PSDs at the various time points.

For very small nanoparticles (typically less than 20 nm in diameter) or for particles whose size approaches the wavelength of the incident light the angular variation of the scattered light intensity may be too small to reliably derive particle size information.

**Documentary standards available:** ISO/TS 21362:2018 Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation (note that the coupling with MALS is not described)

**Main advantages and disadvantages:**

- ☺ Good size resolution
- ☺ Suitable for polydisperse samples
- ☹ Does not yield external dimensions but equivalent hydrodynamic diameters and/or radii of gyration
- ☹ Does not distinguish between constituent particles and aggregates/agglomerates
- ☹ Does not provide a number-based distribution

**Relation of the result to the parts of the definition:**

External dimensions: The detection by MALS does not measure the actual external dimensions of the particle, but determines the hydrodynamic size of. an equivalent sphere, and is more sensitive to larger particles than small ones. However, since A4F provides a separation into quasi monodisperse fractions, this low sensitivity for smaller particles is less problematic than for DLS.

Constituent particles: The A4F technique measures the size of ensembles that move together, so aggregates and agglomerates are seen as single particles. The same is true for MALS, which gives the radius of gyration of the aggregate/agglomerate.

Number-based diameters: AF4-MALS gives results as light intensity-weighted diameters.

**Possible outcome:**

The fact that aggregates and agglomerates are seen as individual particles biases the size information towards high values. In addition, the light intensity-weighted distribution obtained either from the MALS signal, or from any other AF4 detector, gives higher weight to the larger particles than the number-weighted distribution. The median of the number-based distribution of the external dimensions of constituent particles is therefore smaller than the median diameter as obtained by AF4-MALS. This means that a median diameter of less than 100 nm as obtained by AF4-MALS classifies a material as a nanomaterial. No conclusion can be drawn for median diameters larger than 100 nm.

## 5.1.8 Differential electrical mobility analysis on sprayed suspensions (spray-DEMA)

**Applicable to:**

Type of samples: Powders that can be dispersed in air; suspensions with a low load of salts/other non-particle constituents.

*Note: Suspensions are turned into an aerosol and the liquid is evaporated. This means that all solid constituents (salts, sugars, etc.) will either become small particles themselves or increase the diameter of the particles in suspension.*

As for analysis of any suspension, reliable results depend on obtaining stable suspensions. Re-agglomeration or sedimentation of large particles/agglomerates/aggregates makes results invalid.

Particle sizes that can be measured: 3 nm to 1 µm.

**Measurement principle:**

The sample is turned into an aerosol either by direct dispersion of a powder into air or by spraying a suspension of the powder into air. The thus generated particles are then electrically charged and classified according to their mobility in an electric field: larger particles move slower. At the end of the classifier, the particles are detected and counted. Detection is based either on particle counters or by depositing particles inside a faraday cup and measuring the deposited charge.

**Documentary standards available:** ISO 15900

**Main advantages and disadvantages:**

☺ Yields number-based size distributions

☺ Good size resolution

☺ Applicable to broad size distributions from 3 nm to 1 µm

☹ Does not yield external dimensions but equivalent diameters

☹ Measures the size of aggregates/agglomerates rather than constituent particles

☹ Particles larger than 1 µm are removed and therefore not included in the count

☹ Spray-dried aerosol samples may contain nanosized contaminant particles generated from previously dissolved species

**Relation of the result to the parts of the definition:**

External dimensions: spray-DEMA does not measure the external dimensions of particles but measures the equivalent electrical mobility diameter. Dissolved substances in a suspension will add to a particle mass and may lead to biased results, especially for very small particles for which the addition of the dissolved substances changes the mass significantly.

Constituent particles: spray-DEMA measures all particles that move together. Aggregates or agglomerates in the aerosol are regarded as single particles. This means that the size of the constituent particles can never be larger and is usually smaller than the size of the entities measured by spray-DEMA.

Number-based diameters: Results are generally number-based, either because a particle counter is used as detector or because of the equal charge of each particle.

**Possible outcome:**

As spray-DEMA measures aggregates and agglomerates as single particles, the median number-based diameter of the particles measured by spray-DEMA is larger than the median number-based external dimension of the constituent particles. This means that if the number-based median equivalent diameter measured by spray-DEMA is below 100 nm and one is sure that most particles are smaller than 1 µm (and hence not removed in the measurement process), the material is certainly a nanomaterial.

If spray-DEMA gives a median diameter above 100 nm, further measurements by other techniques are needed as this may reflect the diameter of aggregates/agglomerates rather than the constituent particles. In addition, for non-spherical

## 5.1.9 Extrapolating results from screening methods

The sections "possible outcome" in the description of the individual methods stated which conclusions can be derived with certainty from screening methods under the assumption that the sample preparation was adequate.

New scientific knowledge may expand the possibility to use the results from screening methods compared to what can be concluded with certainty today. For example, 15 industrial materials that differed in shape/size and chemical composition were tested with various screening methods. Those materials consisted of particles with near-equiaxial shape, their non-agglomeration/aggregation state could be confirmed (e.g. by descriptive EM) and their equivalent median diameter as determined by various screening methods was larger than 250 nm. By comparison with TEM results they could be reliably classified as not nanomaterials [17]. However, when results are obtained using screening methods the user needs to decide whether the faster analysis and lower measurement cost justifies the risk of false conclusions, as the definitive decision would be based on the evaluation of the number-based distribution of the external dimensions.

## Techniques used in screening methods

- Several methods exist that allow a relatively inexpensive and fast assessment of particle sizes.
- The methods usually do not determine the number-based distribution of the external dimension(s).
- They allow confirming that a material should be classified as a nanomaterial if the measured median diameter is less than 100 nm.
- If the measured median diameter is larger than 100 nm, no conclusion with respect to the EC NM definition can be drawn and confirmatory methods must be used if no further information on the material is available.

## 5.2 Techniques used in confirmatory methods

Confirmatory methods are needed when screening methods yield results which are not suitable for classification according to the EC NM definition and when there is doubt or dispute. In such cases they normally provide a more reliable classification than screening methods. For materials that have an $x_{50,0}$ close to 100 nm, and thus can be considered as borderline cases, confirmatory methods should provide a reliable classification. Confirmatory methods, which are often used for an in-depth characterisation of the particle size distribution of a material, may also simply be chosen from the very beginning or at every step of the classification process.

Confirmatory methods should be applicable to identify particles with non-equiaxial shapes and to determine their external dimensions. They should also be able to identify constituent particles within agglomerates and aggregates and to measure their external dimensions. Materials with polydisperse and multimodal particle size distributions also require more sophisticated methods for a correct assessment against the criteria of the EC NM definition. In such challenging cases mature and very well established techniques are needed for which documentary standards and preferentially also CRMs are available.

Confirmatory methods should yield the particle number-based distribution of the external particle dimensions as raw data to avoid systematic errors and large uncertainties resulting from conversion from another metrics (volume, mass). In all cases, the measured size range should be large enough to allow a classification of the analysed material.

It must be kept in mind that results obtained with confirmatory methods still come with an uncertainty even if they are considered more reliable than screening methods. The uncertainty depends on several factors such as the applied SOP, including sampling, sample preparation, measurement procedure and data analysis, but also on the intrinsic properties of the analysed material. As a consequence there may be cases for which not even these techniques lead to an unambiguous classification whether a material is a nanomaterial or not a nanomaterial according to the EC NM definition.

### 5.2.1 Electron microscopy

**Applicable to:**

Type of samples: Most instruments analyse the sample in high-vacuum chambers; for this the samples must be dry powders. Suspensions can be dried, but with the risk that the particle size distribution is modified. Advanced EM techniques (cryo-TEM, environmental SEM) can be used for biological samples or non-dry samples. All materials can be measured, for reasons of image contrast particles containing heavier atoms are better imaged. Samples need to be compatible with high vacuum (standard EM setup) and electron beam bombardment. TEM can under suitable conditions identify constituent particles in agglomerates and aggregates as well as particles on their own.

Particle sizes that can be measured: Below 1 nm to 1000 µm.

**Measurement principle:**

In EM, the specimen is bombarded with a fine electron beam. In Scanning Electron Microscopy (SEM) the beam is focused on the sample and scanned over a defined area of the sample, whereas in Transmission Electron Microscopy (TEM) part of the electron beam passes through a very thin specimen. Low-energy secondary electrons (SE) are released after inelastic collisions with the atoms in the specimen as well as high-energy backscattered electrons (BSE) after elastic collisions. The effective information range carried by the electrons released from the specimen's interaction volume varies from micrometres (typically for BSE) down to nanometres (typically for SE), depending on their kinetic energy. In SEM, images are constructed based on electrons coming from the sample surface, whereas in TEM, images are constructed based on electrons passing through the samples. Both SEM and TEM give 2-dimensional projections of 3-dimensional particles.

EM images facilitate the determination of number-weighted size distributions by analysing identifiable particles individually. EM also allows an assessment of the morphology of particles. The analysis of flat particles (e.g. disks, flakes) is challenging because the smallest dimension of the particle is not easily accessible for analysis by EM. The determination of a size distribution with EM relies on counting individual particles. Depending on the number of nanoparticles acquired in an image, several images can be necessary for a good counting statistics. Tools such as motorised stage, sequential image acquisition as well as automatic processing of stacks of images should be used to speed up the analysis.

Whereas in an SEM typical beam voltages of up to 30 kV are applied and SE/BSE are collected by various detectors, in TEM the beam voltage reaches 300 kV. For TEM the samples to be analysed must be thin enough to allow the electrons to be transmitted. A spatial resolution of less than 1 nm can be attained if high-performance aberration correctors are used. SEM may be operated in the transmission mode (TSEM), which allows obtaining images of a spatial resolution comparable with TEM, in particular for determination of constituent particles in aggregates and agglomerates, but it is less costly than TEM.

Certain non-commercial software tools specifically developed to determine the number particle size distribution for implementation of the EC NM definition are available, e.g. the ParticleSizer script (plug-in for ImageJ) [58]. The tool provides different splitting methods to handle agglomerates and aggregates, robust handling of different noise levels and adaptability to non-standard images. The script yields a variety of particle size and shape parameters, including the number-based particle size distribution based on the minimum Feret diameter.

The Auto-EM toolbox is an open-source software package that automatically acquires and analyses TEM images [59]. It reduces the user-biased uncertainty of the number-based particle size distribution obtained from image analysis. The only user input consists of a selection of a large area to be imaged in more detail and a set of specific input parameters such as an image overlap or particle number limits.

**Documentary standards available:** see Table A.4

**Main advantages and disadvantages:**

☺ Yields number-weighted size distributions

☺ Size and shape can be measured on 2-dimensional images

☺ In many cases can identify and measure the size of constituent particles in agglomerates and sometimes even in aggregates

☺ Sub-nanometre resolution for TEM, nanometre resolution for SEM

☺ Access to smallest dimension of particles (but only in the projected plane)

☺ Automated image processing available and in further development

☹ Performance strongly dependent on sample preparation

- ☹ Needs vacuum and expensive instrumentation
- ☹ Limited dynamic range (ratio largest size to smallest size < 40) based on images obtained at one magnification only

**Relation of the result to the parts of the definition:**

External dimensions: SEM/TEM measures external dimensions of particles and can provide a variety of descriptors for it.

Constituent particles: SEM/TEM can distinguish individual particles from agglomerates/aggregates and can identify constituent particles within agglomerates/aggregates under favourable conditions.

Number-based diameters: SEM/TEM provides number-based size descriptors, e.g. diameters.

**Possible outcome:**

SEM/TEM can directly provide number-based particle size distributions which allow an assessment whether the material is a nanomaterial.

### 5.2.2 Atomic force microscopy

**Applicable to:**

Type of samples: Dry powders and suspensions (particles need to be immobilised on a flat support surface). Almost any material can be measured. Particles must be well dispersed on a support. The technique cannot identify constituent particles in agglomerates and aggregates. The most reliable measurand is the ‘height’ of a particle in the direction normal to the support surface as the shape of the probe has the least influence on the outcome. The roughness of the substrate must be significantly smaller than the size of the nanoparticles being measured.

Particle sizes that can be measured: 1 nm to <10 µm (particle height, highest resolution in the direction normal to the surface), 10 nm to 100 µm (lateral size, depends on tip geometry)

**Measurement principle:**

Atomic force microscopy (AFM), which belongs to the group of scanning probe microscopy (SPM) techniques is a technique where a sharp tip is fixed on a cantilever and moved along a (support) surface to obtain an image of the surface topology. The AFM can be operated in several modes. In general, imaging modes are divided into contact modes and non-contact modes where the cantilever is vibrated. The shape of the tip as well as the substrate can influence the AFM images.

Particles need to be immobilised on a flat surface in order to be characterised without being moved by the tip. AFM is an imaging technique and can measure the size of particles that are polydisperse in terms of size and/or shape. Organic particles can also be analysed with this technique.

Depending on the mode, forces that are measured in SPM include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces, etc. As well as force, additional quantities may simultaneously be measured through the use of specialised types of probe.

The results of imaging techniques are (mainly) number-weighted particle size distributions. It means that the sample size (number of probed particles) should be sufficiently high for ensuring low uncertainty in size class frequencies. Moreover, the sample size required to achieve a certain confidence level increases with polydispersity.

**Documentary standards available:**
ASTM E2859 - 11(2017)

**Main advantages and disadvantages:**

- ☺ Yields number-weighted size distributions
- ☺ Facilitates determination of particle size and shape as well as surface properties
- ☺ Access to the minimum dimension of a particle
- ☺ Measures a wide range of materials
- ☺ Well suitable for measuring the thickness of platelets
- ☺ Instruments are widely available
- ☹ Strongly dependent on sample preparation (immobilised particles on substrate need to be representative for the material)
- ☹ Lateral size measurements are less reliable than particle height measurements (dependent on tip conditions)
- ☹ Very time consuming: low sample throughput, slow measurement technique
- ☹ Limited dynamic range

**Relation of the result to the parts of the definition:**

External dimensions: AFM measures external dimensions; the best resolution is achieved in the direction normal to the surface on which the particles are immobilised.

Constituent particles: AFM can only qualitatively distinguish between individual particles and agglomerates/aggregates. The size of constituent particles within aggregates/agglomerates cannot be measured reliably.

Number-based diameters: AFM provides number-based size distributions/descriptors.

**Possible outcome:**

AFM can directly provide number-based particle size distributions which allow an assessment whether the material is a nanomaterial.

## Techniques used in confirmatory methods

- Confirmatory methods are used for an in-depth characterisation of the particle size distribution of a material.
- They are needed when screening methods cannot be applied or do not provide the necessary results for a reliable classification.
- They should be able to identify and measure constituent particles within aggregates and agglomerates.
- The methods must be capable to deal with non-spherical particles and polydisperse particle size distributions.
- They should provide the number-based particle size distribution as raw data

## TABLE 5.1

Overview of methods

|  | Method | Size distribution of raw data | Measured sample type[$] | Material type |  |  | Can deal with challenges of particular types of particles* |  |  |  | Documentary standards availability |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  | Inorganic | Organic | Composite‡ | Elongated | Flat | Poly-dispersity | Constituent particles in aggregates / agglomerates |  |
| Screening methods | **TRPS** | number-based | suspension | ++ | ++ |  | -- | -- |  | -- | - |
|  | **Spray-DEMA** | number-based | suspension | ++ | ++ | ++ | -- | -- | + | -- | ++ |
|  | **PTA** | number-based | suspension | ++ | + | ++ | -- | -- | + | -- | + |
|  | **sp-ICP-MS** | number-based | suspension | + | - | o | -- | -- |  | -- | ++ |
|  | **AUC** | mass based | suspension | ++ | o | + | -- | -- | + | -- | ++ |
|  | **SAXS (Guinier)** | mass based | suspension | ++ | o | + | - | - | o | -- | + |
|  | **CLS** | light extinction-based | suspension | ++ | o | + | -- | -- | + | -- | ++ |
|  | **AF4-MALS** | scattered light intensity based | suspension | ++ | ++ | + | -- | -- | + | -- | o |
|  | **DLS** | scattered light intensity-based | suspension | ++ | ++ | + | -- | -- | -- | -- | ++ |
|  | **BET** | no distribution | dry powder | ++ | - | + | -- | -- | -- | -- | ++ |
| Confirmatory methods | **SEM** | number-based | dry powder | ++ | + | + | ++ | o | + | + | + |
|  | **TEM** | number-based | dry powder | ++ | + | + | ++ | o | + | + | ++ |
|  | **AFM** | number-based | dry powder | ++ | o | + | + | + | + | -- | + |

[$] sample preparation issues should be considered, *scale: ++ = very well, + = well, o = moderate, - = not well, -- = not possible, ‡ A composite is a solid material where each particle consists of two or more phase-separated constituents (e.g. core/shell, multi-layered, functionalised particles) [60].

# 6 Volume specific surface area as proxy for nanomaterial identification

The EC NM definition includes a criterion based on the ‘specific surface area by volume’ (VSSA). If the VSSA is above 60 $m^2/cm^3$ then the material may be classified as a nanomaterial.

Using VSSA instead of counting techniques such as electron microscopy to identify nanomaterials is attractive for several reasons. For example, VSSA determination on the basis of gas adsorption measurements has the advantage over imaging and other counting techniques that it does not involve dispersion protocols. Gas adsorption techniques, e.g. the Brunauer-Emmett-Teller (BET) technique, are widely used, and have a relatively low cost, results are already available for many commercial materials, and the same equipment allows a deeper analysis by full gas adsorption isotherm evaluation. Moreover, VSSA (as determined by BET) covers a wide particle size range, from 1 nm to 10 µm diameter, and requires only a relatively straightforward sample preparation procedure. Identification of nanomaterials by VSSA would therefore be an economic approach, particularly for materials for which a determination of the number-based particle size distribution is difficult, e.g. materials containing agglomerates/aggregates or platelet-like particles.

For the purposes of the EC NM definition it is first of all important to understand that VSSA should not be regarded as an independent criterion to identify nanomaterials. This can be seen from Recital (13) of the EC NM definition [1], according to which the VSSA measurement serves as a proxy method to identify a potential nanomaterial. A proxy is an indirect measure of the desired property which is itself strongly correlated to that property. Therefore it is useful to consider the correlation between the number-based size distribution and the VSSA to understand the conditions under which the VSSA is reliably applicable as proxy for the number-based size distribution.

The concepts of Specific Surface Area (SSA) and Volume Specific Surface Area were already outlined elsewhere [9].

The VSSA is defined as

$$VSSA = \frac{SA}{V} = \frac{\Sigma_i\, sa_i}{\Sigma_i\, \mathbf{v}_i} = SSA \times \rho \qquad (1)$$

Here *SA* is the surface area of a sample, *V* the sample volume, $sa_i$ and $\nu_i$ the surface area and the volume of particle number *i*, SSA is the specific surface area and $\rho$ is the appropriate material density value.

It is important to note that the overall VSSA of a powder material is not the statistical average of the VSSA of the individual particles, and in order to calculate the overall sample VSSA it is necessary to sum all the individual particle surface areas and divide this by the sum of all the particle volumes. If the constituent particle shapes and the size distribution are known with sufficient accuracy then the overall sample VSSA can be calculated if there is no particle aggregation (partial fusion of particles). If the ‘degree’ of aggregation is known (i.e. the overall percentage loss of particle surface) a calculation may be made even if the constituent particles are aggregated. The reverse is however not true – a measurement of VSSA contains no detailed information on either the constituent particle shapes or the constituent particle size distribution.

It has to be kept in mind that the definition of the term ‘nanomaterial’ is solely based on the size (i.e. the external dimensions) of the constituent particles [1] and that internal structures are of no relevance [9]. The (total) VSSA in principle can also contain contributions from internal surfaces, such as open pores. Those contributions have to be identified and distinguished from the external VSSA, for which different methods are available [25]. Only the part of the VSSA which is related to the external particle dimensions should be taken into account when using VSSA as a proxy. Classification of a material based on VSSA should in principle be in agreement with classification based on the number based size distribution [9]. In case of non-agreement the classification based on the number-based size distribution takes precedence.

Previous reports and publications have discussed the effects of particle shape on VSSA [7,25,63]. We summarise here the most important points with respect to the EC NM definition and how VSSA can be used in its implementation.

## 6.1 Density values

Assuming that the powder material under investigation is made up of particles with the same composition and crystalline/amorphous structure, the density value used to convert SSA to VSSA is the density of the 'relevant phase'. For example in the case of a solid non-porous $TiO^2$ powder, the density value used to convert the SSA to VSSA should be the density of the $TiO^2$ phase, not the overall 'bulk powder density' which will be considerably lower. Because of the nature of nanomaterials and the fact that their density may differ from tabulated 'true density' values, for example, those in the Handbook of Chemistry and Physics [61], the value often used is the 'skeletal density' as determined by the helium pycnometry method [62,63]. While in many cases this will be equal or close to the known true density value, there are several scenarios where the true density value would be inappropriate. For example,

- if the particles do not all have the same crystalline/amorphous structure the density value to use would be unclear. An example of this is P25 $TiO_2$ where a fraction of the particles have the anatase structure and the rest have the rutile structure, with significantly different densities (rutile: 4.23 g/cm³, anatase: 3.78 g/cm³).
- if the particles are uniformly coated the coated particle density will lie somewhere between the density of the particle core and that of the coating.
- chemical impurities and stoichiometry may also play a role, since the synthesis process of the nanomaterials may result in impure or non-stoichiometric particles, both of which can modify the density of the material.

Therefore, the skeletal density, as determined by gas pycnometry should be used to transform SSA values to VSSA values, unless there is a very strong justification for using another value.

- if there are closed (non-gas-accessible) voids within the particles then these have the effect of reducing the overall particle density.

'Open pores' (i.e. the particles have pores that are not totally enclosed by their walls and are open to the surface either directly or by interconnecting with other pores) increase the surface area and decrease the 'envelope density' of the particles, but they do not in principle affect the skeletal density. The latter is affected only if there is a significant volume fraction of non-gas-accessible voids (or 'closed pores') present within the particles. It may be argued that this would be unlikely for very fine particulate materials. Closed (non-gas-accessible) pores would have the effect of reducing the measured skeletal density and making it diverge from the true density value. However, a simple calculation of the effect of closed pores indicates that these are unlikely to have a large effect on the skeletal density – for example if every particle in a sample of spherical particles with diameter D were to contain ten large closed pores, each of diameter D/10 then the overall effect on the skeletal density would be to reduce it by only 1%. It is unlikely that nanomaterials would exist anywhere approaching such an extreme level of closed void space (unless specifically engineered to include it), so one can assume that the presence of closed pores should not pose a significant difficulty.

## 6.2 Monomodal and monodisperse particles

The threshold of 60 m²/cm³ as given in the EC NM definition is derived for idealised particle systems, where the particles have a specific shape and a known size distribution, such that the criterion that 50% or more of the particles must have one or more external dimensions between 1 nm and 100 nm can be directly translated into a VSSA threshold. A value of 60 m²/cm³ is the theoretical VSSA of a non-porous, perfectly monodisperse material consisting of spherical particles with a diameter of 100 nm or cubic particles with an edge length of 100 nm. In this case, the threshold value of 60 m²/cm³ has a direct relation to the primary (size-based) NM defining criterion and can be considered as the size-based upper VSSA cut-off for such a material: if the VSSA of such a material is larger than 60 m²/cm³, it is a nanomaterial.

Further analysis of the relation (particle size – VSSA) shows that it is possible to derive other, shape specific thresholds. While this is in line with the underlying reasoning for the use of VSSA in the EC NM definition as proxy for particles size, it has to be pointed out that the use of other thresholds is not mentioned explicitly in the EC NM definition and their use to identify nanomaterials is at the discretion of the user.

In order to expand the VSSA concept also to non-spherical particles one can introduce a shape-dependent cut-off value [7,63].

$$VSSA_{cutoff} = 60 \frac{m^2}{cm^3} \ x \ \frac{\delta}{3} \qquad (2)$$

where δ is the number of small dimensions (3 for compact particles, 2 for needles and 1 for platelets). Accordingly, $V_{cutoff}$ is 60 m²/cm³ for spherical particles, 40 m²/cm³ for fibre/needle shaped particles and 20 m²/cm³ for platelets or sheet-like particles. The parameter δ can be adjusted to other values depending on details of the particle shape.

For a monomodal and monodisperse sample of particles with a roughly *spherical shape* it is reasonable to use the relationship VSSA = 6000/D where D is the particle diameter in nanometres and the VSSA is expressed in m²/cm³. In this case D is not the minimum Feret diameter ($x_{Fmin}$) unless the particles are truly spherical. However, the overall effect on the VSSA for a fixed $x_{Fmin}$ of introducing both distortions to an overall spherical shape as well as small topological variations may not be too large and one can assume a rough indicator for $x_{Fmin}$ can be calculated from the VSSA value [25].

For *needle- or fibre-like* shapes with a large aspect ratio (i.e. the length is more than ten times the diameter) which are monodisperse and monomodal in their diameter the relationship VSSA ~ 4000/D can be used, where D is the needle/fibre diameter. In fact if the aspect ratio is 10:1:1 then the VSSA will theoretically be slightly higher than this (42 m²/cm³ for D=100 nm), but any small variations in particle diameter around the median value will result in a slight reduction in VSSA for a fixed D, so the above relationship can be used as a reasonable indicator. In the case of lower aspect ratios one can calculate a 'shape-factor' to relate the VSSA to the needle/fibre minimum cross-sectional diameter, which will usually be the diameter of the minimum inscribed sphere and be equivalent to $x_{Fmin}$.

For *platelet or sheet like* shapes with a large aspect ratio (i.e. the thickness is less than 1/20th of the other two dimensions) and are monodisperse and monomodal in their thickness then the relationship VSSA ~ 2000/D can be used, with D in this case being the platelet/sheet thickness. In fact if the aspect ratio is 20:20:1 then the VSSA will be slightly higher than this (22 m²/cm³ for D=100 nm), but for engineered sheet-like particles with a more extreme aspect ratio (such as those used for some metallic pigments, for example) the above relationship can be used as a reasonable indicator for the average platelet/sheet thickness. As for fibres, in the case of lower aspect ratios one can calculate a 'shape-factor' to relate the VSSA to the platelet/sheet thickness.

There are some particle shapes that have a VSSA higher than that of spherical particles for the same $x_{\mathrm{Fmin}}$ value, most strikingly the tetrahedron, which has a VSSA of about 105 m$^2$/cm$^3$ for $x_{\mathrm{Fmin}}$ =100 nm [25]. Such shapes however require manufacturing using specialised techniques and the classification of such materials against the EC NM definition should be done on a case by case basis, also justifying which dimension has been used for the analysis. In any case, if all the particles in a sample have the same shape and the sample is monomodal and monodisperse, it is straightforward to calculate the relationship between the VSSA and $x_{\mathrm{Fmin}}$.

## 6.3 Polydisperse but monomodal particle size distributions

All the considerations above regarding spherical or needle-/fibre-like or platelet-/sheet-like particle shapes state that the relationships connecting VSSA and external dimension only hold for monomodal and monodisperse particle size distributions. For bi- or multimodal particle size distributions and also for monomodal polydisperse distributions some additional considerations are needed.

For non-porous *spherical* particles, both Gaussian (normal) and lognormal broadening of the particle size distribution around a fixed median value reduce the overall sample VSSA [25]. For a monomodal but broadened (i.e. polydisperse) distribution with either of these distribution shapes, a VSSA of above 60 m$^2$/cm$^3$ will mean that the median value of particle diameter is definitely less than 100 nm. There are distribution shapes that can be constructed that increase VSSA to slightly above 60 m$^2$/cm$^3$ for a median size value of 100 nm or above, but such distribution shapes are unlikely to result from normal particle synthesis processes and the increase of VSSA will be extremely limited if monomodality is maintained.

Conversely, if a VSSA of less than 60 m$^2$/cm$^3$ is measured, one cannot conclude that the material is not a nanomaterial unless an accurate analysis is made of the actual number size distribution and the shape of the particles, and the presence of any population of particles outside those of the main distribution can be excluded. This effectively means that measurement of a VSSA value of less than 60 m$^2$/cm$^3$ on a completely unknown sample cannot be used to show that a material is not a nanomaterial.

A detailed discussion of the relationship between polydisperse but monomodal size distributions of non-porous needle/fibre and platelet/sheet shaped particles can be found in Ref. [25]. It follows from that discussion that if a sample of *fibres* with high aspect ratio (>10:1:1) and monomodal size distribution has a measured VSSA value of above 40 m$^2$/cm$^3$ then it is highly likely that the material fulfils the primary size criterion of the EC NM definition and therefore should be classified as a nanomaterial. For lower aspect ratio fibres, where the size varies but the aspect ratio is fixed, then the threshold above which the material is likely to be a nanomaterial can be calculated. If the VSSA is below the calculated threshold value (i.e. between 40 m$^2$/cm$^3$ and 60 m$^2$/cm$^3$ depending on the aspect ratio) then no definite conclusions can be drawn without additional information.

Furthermore, if a sample of *platelet-/sheet-like* particles with high aspect ratio (>20:20:1) has a measured VSSA value of above 20 m$^2$/cm$^3$ then it is highly likely that the material fulfils the primary size criterion of the EC NM definition and therefore should be classified as a nanomaterial [25]. For lower aspect ratio platelets, where the size varies but the aspect ratio is fixed, then the threshold above which the material is likely to be a nanomaterial can be calculated. If the VSSA is below the calculated threshold value (i.e. between 20 m$^2$/cm$^3$ and 60 m$^2$/cm$^3$ depending on the aspect ratio) then no definite conclusions can be drawn without additional information.

It can additionally be concluded, that for non-porous high aspect ratio platelet/sheet shaped particles that have a reasonably similar thickness, and with random variation in larger dimensions, a measurement of the VSSA can yield a reasonable estimation of the median platelet/sheet thickness. This is true provided that there are no populations of particles with other dimensions (or other shapes) present and that there is no aggregation or significant agglomeration.

## 6.4 Bi- and multi-modal particle size distributions

In the case of multimodal distributions of spherical particles, the VSSA will generally be dominated by the larger size (lower VSSA) mode unless the modes are not too dissimilar in size. Calculations have been published showing how the ratio of overall sample VSSA to the VSSA of the larger size mode alone is affected by the addition of different numbers of smaller particles of various diameters in a perfectly bimodal situation [25]. It can be concluded that also for bi- and multimodal samples of non-porous spherical particles a VSSA of greater than 60 $m^2/cm^3$ almost certainly indicates that the sample is a nanomaterial by the number-based criterion. Similar reasoning applies to bimodal and multimodal size distributions of high aspect ratio needle or platelet shaped particles as for spherical particles. VSSA values above 40 $m^2/cm^3$ (for needle shaped particles) or 20 $m^2/cm^3$ (for platelet shaped particles) may be used as a reasonably reliable indicator that the overall material is a nanomaterial according to the number-based size criterion. Conversely, in general a VSSA value below such a threshold cannot be used to decide that a material is not a nanomaterial. For a detailed discussion on relations between the VSSA and particle size distributions see ref. [25].

## 6.5 Application of VSSA beyond the EC NM definition

Recital (13) of the EC NM definition states that “it should not be possible to use the specific surface area to demonstrate that a material is not a nanomaterial”. On the other hand, the same recital also calls to expand the possibility to use VSSA and other methods in future, as “new scientific knowledge may expand the possibility to use this and other methods … in the future”. Since the adoption of the EC NM definition research has shown that under certain, well defined conditions low or very low VSSA values may give indication that a material is not a nanomaterial. Values of 5 $m^2/cm^3$ or 6 $m^2/cm^3$ [7,63] or even 20 $m^2/cm^3$ [64] were discussed in the literature. The reasoning and approach is detailed in this section. It must be noted that such an approach goes beyond the EC NM definition and should be used with great caution, as it may not be valid in a legal context.

It was already mentioned in [63] that such a low VSSA value is a reliable indicator that a material is not a nanomaterial only if the particle size distribution is monomodal and if the particles are not heavily aggregated. The strict requirement that the size distribution must be monomodal can be explained with the following example. For a monodisperse distribution of spherical particles a VSSA value of 6 $m^2/cm^3$ implies a median diameter of 1000 nm. Adding an equal number of particles (or slightly more) with a diameter of less than 100 nm would not have a significant effect on the measured VSSA value. For example, if the smaller particles had a diameter of 50 nm, the increase in VSSA would be less than 0.25% although such a material is classified as a nanomaterial according to the EC NM definition.

Even if the particle size distribution is monomodal, a low VSSA value alone is not sufficient to show with certainty that a material is not a nanomaterial. Particle size distributions are sometimes assumed to be lognormal to account for skewedness. For spherical particles with a lognormal size distribution, accepting a VSSA of 6 $m^2/cm^3$ as a valid threshold below which the sample would be classified as not being a nanomaterial requires that the mode of the particle size distribution is larger than 38 nm. The reason is that if the median of the sample with a lognormal size distribution is 100 nm it will have a VSSA of less than 6 $m^2/cm^3$ if its maximum (the mode) is smaller than 38 nm and as a consequence it would be falsely classed as not being a nanomaterial. If its mode is larger than 38 nm then (for a median of 100 nm) the VSSA is larger than 6 $m^2/cm^3$. For a VSSA threshold of 20 $m^2/cm^3$ the mode of a lognormal distribution has to be larger than 65 nm to avoid false negative classification, i.e. a nanomaterial as not being a nanomaterial.

An analogous discussion for monomodal materials consisting of elongated or flat particles shows that the corresponding low thresholds below which it can be concluded with reasonable certainty that they are not nanomaterials are 4 $m^2/cm^3$ and 2 $m^2/cm^3$, respectively.

Samples which have a truncated lognormal size distribution, i.e., where there is an upper limit of the particle size, will have larger VSSA values which minimises the risk of false negative classification as ‘not a nanomaterial’. Experience with samples of selected commercial particulate materials did not lead to a false negative classification, if a lower threshold of 6 $m^2/cm^3$ [63] (or even 20 $m^2/cm^3$ [64]) was chosen as limit below which a material would be classified as not a nanomaterial.

It therefore has been proposed that if a material (i) has a strictly monomodal particle size distribution and (ii) is not significantly aggregated and (iii) has a VSSA of less than (a) 6 m²/cm³, (b) 4 m²/cm³, or (c) 2 m²/cm³ and (iv) consists of (a) near-spherical, (b) elongated, or (c) flat particles it is likely that this material is not a nanomaterial. It is up to the user of these empirical findings to decide whether the faster analysis and lower measurement cost for VSSA justifies the risk of false conclusions, as the definitive decision would be based on the evaluation of the number-based distribution of the external dimensions of the material in question.

## 6.6 Possible implications of VSSA measurements

The EC NM definition includes a criterion to use VSSA only for identification of nanomaterials, but it does not contain a VSSA criterion to indicate that a material is not a nanomaterial. Therefore the strategy discussed in the following emphasises the use of VSSA for positive identification of nanomaterials. It also gives suggestions on how to interpret a very low VSSA value in the context of the EC NM definition, while remaining in line with the primary criterion, i. e. the number based particle size distribution.

The VSSA should be measured according to available standards, using validated SOPs [25,65]. If the particles are non-porous and the overall sample VSSA is above 60 m²/cm³ then the sample is classified as a nanomaterial. If porosity of the particles is suspected, standard measurement and data evaluation procedures may be applied to separate the external surface from internal surfaces such as open pores. If the external VSSA is still larger than 60 m²/cm³ then the material is almost certainly a nanomaterial according to the size-based criterion. If the shape, and shape uniformity, of the particles is known, e.g. from electron microscopy, shape dependent thresholds can be applied. If the particles have a very elongated (needle/fibre) shape and the VSSA is larger than 40 m²/cm³ or if the particles are very thin (e.g. flakes) and the VSSA is larger than 20 m²/cm³, then the material is almost certainly a nanomaterial according to the size-based criterion.

A low VSSA value can only be used as an indicator that the material likely is not a nanomaterial if the size distribution is strictly monomodal and is shown not to be very broad, if there is very limited aggregation, and the particle shape is known. Information on the modality of the size distribution and the particle shape may be evaluated from electron microscopy images. As discussed above, if the particle size distribution is monomodal, shape dependent lower thresholds of 6 m²/cm³, 4 m²/cm³ and 2 m²/cm³ for near-spherical, elongated and flat particles indicate that the material likely is not a nanomaterial if its VSSA is below these values.

### Volume specific surface area

- Materials with a VSSA of more than 60 m²/cm³ consisting of non-porous but otherwise unknown particles are classified as nanomaterials.
- A VSSA measurement of less than 60 m²/cm³ on a completely unknown sample cannot be used to conclude that a material is not a nanomaterial according to the EC NM definition.
- In case of doubt classification should be made based on the number-based particle size distribution. The latter overrides the VSSA criterion.
- Materials with high aspect ratios can be considered nanomaterials if they have a VSSA of more than 40 m²/cm³ (needles/fibres) or 20 m²/cm³ (platelets/sheets).
- Lower limits for the VSSA, e.g. 6 m²/cm³, have been proposed for a classification that a material likely is not a nanomaterial.

# 7 Approach to identify nanomaterials

## 7.1 Main steps to be considered in the nanomaterial identification process

Although particle size can be determined by a large variety of analytical techniques, each technique has its region of applicability in terms of material classes, material properties and the accessible size range, including the medium in which the particles are dispersed. None of the available techniques is suitable for all materials. However, if size measurements are performed to fulfil regulatory obligations, the results must be relevant and reliable. To cope with all these challenges it is necessary to understand which techniques can be used for which materials and for which purpose. To select the most appropriate technique(s) one should match material properties with the regions of applicability (Figure 2.7) and the performance profile of the size measurement techniques. Finally, to reach a decision on whether a material meets the requirement of the EC Recommendation a process customised to a given material has to be followed. The main steps of such a process are presented in Figure 7.1 and discussed below.

**FIGURE 7.1**

The main steps to be considered in the nanomaterial identification process.

### 7.1.1 Information on the material

Before assessing a material according to the EC NM definition, as much information as possible on the given material should be collected. This will allow selecting the most appropriate particle size analysis method(s) and will help identifying methods which are incompatible with the material to be analysed. The following material properties are most crucial for the choice of an appropriate technique:

- **Chemical nature (composition and internal structure)**

  The chemical nature of the particles strongly influences the choice of the appropriate characterisation methods. Certain methods are very sensitive to the elemental composition e.g. due to element-specific detection (e.g. the use of sp-ICP-MS is not applicable to organic materials) or to the very nature of the sample and may be applied only to a limited variety of chemicals. Some of the techniques can analyse samples consisting only of particles of the same chemistry (i.e. all particles homogenously consisting of (i) a single chemical element (e.g. Au) or compound (e.g. SiO2) or (ii) different elements or compounds) due to e.g. element/compound-specific sensitivity (e.g. sp-ICP-MS, DLS) and some can deal with samples consisting of particles with different chemistries (e.g. material consisting of both TiO2 and SiO2 particles). The basic principles behind certain techniques mean that they can reliably assess only the size of particles of the same chemical composition during the same measurement (e.g. the scattering signal of a mixture is dominated by the component with highest optical or electron density contrast e.g. DLS or SAXS), whereas some methods can be applied also to a mix of different particles (e.g. TEM and SEM). Consequently in addition to knowledge of the technique, a good knowledge of the chemical composition and internal structure of the particles is crucial for reliable analysis of particle size.

  When collecting the information on the chemical nature of a sample special attention should be dedicated to the internal structure of particles as it may influence the determination of the particle size in different techniques. In principle particles may consist of different elements and/or compounds arranged internally in various ways. For instance core-shell particles consist of two components, one of which (the core) lies within the other that forms the outer layer (the shell). Multilayer (or multishell) particles are core-shell particles with more than one outer layer (shell). Particles with inclusions are particles in which the components are phase-separated from each other and one phase is dispersed in the other and forms the inclusions. The number and size of the domains can vary, and their spatial distribution within the particles is often not uniform. Materials with such complex structure may require particular attention when choosing the method for analysis.

- **Shape**

  Solid particles may have a wide variety of shapes, such as spheres, cubes, tubes, wires, plates, etc. and the shape strongly affects the choice of characterisation methods. As discussed in Section 5 many of the currently used characterisation methods assume that particles are spherical or yield an equivalent spherical size, which limits their applicability for particles having a non-spherical shape. Moreover if the particles are plate- or fibre-like, methods need to be suitable to specifically measure their smallest dimensions. Samples consisting of a mixture of particles of different shapes may pose additional difficulties in the analysis, and in these cases only electron microscopy, and to a limited extent also atomic force microscopy, may yield reliable results. Knowledge of the particle shape is therefore necessary to ensure correct interpretation of data.

- **Dispersibility**

  Some characterisation methods require the particles to be dispersed in a liquid phase (e.g. DLS, AUC, CLS, sp-ICP-MS), or a gas phase (spray-DEMA), whereas others only work for dry powders (e.g. BET). To be able to choose an appropriate method to determine particle size (distribution) it is necessary to know if the material can be analysed as-received or whether it needs to be dispersed in a suitable dispersant. For instance it may be useful to establish solubility/dispersibility parameters to determine the optimal dispersant for the analysed material [66,67]. The information on the dispersing media (e.g. polar, non-polar, etc.) and specific protocols to be used is very helpful for a successful sample preparation as discussed in Section 4, and is crucial for reliable particle size determination. Therefore, it is generally necessary to know if the trade form and dispersibility of the material matches the technical requirements of a chosen method.

- **Other properties**

  Specific properties of the particles such as electrical, optical, magnetic and surface properties may affect the results of the particle size measurement as these properties may interfere or, on the contrary, facilitate the use of certain measurement methods. Stability of the material during preparation and measurement also needs to be considered. For instance, if a sample is sensitive to damage by irradiation with high-energy electrons the use of electron microscopy methods can be challenging. Other particles may be stable only in a narrow temperature range, which hampers the use of certain methods or requires specific protocols to be used to obtain reliable results. For example, in preparation of a BET measurement heating the sample up to a temperature high enough to remove free water and gas residues is applied. Therefore, it is necessary to know if applying a specific measurement technique can damage a sensitive material. Specific material properties must therefore be taken into account to avoid the use of inappropriate methods.

### 7.1.2 Knowledge on techniques and their measurement capabilities

For a reliable classification of a material according to the EC NM definition good knowledge of the measurement techniques' working ranges (Figure 2.7) is required. Information on the physical principles, distinctive features and applicability domains of different particle size measurement techniques is available in the scientific and technical literature. In addition, technical guidelines and documentary standards on particle characterisation developed by standardisation bodies are also available and should be used where possible to underpin the reliability of measurement results (see Annex). It is particularly important to relate the features of a specific technique to its potential suitability for performing measurements for material classification. Section 5 provides suggestions on what should be considered when choosing a method for this purpose, indicating which properties of the methods should be considered when trying to identify nanomaterials.

Key questions that affect the applicability of a method for identifying nanomaterials are, e.g. whether the method provides information on constituent particles, and whether it provides a particle size distribution by number. Section 5 of this report, as well as a previous JRC Reference Report [20] provide an overview of measurement techniques with an evaluation of their applicability to the requirements of the EC NM definition. Recently the performance of widely available and frequently used techniques to determine particle size was systematically evaluated within the NanoDefine [68] project with focus on the EC NM definition requirements, using a set of representative examples, including well-defined quality control materials as well as industrial materials of complex particle shapes and considerable polydispersity. In this way, specific regions of applicability of the individual methods in terms of the materials classification criteria, e.g. material classes, chemical composition, size range, trade forms, etc., were established [17,63]. The results of this evaluation along with criteria applied by the experts can be found in the NanoDefine Methods Manual [24].

### 7.1.3 Matching material and appropriate technique

Only a good match between the material properties and the validated performance of the method will lead to reliable and robust data which allow classification of a particulate material according to EC NM definition.

Materials can be classified according to the criteria that are most crucial for the selection of an appropriate experimental method for particle size measurement [18]. Applying such a material classification scheme allows the selection of methods that are compatible with the material and well suited to measure materials with specific characteristics. This in turn helps to obtain reliable data that are fit for the purpose of classifying the material as nano or not nanomaterial.

Taking into account the method performance characteristics [17] a matching matrix has been proposed [18,24] which may be used to link materials with known specific physicochemical properties with the methods best suitable to analyse particle size distributions according to the EC NM definition.

### 7.1.4 Sampling and sample preparation

As described in Section 4 appropriate sample preparation is crucial for achieving reliable and unbiased particle size distribution results. The sample preparation can be seen as a twofold exercise. Firstly samples need to be drawn from a material (from a batch) according to an accepted sampling plan. As these samples are intended to serve as basis for a decision about the original material, they must be fully representative. This ensures that the measurement results obtained for the sample applies to the complete batch. Guidance on taking representative samples are given, for instance, in ISO 14488:2007 [69] and in Ref [70].

Secondly, depending on the chosen measurement technique the sample may require additional treatment before it can be analysed and this is strongly method and material dependent. There are many SOPs and documentary standards (see Table A.2 in the Annex) for sample preparation

in the Annex) for sample preparation

available in the literature suitable for a specific type of a material or for a specific technique. Recently, also a catalogue of SOPs dedicated to sample preparation and measurements of particle size distribution by various techniques was published [65].

### 7.1.5 Data quality and reliability

As described in detail in Section 3 reliable measurements require documented and validated methods which guarantee that the method is fit for purpose and its results can support a decision in a legal or regulatory context. If available, documentary standards applicable to a specific method should be used. When performing measurements the principles of good laboratory practice (GLP) and good measurement practice (as described in ISO/IEC 17025) should be applied to assure that the laboratory's measurement results meet the required level of quality. The methods used and the outcomes of the measurements need to be documented and reported in a way which guarantees the reproducibility of the data and the analytical procedure.

Guidelines issued by authorities often list and describe methods recommended for regulatory purposes. A decision whether a material meets the criteria for a nanomaterial as defined under a specific regulatory provision not only requires selecting the appropriate method(s) for a specific material, but must also considers the measurement uncertainty associated to the result. If a measurement result indicates that a material is far away from the threshold separating nanomaterials from materials which are not nanomaterials a larger measurement uncertainty can be accepted as compared to when it is a borderline material. In the latter, classification as nanomaterial or not a nanomaterial is more difficult. Therefore the estimation of measurement uncertainty is necessary for the assessment of compliance, or non-compliance, with the EC NM definition because measurements without any indication of their uncertainty are unusable for regulatory purposes [65].

### 7.1.6 Decision: nanomaterial or not a nanomaterial

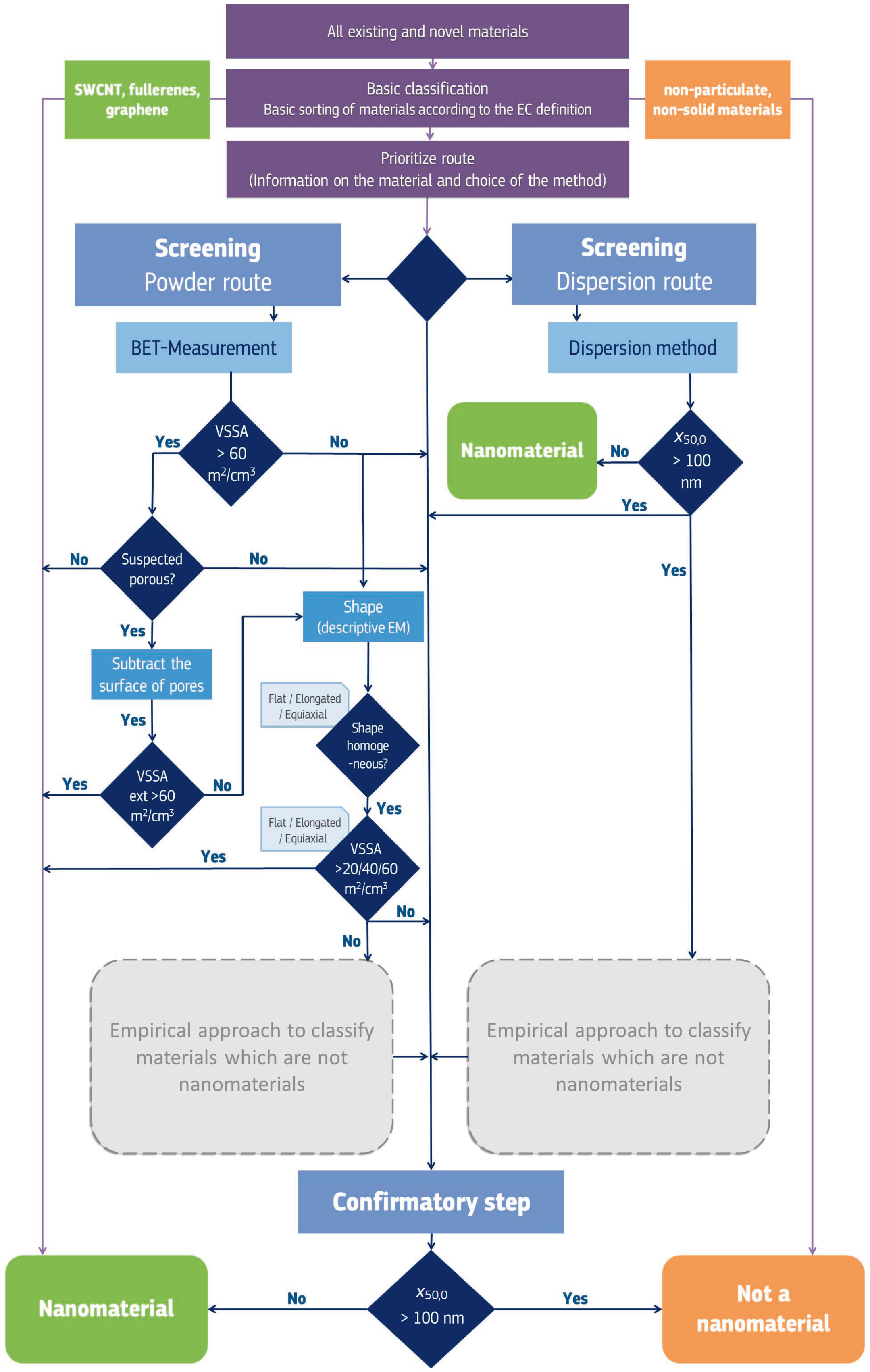
Unambiguous classification of a material as nanomaterial is of high importance as it otherwise may trigger additional tests for industry which needs to assure the compliance of their products with the EU legislation and for the regulators who need to control and evaluate additional information. Thus to understand if a material meets the requirements of the EC NM definition, a fit-for-purpose measurement strategy should be created, which may be strengthened by a flowchart in which scientifically valid criteria would be applied at each decision node. The strategy should allow to reach, as easily as possible, a decision that reflects the true nature of the material to fulfil regulatory obligations on the one hand and be economically viable on the other hand.

In the following section a flowchart for the classification of materials according to the EC NM definition is proposed and discussed. The flowchart is based on the assumption that many particulate materials can be classified relatively easily by using screening methods and only in specific cases, where these methods cannot be considered reliable, more complex (confirmatory) methods should be used to unambiguously classify a material as nanomaterial or not a nanomaterial.

## 7.2 Strategy towards classification

A flowchart can be a useful tool to produce robust decisions based on a systematic assessment of different system conditions and their impacts. The flowchart, presented in Figure 7.2, and explained in detail in sections 7.2.1-7.2.6, aims at guiding the applicant through the decision process in a straightforward manner while avoiding, as much as possible, time-consuming and expensive analyses and may thus provide an effective and cost-efficient strategy for the classification of a material according to the EC Recommendation. The flowchart starts with a single node, which further branches into different routes and decision nodes, each applying a different decision criterion. The main decision criteria used in the flowchart either stem directly from the EC NM definition (i.e. $x_{50,0} \leq 100$ nm, VSSA > 60 m²/cm³), or are derived theoretically (e.g. VSSA > 20 m²/cm³ or VSSA > 40 m²/cm³, depending on particle shape). Lately, scientific knowledge has expanded and new, science-based possibilities to use BET and dispersion screening methods to classify a material as not a nanomaterial have emerged [17,25,63,71]. Consequently the flowchart also includes options based on additional criteria and thresholds not explicitly outlined in the scope of the EC Recommendation, but which aim at facilitating the decision making process (grey shaded boxes with dashed lines in the flowchart). These criteria are discussed in Sections 6.5 and 5 (see also [17,63]). For specific materials and under certain well-defined conditions they allow classifying a material as likely not a nanomaterial based on the outcomes of screening methods. It should, however, be kept in mind that the presented flowchart by no means provides the only possible strategy for nanomaterial classification, and that other scientifically valid approaches can also be used provided that they allow a reliable assessment of materials according to the requirements laid down in the EC NM definition. The different parts of the flowchart are explained in more detail in the following subsections.

**FIGURE 7.2**



Flowchart for material classification according to the EC NM definition. The grey shaded boxes with dashed lines represent empirical and theoretical criteria for classifying materials which are likely not a nanomaterial (see Figures. 7.4 and 7.5).

### 7.2.1 Basic classification

The EC NM definition explicitly includes some and excludes other materials. By derogation fullerenes, graphene flakes and single wall carbon nanotubes (SWCNTs) with one or more external dimensions below 1 nm are considered as nanomaterials. As explained in an earlier JRC Science for Policy Report [9], the EC NM definition should only apply to particulate materials which consist of solid particles with external dimensions in the range of 1 nm to 100 nm. Hence, the definition is not applicable to materials consisting of liquid particles (e.g. emulsions and aerosols of liquid droplets). Similarly the definition does not cover nanostructured materials unless the external dimensions fulfil the EC NM definition. A basic classification based on these provisions is made in the first step of the flowchart (Figure 7.3). Non-solid and/or non-particulate materials or nanostructured particles with all external dimensions bigger than 100 nm are immediately excluded as nanomaterials whereas fullerenes, graphene flakes and SWCNTs with one or more external dimensions below 1 nm are identified as nanomaterials. In such cases, no further analysis of the material is required .

**FIGURE 7.3**

Basic classification of materials

### 7.2.2 Prioritisation route: information on the material and method matching

Materials which cannot be conclusively classified based on their basic material properties (Section 7.1.1) can be further evaluated by following specific branches in the flowchart. The prioritisation step collects some crucial information on the sample to be analysed. It is recommended to collect information on the physical and chemical nature and structure of the material as well as some other material specific properties (see Section 7.1). If possible, the shape of the particles should be assessed by descriptive EM and it is suggested to acquire information on the dispersibility if such information is not already available elsewhere. Although at this stage the decision flow chart itself does not require this information it is crucial for the choice of the appropriate identification route (powder or dispersion) and specific measurement method(s). As discussed in this report, thorough knowledge on the measurement technique is necessary to choose the most suitable method for the analysis of the material in question. Information on the applicability of methods is widely available. In particular, the NanoDefine Methods Manual [24,71] and the work conducted by Gaillard and colleagues [18] could be of help. The latter provides information on how to match the most suitable characterisation method to a specific material and is explicitly tailored to meet the requirements of the EC NM definition.

### 7.2.3 Screening

Among the screening methods described in Section 5.1 the flowchart distinguishes techniques that can characterise a material in a powder state ("powder route") or in dispersion ("dispersion route"). Which of the two branches to follow depends on several criteria such as dispersibility of the material, type of material, availability of suitable techniques, regulatory requirements, etc. For instance, some dispersion procedures can degrade the material when inappropriate conditions are applied. In such cases the dispersion route should be avoided. Furthermore, the selection of the technique can also depend on its availability and the expertise the user may have in-house. Authorities may require results obtained with a specific technique or method and this should also be considered when deciding whether to choose the powder or dispersion route. In any case, when selecting a screening technique it is of utmost importance that the material properties and the technique's measurement capabilities match (Section 7.1.3). The availability of information on material synthesis/production and provenance can be of great help when selecting a suitable screening method. It has also to be kept in mind that most of the screening techniques require additional material information to allow a reliable (accurate) calculation of the equivalent $x_{50,0}$ (e.g. optical properties, (effective) densities, particle shape). If a material is available as suspension (e.g. colloidal material), the user should follow the "dispersion route", which is the right branch of the flowchart shown in Figure 7.2. For such materials the dry powder analysis on the dried out particulate component should not be performed as its outcomes are not reliable. If the material is in powder form and can be dispersed, the analysis can be performed by either route. If the material cannot be dispersed, or the user decides not to disperse it, the "powder route" (the left path in Figure 7.2) should be followed.

The outcome of a screening method may be inadequate for concluding that a material is not a nanomaterial. As explained in Section 5, this is mostly due to the physical principles of the screening techniques, which often do not provide direct information on the external dimension of the particles, but instead measure other properties that can be correlated with the particle dimensions. Screening methods are not able to distinguish constituent particles from aggregates/agglomerates and, therefore, risk in overestimating the measured particle size. However, under certain well-defined conditions empirical criteria based on results obtained with screening methods can be established to indicate with acceptable uncertainty that a material is not a nanomaterial. These criteria are discussed in Section 6 for the powder route and in detail in the literature [17] for the dispersion route. For example, findings reported from the NanoDefine project show that if the equivalent median particle diameter of a material obtained from dispersion screening methods is larger than 250 nm, and provided that descriptive EM confirms that the particles have a near equiaxial shape and are not agglomerated or aggregated, then the material may be classified as not a nanomaterial (any significant population of extremely small particles below the limits of the descriptive EM detection should be excluded). In such cases an additional plausibility check with another method can also be helpful. In support to the material classification process an empirical approach to classify a material according to these criteria is included in the flowchart in a grey shaded box with dashed lines (see Figures 7.4 and 7.5). However, as these criteria do not explicitly originate from the EC recommendation it is always possible to skip this option and perform a confirmatory analysis, for example, by means of electron microscopy techniques which can be applied to most materials.

### 7.2.4 Screening: dispersion route

Figure 7.4 shows the path that is applicable to materials which are either provided as a suspension or which need to be dispersed prior to analysis (see also Section 4).

After measurements performed with an appropriate method (technique and material must be compatible), the user continues depending on the outcome of the screening.

1. If the material has a $x_{50,0}$ smaller than 100 nm and if the results are judged acceptable, this material is considered as nanomaterial without the need for additional confirmatory or plausibility checks. However, if the reliability of the result is questionable the user may perform further measurements either using other screening techniques to check the plausibility of the results, or by using a suitable confirmatory method.

As discussed in Section 5.1, the result of a screening method may be significantly biased due to particle aggregation\agglomeration, a high polydispersity or when the particles have a strong non-equiaxial shape. Under such conditions the validity of the physical measurement principles of the screening technique can be compromised, hence increasing the risk of false-negative classifications. Despite the measurement limitations of screening techniques mentioned above, NanoDefine researchers found that results from screening methods, complemented with results from, for example, descriptive EM or BET, can provide a strong indication that a material is likely not a nanomaterial. Based on a selection of 15 industrial powder materials, an empirical size criterion of 250 nm has been proposed and is included in the flowchart (grey shaded box with dashed lines).

2. If the material has a $x_{50,0}$ larger than 250 nm the plausibility of the screening result may be verified by either (i) descriptive electron microscopy analysis or (ii) by BET (by following the powder route). The purpose of option (i) is to estimate whether the shape of the particles in the sample is approximately equiaxial, and whether there are no indications of excessive aggregation/agglomeration or strong polydispersity. In the absence of such indication, the material can be considered as likely not being a nanomaterial. If this is not the case, then results obtained by the screening method can be biased and a conclusive classification can only be obtained by applying a confirmatory method.

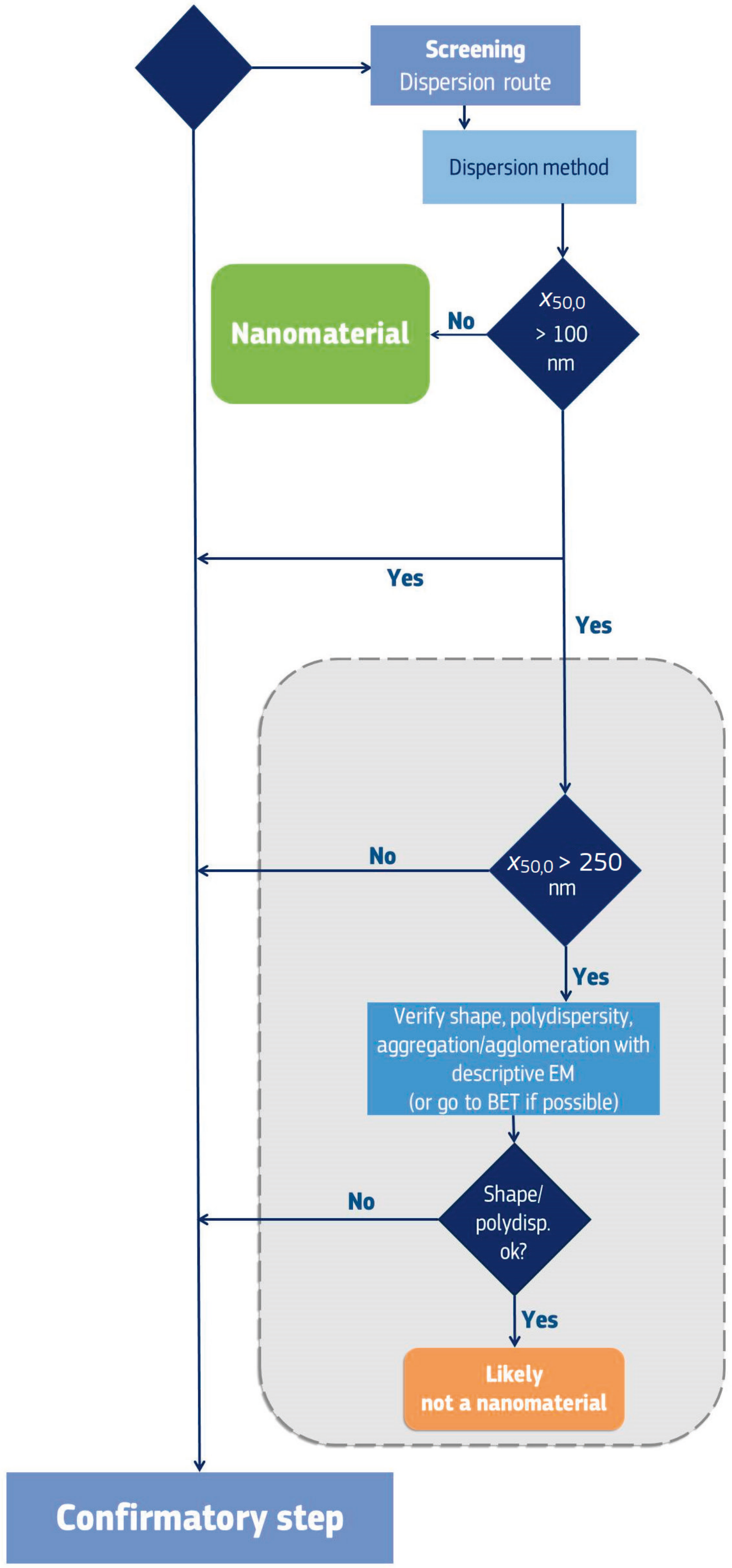
   Option (ii) can be used to verify the outcome of the dispersion screening method if the original form of a material is a dry powder. In such a case, the user should follow the entire powder route. In case of doubt it is recommended to move directly to the confirmatory methods. In both cases the results of the initial analysis in dispersion should not be considered anymore.

3. If the material has a $x_{50,0}$ in the range 100 nm to 250 nm the use of confirmatory methods is necessary.

At any stage of the analysis via the dispersion route the user can decide to switch to a confirmatory method.

**FIGURE 7.4**

Dispersion route for screening by particle size analysis methods. The grey shaded box with dashed lines represents empirical criteria for classifying materials which are likely not nanomaterials.



## 7.2.5 Screening: powder route

As described in Section 6, if technically feasible and requested under specific legislation, compliance with the EC NM definition may be determined on the basis of the VSSA, which can be used as a proxy (for the number-based criterion). In such a case a (non-porous) material with a VSSA larger than 60 $m^2/cm^3$ is considered as nanomaterial. However, if the VSSA is smaller than 60 $m^2/cm^3$ one cannot conclude, at least not without further information, that the material is not a nanomaterial. As the VSSA is considered as a proxy for the number-based particle size distribution it should be strongly correlated to the external dimensions of the analysed particles and therefore any contribution to the surface area resulting from accessible pores in the particles must be subtracted from the total VSSA. Furthermore, if the particles have a specific shape, this can be taken into account (see Section 6) to apply shape-specific VSSA thresholds for the identification of nanomaterials.

The use of very low VSSA values as proxy to identify materials which are not nanomaterials is discussed in Section 6. It must be kept in mind that the conditions outlined in that section must be fulfilled in order to come to a reliable classification. Although this possibility is not explicitly addressed in the EC NM definition, it is included in the flowchart as an empirical approach as it has the potential to considerably simplify the identification of materials which do not fall under the EC NM definition. In case of doubt one should use the number-based particle size distribution for classification, as this takes precedence.

Figure 7.5 presents the detailed powder route of the flowchart for identifying a material as nanomaterial or not a nanomaterial when applying BET analysis. The chart is divided into three main blocks with increasing levels of complexity during the identification process: i) screening: this gives the opportunity to classify a material clearly as nanomaterial, ii) further evaluation by taking into account the shape of the particles and sample modality (of the size distribution) iii) verification of borderline cases. The latter results in application of confirmatory methods for final decision.

Keeping in mind that the sample for BET analysis has to be in powder form, the flowchart could be applied as follows way:

The first step in the powder route is to determine the VSSA of the material:

1. If a material has a VSSA larger than 60 $m^2/cm^3$:

   A. If non-porous porosity particles can be assumed, the material can be identified as nanomaterial according to the EC NM definition.

   B. If the material is suspected to be porous, appropriate method(s), e.g. t-plot [72,73], should be applied to separate the surface area resulting from the presence of open micro- and mesopores from the outer surface which is solely related to the external dimensions of the particle.

   If the resulting outer surface $VSSA_{ext}$ is larger than 60 $m^2/cm^3$ the material can be classified as a nanomaterial.

2. If the VSSA or $VSSA_{ext}$ of the analysed material is lower than 60 $m^2/cm^3$ then descriptive EM analysis has to be performed to determine the general particle shape.

   If the particles have homogenous shapes (elongated or flat and a high aspect ratio) the following criteria can be applied (see Section 6.2):

   - VSSA > 40 $m^2/cm^3$ for elongated, fibre-like particles, $\delta$ =2
   - VSSA > 20 $m^2/cm^3$ for flat, platelet-like particles, $\delta$ =1

   The analysed material can be classified as nanomaterial, provided that the conditions for the applicability of the VSSA criteria are fulfilled. If the material does not consist of particles of homogenous (elongated or flat) shape the use of a confirmatory method is necessary.

3. If the VSSA value is smaller than 6 $m^2/cm^3$ and the user would like to use the empirical approach to classify a material as likely not a nanomaterial:

   In such a case the modality of the sample has to be determined and if it is bi-modal the use of confirmatory methods is necessary. If the sample is monomodal, the following shape specific cut-off criteria discussed in Section 6.5 can be applied:

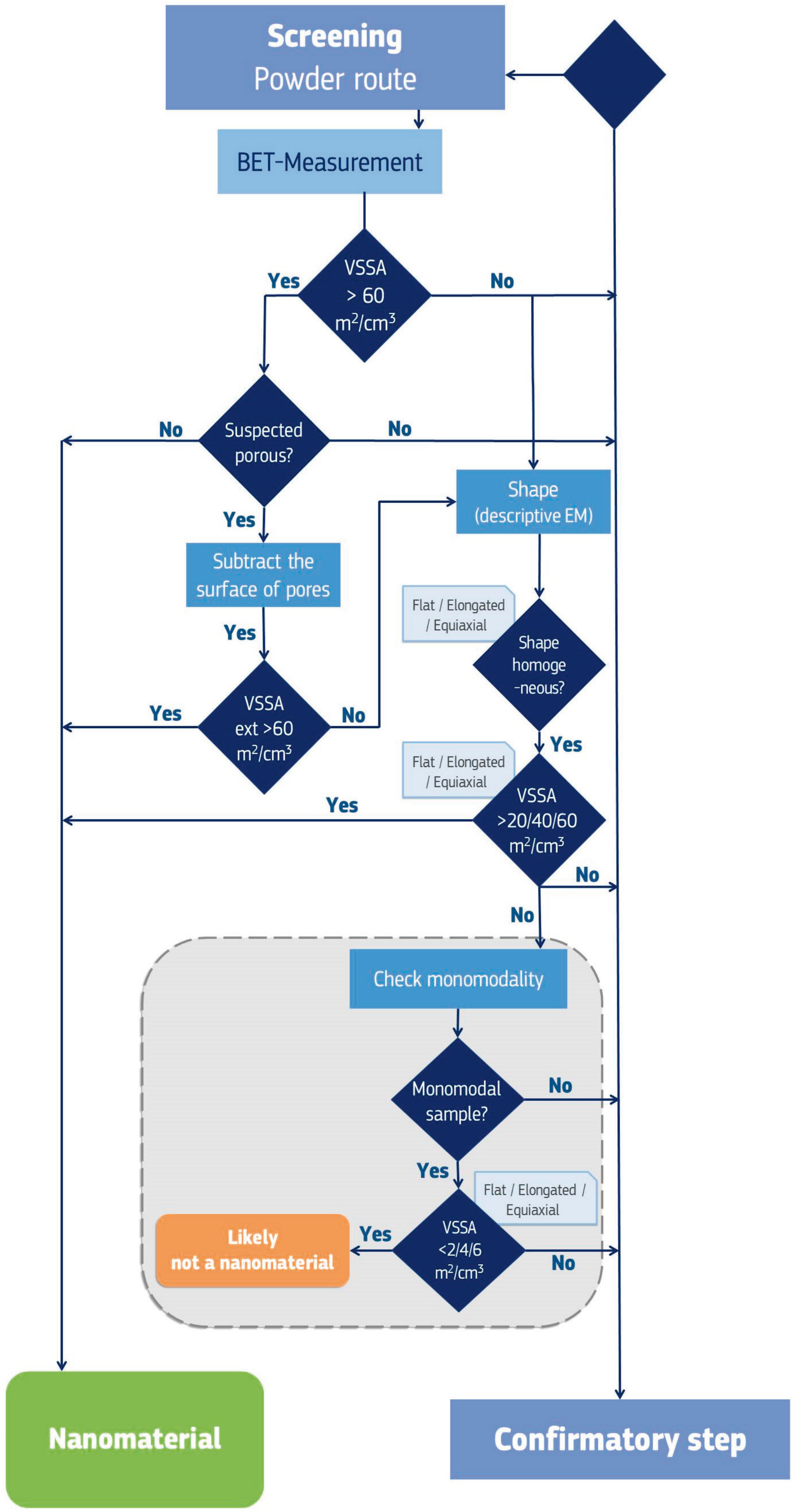
- Spherical shape: VSSA < 6 $m^2/cm^3$ (equivalent to a $d_{VSSA}$ =1000 nm of spherical particles)
- Elongated, fibre-like particles: VSSA < 4 $m^2/cm^3$ (equivalent to a $d_{VSSA}$ =1000 nm of elongated particles)
- Flat, platelet-like particles: VSSA < 2 $m^2/cm^3$ (equivalent to a $d_{VSSA}$ =1000 nm of flat particles)

If the results meet the criteria, the material is likely not a nanomaterial within an acceptable uncertainty. If multimodality is detected, if there is a mixture of different shapes or the shapes cannot be approximated by spherical, rod or platelet shapes, the user should use a confirmatory method to unambiguously classify the analysed material.

4. If the VSSA of a material is outside of the range of values discussed above it should be regarded as a borderline case (even if the sample is monomodal), i.e. a particulate material with particles of:

- Spherical shape:
  $\delta=3$ and 6 < VSSA < 60 $m^2/cm^3$ or
- Elongated, fibre-like particles:
  $\delta=2$, and 4 < VSSA < 40 $m^2/cm^3$ or
- Flat, platelet-like particles:
  $\delta=1$, and 2 < VSSA < 20 $m^2/cm^3$

and a confirmatory method should be applied to reach a reliable decision.



## FIGURE 7.5

Powder route for screening by BET. The grey shaded box with dashed lines represents empirical and theoretical criteria for classifying materials which are likely not nanomaterials.

### 7.2.6 Confirmatory step

Figure 7.6 presents the final step of the flowchart where confirmatory methods are used for material classification.

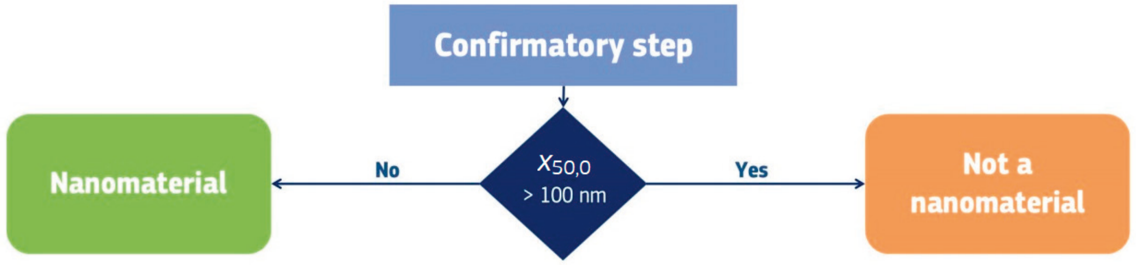
An analysis using confirmatory methods is needed when the screening method approach is inconclusive or when it cannot be applied to the material. Additionally, confirmatory methods may also simply be chosen from the very beginning or at every step of the classification process.

If the EM image analysis results in $x_{50,0}$ larger than 100 nm, then the material is classified as not a nanomaterial. If the resulting $x_{50,0}$ is smaller than 100 nm, the material is classified as a nanomaterial.

Almost any particulate material can be analysed by EM unless it cannot withstand the conditions (e.g. electron beams and/or high vacuum) in the sample chamber of the EM instrument. Such materials may possibly be analysed by variable pressure and/or low vacuum EM or by applying low probe currents. Nevertheless, sample preparation may still be an issue.

**FIGURE 7.6**

Confirmatory step for material classification based on the median particle size $x_{50,0}$.



# 8 Summary and concluding remarks

The methods and measurement techniques that can be used to measure particle size distributions can be based on considerably different measurement principles, and the level of detail that they provide and their region of applicability may also differ considerably. Likewise, there is an overwhelming diversity of potential nanomaterials with a wide spectrum of physical-chemical properties. For most materials, methods are available which allow their classification as nanomaterial or not a nanomaterial. Still, there are cases for which this is too challenging with the methods currently available. As the EC NM definition is comprehensive it can potentially cover any particulate material, and therefore an assessment of whether they fall under the EC NM definition may be required for materials with entirely different properties.

Therefore, in order to select one or more suitable methods for an experimental assessment of whether a specific material falls under the EC NM definition the following considerations need to be made:

- Is there sufficient physicochemical information on the material?

  Basic physical-chemical information on the material to be assessed should be obtained and available before a measurement of the particle size distribution is performed, including chemical composition, dispersibility, particle shape, expected size range and other properties that could affect the choice of size measurement techniques.

- Is the selected technique compatible with the material?

  The measurement technique(s) to be applied must be compatible with the material. For example, if the measurement or preparation conditions induce changes in the particle size distribution of the material, the method should not be used to assess whether that material falls under the EC NM definition. The physical-chemical properties of a material should therefore be matched with the capabilities of the techniques and the measurement conditions.

- What is the purpose of the analysis?

  The analyst should consider whether the purpose of the analysis is screening or confirmation. Screening may provide a more affordable, cost-efficient identification of a nanomaterial in certain cases or it may provide indications that the material indeed is a nanomaterial, but in other cases, if a conclusive assessment cannot be obtained by screening, a more elaborate analysis involving a confirmatory method may be required.

- Is the method validated?

  When available, validated methods should be used; methods for which (international) documentary standards exist or are otherwise internationally recognised should be preferred. The user should also be familiar with the robustness and reliability of the method and should be able to estimate the uncertainty of the measurement result.

- Which type of raw data does the method produce?

  When choosing a method, the analyst must be aware of the type of size distribution of raw (primary) data a technique yields, i.e., whether the technique provides a number-, mass- or scattered light intensity-based distribution or any other type of quantity, and whether the technique counts individual particles or measures ensembles of particles instead.

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

- Are there specific regulatory requirements to be met?

  One should also be aware of the requirements of any specific regulatory provision according to which nanomaterials need to be identified. For example, it may be necessary to use more than one technique, or the use of a specific technique may be compulsory.

- Is the sample to be analysed representative for the original material?

  The outcome of the sample analysis should be applicable to the whole material. Nearly all particle measurement techniques involve a degree of subsampling to perform the analysis. It is therefore essential to ensure appropriate subsampling of the material from the bigger batch so the analysed sample can be considered representative.

- Is the applied sample preparation appropriate for the intended method?

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

- Is the outcome of the analysis reliable?

  The outcome of the analysis will serve as a base for decisiding whether a material is a nanomaterial or not according to the EC NM definition. Therefore the reliability and robustness of the measurement result should be ensured by applying good measurement practices.

- Is the outcome of the analysis accompanied by all meta-data necessary for assessing the reliability of the data?

  The analysis outcome needs to be supported with the information on how the analysis was performed. Precise and detailed information on meta-data are pivotal to ensure the proper assessment of the robustness and reliability of the generated data and is necessary to guarantee reproducibility and comparability of the measurement outcomes. For instance for the purpose of data comparability, the exact details of all steps involved in a dispersion protocol, the equipment and measurement procedure applied should be reported.

- Is the size range of the analysis performed suitable for the analysed sample?

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

- Is the outcome of the analysis sufficient for reaching a decision on whether the analysed material is a nanomaterial or not according to the EC NM definition?

  The decision on whether a material is a nanomaterial or not according to the EC NM definition may have legal consequences and should therefore be based on robust data and be supported by applying scientifically sound criteria to the outcomes of the analysis. There are a number of issues which should be considered when assessing the appropriateness of the analysis outcome. For instance the EC Recommendation requires the median value of the constituent particle size distribution on a number basis. However the outcome of the confirmatory methods resulting in $x_{50,0}$ higher than 100 nm cannot be easily interpreted and additional information on the sample such as agglomeration/aggregation state, polydispersity, particle shape, etc., may be needed to draw a valid conclusion. In such cases, it is useful to perform a plausibility check with another method, which is based on different physical principles and involves different sample preparation.

# Annex

## Abbreviations and acronyms

| | |
|---|---|
| AF4 | Asymmetrical Flow Field-Flow Fractionation |
| AFM | Atomic Force Microscopy |
| ASTM | ASTM International, formerly known as American Society for Testing and Materials |
| AUC | Analytical Ultra Centrifugation |
| BAM | Bundesanstalt für Materialforschung und -prüfung |
| BET | Brunauer-Emmett-Teller |
| BSE | Backscattered electrons |
| CEN | European Committee for Standardization |
| CLS | Centrifugal Liquid Sedimentation |
| CRM | Certified Reference Material |
| DEMA | Differential Electrical Mobility Analysis (also spray-DEMA) |
| DLS | Dynamic Light Scattering |
| EC | European Commission |
| ECD | Equivalent Circular Diameter |
| EC NM definition | EC Recommendation on the Definition of a Nanomaterial |
| EFSA | European Food Safety Authority |
| EM | Electron Microscopy |
| EU | European Union |
| GLP | Good Laboratory Practice |
| ICP-MS | Inductively Coupled Plasma - Mass Spectrometry |
| ISO | International Organization for Standardization |
| JRC | Joint Research Centre of the European Commission |
| LOQ | Limit of Quantification |
| MAD | Mutual acceptance of data |
| MALS | Multi-Angle Light Scattering |
| NM | Nanomaterial |
| OECD | Organisation for Economic Co-operation and Development |
| PSD | Particle Size Distribution |
| PT | Proficiency Testing |
| PTA | Particle Tracking Analysis |
| RM | Reference Material |
| SAXS | Small-Angle X-ray Scattering |
| SE | Secondary electrons |
| SEM | Scanning Electron Microscopy |
| SOP | Standard Operating Procedure |
| sp-ICP-MS | Single Particle ICP-MS |
| SPM | Scanning Probe Microscopy |
| SSA | Specific Surface Area |
| SWCNT | Single Wall Carbon Nanotube |
| TEM | Transmission Electron Microscopy |
| TG | Test Guideline |
| TRPS | Tuneable Resistive Pulse Sensing |
| TSEM | Scanning Electron Microscopy operated in Transmission Mode |
| VAMAS | Versailles Project on Advanced Materials and Standards |
| vdW | van der Waals |
| VIM | International Vocabulary of Metrology |
| VSSA | Volume-Specific Specific Surface Area |
| $x_{50,0}$ | Median particle size |
| $x_{Fmin}$ | Minimum Feret diameter |
| $x_{Fmax}$ | Maximum Feret diameter |
| XRD | X-ray Diffraction |

## Documentary standards

**TABLE A.1**

Non-exhaustive list of relevant documentary standards and guidance documents related to vocabulary and terminology of nanomaterials and nano-objects and the available characterisation methods

| Standardisation body and technical committee | Reference number | Title |
|---|---|---|
| **ISO/TC 229** | ISO/TS 80004-1:2015 | Nanotechnologies – Vocabulary – Part 1: Core terms |
| **ISO/TC 229** | ISO/TS 80004-2:2015 | Nanotechnologies – Vocabulary – Part 2: Nano-objects |
| **ISO/TC 229** | ISO/TS 80004-3:2010 | Nanotechnologies – Vocabulary – Part 3: Carbon nano-objects |
| **ISO/TC 229** | ISO/TS 80004-4:2011 | Nanotechnologies – Vocabulary – Part 4: Nanostructured materials |
| **ISO/TC 229** | ISO/TS 80004-6:2013 | Nanotechnologies – Vocabulary – Part 6: Nano-object characterization |
| **ISO/TC 24/SC 4** | ISO 26824:2013 | Particle characterization of particulate systems – Vocabulary |
| **ISO/TC 229** | ISO/TR 18196:2016 | Nanotechnologies – Measurement technique matrix for the characterization of nano-objects |
| **CEN/TC 352** | CEN/TS 17010:2016 | Nanotechnologies – Guidance on measurands for characterising nano-objects and materials that contain them |

**TABLE A.2**

Non-exhaustive list of relevant documentary standards and guidance documents related to sampling, sample splitting and sample preparation

| Standardisation body and technical committee | Reference number | Title |
|---|---|---|
| **ISO/TC 47** | ISO 8213:1986 | Chemical products for industrial use – Sampling techniques – Solid chemical products in the form of particles varying from powders to coarse lumps |
| **ISO/TC 24/SC 4** | ISO 14488:2007 | Particulate materials – Sampling and sample splitting for the determination of particulate properties |
| **ASTM C09.20** | ASTM C702 / C702M - 18 | Standard practice for reducing samples of aggregate to testing size |
| **ASTM D04.30** | ASTM D75 / D75M - 14 | Standard practice for sampling aggregates |
| **BSI LBI/37** | BS 3406-1:1986 | Methods for determination of particle size distribution. Guide to powder sampling |
| **ISO/TC 24/SC 4** | ISO 14887:2000 | Sample preparation – Dispersing procedures for powders in liquids |
| **ISO/TC 229** | ISO/TR 20489:2018 | Nanotechnologies – Sample preparation for the characterization of metal and metal-oxide nano-objects in water samples |
| **OECD** | ENV/JM/MONO(2012)40 | Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials |
| **OECD** | Test No. 318 | Dispersion stability of nanomaterials in simulated environmental media |

## TABLE A.3

Non-exhaustive list of relevant documentary standards and guidance documents related screening measurement techniques and techniques used in conjunction

| Technique | Standardisation body and technical committee | Reference number | Title |
|---|---|---|---|
| **Brunauer–Emmett–Teller (BET) (Specific surface area)** | ISO/TC 24/SC 4 | ISO 9277:2010 | Determination of the specific surface area of solids by gas adsorption – BET method |
| | ISO/TC 206 | ISO 18757:2003 | Fine ceramics (advanced ceramics, advanced technical ceramics) – Determination of specific surface area of ceramic powders by gas adsorption using the BET method |
| | ISO/TC 24/SC 4 | ISO 15901-1:2016 | Evaluation of pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption – Part 1: Mercury porosimetry |
| | ISO/TC 24/SC 4 | ISO 15901-2:2006 | Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption – Part 2: Analysis of mesopores and macropores by gas adsorption |
| | ISO/TC 24/SC 4 | ISO 15901-3:2007 | Pore size distribution and porosity of solid materials by mercury porosimetry and gas adsorption – Part 3: Analysis of micropores by gas adsorption |
| | ISO/TC 24/SC 4 | ISO 12154:2014 | Determination of density by volumetric displacement – Skeleton density by gas pycnometry |
| | ISO/TC 229 | ISO/TS 11937:2012 | Nanotechnologies – Nanoscale titanium dioxide in powder form – Characteristics and measurement |
| **Differential mobility analysis (spray-DEMA)** | ISO/TC 92/SC 3 | ISO 29904:2013 | Fire chemistry – Generation and measurement of aerosols |
| | ISO/TC 24/SC 4 | ISO 15900:2009 | Determination of particle size distribution – Differential electrical mobility analysis for aerosol particles |
| **Differential mobility analysis (DMA)** | ISO/TC 24/SC 4 | ISO 27891:2015 | Aerosol particle number concentration – Calibration of condensation particle counters |
| | ISO/TC 146/SC 2 | ISO 28439:2011 | Workplace atmospheres – Characterization of ultrafine aerosols/nanoaerosols – Determination of the size distribution and number concentration using differential electrical mobility analysing systems |
| **Light scattering aerosol spectrometer (LSAS)** | ISO/TC 24/SC 4 | ISO 21501-1:2009 | Determination of particle size distribution – Single particle light interaction methods – Part 1: Light scattering aerosol spectrometer |
| **Light scattering liquid-borne particle counting (LSLPC)** | ISO/TC 24/SC 4 | ISO 21501-2:2007 | Determination of particle size distribution – Single particle light interaction methods – Part 2: Light scattering liquid-borne particle counter |
| **Laser diffraction (LD)** | ISO/TC 24/SC 4 | ISO 13320:2009 | Particle size analysis – Laser diffraction methods |
| **X-ray diffraction (XRD)** | ISO/TC 229 | ISO/TS 11937:2012 | Nanotechnologies – Nanoscale titanium dioxide in powder form – Characteristics and measurement |
| **Single particle inductively coupled plasma mass spectrometry (sp-ICP-MS)** | ISO/TC 229 | ISO/TS 19590:2017 | Nanotechnologies – Size distribution and concentration of inorganic nanoparticles in aqueous media via single particle inductively coupled plasma mass spectrometry |
| **Dynamic light scattering (DLS)** | ISO/TC 24/SC 4 | ISO 22412:2017 | Particle size analysis – Dynamic light scattering (DLS) |
| | ISO/TC 24/SC 4 | ISO/DTR 2218 | Good practice for dynamic light scattering (DLS) measurements |
| **Particle tracking analysis (PTA)** | ISO/TC 24/SC 4 | ISO 19430:2016 | Particle size analysis – Particle tracking analysis (PTA) method |
| **Centrifugal liquid sedimentation (CLS)** | ISO/TC 24/SC 4 | ISO 13318-1:2001 | Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines |
| | ISO/TC 24/SC 4 | ISO 13318-2:2007 | Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method |

| | | | |
|---|---|---|---|
| | ISO/TC 24/SC 4 | ISO 13318-3:2004 | Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 3: Centrifugal X-ray method |
| | ISO/TC 24/SC 4 | ISO 18747-1:2018 | Determination of particle density by sedimentation methods – Part 1: Isopycnic interpolation approach |
| | ISO/TC 24/SC 4 | ISO 18747-2:2019 | Determination of particle density by sedimentation methods – Part 2: Multi-velocity approach |
| | ISO/TC 45/SC 3 | ISO 15825:2017 | Rubber compounding ingredients – Carbon black – Determination of aggregate size distribution by disc centrifuge photosedimentometry |
| **Small-angle X-ray scattering (SAXS)** | ISO/TC 24/SC 4 | ISO 17867:2015 | Particle size analysis – Small-angle X-ray scattering |
| **Field-flow fractionation (AF4)** | ISO/TC 229 | ISO/TS 21362:2018 | Nanotechnologies – Analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation |
| | CEN/TC 352 | CEN/TS 17273:2018 | Nanotechnologies – Guidance on detection and identification of nano-objects in complex matrices |
| **Ultrasonic spectroscopy (USSp)** | ISO/TC 24/SC 4 | ISO 20998-1:2006 | Measurement and characterization of particles by acoustic methods – Part 1: Concepts and procedures in ultrasonic attenuation spectroscopy |
| | ISO/TC 24/SC 4 | ISO 20998-2:2013 | Measurement and characterization of particles by acoustic methods – Part 2: Guidelines for linear theory |
| | ISO/TC 24/SC 4 | ISO 20998-3:2017 | Measurement and characterization of particles by acoustic methods – Part 3: Guidelines for non-linear theory |

## TABLE A.4

Non-exhaustive list of relevant documentary standards and guidance documents related to confirmatory measurement techniques and techniques used in conjunction

| Standardisation body and technical committee | Reference number | Title |
|---|---|---|
| **ISO/TC 24/SC 4** | ISO 13322-1:2014 | Particle size analysis – Image analysis methods – Part 1: Static image analysis methods |
| **ISO/TC 201/SC 1** | ISO 18115-2:2013 | Surface chemical analysis – Vocabulary – Part 2: Terms used in scanning-probe microscopy |
| **ISO/TC 201/SC 7** | ISO/TR 14187:2011 | Surface chemical analysis – Characterization of nanostructured materials |
| **ISO/TC 201/SC 9** | ISO 11952:2019 | Surface chemical analysis – Scanning-probe microscopy – Determination of geometric quantities using SPM: Calibration of measuring systems |
| **ISO/TC 202/SC 1** | ISO 22493:2014 | Microbeam analysis – Scanning electron microscopy – Vocabulary |
| **ISO/TC 202/SC 3** | ISO 29301:2017 | Microbeam analysis – Analytical electron microscopy – Methods for calibrating image magnification by using reference materials with periodic structures |
| **ISO/TC 202/SC 4** | ISO 16700:2016 | Microbeam analysis – Scanning electron microscopy – Guidelines for calibrating image magnification |
| **ISO/TC 202/SC 4** | ISO/TS 24597:2011 | Microbeam analysis – Scanning electron microscopy – Methods of evaluating image sharpness |
| **ISO/TC 229** | ISO/DIS 19749<br>(Under development) | Nanotechnologies – Measurements of particle size and shape distributions by scanning electron microscopy |
| **ISO/TC 229** | ISO/DIS 21363<br>(Under development) | Nanotechnologies – Measurements of particle size and shape distributions by transmission electron microscopy |
| **ASTM E04.11** | ASTM E766 - 14e1 | Standard practice for calibrating the magnification of a scanning Electron microscope |
| **BSI LBI/37** | BS 3406-4:1993 | Methods for determination of particle size distribution. Guide to microscope and image analysis methods |
| **DIN** | DIN SPEC 52407 | Nanotechnologies – Methods for preparation and assessment for particle measurements with atomic force microscopy (AFM) and transmission scanning electron microscopy (TSEM) |
| **ASTM E56.02** | ASTM E2859 - 11(2017) | Standard guide for size measurement of nanoparticles using atomic force microscopy |

## TABLE A.5

Non-exhaustive list of relevant documentary standards and guidance documents related to representation of measurement data and reporting requirements

| Standardisation body | Reference number | Title |
|---|---|---|
| **ISO/CASCO** | ISO/IEC 17025:2017 | General requirements for the competence of testing and calibration laboratories |
| **ISO/TMBG** | ISO/IEC Guide 98-3:2008 | Uncertainty of measurement – Part 3: Guide to the expression of uncertainty in measurement (GUM:1995) |
| **ISO/TC 201/SC 2** | ISO 20579-4:2018 | Surface chemical analysis – Guidelines to sample handling, preparation and mounting – Part 4: Reporting information related to the history, preparation, handling and mounting of nano-objects prior to surface analysis |
| **ISO/TC 24/SC 4** | ISO 9276-1:1998<br>(Cor 1:2004) | Representation of results of particle size analysis – Part 1: Graphical representation |
| **ISO/TC 24/SC 4** | ISO 9276-2:2014 | Representation of results of particle size analysis – Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions |
| **ISO/TC 24/SC 4** | ISO 9276-3:2008 | Representation of results of particle size analysis – Part 3: Adjustment of an experimental curve to a reference model |
| **ISO/TC 24/SC 4** | ISO 9276-5:2005 | Representation of results of particle size analysis – Part 5: Methods of calculation relating to particle size analyses using logarithmic normal probability distribution |
| **ISO/TC 24/SC 4** | ISO 9276-6:2008 | Representation of results of particle size analysis – Part 6: Descriptive and quantitative representation of particle shape and morphology |

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## Abstract

This report addresses identification of nanomaterials according to the European Commission's Recommendation on the definition of nanomaterial (2011/696/EU) by measurements and discusses options and points to consider when assessing whether a particulate material is a nanomaterial or not. The primary criterion to identify nanomaterials is the median of the number-based distribution of the constituent particles' external dimensions, regardless of whether these particles appear separate from one another or are parts of aggregates or agglomerates. The main steps in the nanomaterial identification process are collecting information on the material, acquiring knowledge of the measurement method(s), matching method(s) and material, sample preparation, measurement/analysis and decision (nanomaterial / no nanomaterial).

Assessment of particle size measurements requires specification of the measurand, the physical principle of the measurement technique, the applied sample preparation protocol, the covered size range and the data analysis procedure to allow a reliable classification of a material according to the EC nanomaterial definition. A variety of screening and confirmatory techniques is available to analyse particle size distributions. Screening techniques do not measure directly the number-based distribution of the external particle dimensions, but they are fast and inexpensive and still allow to positively identify a material as a nanomaterial. Confirmatory techniques are usually more costly and time-intensive, but may provide a more reliable classification and allow resolving doubts or disputes. The volume specific surface area can serve as proxy to identify nanomaterials, provided that certain requirements are fulfilled. For a correct classification whether a material is a nanomaterial or not, a thorough knowledge of the applied measurement method is needed to correctly interpret the outcome of a measurement and to understand whether a specific technique is fit for the purpose.

Reliable measurement results can be obtained if a reference measurement system is implemented, which is typically based on documented and validated methods and reference materials. Best practices should be applied when reference measurement systems are not available. This report provides examples and practical options for consideration, including a flowchart that can assist users with relevant technical knowledge in the identification of nanomaterials.

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