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The NanoDefine Methods Manual

*Part 1: The NanoDefiner
Framework and Tools*



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Legal Note

This document contains general recommendations supporting the user in the decision whether a material is a nanomaterial according to the EC Recommendation on the Definition of Nanomaterial (Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). OJ L 275, pp. 38-40). However, users are reminded that the texts of the appropriate EC legal acts are the only authentic legal reference and that the information in this document does not constitute legal advice. Usage of the information remains under the sole responsibility of the user. The NanoDefine Consortium Partners do not accept any liability with regard to the contents of this document.

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NanoDefine

Development of an integrated approach based on validated and standardised methods to support the implementation of the EC recommendation for a definition of nanomaterial

The NanoDefine Methods Manual

Part 1: The NanoDefiner Framework and Tools

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement n° 604347

Website: <http://www.nanodefine.eu/>
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About the NanoDefine Methods Manual

The present series of reports, **the NanoDefine Methods Manual**, has been developed within the NanoDefine project 'Development of an integrated approach based on validated and standardized methods to support the implementation of the EC recommendation for a definition of nanomaterial', funded by the European Union's 7th Framework Programme, under grant agreement 604347.

In 2011 the European Commission (EC) published a recommendation for a definition of the term 'nanomaterial', the EC NM Definition, as a reference to determine whether an unknown material can be considered as a 'nanomaterial' for regulatory purposes¹. One challenge is the development of methods that reliably identify, characterize and quantify nanomaterials (NM) both as substances and in various products and matrices.

The overall goal of NanoDefine was to support the implementation of the EC NM Definition. It can also support the implementation of any NM definition based on particle size. The project has developed an integrated approach, which allows identifying any material as a nano- or not a nanomaterial according to the EC NM Definition. NanoDefine explicitly supported the governance challenges associated with the implementation of legislation concerning nanomaterials by:

- addressing the issues on availability of suitable measuring techniques, reference materials, validated methods, acceptable to all stakeholders (authorities, policy makers, commercial firms),
- developing an integrated and interdisciplinary approach and a close international co-operation and networking with academia, commercial firms and standardization bodies.

Thus, the NanoDefine Methods Manual provides guidance on practical implementation of the EC NM Definition throughout the nanomaterial characterization process, and on the characterization techniques employed as well as their application range and limits. It assists the user in choosing the most appropriate measurement method(s) to identify any substance or mixture for a specific purpose, according to the EC NM Definition of a nanomaterial. The NanoDefine project also explored how to assess a material against the criteria of the definition through proxy solutions, i.e. by applying measurement techniques that indirectly determine the x_{50} . Those findings were developed through empirically based scientific work and are included in Part 1 of this Manual. As they go beyond the text of the EC NM Definition, they may be used as practical approach to indicate whether a material is a nanomaterial or not, but keeping in mind that they should not be taken as recommendation for the implementation of the EC NM Definition in a regulatory context.

The NanoDefine Methods Manual consists of the following three parts:

- Part 1: The NanoDefiner Framework and Tools
- Part 2: Evaluation of Methods
- Part 3: Standard Operating Procedures (SOPs)

Part 1 covers the NanoDefiner framework, general information on measurement methods and performance criteria and tools developed by NanoDefine such as a materials categorisation system, a decision support flow scheme and an e-tool.

Part 2 discusses the outcome of the evaluation of the nanomaterials characterisation methods for measuring size.

Part 3 presents the 23 Standard Operating Procedures developed within the NanoDefine project.

The current document is part 1.

Abbreviations and acronyms used in the Manual

AC	Analytical Centrifugation
AF4	Asymmetrical Flow Field-Flow Fractionation
AFM	Atomic Force Microscopy
ALS	Angular Light Scattering
Aq.	Aqueous
AR	Aspect Ratio
AUC	Analytical Ultra Centrifugation
BET	Brunauer-Emmett-Teller
BSA	Bovine Serum Albumin
CM	Characterisation Method
CEN	European Committee for Standardization
CLS	Centrifugal Liquid Sedimentation
CPC	Condensation Particle Counter
DEMA	Differential Electrical Mobility Analysis (also spray-DEMA)
DMA	Differential Mobility Analyser
DUM	Dynamic Ultramicroscopy
DLS	Dynamic Light Scattering
DSFS	Decision Support Flow Scheme
DUM	Dynamic Ultramicroscopy
EC	European Commission
EC NM Definition	EC Recommendation on the Definition of a Nanomaterial
EDX / EDS	Energy Dispersive X-ray spectrometry
EELS	Electron Energy Loss Spectroscopy
EFTEM	Energy-Filtered Transmission Electron Microscopy
EHS	Environment, Health and Safety
EM	Electron Microscopy
ESD	Equivalent Spherical Diameter
ESI-SMPS	<i>Engineering System International</i> SMPS
ESZ	Electrical Sensing Zone
FFF	Field-Flow-Fractionation
FTIR	Fourier-transform infrared spectroscopy
HSE	Health, Safety and Environment
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
KB	Knowledge Base
LD	Laser Diffraction
LS	Light Scattering

MALS	Multi-Angle Light Scattering
MALLS	Multi angle laser light scattering
MCS	Material Categorisation Scheme
MT	Measurement Technique
MWCNT	Multi-walled Carbon Nanotube
m/z	Mass-to-Charge Ratio
NaDS	Sodium Dodecyl Sulphate
NM	Nanomaterial
NTA	Nanoparticle Tracking Analysis
NP	Nanoparticle
PSD	Particle Size Distribution
PTA	Particle Tracking Analysis
QELS	Quasi Elastic Light Scattering
RI	Refractive index
SAXS	Small-Angle X-ray Scattering
SDS	Safety Data Sheet
SEM	Scanning Electron Microscopy
SEM-EDX	SEM-Energy Dispersive X-ray Analysis
SedFFF	Sedimentation field-flow-fractionation
SFM	Scanning Force Microscopy
SLS	Static Light Scattering
SMPS	Scanning Mobility Particle Sizer
SOP	Standard Operating Procedure
spICP-MS	Single Particle ICP-MS
TEM	Transmission Electron Microscopy
TRPS	Tuneable Resistive Pulse Sensing
UF	Ultrafine
USB	Ultrasonic Bath Sonicator
USP	Ultrasonic Probe Sonicator
USSp	Ultrasonic Spectroscopy
UV	Ultra Violet
UV-vis	Ultra Violet - Visible
VS	Vial Sonicator
VSSA	Volume-Specific Specific Surface Area
XRD	X-ray Diffraction

Executive Summary

The overall goal of the NanoDefine project was to support the implementation of the EC Recommendation for a Definition of Nanomaterial (2011/696/EU) (EC NM Definition)¹. The project has developed an integrated approach, which allows identifying any material as falling within or outside the EC NM Definition.

Data, knowledge and tools developed, generated and/or evaluated in the project form the bases of the NanoDefiner Framework, e-tool and the NanoDefine Methods Manual. All these instruments are a result of a collaborative work of project partners, and development of NanoDefine Methods Manual and the NanoDefiner Framework were led by the JRC.

The NanoDefiner Framework, e-tool and Methods Manual were developed in the context of the EC Recommendation for a definition of nanomaterial¹, which provides a common basis for regulatory purposes across all areas of European Union policy. The definition or core parts of it have been enacted in EU legislation, (e.g. REACH, Biocidal Products Regulation, Medical Devices Regulation). Therefore development of appropriate methods and approaches to understand if a material meets the criteria laid down in the EC NM Definition is of key importance both for industry, stakeholders and regulators.

The objective of the NanoDefiner Framework is to provide the industry, stakeholders and regulators with information and procedures to decide, for particulate materials, whether they fulfil the EC's Recommendation on a Definition of Nanomaterial (2011/696/EU).

The NanoDefiner Framework relies on three pillars: (i) knowledge base (methods performance evaluation and development), (ii) Materials Categorisation Scheme and (iii) Decision Support Flow Scheme, and the framework is implemented in the NanoDefiner e-tool software.

The developed framework and its tools are:

- easy to implement: they integrate end-users' current practice/facilities/expertise with new developments
- cost efficient: they offer a tiered approach to selecting the most adequate analytical route to arrive at an identification according to the EC NM Definition with the least possible effort
- flexible: they define criteria for the inclusion of novel technologies and can be adapted easily to changing regulatory requirements
- sustainable: part of the developed approach has already been implemented in structures that persist beyond the duration of the project

By applying the developed tools and following the logic of the NanoDefiner Framework, the user is provided with recommendations on the most suitable method(s) to characterise specific particulate materials. Based on the data input, the user is provided with a decision whether the analysed material is a nano- or not a nanomaterial according to the EC NM Definition. The NanoDefiner decision framework allows expert judgement at every decision node, with options based on material type, purpose, required data quality and economic parameters. It guides the user to the most reliable and cost-efficient measurement method and provides recommendations to identify any substance according to the EC NM Definition. The NanoDefiner Framework and its tools are tested best practice procedures that allow industrial and regulatory stakeholders the identification of particulate materials and products containing such materials.

1 Definition of Nanomaterial

1.1 European Commission's Recommendation on the definition of nanomaterial

In October 2011 the European Commission (EC) published a 'Recommendation on the definition of nanomaterial'¹ (here subsequently referred to as the EC NM Definition), to promote consistency in the interpretation of the term 'nanomaterial' for legislative and policy purposes in the EU. The purpose of the EC NM Definition is to allow determination of when a material should be considered a nanomaterial (NM) for regulatory purposes in the European Union. The EC NM Definition uses size (i.e. size range 1 – 100 nm) as the only defining property of the material. The size refers to the external dimensions of the constituent particles of a material which can be unbound but also may be in a form of agglomerates and/or aggregates.

The European Commission recommends the following definition of the term 'nanomaterial':

'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 Recommendation additionally 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 is not legally binding and does not entail a direct obligation for Member States or stakeholders. Therefore it can be assumed that its implementation will happen through different pieces of specific product legislation. In this process the overarching broad definition can be adjusted to the scope and precise needs of a specific regulation. Examples for this are the Biocidal Products Regulation, the Regulation on Medical Devices, the Cosmetic Products Regulation and the Novel Food Regulation (see Annex 6). It is expected that some of these Regulations will be amended with the intention to harmonise

the legally binding definitions of nanomaterials with the EC NM Definition. This way the EC NM Definition, although being legally non-binding, has an effect on specific legislation.

1.2 Legal status of nanomaterials in the EU (REACH, CLP and product specific legislation)

In the European Union there is no dedicated nano-specific regulation. However, horizontal and sector-specific legislation provides a binding framework for manufacturers, importers and users to ensure the safety of substances and products on the market. Annex 6 lists the most relevant EU legislation.

In the EU chemical substances are regulated under the Regulation (EC) No 1907/2006 concerning Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). REACH provides an overarching legislation applicable to the manufacturing, placing on the market and use of substances on their own, in preparations or in articles. Another horizontal regulation related to chemical substances in Europe is the Regulation on Classification, Labelling and Packaging of chemical substances and mixtures (CLP Regulation, Regulation (EC) No 1272/2008). The regulation introduces a system for classifying and labelling chemicals based on the United Nations' Globally Harmonised System (UN GHS). Both regulations use the same terminology and are coherent in requirements.

On December 4th 2018 the Commission adopted amendments to the REACH annexes, which now include nano-specific requirements. The EC NM Definition is included in the amended REACH annexes and is applicable for identifying if a substance is in a nanoform under the framework of REACH thus triggering the application of the nano-specific provisions for its registration.

Currently, several pieces of sector-specific EU legislation explicitly address NMs. This includes the Regulation on the Provision of Food Information to Consumers (1169/2011), the Regulation on Plastic Food Contact Materials and Articles (10/2011), the Regulation on Active and Intelligent Materials and Articles (450/2009), the Biocidal Products Regulation (528/2012), the Novel Food Regulation (2015/2283), the Medical Devices Regulation (2017/745) and the Cosmetic Products Regulation (1223/2009).

It is worth to notice that several EU member states, e.g. France, Belgium and Denmark, have created their own registration scheme and have put an obligation on the producers and importers of nanomaterials to notify any foreseen use of these materials on the national market.

2 NanoDefiner framework concept

Nanotechnology is a key enabling technology, but the existing uncertainties concerning Environment, Health and Safety (EHS) need to be addressed to explore the full potential of this new technology. The constant increase of the use of nanomaterials has triggered the need for their regulation; therefore, worldwide, a variety of legislative provisions in different sectors address nanomaterials and require their identification, characterisation, quantification, and often a particular safety assessment. Specific regulatory provisions include definitions of the term 'nanomaterial' to identify a material as nanomaterial or not a nanomaterial according to certain criteria, and to decide if nanomaterial specific provisions apply. Regardless of differences in scope and implementation, all definitions of the term 'nanomaterial' share one common feature as the fundamental defining element: particle size. Consequently, in any context for a decision on whether a material is a nanomaterial or not, it is always necessary to determine its particle size distribution. This involves the measurement of particle size from few nanometres up to several micrometres. 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 such size measurements are to be done to fulfil regulatory obligations, the results must be relevant, reliable and transparent so that the involved parties, i.e. commercial firms and regulators, including non-specialists in the metrology field, mutually can accept the conclusions drawn from them. To cope with all these challenges, it is necessary to come to an agreement on 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 and the performance profile of size measurement techniques (MT). In the case of particulate materials, the availability of a knowledge base (KB) consisting of size measurement techniques matched to specific material properties would greatly facilitate a reliable regulatory valid identification as a nanomaterial or not a nanomaterial. In this context a consortium of European research institutes and universities, metrology institutes and nanomaterials and instrument manufacturers was established to mobilise the critical mass of expertise required to support the implementation of the definition. The NanoDefine project was founded and it provided a framework that supports the implementation of the definition. The framework builds on three pillars: a knowledge base (methods performance evaluation and development), a technique-driven Material Categorisation Scheme (MCS) and a Decision Support Flow Scheme (DSFS), and it is also implemented in a software, the NanoDefiner e-tool (see Figure 1).

Part 1 of the Methods Manual presents the basic information on the pillars of the NanoDefiner Framework as well as general information on the framework. Section 3 specifically introduces the criteria applied to the evaluation of characterisation methods and presents its results in tables. Detailed information on the methods evaluation and description of each method including its advantages and limitations regarding particle size determination are presented in Part 2 of this manual. The detailed Standard Operating Procedures of the sample preparation and dispersion are included in a separate document (Part 3 of the manual). All this information comprises the basis of the first pillar of the NanoDefiner Framework: the Knowledge Base.

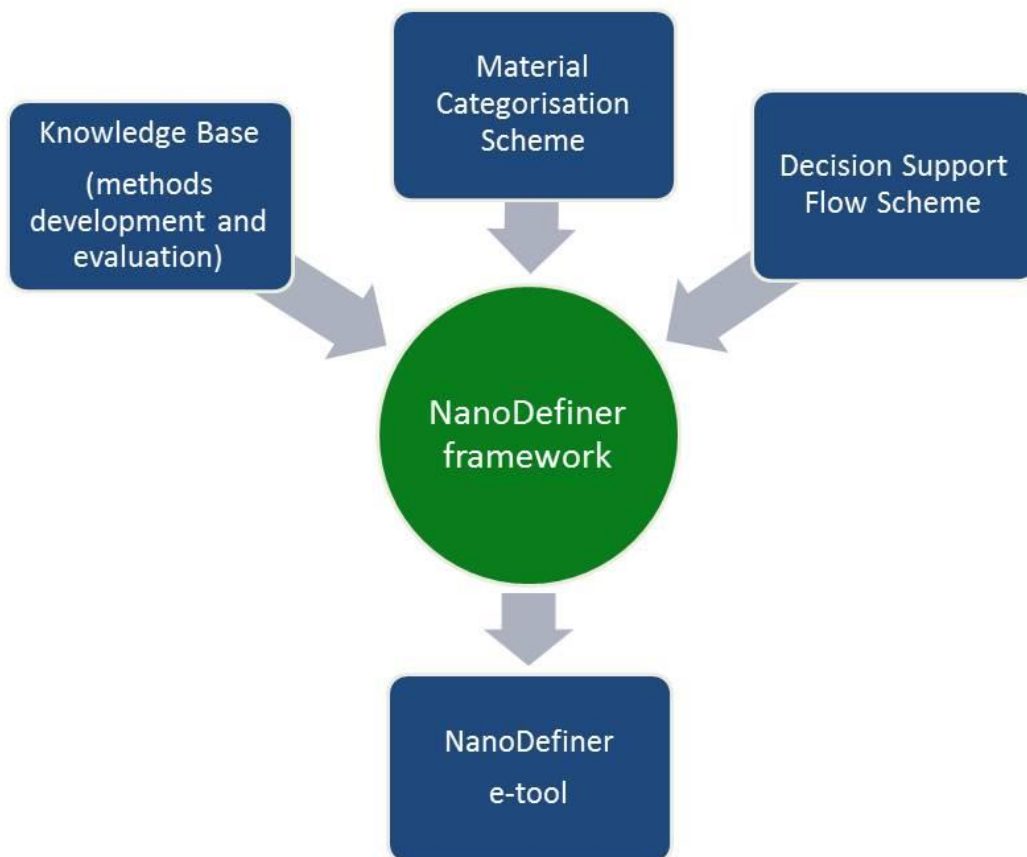


Figure 1: NanoDefiner Framework. Pillars and implementation in the e-tool.

The **Knowledge Base** contains attributes which describe the performance profiles of measurement techniques and the material profiles in terms of physicochemical properties. The KB contains also administrative information, explanations for certain cases in the MT recommendation, and MT-specific weighting of attributes for the decision making. Measurement techniques are described via 83 attributes on supported material properties, measurement performance and technical/economic aspects (e.g. particle shape, working size range, cost efficiency). Materials are currently described via 21 attributes on physicochemical features (e.g. stable temperature range). The attributes in the knowledge base were derived from templates for the description of the measurement technique performance profiles and from the MCS for the description of material property profiles. The KB is spreadsheet-based and maintainable by non-computer scientists. The KB was developed through comprehensive analysis of available particle measurement techniques and characterisation methods (CMs)^a that were previously identified as candidates for a reliable analysis of the number-based size distribution of a particulate material. The performance of measurement techniques was assessed by experts from industry and academia.¹⁰ During the project the KB was continuously optimised via multiple revision cycles to ensure

^a A 'characterisation method' includes sample preparation, measurement procedure and data evaluation. A 'measurement technique' refers only to the measurement itself. However, sometimes the two terms are used interchangeably.

the validity of its structure and the configured profiles. Based on the evaluated capabilities, characterisation methods for tier 1 (screening) and tier 2 (confirmatory) are recommended when identifying nanomaterials (and also materials falling outside the EC NM Definition) according to the EC NM Definition while using the NanoDefiner Framework. The KB also includes the default property profiles of 17 specific materials tested in NanoDefine as well as the default performance profiles of 16 measurement techniques (see Section 3). The structure of the KB allows deriving material group-/type-dependent measurement technique configurations from their default configuration set-up.

The **Material Categorisation Scheme²** (MCS) is a practical categorisation system for the fundamental task to select appropriate particle sizing methods for all kinds of particulate materials. It is technique-driven and pragmatic facilitating the regulatory identification of nanomaterials. In this scheme, materials are categorised according to criteria linked to the capabilities of experimental methods for particle size measurement. This allows the selection of methods that are compatible and suited to measure materials with specific characteristics, which in turn helps to assess the reliability of the obtained data. The MCS is described in detail in section 4 supported by the detailed information presented in Annex 1. The information which allows matching specific materials with the suitable methods is described in section 5 and summarised in Table 7.

The **Decision Support Flow Scheme** (DSFS) is a logical sequence of tasks, decision nodes and options to guide the user in order to decide whether a material is a nanomaterial according to the EC NM Definition. The flow scheme takes into account already available information on the material as well as the requirements for the quality of the result and the availability of instruments and methods in the laboratory. At each decision node the DSFS evaluates the obtained information and data and guides the user through the next steps. This can either be an additional measurement by a tier 1 method or a tier 2 method or may directly lead to the recommendation for a decision on whether a given material should be considered as a nanomaterial or not. The DSFS is described in some detail in section 6.

The **NanoDefiner e-tool** is free, open source specific software which is based on the decision support framework (knowledge base, material categorisation scheme, decision support flow scheme). It pools results and conclusions together from method evaluation and developments in NanoDefine with findings obtained from validation and case studies. This tool, with options based on material type, purpose, required data quality (including and economic parameters), guides the user to the measurement technique that is expected to be most reliable and provides recommendations to categorise any substance according to the EC NM Definition. The e-tool is described in section 7, accompanied by a software specific guidance in Annex 4.

Building on the elements above, the NanoDefiner framework uses a tiered approach of measurement methods as it is expected that many materials can be already categorised by rather simple, robust and cost-efficient methods (tier 1). Only in cases where these methods do not produce the required information or are not sufficiently reliable, one has to proceed to tier 2 of more sophisticated methods (confirmatory) to reliably assess the size of nanoparticles with different shapes, agglomeration/aggregation states or specific composition. This concept allows to (i) align with cost-efficient methods that are currently available/used in stakeholders' laboratories, and to (ii) limit the use of more labour intensive methods and high-end instrumentation to the cases when tier 1 methods fail.

The identification procedure guides the selection of the measurement techniques that are expected to be most appropriate for a specific case taking into account already available information on the material, requirements on the quality of the result and the availability of instruments and methods in the

laboratory. At each decision node the framework evaluates the obtained information and data and guides the user through the next steps. This can either be an additional measurement by a tier 1 or a tier 2 method or may directly lead to the decision if a given material should be considered as nanomaterial.

By following the logic of the NanoDefine Framework (see Figure 2) manually or through the e-tool, the user is provided with recommendations on the most appropriate method to characterise a specific particulate material. Furthermore, based on the data input, the user is provided with a suggestion of whether the analysed material is a nanomaterial according to the EC NM Definition. Therefore the NanoDefiner framework can be seen as a set of tools which supports users who for regulatory purposes need to identify nanomaterials and materials falling outside the EC NM Definition in a fast and economical way. The major outcome of the NanoDefiner Framework is a tested best practice procedure that allows industrial and regulatory stakeholders to do this.

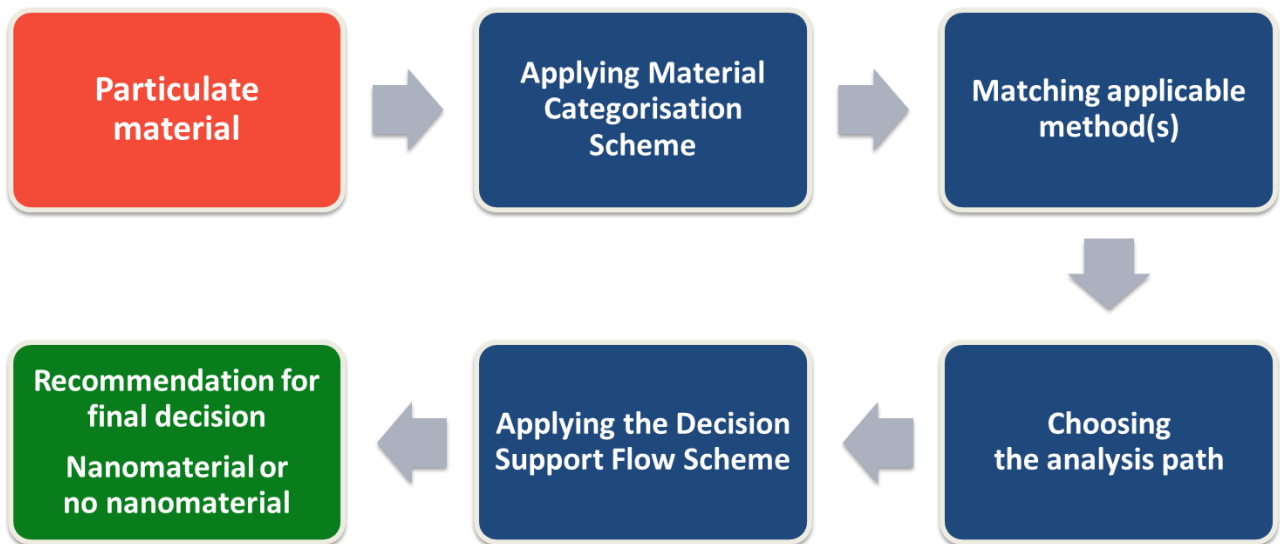


Figure 2: Logical sequence within the NanoDefiner Framework

3 Measurement Methods

This chapter introduces the criteria according to which the Characterisation Methods (CM) available in NanoDefine were evaluated. These methods were candidates for the reliable analysis of the number-based size distribution of a particulate material, with the goal to identify nanomaterials according to the EC NM Definition.

Detailed information on different types of methods and their evaluation which allows for the determination of size and size distributions are presented in a separate document: 'The NanoDefine Methods Manual. Part 2: Evaluation of methods.'³ Part 2 presents the results of the evaluation of the methods performance, which constitute the first pillar of the NanoDefiner framework: the Knowledge Base.

The following measurement techniques were evaluated, see Table 1.

Table 1: List of the measurement techniques evaluated in the NanoDefine project

	Tier 1 methods (screening)	Tier 2 methods (confirmatory)
Recommended by NanoDefine for general use (Based on the results obtained on the NanoDefine Training Set materials)	DLS AC BET Spray DEMA Mini TEM	TEM SEM
Not applicable for general use, but potentially suitable for specific materials (Based on the results obtained on the NanoDefine Training Set materials)	PTA LD SAXS XRD spICP-MS USSP ALS AF4-ICP-MS AF4-LS TRPS*	AFM*

*Method not experimentally evaluated in NanoDefine

ABBREVIATIONS: AC: Analytical centrifugation. AF4: Asymmetrical flow field-flow fractionation, AFM: atomic force microscopy, ALS: Angular light scattering BET for determination of volume specific surface area (VSSA), DEMA: Differential electrical mobility analysis, DLS: Dynamic light scattering. LD: Laser diffraction, LS: Light scattering, PTA: Particle tracking analysis, SAXS: Small-angle X-ray scattering, SEM: Scanning electron microscopy, spICP-MS: Single particle Inductively coupled plasma –Mass spectrometry, TEM: Transmission electron microscopy, USSP: Ultrasonic spectroscopy, TRPS: Tunable Resistive Pulse Sensing

An overview of the performance of the techniques is presented in this document (Part 1) in the form of tables. Such tables provide the user with a possibility for quick selection of the method which may be appropriate for the characterisation of given material. However for final selection of the method to be

employed it is highly recommended to consult the detailed performance tables found in Part 2 of the NanoDefine Methods Manual. Part 2 also includes a non-exhaustive list of relevant international standards on particle sizing.

Determination of size and size distributions can be based on different principles and approaches (e.g. imaging, sedimentation, light extinction). Generally, particle sizing techniques can be grouped according to the following principles:

- counting methods (measuring particle properties at individual particles)
- (spectroscopic) ensemble methods (measuring the spectral or parametric response of a representative particle ensemble of the total particle system)
- fractionating methods (measuring the amount or concentration of size/property classes after fractionating the particle system).
- integral methods.

Please see Part 2 of this manual for a detailed explanation of these principles.³

Table 1 gives an overview of the evaluated methods and whether they are tier 1 (screening) or tier 2 (confirmatory).

3.1 Performance criteria

For the purpose of the NanoDefiner framework performance criteria of each measurement method were elaborated in detail. The performance criteria either relate (i) to the materials to be analysed or (ii) to the technical capability of the method. The criteria include:

Applicability to different types of substances

- Powder or liquid suspensions or embedded in a matrix
- Dispersibility according to dispersion protocols
- Nature of the Substance
- Inorganic materials such as metals, ceramics, salts, oxides (significant content of inorganic elements homogeneously incorporated in all constituent particles)
- Particles which exhibit size-dependent absorption of photons / fluorescence (metals, quantum dots ...)
- Carbon-based (CNT, nanodiamond, carbon black...)
- Organic, particular (polymers, dyes, etc., nanonized, precipitated)
- Organic, non-particular (dendrimers, liposomes, supramolecular assemblies...)
- Biological (nucleic acid, peptide, protein)
- Composite particles
- Other
- Particle shape and number of small dimensions
- Thermal degradation sensitivity
- Cooling degradation sensitivity

- Sensitivity to an electron beam (E-beam sensitivity)
- Sample dispersity and modality
- Optional criteria: electrical conductivity, magnetic properties, functionalisation/no functionalisation on the surface,
- Agglomeration/aggregation state

Technical factors of the method

- Type of measurement technique (Counting, fractionating or [spectroscopic] ensemble techniques)
- Working range
- Trueness
- Robustness
- Precision
- Resolution
- Size distribution
- Selectivity
- Capability to measure aggregation
- Counting constituent particles in aggregates
- Composition
- Specification of the measurand
- Non-destructive/destructive

These criteria as well as the detailed outcomes of the methods evaluation along with the method description are presented in a separate document, 'The NanoDefine Methods Manual. Part 2: Evaluation of methods'.³

Each characterisation method was evaluated against these criteria depending on the substance to analyse (if the characterisation method is suitable for this type of substance) and on the technical factors. The applied criteria are not listed according to their priority.

3.2 Performance table

The outcomes of the ranking of each characterisation method are presented in a dedicated table. For clarity each table was divided into two sections: the first (blue rows) is related to the type of substance to analyse. For each method it indicates whether that method is suitable to characterise materials with specific properties. The second section (yellow rows) is related to the technique. It indicates the characteristics of each characterisation method according to the specified criteria. Table 2 represents an empty method performance table; Chapter 2 of Part 2 describes the performance table content in more detail. Performance tables filled in for characterisation methods (see section 3.3 for an overview of the methods) can be found in the Part 2 of NanoDefine Methods Manual.³

Table 2 Performance table for assessment of measurement techniques.

Colour code: material-related technical characteristics and metrological aspects of the technique

Criteria (general)	Criteria (more specific)	Characterisation (Yes/No)	Notes
Nanoparticles in powder or liquid suspensions or embedded in a matrix	Dispersed in liquids		
	Solid particulate form		
	Dispersed or embedded in matrices		
Dispersibility by dispersion protocols	Dispersible in aqueous media		
	Dispersible in non-polar liquids		
	Dispersible in polar liquids other than water		
	Dispersible in material-specific media		
	Can be aerosolised		
Substance Nature	Inorganic		
	Size-dependent absorption / fluorescence		
	Carbon based		
	Organic, particulate		
	Organic, non-particulate		
	Biological		
	Composite		
	Other		
Composite particles (see section 2.3.3.1)	Core/shell		
	Multiple coatings		
	A mix of two or more different materials		
Number of small dimensions	1 (e.g. thickness of nanoplates)		
	2 (e.g. diameter of nanofibres)		
	3		
Shape of nanoparticles	Sphere or similar		
	Equiaxial		
	Tubes, fibres, rods (length:diameter ≥ 3)		
	Flakes and discs (thickness: lateral extension ≤ 0.25)		
	Other		
Thermal degradation sensitivity (Must be compatible with Measurement Technique working range: x-y °C)	Above 0 °C		
	Sensitive above 25 °C		
	Sensitive above 37 °C		
	Sensitive above 50 °C		
	Sensitive above 100 °C		
	Sensitive above 150 °C		
	Sensitive above 500 °C		

	Sensitive above 1000 °C		
Cooling degradation sensitivity (Must be compatible with Measurement Technique working range: x-y °C)	Sensitive below 25 °C		
	Sensitive below 0 °C		
	Sensitive below -18 °C		
	Sensitive below -35 °C		
	Sensitive below -78 °C		
	Sensitive below -195 °C		
Electron beam sensitivity	Electron beam sensitive		
	Not electron beam sensitive		
Particle size dispersity and modality	Monodisperse		
	Polydisperse		
	Monomodal		
	Multimodal		
Conductivity properties (electrical)	Conductive		
	Semiconductive		
	Insulator		
Magnetic properties	Magnetic		
	Non magnetic		
Functionalization/no functionalisation	Functionalised		
	Not functionalised		
Agglomeration/aggregation state	Nanoparticles are aggregated		
	Nanoparticles are not aggregated		
	Nanoparticles are agglomerated		
	Nanoparticles are not agglomerated		
Counting, fractionating or ensemble technique	Single particle counting		
	Measures or calculates number or number concentration from fractionating techniques		
	Calculates number or number concentration from spectroscopic ensemble techniques		
	Integral technique		
	Used in hyphenated methods		
Working range	Size range		
	Concentration range		
	Minimum needed sample amount		
	Linearity/proportionality		
	Limits of detection/quantification		
	Sensitivity (Counting efficiency) as a function of size		
Trueness	Indicate the trueness of this measurement technique in measuring the particle size		

Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique		
Robustness	Specify the robustness of this measurement technique		
Precision	Specify the precision of the measurement technique		
Resolution	Specify the resolution of this measurement technique		
Size distribution	Is it possible to measure size distribution?		
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition		
	Discrimination between NPs and non-NPs of another chemical composition		
	Discrimination from NPs of another chemical composition		
	Impurities		
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?		
Measurement of individual particles	Does this measurement technique characterise individual particles?		
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?		
Chemical composition	Does this measurement technique analyse chemical composition?		
Specification of the type of size (diameter)	Specify: for example hydrodynamic...		
Destructive measurement technique or not	Is it a destructive measurement technique?		
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?		
Sample support	Does this measurement technique need preparation on suitable supports?		

3.3 Evaluation tables

Details on the evaluation of individual characterisation methods can be found in Part 2 of this Report.³ A general overview of the recommended characterisation methods and with their capabilities is shown in Table 3 to Table 6. For the methods, Table 3 gives an overview of the size range within for the measurements with the various methods, Table 4 gives the suitability for material types, Table 5 capabilities related to particles, agglomerates and aggregates and Table 6 presents additional information relevant for the methods capabilities. For clarity only the scores fair, good and very good are highlighted in the tables. It should be noted that these tables give only a general overview of the recommended methods. For an appropriate selection of suitable methods the detailed performance tables should be consulted. Table 1 lists the evaluated measurement techniques.

Table 3: Evaluation of the methods: Size range

Type of method	Method		Size range					
			nm			µm		
			1-10	10-30	30-100	0.1-1	1-10	>10
Counting	EM	SEM		Good	Very good	Very good	Very good	Very good
		TSEM	Good	Very good	Very good	Very good	Very good	Very good
		TEM	Very good	Very good	Very good	Very good	Very good	Good
		SFM/AFM	Good	Very good	Very good	Good	Fair	
		PTA		Fair	Good	Very good		
		TRPS			Fair	Very good	Very good	
		spICP-MS		Fair	Good	Very good		
Ensemble		DLS	Good	Very good	Very good	Very good	Fair	
		SAXS	Good	Very good	Very good			
		USSP		Good	Very good	Good	Fair	Fair
		ALS			Fair	Very good	Very good	Very good
Fractionating		FFF	Good	Very good	Very good	Very good	Fair	
		AC	Fair	Good	Very good	Very good	Good	
		DEMA	Good	Very good	Very good			
Integral		BET	Good	Very good	Very good	Very good	Very good	Fair
Legend:			Not covered	Fair	Good	Very good		

Table 4: Evaluation of the methods: Material

Type of method	Method		Sample			Type of material						Shape					
			Dispersed in liquids	Solid particulate form	Embedded in matrix	Inorganic	Carbon based	Organic, particulate	Biological	Core/Shell	Multiple coatings	Inclusion	Sphere	Equiaxial	Tubes, fibres, rods	Flakes discs	
Counting	EM	SEM															
		TSEM															
		TEM															
	SFM/AFM																
	PTA																
	TRPS																
	spICP-MS																
Ensemble	DLS																
	SAXS																
	USSP																
	ALS																
Fractionating	FFF																
	AC																
	DEMA																
Integral	BET																
Legend:		Not covered	Fair	Good	Very good												

Table 5: Evaluation of the methods: Particles, aggregates and agglomerates

Type of method	Method		Size distribution	Measures aggregates/agglomerates	Measures individual particles	Counting constituent particles in aggregates	Measures constituent particles in aggregated/agglomerated samples	Measures constituent particles in not aggregated/agglomerated samples
Counting	EM	SEM	Very good	Fair	Very good	Very good	Very good	Very good
		TSEM	Very good	Fair	Very good	Very good	Very good	Very good
		TEM	Very good	Fair	Very good	Very good	Very good	Very good
	SFM/AFM		Fair	Not covered	Fair	Not covered	Not covered	Very good
	PTA		Very good	Fair	Very good	Not covered	Not covered	Very good
	TRPS		Very good	Not covered	Very good	Not covered	Not covered	Very good
	spICP-MS		Very good	Not covered	Very good	Not covered	Not covered	Very good
Ensemble	DLS		Very good	Fair	Not covered	Not covered	Not covered	Very good
	SAXS		Very good	Fair	Not covered	Not covered	Not covered	Very good
	USSP		Very good	Not covered	Not covered	Very good	Very good	Very good
	ALS		Not covered	Not covered	Not covered	Not covered	Not covered	Not covered
Fractionating	FFF		Very good	Not covered	Not covered	Not covered	Not covered	Very good
	AC		Very good	Not covered	Not covered	Not covered	Not covered	Very good
	DEMA		Very good	Not covered	Very good	Not covered	Not covered	Very good
Integral	BET		Not covered	Not covered	Not covered	Not covered	Fair	Very good
Legend:		Not covered	Fair	Good	Very good			

Table 6: Evaluation of the methods: Additional information

Type of method	Method		Direct counting technique	Access to the smallest dimension of each particle	Measurement of the material as it is	ISO standards available	Size Accuracy	Chemical selectivity	Access to constituent particles?
Counting	EM	SEM	Very good	Good	Not covered	Good	Very good	(+ EDX)	Very good
		TSEM	Very good	Good	Not covered	Good	Very good	(+EDX)	Very good
		TEM	Very good	Good	Not covered	Good	Very good	(+EDX)	Very good
	SFM/AFM	Not covered	Very good	Not covered	Not covered	Good	Very good	Not covered	Good
	PTA	Not covered	Very good	Not covered	Not covered	Good	Very good	Not covered	Not covered
	TRPS	Not covered	Very good	Not covered	Not covered	Good	Very good	Not covered	Not covered
	spICP-MS	Not covered	Very good	Not covered	Not covered	Good	Very good	Very good	Not covered
Ensemble	DLS	Not covered	Not covered	Not covered	Not covered	Good	Very good	Not covered	Not covered
	SAXS	Not covered	Not covered	Good	Very good	Good	Very good	Not covered	Very good
	USSP	Not covered	Not covered	Not covered	Not covered	Good	Very good	Not covered	Not covered
	ALS	Not covered	Not covered	Not covered	Not covered	Good	Very good	Not covered	Not covered
Fractionating	FFF	Not covered	Not covered	Not covered	Not covered	Good	Very good	(+Detector)	Not covered
	AC	Not covered	Not covered	Not covered	Not covered	Good	Very good	Not covered	Not covered
	DEMA	Not covered	Good	Not covered	Not covered	Good	Very good	Not covered	Not covered
Integral	BET	Not covered	Not covered	Not covered	Good	Very good	Not covered	Good	
Legend:									
Legend:		Not covered	Fair	Good	Very good				

4 Material Categorisation Scheme

The Material Categorisation Scheme developed within the NanoDefine project supports selecting appropriate particle sizing methods for all kinds of solid particulate materials². It is technique-driven and pragmatic to facilitate the regulatory identification of nanomaterials.

In this scheme materials are categorised according to criteria presented, see Ref. [3], which are linked to the capabilities of experimental methods for particle size measurement. This allows the selection of methods that are compatible and suited to measure materials with specific characteristics, which in turn assures the reliability and general acceptance of the obtained data.

Based on the performance characteristics of specific techniques, i.e. what kind of material they actually can characterise in a reliable way, the MCS uses three principal categories of particulate materials (see Figure 3).

Material with monotype particles: all particles have essentially the same chemical and structural composition. For the purposes of specific legislation an ensemble of such particles can constitute a 'nanofom' (Commission Regulation (EU) 2018/1881) and a 'discrete form' of substance in nanoscale as defined by US EPA under specific conditions. The particles can consist of (i) a single chemical element (e.g. Au) or compound (e.g. SiO₂) or (ii) different elements or compounds, but with the same internal structure. In the latter case, the particles are composite⁴ particles. Composite particles can be present in different types as well. *Core-shell particles* consist of at least two components, one of which (the core) lies within the other that forms the outer layer (the shell). *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. The internal structure of a composite particle can be important for selection of the measurement technique. For example, certain techniques such as spICP-MS (unless the structure is known and multi-element spICP-MS is used) or XRD cannot accurately measure the size of multilayer particles.

Materials with multiple types of particles: a material that contains particles of different types, i.e. different chemical or structural compositions. It can be visualised as a mixture of different materials with monotype particles.

Articles and formulations that contain particles of the same or different types: an article is an object which, during production, is given a special shape, surface or design which determines its function to a greater degree than does its chemical composition.⁵ An article may consist of different chemical substances in different physical phases (liquid/solid/gaseous) and forms, including nanoparticles of one or several types. A formulation is a particular combination of chemicals (prepared according to a formula) that do not chemically react with each other. The chemicals in a formulation are chosen because of their specific properties, and, when combined, result in a product with desirable characteristics. This includes also certain consumer products, which are defined according to CEN as items intended for consumers or likely to be used by consumers.⁶ For example, a sunscreen that contains titanium dioxide nanoparticles is a formulation and a tennis racquet with incorporated carbon nanotubes is an article. Both of them are consumer products.

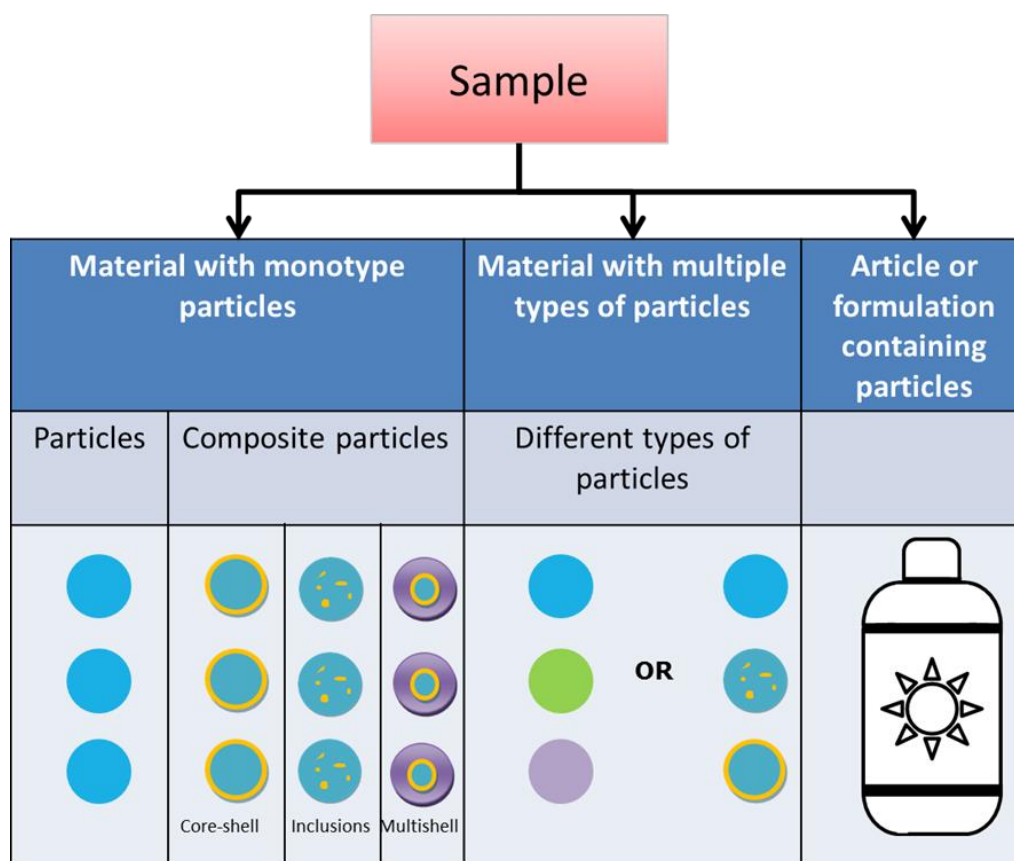


Figure 3: Different types of particles and particulate materials are considered in the categorisation scheme. 'Sample' is the generic term for the material to be analysed (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

If the particles are all of the same type, a characterisation method needs to be suitable for that type only, whereas if a material consists of different particle types, the chosen technique should be applicable to all particle types present.

The choice of the most appropriate method(s) to measure particle size is further determined by the properties of the material to measure since the latter may determine the methods' limits of applicability. Hence, only a good match between the material properties and the performance of the method will lead to reliable and robust results.

After the determination of the material type, the material is further categorised according to the following sub-categories that describe the most relevant particle parameters which dominate the choice of the analytical technique(s) for particle size determination (see Figure 8 and also Annex 1 for detailed information).

Each of these main criteria further is further sub-divided to specify in detail material characteristics that are relevant for particle sizing methods. A detailed discussion on the selection and further subdivision of the categorisation criteria for the proposed scheme can be found in Annex 1. The resulting proposed MCS is presented in Figure 8. Not only can it serve as a powerful tool supporting regulatory identification of nanomaterials but it should be also helpful for academia, industry and

other stakeholders when choosing the most appropriate method for development, research or quality control (QC) purposes involving particulate materials, including nanomaterials.

Chemical composition (or chemical nature): The chemical nature of the particles strongly influences the choice of the appropriate characterisation methods. This may be due to a composition-dependent sensitivity, or because some methods may be applied only to a limited variety of chemicals, e.g. due to element-specific detection.

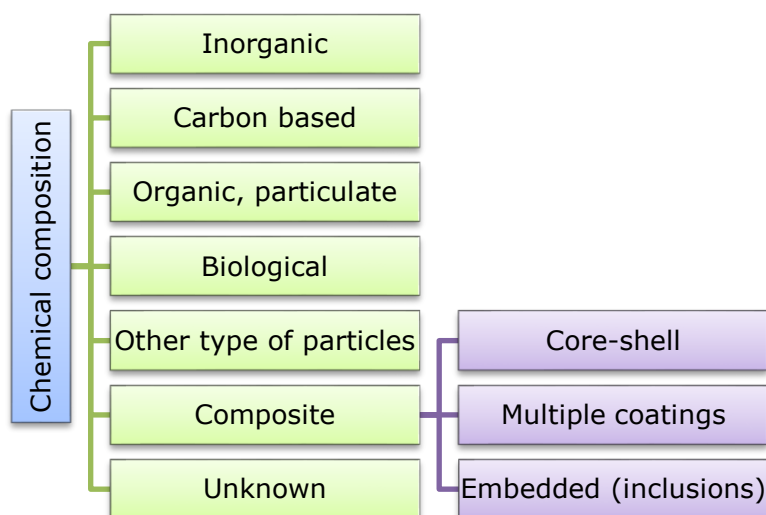


Figure 4: Chemical composition (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

Number of small dimensions and shape: Many of the currently employed characterisation methods implicitly assume that the particles are spherical or yield an equivalent spherical size, which limits their applicability to particles with non-spherical shape.⁷ Furthermore, methods need to be specifically suitable to measure the smallest dimensions of plate- or fibre-like particles.

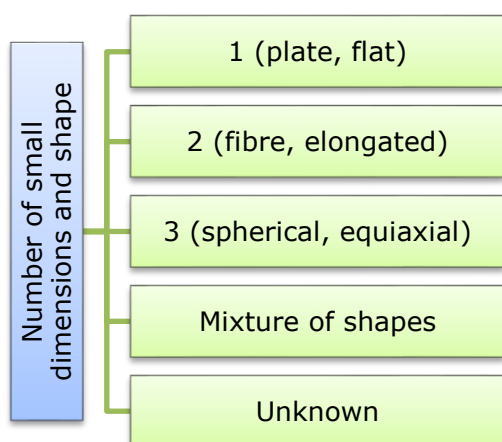


Figure 5: Number of small dimensions and shape (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

Size range: Methods for particle sizing have a range within which they can measure particle sizes. That size range can depend on further criteria, e.g. the chemical composition or the polydispersity of the material. On the other hand, an analysis needs to cover the entire size range of the particulate material in order to get an accurate result for the size distribution.⁷

Trade form and dispersibility: Some characterisation methods require the particles to be dispersed in a liquid phase, whereas others only work for powders. Therefore, it is essential to have information on the analysed trade form of the material and to know if the material to be analysed is pre-dispersed or can be dispersed. This should also include information on the dispersing media and specific protocols to be used.

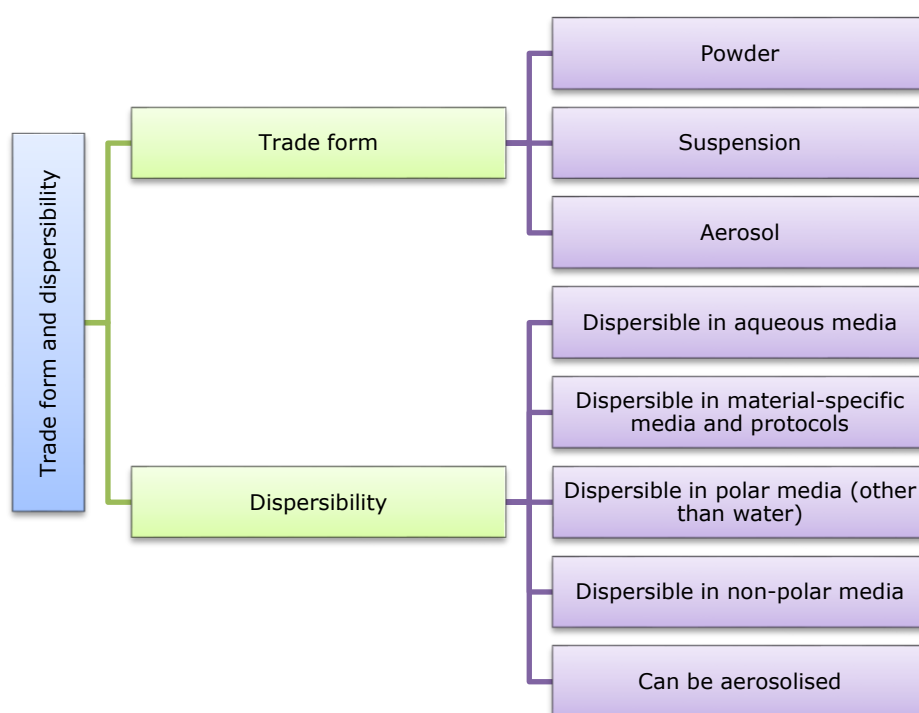


Figure 6: Trade form and dispersibility. (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

Stability during testing: Some materials may be incompatible with the conditions of certain measurement techniques, e.g. they may be sensitive to irradiation by electrons. Other materials may be stable only in a narrow temperature range. Thus, it is generally necessary to know if characterisation methods through their probes can cause damages to materials.

Specific properties: Specific electrical, optical, magnetic and surface properties may interfere with or, on the contrary, facilitate certain measurement methods.⁷ Specific material properties are therefore to be taken into account in order to avoid inappropriate methods.

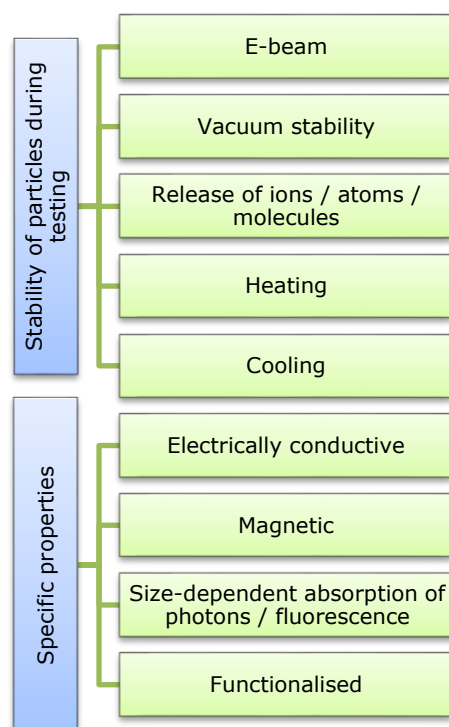


Figure 7: Stability of particles during the testing and specific properties (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

For illustration of the categorisation system, let us consider a hypothetical material that consists of monotype particles. That material can either directly match one of the sub-criteria (e.g. main criterion: chemical composition → sub-criterion: carbon based) or assume a value associated with a sub-criterion, which can be non-numerical (shape → spherical (3 small dimensions)) or numerical (stability of particles during testing → heating → stable up to ...K). A material fully categorised this way can then be matched to the performance of available particle size measurement methods, which finally allows the selection of the appropriate and most suitable methods for a given material.

The MCS can be applied also to materials with multiple particle types, i.e. where particles do not have the same chemical composition and internal structure, by applying the categorisation to each individual type of particles of the material. Ideally, the analysis of such a material employs selective techniques to measure the size distribution of each particle type independently of the other type(s). This is typically possible only if one deals with a mixture of different substances or a mixture of different types of nanoparticles with non-overlapping distributions of probed properties (e.g. minimum Feret diameter with TEM or settling velocity with analytical centrifugation).

Similarly, categorisation of particles in an article or a formulation is also possible (see Annex 1). In that case, criteria for the possibility to remove the non-particulate matrix (defined here as a non-particulate constituent or component of a material, including additives) are added while other criteria remain the same. Addressing also particulate materials in the MCS which are incorporated in articles or products aims to facilitate the identification of the particles for regulatory enforcement when required.

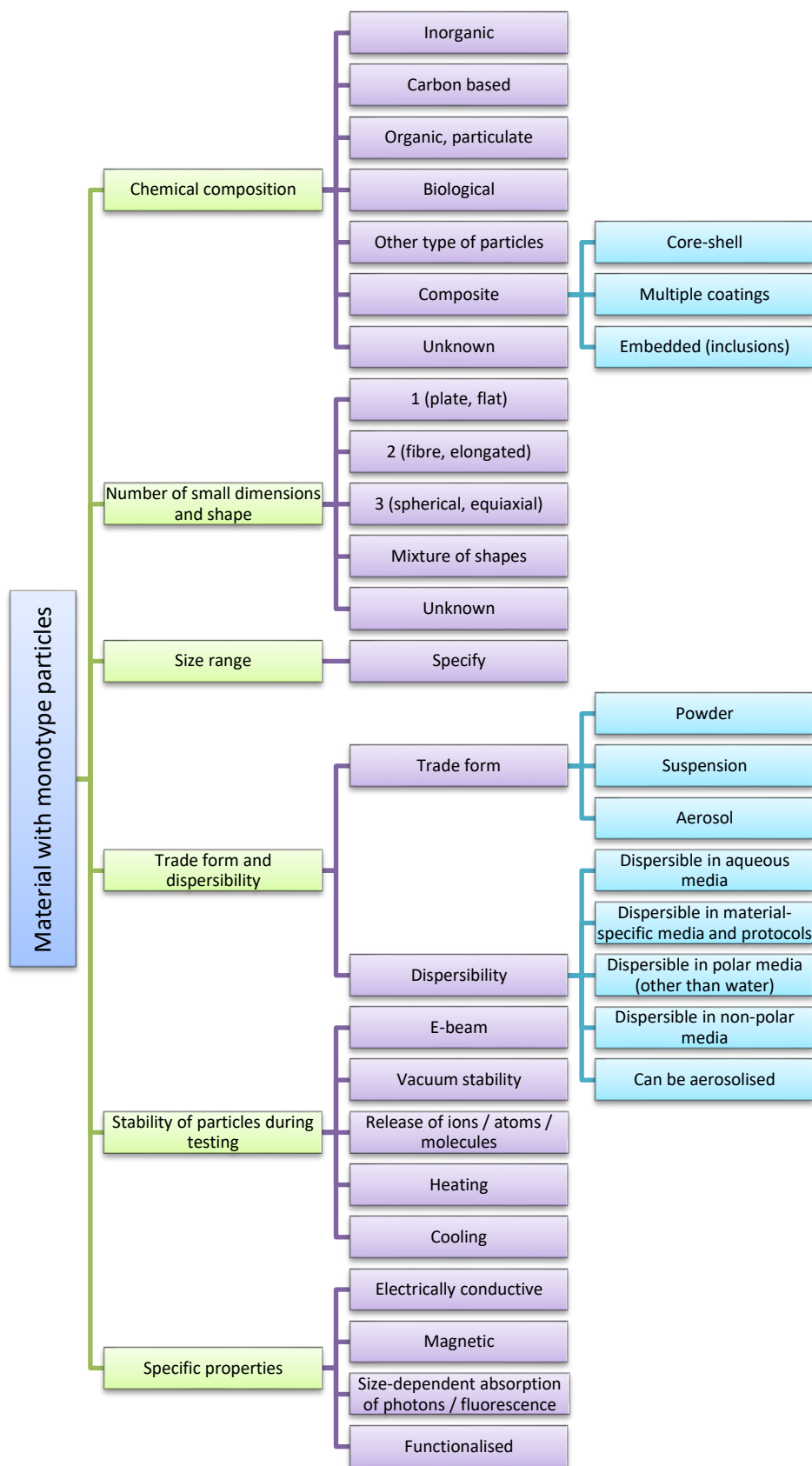


Figure 8: Material Categorisation Scheme (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

5 Matching material properties and experimental capabilities

The performance of a broad range of widely available and frequently used methods to determine particle size was systematically and experimentally evaluated against the criteria of the MCS⁸ within the NanoDefine⁹ project (see section 3.1 and Annex 1). This was done using a set of representative examples, including well-defined quality control materials as well as industrial materials of complex shapes and considerable polydispersity. This way, specific regions of applicability of the individual methods in terms of the materials categorisation criteria, e.g. material classes, chemical composition, size range, trade forms etc.,¹⁰ were established.

The resulting identification matrix can be used to match materials with known specific properties listed in the MSC to the methods best suitable to analyse particle size. Table 7 shows the results of this evaluation.

Guidelines issued by authorities usually list and describe methods recommended for regulatory purposes. Including both the MCS presented here and the mapping of the method performance criteria in such guidance would help to harmonise nanomaterial identification and improve its robustness. This would help in cases where a regulatory decision on the identification of a material as a nanomaterial (with all its regulatory consequences) is necessary. However, a final decision on whether a material meets the size criteria for a nanomaterial as defined under a specific legislation not only requires selecting the appropriate method(s) for a specific material, but also considerations on the measurement uncertainty associated to the result obtained with a method in combination with a particular material. A greater measurement uncertainty can be accepted for materials far away from the borderline (i.e. close to the threshold) separating nanomaterials and materials falling outside the EC NM Definition compared to borderline materials, for which identification as nanomaterial or not is more difficult. The latter would require in-depth confirmatory methods to achieve a reliable identification. Such different levels of complexity in the analysis could be taken into account in a tiered method approach as discussed by Babick et al.¹⁰

An example – case study: gold nanorods

In the following, a simple example where we apply the MCS to a material consisting of monotype particles is presented, and the material consists of a suspension of gold nanorods. The task is to identify the most suitable characterisation method(s) that would allow determining whether this is a nanomaterial according to the EC NM Definition. Another, more complex example for categorisation of a material where the particles are embedded in a matrix can be found in Annex 1.

We assume that the following information on the material is available. The chemical composition is inorganic, two dimensions are expected to be smaller than the third dimension (hence the shape is elongated), the size range of the smallest dimension is expected to be between 40 nm and 90 nm and the trade form is a suspension. The gold nanorods are dispersible in aqueous media, stable under e-beam irradiation and in vacuum. Release of molecules, atoms or ions is not expected. The particles are stable at least between -100°C and 400°C. They are electrically conductive and have unknown magnetic properties. There may be a size-dependent absorption of photons. They are not functionalised. With this information, the categorisation scheme for this material is filled in, see Figure 5 (see darker cells). Matching these material properties with the methods' performance characteristics (Table 7) gives SEM, TEM and AFM as recommended methods for analysis. For this material, the elongated shape of the particles is the most restrictive property, and hence methods which give as result an equivalent sphere diameter are not recommended for its analysis.

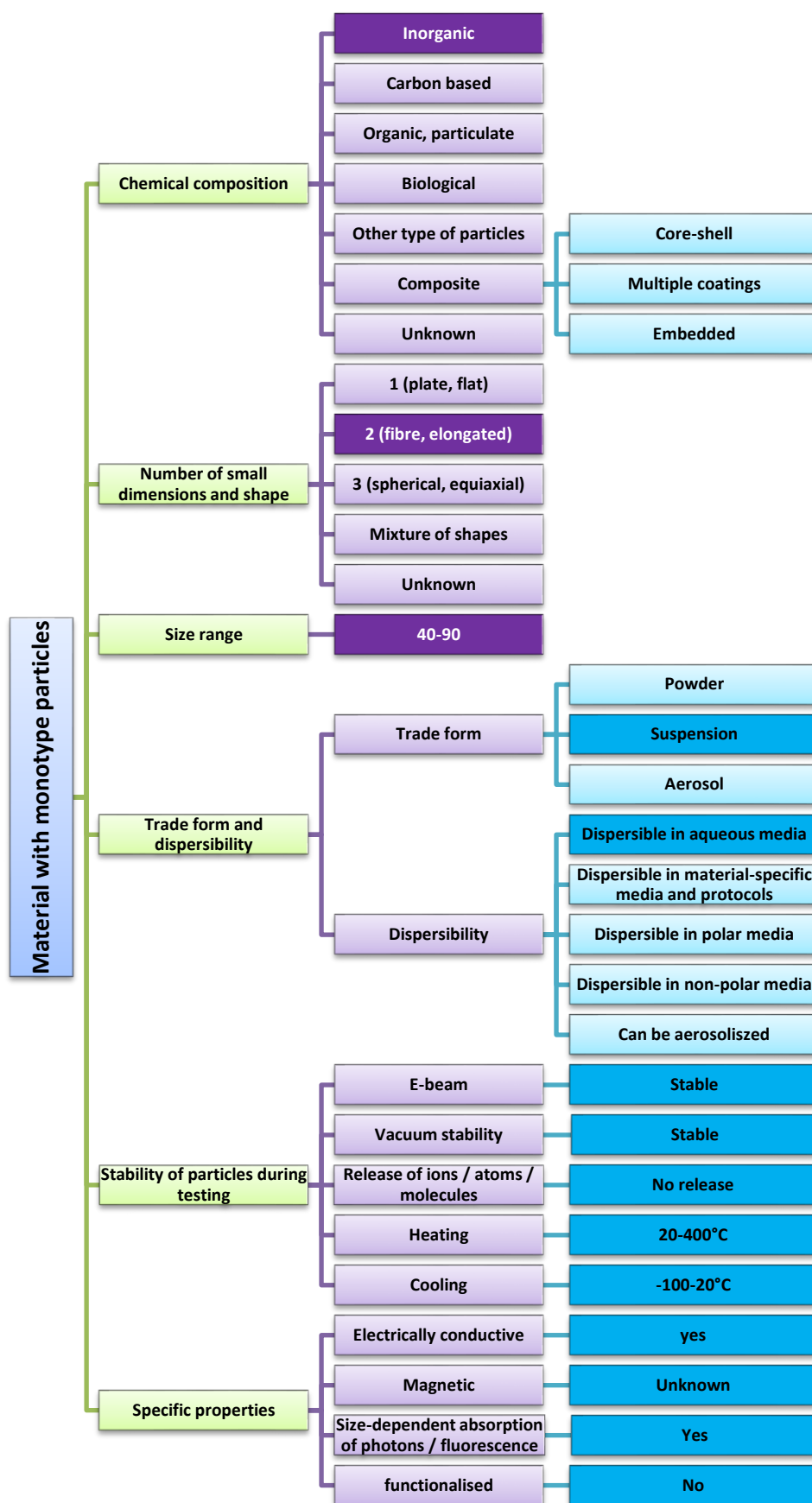


Figure 9: The material categorisation scheme applied to gold rods (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

Table 7: Suitability of methods for particle size measurements for the analysis of materials with specific properties (Adapted from Ref. [2] with permission from the Royal Society of Chemistry).

Techniques recommended by NanoDefine technique evaluation ¹⁰								Techniques currently not recommended by NanoDefine technique evaluation						Techniques not evaluated by NanoDefine		
	TEM	SEM	BET	DEMA spray	AC - turb	AC-RI	DLS	spICP-MS	PTA	USSp	AF4-MALS	ALS	SAXS	TRPS	AFM/SFM	
MAIN CRITERIA FOR MONOTYPE AND MULTITYPE PARTICLES AND PRODUCTS\ARTICLES CONTAINING PARTICLES																
CHEMICAL COMPOSITION	Inorganic	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Carbon based	X	X	X	X	X	X		X	X	X	X	X	X	X	X
	Organic particulate	X	X	X	X	X	X		X	X	X	X	X	X	X	X
	Biological	X	X		X	X	X		X		X	X	X	X	X	X
	Other	X	X		X				X				X		X	
	Unknown	X	X		X								X		X	
COMPOSITE	Core shell	X	X	X	X	X ^b	X ^b	X	X ^b	X	X ^b	X	X ^b	X ^b	X ^b	X
	Multishell coating	X	X	X	X	X ^b	X ^b	X	X ^b	X	X ^b	X	X ^b	X ^b	X ^b	X
	Inclusions	X	X	X	X	X ^b	X ^b	X	X ^b	X		X	X ^b	X ^b	X ^b	X

^b Depends on the material

NUMBER OF SMALL DIMENSIONS / SHAPE	1 (plate, flat)	X ^c	X ^c	X													X
	2 (fibre, elongated)	X	X	X													X
	3 (Spherical or equiaxial)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Mixture of different dimensioned particles (Mixture of shapes)	X ^{d, e}	X ^{d, e}														X
NUMBER OF SMALL DIMENSIONS	1 (plate, flat)	X ^f	X ^d	X													X
	2 (fibre, elongated)	X	X	X													X
	3 (Spherical or equiaxial)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

^c Although not recommended by NanoDefine, under specific conditions the method is capable of determining the thickness of objects with one small dimension (e.g. platelets).

^d if specific protocols are used.

^e Mixture of shapes with 2 and 3 small dimensions.

^f Although not recommended by NanoDefine, under specific conditions the method is capable of determining the thickness of objects with one small dimension (e.g. platelets).

	Mixture of different dimensioned particles (Mixture of shapes)	X ^{d, e}	X ^{d, e}													X
Size range (nm) (approximate borders chosen for ease of use)		1 nm-1000 μm	10 nm - 1000 μm	1 nm - 10 μm	2 nm - 1 μm	5 nm-100 μm	1 nm - 1 μm	3 nm - 5 μm	15 nm - 1 μm	10 nm - 1 μm	1 nm-100 μm	1 nm - 1 μm	70 nm - 10 nm	1 nm - 100 nm	50 nm - 10 μm	1 nm - 10 μm
TRADE FORM	Powder	X ^d	X	X							X			X		X ^c
	Suspension	X ^d	X ^d		X	X	X	X	X	X	X	X	X	X	X	X ^c
	Aerosol				X											
DISPERSIBILITY	Aqueous Polar	X ^d	X ^d		X	X	X	X	X	X	X	X	X	X	X	X ^d
	Non polar	X ^d	X ^d		X	X	X	X		X	X	X	X	X		X ^d
	Specific media	X ^d	X ^d			X	X					X	X	X	X	X ^d
	Can be aerosolised												X			
Electron beam sensitive				X	X	X	X	X	X	X	X	X	X	X	X	X
Sensitivity to vacuum					X	X	X	X	X	X	X	X	X	X	X	X

Release of ions/atoms/molecules	X	X	X	X	X	X	X		X	X	X	X	X	X	X
Analysis temperature (°C)	15-25	15-25	15-40	10-40	5-60	5-60	5-60	15-40	10-40	-10-60	10-40	-40-100	-10-60	15-25	15-40
Conductive materials	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Magnetic materials	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Absorption fluorescence	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Functionalisation	X	X						X				X	X		X
MULTITYPE PARTICLES															
Can measure multitype material	X	X	X	X											X
PRODUCTS AND ARTICLES CONTAINING PARTICLES															
In case the matrix components can be removed	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

TYPE OF MATRIX	embedded in a solid matrix	X ^d	X ^d										X ^d	X ^d		X ^d
	embedded in a liquid/gel matrix	X ^d	X ^d		X	X	X	X	X	X	X	X	X	X	X	X ^d
	suspended in a gas				X											

Abbreviations: TEM Transmission Electron Microscopy, SEM Scanning Electron Microscopy, BET Brunauer–Emmett–Teller (gas physisorption) method, DEMA Spray Differential electrical mobility analysis, AC TURB Analytical centrifugation with light turbidity measurement, AC RI Analytical centrifugation with refractive index detector, DLS Dynamic light scattering, spICP-MS Single particle inductively coupled plasma mass spectrometry, PTA Particle tracking analysis, USSp Ultrasonic spectroscopy, AF4-MALS Asymmetric flow field-flow-fractionation coupled to Multi-Angle Light Scattering, ALS Angular light scattering, SAXS Small-angle X-ray scattering, XRD X-Ray Diffraction, TRPS Tuneable resistive pulse sensing, AFM Atomic Force Microscopy

6 Decision Support Flow Scheme

The Decision Support Flow Scheme is a core of the NanoDefiner Framework and it aims at providing the most reliable and fastest way to identify a material (according to the EC NM Definition) so that time-consuming and expensive analyses can be avoided as much as possible. At the same time, the flow scheme with its decision criteria is designed in a way which is pragmatic and allows a reasonably certain decision so as to fulfil regulatory obligations on the one hand and be economically viable on the other hand.

The NanoDefine project also explored how to assess a material against the criteria of the definition through proxy solutions, i.e. by applying measurement techniques that indirectly determine the x_{50} ⁹. Those findings developed through empirically based scientific work are included in the Decision Support flow Scheme. As they go beyond the text of the EC NM Definition, they may be used as practical approach to indicate whether a material is a nanomaterial or not, but keeping in mind that they should not be taken as recommendation for the implementation of the EC NM Definition in a regulatory context without.

Figure 10 presents the decision support flow scheme (DSFS) that was built on the results of work performed by the NanoDefine consortium. It starts with a basic categorisation of existing and novel materials, after which it guides the user through the decision process to reach the conclusion on the material classification as a nanomaterial or not.

The first step addresses materials which are explicitly included or excluded in the Commission Recommendation on the definition of nanomaterials (Section 6.1). The majority of materials do not belong to one of these groups, and therefore in most cases the user continues with the flow scheme.

If the material is in dry powder form the user can verify whether the material can be dispersed to prioritise the route of analysis, that is, continue with the powder or with the material in liquid dispersion. This information may facilitate the choice of the most appropriate measurement technique. The next step requires from the user to apply the material categorisation scheme (Section 4) and match it with the methods performance tables (section 3 and Part 2 of the manual³). This allows creating a list of techniques which can be applied to the specific material in question. Taking into account the material characteristics and the method performance table the user has the possibility to choose which path to follow in the decision support flow scheme. Depending on the trade form (powder or dispersion), and the material properties known to the user, in principle three pathways can be followed (see Figure 10): the one applicable to dispersed materials ('dispersion route', section 6.3.1), the one applicable to powders ('powder route', section 6.3.2), or the user can decide to skip tier 1 and go directly to tier 2 (section 6.4).

The following sections provide a step-by-step description of the decision support flow scheme.

⁹ x_{50} – median; is the size at which 50% of the particles are larger and the other 50% are smaller than a 100 nm

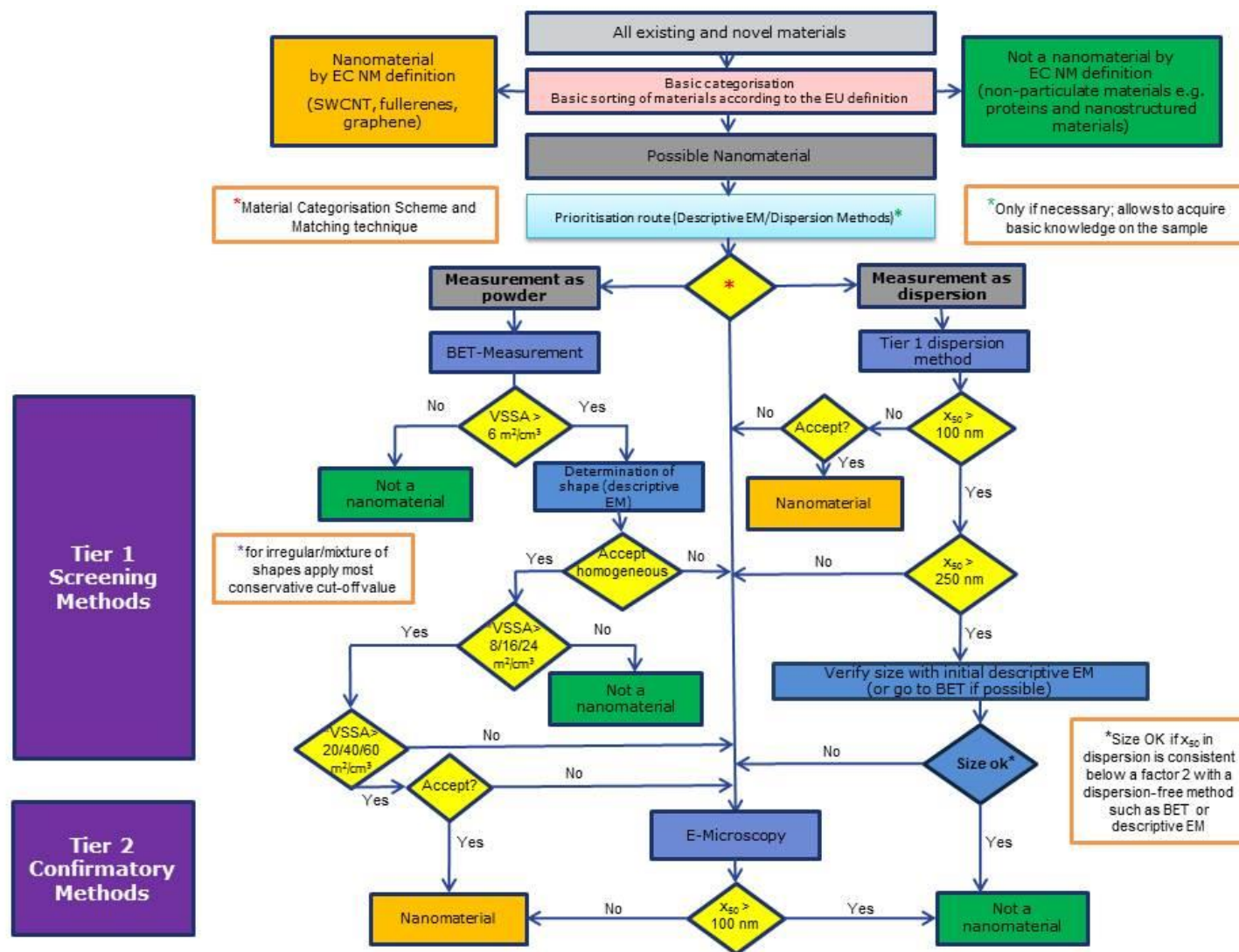


Figure 10: Decision support flow scheme for identification of nanomaterials. Units have been omitted for clarity. x_{50} designates the number based median size of particles.

6.1 Basic categorisation

The EC NM Definition explicitly includes some and excludes other materials. It covers only particulate materials. Fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm are considered to be nanomaterials. According to the Commission Staff working paper¹¹ and Ref. [12], materials composed of non-solid particles ('soft materials'), such as nano-emulsions, should not be considered as nanomaterials. Moreover, the definition does not explicitly address nanostructured materials. This has to be considered when performing the assessment of the information to be used for basic categorisation of a material as it may lead to the direct identification of two classes of material; i) non-particulate materials, nanostructured materials (see Ref. [12]) and materials composed of non-solid particles (e.g. liquid particles or micelles and liposomes): these are not nanomaterials and ii) fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm: these are nanomaterials. In such cases further analysis of the material for identification purposes is not needed (see Figure 11).

After the exclusion of those two cases the remaining materials may possibly be nanomaterials and need to be further evaluated.



Figure 11: Basic categorisation of nanomaterials

6.2 Prioritisation route, material categorisation scheme and technique matching

Some basic knowledge of the properties of the material under investigation is needed to enable the correct choice of the most appropriate technique(s). Thus the prioritisation route step aims at collecting some crucial information on the sample to be analysed. Shape (by descriptive EM) and dispersion analysis (if needed) are should be performed if such information is not already available elsewhere. Although at this stage the decision support flow scheme and NanoDefiner e-tool do not require this information it will facilitate greatly the appropriate choice of the identification path. Furthermore, knowledge of particle shape(s) and (multi-)modality are necessary at a more advanced stage of the flow scheme.

To be able to decide which path to follow the material categorisation scheme (see section 4 and Annex 1) and methods matching performance table (section 3 and Ref. [3]) should be applied at this step. This allows identifying the most appropriate technique which can be used to measure the particle size distribution of the analysed material and enables the correct choice of the identification path (i.e.: Tier 1: powder or dispersion route or Tier 2). The information on how to

apply the MCS and method matching tables can be found in Refs. [2,3]. While applying the Decision Support Flow Scheme the NanoDefine Methods Manual should be consulted whenever needed.

6.3 Tier 1 assessment

Tier 1 techniques are divided into techniques that can characterise a material in powder state or in liquid dispersion. Thus, after basic categorisation of the material, the next piece of information needed to enter tier 1 is the state in which material is available, i.e. as powder or in dispersion.

If a material is already in dispersion (e.g. colloidal material), the user should follow the pathway along the analysis of dispersions, the 'dispersion route', (section 6.3.1) which is the right branch in Figure 10 as the analysis of a powder from a dried dispersion is not reliable and thus not recommended.

If the material is in powder form and can be dispersed, the user can choose to analyse the material in two ways: as powder or as dispersion. That choice may depend on several criteria such as dispersibility of the material, type of material, availability of the mobility-based techniques, regulatory purposes etc.

Some dispersion procedures required for a reliable measurement can degrade the material. It has to be also kept in mind that the decision support flow scheme is linked to the material categorisation scheme and the methods performance evaluation. Some of the techniques may simply not be suitable for certain types of nanomaterials. Furthermore the decision can also depend on the availability of the techniques and the expertise the user may have in-house and would prefer to use if possible. Certain legislation explicitly require providing results obtained with a specific technique or method and this should also be considered when deciding whether to choose the powder or dispersion route.

These issues were previously discussed² ('Material categorisation scheme') and the NanoDefine document 'Set of criteria with ranking system to steer the decision process'¹³. Further information is available on the NanoDefine project website⁹.

If the material is analysed as dispersion, most of the techniques evaluated in Tier 1 can be applied (see Table 1). Nevertheless, the user should follow the outcome of the techniques-matching (see Table 7) to be certain that a chosen technique is applicable to the material under investigation.

If the material cannot be dispersed or the user decides not to disperse it, the left path on Figure 13 (headlined 'powder route') needs to be applied. This path is described in detail in section 6.3.2.

The user may always choose to skip the tier 1 step and go directly to tier 2 which involves the analysis of the material by applying confirmatory methods (see Table 1).

Electron Microscopy techniques that are the bases of tier 2 are considered as expensive and require some effort to prepare specimens that provide representative samples of the particle system. However these methods can be applied to most materials, even to those with complex particle shapes or structures. Moreover, if the user has EM equipment already in-house and has the necessary expertise in the EM analysis, these techniques may be the preferred choice in any case. More information on EM techniques is provided in section 6.4 and Ref. [3].

6.3.1 Tier 1 - Analysis of dispersions ('dispersion route')

Figure 12 shows the path that is applicable to a material which is already in dispersion form or when the user decided to disperse a powder material, following specific SOPs which are available for certain materials¹⁴.

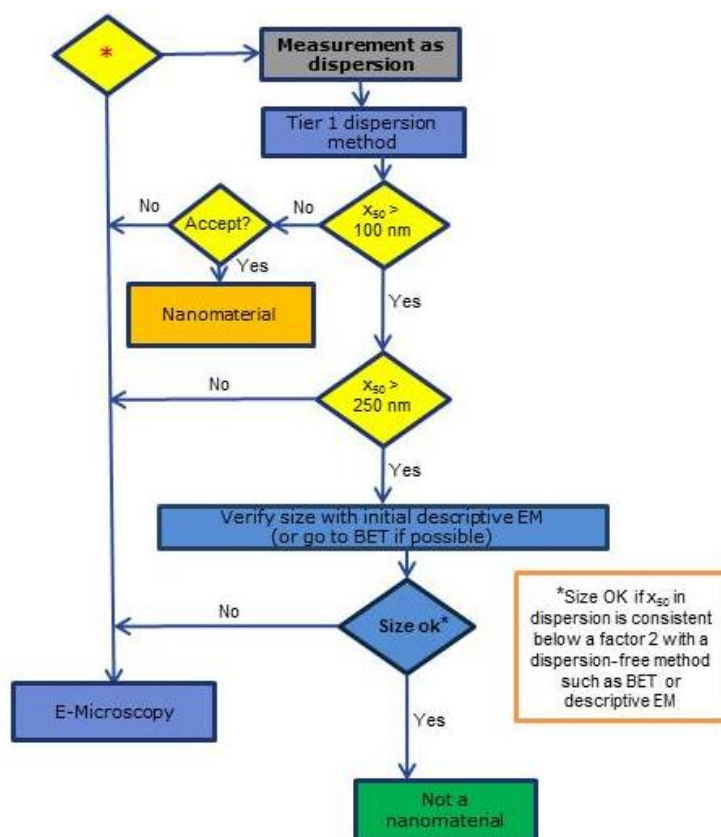


Figure 12: Tier 1 dispersion route flow scheme.

The list of tier 1 methods is provided in Table 1. As some of these methods showed limitations during the testing procedure that was applied to NanoDefine training set materials it is recommended to verify the applicability to the material to be tested.

Based on the outcomes of the measurement techniques performance evaluation carried out within the NanoDefine project on a set of training materials¹⁰ the generally recommended methods for the analysis of dispersion in tier 1 are: spray-DEMA, all AC techniques and DLS (see Table 1). However, for specific materials other techniques may still be suitable as screening methods, as also discussed in Ref.10.

After the screening performed with an appropriate tier 1 method, the user continues depending on the outcome of the screening.

- A. If the material is found to have a x_{50} smaller than 100 nm and if the results are judged acceptable, this material is considered to be a nanomaterial without the need to apply tier 2 methods. If a doubt about the reliability of the result arises the user should

perform further measurements either with other techniques from Tier 1 to check the plausibility of the results, or move to Tier 2 (EM).

- B. If the material is found to have a x_{50} larger than 100 nm or if there are some doubts regarding the reliability of the outcomes, the plausibility of the results should be verified by either (i) descriptive electron microscopy analysis or (ii) by BET. Option (i) can be used to estimate whether in the sample the general particle shape is compact (i.e. approximately spherical or equiaxial) and whether the particle size is in reasonable agreement with the results of the tier 1 method or if there are indications of aggregation/agglomeration. Within the NanoDefine approach size is considered to be correct if x_{50} in dispersion is consistent within a factor of 2.5¹⁰ with a dispersion-free method such as BET or descriptive EM.

A strong disagreement regarding the particle size between tier 1 and descriptive EM screening could indicate aggregation or a significant deviation from a spherical shape, which would make it necessary to escalate to tier 2. Option (ii) to verify the outcome of tier 1 can be applied if the original trade form of the material is a dry powder. In such case, the user can follow the BET analysis pathway (see also section 6.3.2). In case of a negative result the user can choose either to follow the BET approach path to reach the decision or to go directly to tier 2 methods. In both cases the results of the initial analysis in dispersion should not be considered anymore.

If a descriptive EM scan reveals that the particle shape is not compact, the user should go directly to tier 2 for confirmatory methods e.g. detailed EM.

On the other hand, if the particle shape is (i) compact and (ii) the material is sufficiently dispersed (the particle size values obtained from tier 1 method and EM scan matches reasonably well) and (iii) the x_{50} is larger than 250 nm, it is likely that the material is not a nanomaterial.

- C. If the x_{50} is smaller than 250 nm, one should proceed to the tier 2 to perform an analysis by applying confirmatory methods. In this case one could also carry out the analysis by applying another tier 1 technique and check for plausibility of the obtained results.

The empirical threshold of >250 nm for both powder and dispersion paths is based on experience gained within the NanoDefine project. The results obtained by testing a set of materials in NanoDefine using the techniques of tier 1 were compared with results from EM analysis¹⁰. After confirmation of plausibility, aggregation and shape as explained above, it was found that basically all of the x_{50} values determined by a tier 1 method which would lead to identification as not being a nanomaterial agree with the corresponding values of EM within a factor of 2.5. This means, if, after checks for plausibility, aggregation and particle shape, one of the Tier 1 methods gives a d_{50} above 250 nm, this means for most materials that they are likely not nanomaterials according to the EC NM Definition.

Likewise (and an important proposition in the implementation of the EC NM Definition) if the volume, extinction, or intensity-weighted median size value of a material, as measured by AC, DLS, or AF4-LS, is smaller than 100 nm, the material can be classified as nanomaterial without the need for conversion to a number-weighted median.

6.3.2 Tier 1 - Analysis of powders ('powder route')

If the user decides to analyse the material in powder form the BET method should be applied. Application of the BET method for this purpose requires basic knowledge of the size modality of the

sample. If the material seems fairly monomodal then BET may be applied. On the other hand, if the sample is multimodal, BET should not be used and tier 2 methods should be applied.

The DSFS for powder analysis is presented in Figure 13.

BET analysis

The BET method allows determining the volume-specific surface area (VSSA) of the material.

VSSA is one of two different metrics suggested in the EC NM Definition to be used for nanomaterial classification. Nonetheless currently VSSA cannot be used as a tool to categorise a material as being not a nanomaterial and the particle size distribution remains the only means. Refs. [15, 16] discuss the applicability ranges of the VSSA method and the quantitative relation to number-based particle size distribution for real-world samples. Furthermore, the possibility to classify a material according to the EC NM Definition by measuring the VSSA and the conditions of applicability to use VSSA as identification criteria are also discussed in Ref. [15]. For correct interpretation of BET results, further information on the material's porosity, particle size distribution and shape is needed.

For dry powders, VSSA can be calculated by multiplying the value of the mass-specific SSA obtained from BET analysis¹⁷ by ρ (skeletal density¹⁸) that may be determined for example by gas pycnometry. In many cases BET is routinely applied to characterise the manufactured material in house, thus if VSSA would be accepted by the EC for NM or not NM identification the analysis could be performed at low cost, without the need for additional measurements. BET is the only method apart from SEM and TEM to cover the entire particle size range from 1 nm to 10 μm [3], with limitations of SEM and TEM to reach the lower and upper limits, respectively. Therefore under specific conditions BET analysis may be used when applying specific criteria of cut-off limits for identification of powder materials without the need to actually measure particle size distributions by number (see Figure 13).

Based on the value of VSSA and knowing the particle shape, the size of the particle can be estimated. This provides the basis for establishing the shape-dependent VSSA cut-off values for a given particle size. The quantitative relationship between the VSSA and the smallest particle dimension can be expressed as (with d_{minVSSA} in μm when using the VSSA in m^2/cm^3):

$$d_{\text{minVSSA}}(D) = \frac{2D}{VSSA} \quad (1)$$

where D corresponds to the number of small external particle dimensions (1, 2 or 3).

A detailed description of the quantity d_{minVSSA} is presented in Annex 2 and Annex 3.

Based on existing data obtained from testing a NanoDefine material training set on the BET method, the following screening strategy addressing VSSA was developed. For most of NanoDefine materials this approach resulted in the same material identification based on BET and EM analysis results, leaving only borderline materials for tier 2 assessments.

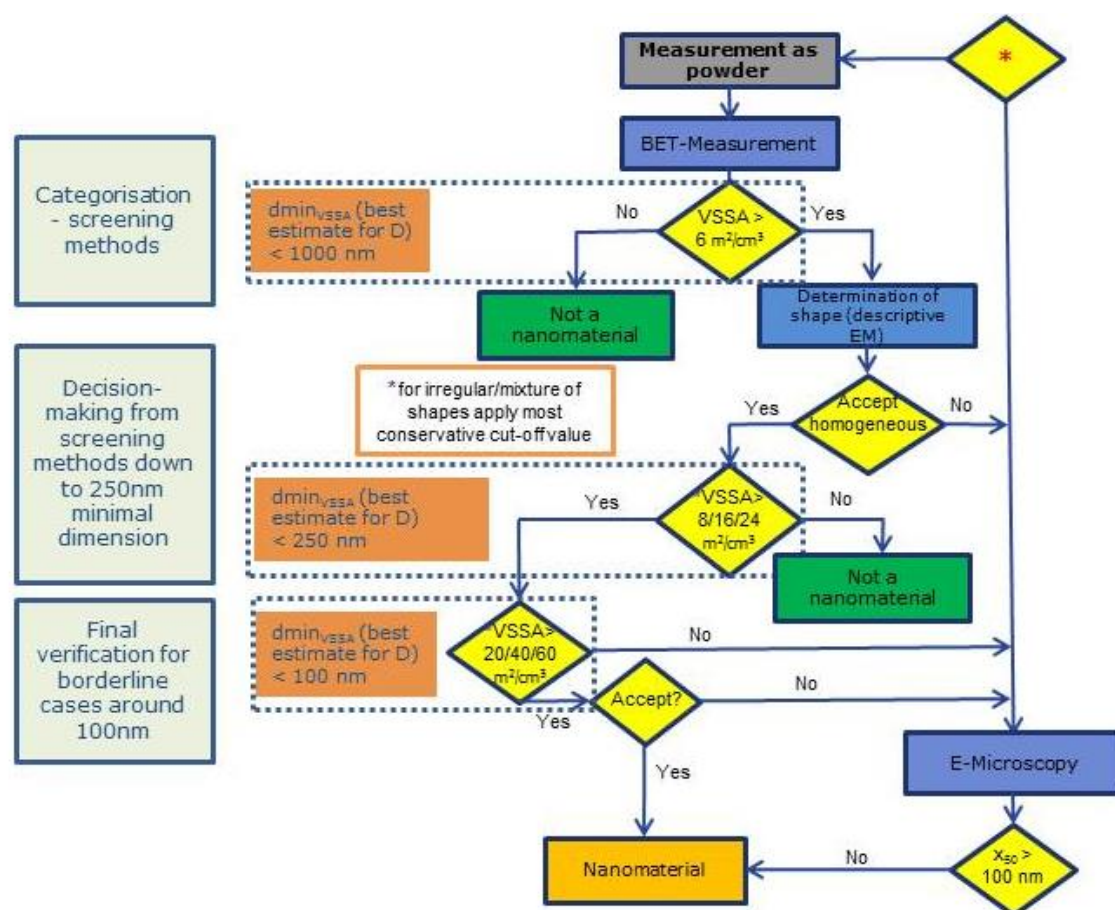


Figure 13: Tier 1 powder route decision tree involving BET measurements for nanomaterial identification. Homogenous: same shape, same composition of the particles

Figure 13 shows the detailed decision tree for identification as a nano or not a nanomaterial when applying BET analysis. The predictive model is divided into three main blocks with increasing levels of difficulty encountered during the identification process: i) screening: this gives the opportunity to identify materials that are not nanomaterials based on a very low VSSA, ii) further decision making by taking into account the particle shape, iii) verification of borderline cases: this leads to application of tier 2 methods for final decision.

Keeping in mind that the sample for BET analysis has to be originally in powder form the decision tree should be applied in the following way:

To calculate VSSA, the specific surface area resulting from the BET analysis, i.e. SSA, has to be multiplied by the skeletal density of the material.

- A. If the VSSA value is smaller than $6 \text{ m}^2/\text{cm}^3$ (equivalent to a $d_{VSSA} = 1000 \text{ nm}$ for spherical particles) the material likely is not a nanomaterial.

The $6 \text{ m}^2/\text{cm}^3$ VSSA value corresponds to a factor of 10 below the cut-off given by the theoretical VSSA of a material consisting of perfectly monodisperse, spherical particles with a diameter of 100 nm. For such a material the value is $60 \text{ m}^2/\text{cm}^3$. The maximum deviation between d_{VSSA} and $d_{minVSSA}$ due to the unknown particle shape is a factor of 3 ($60 \text{ m}^2/\text{cm}^3$ vs. $20 \text{ m}^2/\text{cm}^3$). Furthermore, a mismatch between $d_{minVSSA}$ and EM by not more than a factor of 2.5 was observed in the

NanoDefine training set materials. Combining these two effects, the overall disagreement should be a factor of 7.5 at most. Thus, using 10 times the size-based cut-off value ($10 \times 100 \text{ nm} = 1000 \text{ nm}$) may be considered sufficiently conservative to exclude classification of a material as falling outside the EC NM Definition, when in fact it is a nanomaterial (false negative classification).

- B. If the value of VSSA is larger than $6 \text{ m}^2/\text{cm}^3$, the particle shape should be determined by means of simple, descriptive EM analysis. This also provides a possibility to identify high levels of aggregation and multimodality of the analysed sample. In such cases BET analysis should be terminated and tier 2 methods should be applied for the final material identification.

If the analysed sample is not aggregated and the size distribution is not multimodal, a re-evaluation of the BET results has to be done with pragmatic aspect ratio criteria to select the appropriate shape-specific cut-off value, see also Ref. [19].

Accordingly, the next step of the decision tree assumes knowledge of the particle shape, and the cut-off $d_{min_{VSSA}}$ is 250 nm. As previously explained 250 nm originates from the maximum mismatch between $d_{min_{VSSA}}$ and EM within a factor of 2.5 that was observed in the entire tested material training set. Details of calculations and the maximum deviations induced by the aspect ratio cut-offs are explained in Annex 3.

Consequently if the particle shape can be identified, the following shape-dependent thresholds should be applied to the VSSA values obtained in the screening step:

If the particle shape of the material is

- Spherical (aspect ratio $<3:1:1$, $D=3$) the threshold for VSSA = $24 \text{ m}^2/\text{cm}^3$
- Elongated, fibre-like (aspect ratio $>3:1:1$, $D=2$) the threshold for VSSA = $16 \text{ m}^2/\text{cm}^3$
- Flat, platelet (aspect ratio $>3:3:1$, $D=1$) the threshold for VSSA = $8 \text{ m}^2/\text{cm}^3$

Therefore the material most likely is not a nanomaterial if the obtained VSSA value is smaller than:

- VSSA $< 24 \text{ m}^2/\text{cm}^3$ for spherical particles
- VSSA $< 16 \text{ m}^2/\text{cm}^3$ for elongated, fibre-like particles
- VSSA $< 8 \text{ m}^2/\text{cm}^3$ for flat, platelet-like particles.

If the obtained VSSA value for a given shape is larger than the corresponding cut-off value the results should be compared with the maximum value possible for the VSSA for a given shape considering that the smallest size of the particle is 100 nm. Consequently if the value of VSSA obtained in the screening step is larger than

- VSSA $> 60 \text{ m}^2/\text{cm}^3$ for spherical particle (aspect ratio $<3:1$) $D=3$
- VSSA $> 40 \text{ m}^2/\text{cm}^3$ for elongated, fibre-like particles (aspect ratio $>3:1:1$) $D=2$
- VSSA $> 20 \text{ m}^2/\text{cm}^3$ for flat, platelet-like particles (aspect ratio $>3:3:1$) $D=1$

then the analysed material is identified as nanomaterial, provided that the conditions for the applicability of the VSSA criteria are fulfilled^{15, 16}.

If the VSSA of a material is outside of the range of values discussed above it should be regarded as borderline case, i.e. a particulate material with particles of:

- Spherical shape (aspect ratio $<3:1$) and $24 < \text{VSSA} < 60 \text{ m}^2/\text{cm}^3$ or
- Elongated, fibre-like particles (aspect ratio $>3:1:1$), $D=2$, and $16 < \text{VSSA} < 40 \text{ m}^2/\text{cm}^3$ or

- Flat, platelet-like particles (aspect ratio >3:3:1), $D=1$, and $8 < VSSA < 20 \text{ m}^2/\text{cm}^3$

In such cases application of the tier 2 methods is necessary for final identification of the material. On the other hand if the shape cannot be assigned to any of the groups mentioned above but the sample is homogenous (same shape, same composition of the particles) it may be possible to still follow the BET path by applying the most restrictive cut-of values.

As already mentioned, if multimodality is detected, if there is a mixture of different shapes or the shapes cannot be approximated by spherical, rod or platelet, the user should escalate to tier 2 and perform EM based analysis.

6.4 Tier 2 Classification

Figure 14 shows the scheme of the tier 2 in the material classification.

Analysis based on tier 2 methods has to be performed if the tier 1 method approach is inconclusive; however tier 2 methods may also be chosen from the beginning of the classification process without necessary going through tier 1 methods.

If the EM image analysis gives x_{50} larger than 100 nm, the material is not classified as nanomaterial. Otherwise, if the resulted x_{50} is smaller than 100 nm, the material will be classified as a nanomaterial.

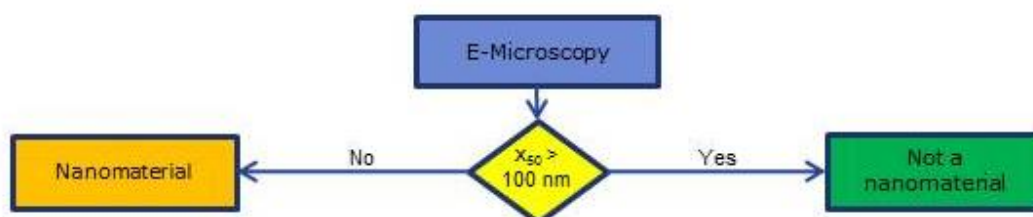


Figure 14: Tier 2 classification

Almost any particulate material can be analysed by EM unless it is sensitive to electron beams and/or to vacuum. It may however be possible to analyse such materials by variable pressure and/or low current EM). Nevertheless, sample preparation may still be an issue, because the EM also requires dispersion. Furthermore, platelet shapes cannot be assessed reliably in TEM, often also not in SEM. Finally, constituent particles within aggregates are sometimes not accessible, and may be better accessed by gas adsorption (powder route, see above).

To obtain quantitative EM data, an automated image analysis software ParticleSizer was developed in the NanoDefine project²⁰, by improving and tailoring existing software packages in order to obtain number-based particle size distributions based on recorded images. The results are described in detail in Ref. [21]. The output data format of the ParticleSizer can be imported into the NanoDefiner e-tool as an analysis result with automated categorisation.

Furthermore, a NanoDefine - auto EM – toolbox to allow rapid particle sizing and elemental identification was developed^{h,22}.

The NanoDefine decision flow scheme should be seen as a tool that allows in a fast and economical way to identify any particulate material as nanomaterial or not according to the EC NM Definition.

This Decision Support Flow Scheme integrates the material categorisation scheme², the method (or technique) performance tables³, the results obtained on the NanoDefine training set materials¹⁰ and results regarding the use of VSSA measurements¹⁵. The scheme is implemented the NanoDefiner software workflow. Decision Support Flow Scheme, material categorisation scheme and the e-tool were tested in practice, the results can be found in Annex 5 Case studies, confirming the applicability of these tools.

^h <https://github.com/AutoEM/AutoEM-toolbox>

7 NanoDefiner e-tool

The NanoDefiner e-tool is a specific software which implements the NanoDefine decision flow scheme²³. It pools results and conclusions together from method/technique evaluation and developments within NanoDefine with findings obtained from validation and case studies. This tool, with options based on material type, purpose, required data quality and economic parameters, guides the user to the most reliable and cost-efficient measurement techniques and provides recommendations to identify/categorise any substance or mixture according to the EC NM Definition. It includes also extensive reporting options including the particulate component attributes, suitability of the selected measurement technique(s) and uncertainty information. Depending on the dossier purpose, different report templates can be used to include specific information; attachments are possible as well.

The NanoDefiner e-tool relies on the NanoDefiner framework pillars (see Figure 15): Knowledge database (see also Ref. [3]), Material Categorisation Scheme (section 4 and Ref. [2]), Decision Support Flow Scheme (section 6). Furthermore, input from the user on properties of the specific material(s) to be analysed and measurement results (the latter may be exported from the ParticleSizer²¹ or Single Particle Calculator toolⁱ (spICP-MS)) is fed into the e-tool. The scheme of the logical workflow of the e-tool is presented in Figure 16.

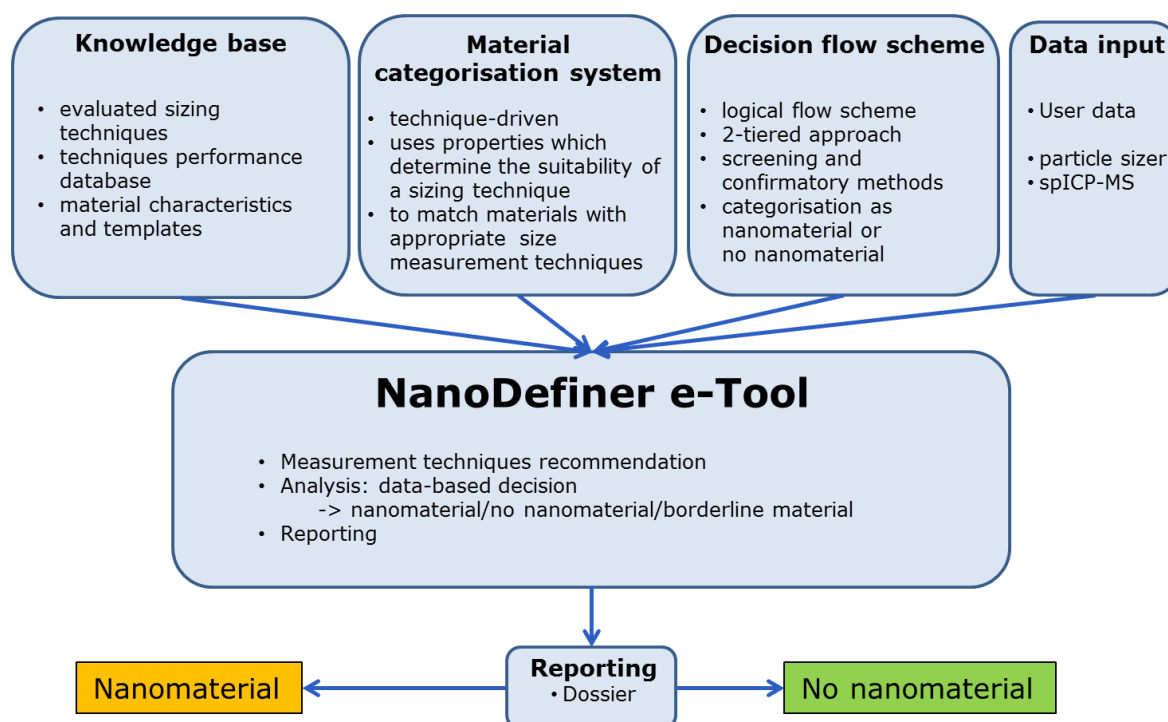


Figure 15: Implementation of the NanoDefiner framework in the NanoDefiner e-tool

ⁱ <https://www.wur.nl/nl/show/Single-Particle-Calculation-tool.htm>

The NanoDefiner e-tool is publicly available at <https://labs.inf.fh-dortmund.de/NanoDefiner/> for online application or download for local deployment. Its source code is available on GitHub^j and was published under the MIT license^k.

Users need to be registered and the account requires manual activation by the development and administrator team before being usable. For productive use of the NanoDefiner e-tool, the local deployment is suggested.

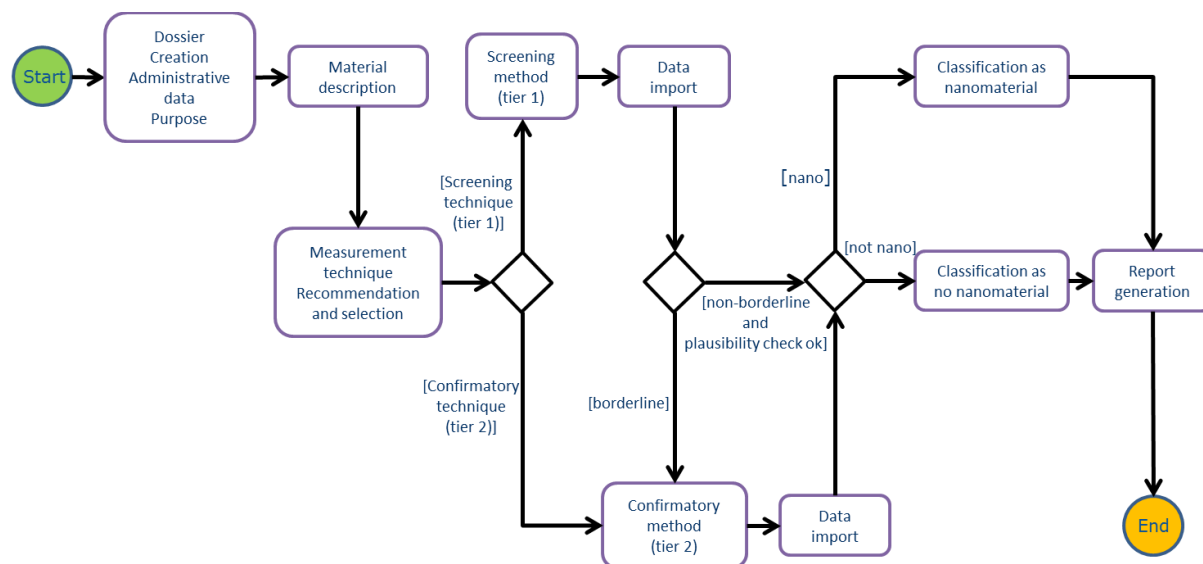


Figure 16: Workflow within the NanoDefiner e-tool (adapted from [23])

7.1 Features of NanoDefiner e-tool

The first public version (1.0.0) of the NanoDefiner e-tool implementation includes the following primary features:

- **Evaluated and guided workflow:** With the help of experts an evaluated and guided workflow was established that leads the user through the following series of work stages: i) dossier and sample creation, ii) material definition via the material categorisation scheme, iii) method construction by choosing recommended measurement techniques, iv) conduction of (external) laboratory analysis, v) analysis data import and (automated) nano/not nano/borderline decision and vi) dossier report generation by choosing applied methods.
- **NanoDefine Methods Manual:** The NanoDefine Methods Manual is integrated into the NanoDefiner e-tool implementation and can be accessed via a dedicated menu item. Also, the NanoDefiner e-tool is enriched with help links (e.g. in the material categorisation scheme during material description) that refer to specific content of the manual. When accessing these, the related positions in the manual are directly displayed. These references can be

j <https://github.com/NanoDefiner/NanoDefiner> (access 2019-08-16)

k <https://opensource.org/licenses/MIT> (access 2019-08-16)

managed via the knowledge base of the NanoDefiner, which allows linking every attribute to a specific manual item.

- **Dossier:** All information relating to each individual material categorisation process, including particulate component definition and method conduction, is collected in a dossier which concludes in a report specific to a purpose (e.g. a certain piece of legislation) for further use.
- **Material categorisation scheme:** The particulate components of the material to be categorised can be described using an extensive set of attributes. During material description, live feedback on the suitability of configured measurement techniques is provided, including information on the completeness of the general particulate component description and its technique-specific impact. Also explanations on techniques that are not recommended are provided. Furthermore, the user is given information on the completeness of material descriptions and on the impact of unknown values on MT recommendations.
- **Methods:** To assist in choosing MTs, the user is provided with detailed information on the suitability of the MT for the particulate components, including warnings about unknown particulate component attributes and material and method uncertainty information. Analysis data (e.g. exported distribution data from the ParticleSizer^l also developed in NanoDefine, a volume-specific surface area (VSSA) value, or Single Particle Calculation tool^m (SPC) data) can be uploaded and are used to determine a nanomaterial/no nanomaterial/borderline material decision for each method. For documentation and transparency purpose, the MT-specific uncertainty can be stated as percentage value, oriented towards the Guide to the Expression of Uncertainty in Measurement (GUM).
- **Reports:** Reports are created as Portable Document Format (PDF) files and can be generated based on a selection of conducted methods and combine all relevant dossier information, including the particulate component attributes, method results and MT suitability and uncertainty information. Depending on the dossier purpose, different report templates can be used to include information required by the specific registration authority. Attachments are possible, thus files supporting evidence (e.g. raw analysis data and images) can be embedded into reports.
- **Explanation of recommendations:** Throughout the dossier creation process, detailed explanations on the MT recommendation process are provided (e.g. during material or method description), giving the user insight into which specific material attributes led to the MT recommendation result.
- **User management:** Each user has a profile containing a basic set of personal information for inclusion in the report. Lab settings allow managing availability, cost, duration, and default measurement uncertainty of MTs, which are taken into account during MT recommendation.
- **Knowledge base:** MT recommendations are based on a knowledge base, built with the help of experts. The main entities it comprises are performance profiles of MTs and test materials for the use as templates.

^l <https://imagej.net/ParticleSizer> (last accessed 2019-08-16)

^m <https://www.wur.nl/en/show/Single-Particle-Calculation-tool.htm> (last accessed 2019-08-16)

- **Institutionalisation:** Inclusion of a custom logo in the reports is available as the first step towards institutional customisation of the NanoDefiner e-tool. Developers and administrators may also configure custom colour profiles to extend the corporate identity representation.
- **Internationalisation:** The NanoDefiner features internationalisation, given that translations of used locale configurations and knowledge base components are entered. The innately provided locale configurations cover British English.
- **Feedback channel:** Users can provide feedback using a simple form within the NanoDefiner e-tool to ease the process of communicating improvement suggestions to the development team.

What the NanoDefiner e-tool does and how it can help in identifying nanomaterials according to the EC NM definition. It

- **supports** users who need to identify nanomaterials, e.g. for registration purposes
- is addressed to users with **knowledge** of particle size measurements
- **provides a guided** workflow (applied decision flow scheme)
- **matches** material properties with method performance
- **provides transparent recommendation** for a method
- **supports economic decisions**
- provides **results** with comprehensive and transparent reporting
- includes online manual and help
- is **configurable** by expert user administrator

What is outside the scope of the NanoDefiner e-tool? **It does not**

- provide details on sample preparation
- give detailed instructions on methods
- consider methods in development
- provide legally binding identification of nanomaterials or no nanomaterials
- substitute expert assessment

These points should always be kept in mind while applying the NanoDefiner e-tool.

7.2 Software packages

All additionally used software packages are free and of open source. The following listing provides an overview over a collection of used software packages together with their version, license, and project page.

- **Java** in version 8 (GPL, <https://www.oracle.com/java/>)
- **Apache Tomcat** in version 8 (Apache License 2.0, <http://tomcat.apache.org/>)
- **Spring Framework** in version 4.3 (Apache License 2.0, <https://spring.io/>)
- **DBMS** (such as MySQL, <http://www.mysql.com/>)
- **Hibernate** in version 5.2 (LGPL 2.1, <http://hibernate.org/>)
- **Drools** in version 6.4 (Apache License 2.0, <http://www.drools.org/>)
- **Apache Maven** in version 3 (Apache License 2.0, <https://maven.apache.org/>)
- **Apache Shiro** in version 1.3 (Apache License 2.0, <http://shiro.apache.org/>)
- **Thymeleaf** in version 3.0 (Apache License 2.0, <http://www.thymeleaf.org/>)
- **DynamicReports** in version 5.0 (LGPL 3, <http://www.dynamicreports.org/>)
- **OpenCSV** in version 3.9 (Apache License 2.0, <http://opencsv.sourceforge.net/>)
- **Guava** in version 21.0 (Apache License 2.0, <https://github.com/google/guava>)

Relevant licenses:

- **Apache License:** <https://www.apache.org/licenses>
- **GNU General Public License (GPL):**
<https://www.gnu.org/licenses/gpl-3.0.en.html>
- **GNU Lesser General Public License (LGPL):** <https://www.gnu.org/copyleft/lesser.html>

7.3 NanoDefiner e-tool guide for version 1.0.0

The NanoDefiner e-tool is accompanied by a specific guidance document which assists the user in the practical application of the software. The guidance document is included in this manual as Annex in a form as included in the e-tool. A video tutorial on how to use the NanoDefiner e-tool is publically available from the NanoDefine project official website www.nanodefine.eu.

8 Conclusions

The NanoDefiner Framework provides industry and regulatory agencies with the tools that support the implementation of the EC NM Definition.

The NanoDefine Methods Manual provides practical advice for the user of the NanoDefiner Framework and its tools and aims at providing clear guidance on each step in the process of identifying a material as a nanomaterial according to EC NM Definition or as being not a nanomaterial. The NanoDefine Methods Manual consists of three parts: Part 1 covers (i) the NanoDefiner framework, (ii) general information on measurement methods and performance criteria and (iii) tools (Materials Categorisation Scheme, Decision Support Flow Scheme and an e-tool); Part 2 discusses the outcome of the evaluation of the nanomaterials characterisation methods and Part 3 gathers Standard Operating Procedures (SOPs) developed within NanoDefine.

The NanoDefiner Framework relies on the following pillars: (i) knowledge base (methods performance evaluation and development), (ii) technique-driven Materials Categorisation Scheme and (iii) Decision Support Flow Scheme, and it is implemented in the NanoDefiner e-tool software.

The developed framework and its tools are:

- easy to implement: they integrate the current practice/facilities/expertise present at end-users with new developments
- cost efficient: they offer a tiered approach for the selection of the most adequate analytical route to get to an identification according to the EC NM Definition with the least possible effort
- flexible: they define criteria for the inclusion of novel technologies and can be adapted easily to changing regulatory requirements
- sustainable: the developed approach is to be implemented in structures that persist beyond the duration of the project

By applying the developed tools and following the logic of the NanoDefiner Framework manually or through the e-tool, the user is provided with recommendations on the most suitable method(s) to characterise specific particulate materials. Based on the data input, the user is provided with a decision whether the analysed material is a nanomaterial or not a nanomaterial according to the EC NM Definition. If the material turns out to be a borderline material according to the decision support flow scheme, re-categorisation via tier 2 methods is necessary. The NanoDefiner Framework and its tools are tested best practice procedures that allow industrial and regulatory stakeholders the identification of particulate materials and products containing such materials according to the EC NM Definition.

The e-tool is available as free software which can be downloaded at the following public service:

<https://labs.inf.fh-dortmund.de/NanoDefiner/> as well as on the project website: <http://www.nanodefine.eu/> (sites accessed 16/08/2019).

Its open source code is available from the GitHub repository.

The NanoDefiner framework with its tools fully supports the implementation of the EC NM Definition, facilitating the decision making process that leads to the identification of nanomaterials for regulatory purposes.

Annex 1 Material Categorisation Scheme details

a. Selection of the criteria

Chemical composition

The choice of the method for accurate particle size characterisation often depends very much on the chemical composition.²⁴ Taking into account the performance of methods for particle size measurement, this criterion is further divided into sub-classes which allow categorising most of the currently available particulate materials:

- Inorganic materials (e.g. metals and their alloys, oxides and sulphides, salts, silicates), except carbon
- Pure carbon-based materials (CNTs, nanodiamonds, carbon black...)
- Organic particulate materials (polymers, pigments, etc.,)
- Biological materials, including synthetic biological materials
- Other types of particles
- Materials consisting of composite particles
- Unknown

The above-shown division is based on the assumption that in all sub-classes, except for composite and unknown particles, the elements are homogeneously distributed across the particles. Consequently e.g. a core-shell particle that consists of a Ag core and Au shell cannot be categorised as inorganic even though it consists of inorganic elements, but it is categorised as a composite particle.

Organic particulate materials such as polymer coils or pigments are relatively straightforward to measure due to their constituent particle boundaries, despite potentially challenging aggregate structures and shape issues. On the other hand, the category called 'other' such as dendrimers or supramolecular assemblies can be challenging due to their structure and complex chemical composition. For instance, sizing by Ultrasonic Spectroscopy (USSp), where the diameter (acoustophoretic diameter) is calculated based on spherical particle estimation, cannot measure properly these types of materials.

Biological materials such as nucleic acids or proteins were grouped separately because of their possible sensitivity to some testing procedures. Their spatial conformations are sensitive to pH and temperature, and a denaturation of their structure and functions due to experimental conditions would render them different in comparison to the original sample.

The chemical nature of the particles strongly influences the choice of the appropriate characterisation techniques. Actually, certain techniques, e.g. spICP-MS,²⁵ are very sensitive to the elemental composition of the analysed sample and thus the use of spICP-MS for instance is limited to inorganic materials with sufficiently high atomic weight.

Composite particles deserve specific attention when their size is analysed. It is therefore necessary to know if a specific technique is able to determine the particle size without interference caused by the individual composite structure.

Small dimensions and shape

Particle shape and the number of small external particle dimensions also affect the choice of characterisation methods. Many of the currently employed characterisation methods implicitly assume that the particles are spherical or yield an equivalent spherical size (for example DLS)²⁴. This limits their use for analysing materials with non-spherical particles. An additional difficulty occurs if the analysed sample consists of a mixture of particle forms of different shapes. In such case, only electron microscopy (EM) and possibly atomic force microscopy may yield reliable results. Even with EM, the analysis of plate-like particles is problematic as the smallest dimension (thickness) could be difficult to access.

Considering the characteristics of available analytical methods, the criteria shape and number of nanoscale dimensions of nanoparticles can assume the following values:

Number of nanoscale dimensions:

- 1
- 2
- 3
- Mixture of nanoparticles with different shapes

In principle, indication of the number of small dimensions is sufficient for the purposes of the categorisation scheme, but often it is helpful to characterise particle shape with more descriptive terms. They are also included here regardless of some redundancy with the criterion of number of small dimensions. Descriptive criteria for particle shape are:

- One small dimension: plates (flat shapes incl. irregular flakes)ⁿ
- Two small dimensions: fibres (elongated shapes such as tubes, fibres, rods)ⁿ
- Three small dimensions: Spherical, equiaxial or similar (e.g. prismatic, cubic, tetrahedral)
- Mixture of nanoparticles with different shapes
- Other (incl. unknown)

Size range

Techniques for particle sizing have their own measurement range. The size range therefore is definitely a criterion which can limit the choice of the techniques to obtain an accurate result.¹⁰ That size range can depend on further criteria, e.g. the chemical composition or the polydispersity of the material. In practice, the analysis needs to cover the entire size range of the particulate material in order to get an accurate result for the size distribution.²⁶ If the particle size range in a sample is too large, certain techniques cannot determine the actual particle size distribution. DLS, for example, is much more sensitive to large particles than to small ones, which can easily lead to inaccurate size distribution results for particulate materials with a broad particle size distribution. Moreover, other techniques such as Tunable Resistive Pulse Sensing (TRPS) are not able to measure very small particles.²⁴ In both cases, an overestimation of the measured particle size is the consequence. Conversely, small angle X-ray scattering (SAXS) is not able to measure particles

ⁿ According to ISO/TS 80004-2:2015(en), terms such as nanofibre or nanoplate may be preferred to the term nanoparticle if the dimensions differ significantly (typically by more than 3 times)

above 100 nm, this making the technique prone to underestimate the median particle size for broad size distributions well above 100 nm; this finally results in possible false-positive results, i.e. identification of materials as nanomaterials when they are not.

Trade Form and Dispersibility

Some characterisation techniques require the particles to be dispersed in a liquid phase, whereas others only work for powders. Therefore, it is essential to know if the substance to analyse is pre-dispersed or can be dispersed, including information on the dispersing media and specific protocols to be used, in order to determine which characterisation technique could be suitable for the analysis. As recently discussed by Hartmann et al.,²⁷ particle size distributions can be affected by many factors, such as sample preparation protocols, including the choice of the dispersion media, particle concentration or material tendency to aggregation and agglomeration.

The user should therefore first indicate the trade form of the sample, which can be

- powder
- suspension
- aerosol

Considering the characteristics of the evaluated techniques, materials are categorised further as:

- Dispersible in aqueous media (by generalised protocols)
- Dispersible in material-specific media and protocols
- Can be aerosolised

Stability of particles during testing

Some materials may be incompatible with the conditions of certain measurement techniques, e.g., they may be sensitive to irradiation by electrons. If this is the case, they cannot be characterised reliably with EM, or require more sophisticated EM techniques, such as cryo-EM or soft excitation conditions (low beam current or voltage) techniques. Particularly polymers or organic solids may be degraded by electron beam irradiation.^{28,29,30} Other substances may be stable in a narrow temperature range.³¹ Therefore, it is generally necessary to know if characterisation techniques can induce damages to sensitive materials by their probes or by operating conditions (for example vacuum in EM).

The criteria addressed here relate to sensitivity against

- Electron beam irradiation
- vacuum conditions
- heating (with specification of the maximum acceptable temperature)
- cooling (with specification of the lowest acceptable temperature)
- If a material releases ions, atoms or molecules in its environment, this can also interfere with measurements, therefore this criterion is also included. For instance, as Ag particles dissolve, nanoparticles can actually remain undetected.

Specific properties

Specific electrical, optical, magnetic and surface properties may interfere with or, on the contrary, facilitate certain measurement techniques.⁷ Specific material properties are therefore to be taken into account in order to avoid inappropriate techniques.

For instance, an electrically insulating material cannot be analysed in conventional SEM unless it is coated with a thin layer of conductive material.³² The charging effect arising from electron/ion irradiation can be otherwise avoided to a certain extent if the SEM instrument used has a low voltage option or a variable pressure option.³³

In addition to standard techniques, magnetic particles may also be easily characterised with some particular techniques, e.g. magnetic force microscopy (MFM), in which a sharp magnetised tip maps the magnetic force gradient above the sample surface while simultaneously acquires topographical data.³⁴

Some materials have also specific spectroscopic properties. For instance, Raman spectroscopy can be used to measure the diameter of Single Walled Carbon Nanotubes.³⁵ UV-Vis spectrometry can also serve as a size measurement technique if the material exhibits surface plasmon resonances,³⁶ e.g. silver or gold nanoparticles.

Another criterion is the presence of a functionalisation or coating of the particles, i.e. if the outermost layer has a different chemical nature than the core of the particles. This modifies the characteristics of the particle surface e.g. the presence of hydrophobic, hydrophilic, reactive groups. Some characterisation techniques - especially those where the measurements are performed in a liquid dispersion - can be affected by these features. For example, the analysis may lead to artefacts especially when the hydrodynamic diameter is measured, and this fact also needs to be taken into consideration for the sample preparation.

Functionalisation is distinguished in this MCS from the coating which is defined as a uniform layer. For instance, a particle completely covered by a layer (the coating) will belong to the composite category. On the other hand, a particle with molecules bound to the surface in a less dense layer, where the surface of the particle is still accessible by other molecules, is considered here as a functionalised particle. Characterisation techniques such as DLS (measuring hydrodynamic diameter) or USSp (measuring acoustophoretic diameter)²⁴ will measure the whole ensemble and will give a false estimation of the particle size.

For the reasons described above, it can be important to be aware of the following specific properties of the sample:

b. Particles embedded in a matrix

The materials categorisation system can also be applied, in a slightly extended form, to nanoparticles embedded in an article or in a formulation. In the example shown in Figure 17, this is done for a sunscreen lotion which contains titanium dioxide nanoparticles. In that case, the categorisation system is extended by two main criteria: (i) type of matrix and (ii) removal of non-particulate components. 'Removal' includes all means of separating the particulate components from the matrix, including filtering, digestion and ashing.

Type of matrix

The type of matrixes in which particles are embedded or suspended is also a necessary criterion to be taken into account in order to know which techniques can be applied. Three cases can be selected:

- Particles are embedded in a solid matrix
- Particles are embedded in a liquid/gel matrix
- Particles are suspended in a gas

If the particles cannot be removed from the matrix, the analysis must be performed directly on the embedded particles.

Removal of the non-particulate components and particles extraction

If the matrix can be separated without altering the particulate components, the techniques used to measure the latter can be the same as those used for materials with monotype and multiple types of particles. For instance, if the matrix of a sunscreen which contains particles of titanium dioxide can be removed, the techniques to analyse the remaining particles would be the same as for pristine (or coated, if applicable) titanium dioxide. A variety of procedures to separate the matrix in order to extract nanoparticles are described in the literature,^{37,38} including digestion methods to remove food matrices. However, such procedures must be compatible with the particles to extract them without modifying the particles during the extraction process.

The criterion of matrix removal can then be selected as follows:

- the non-particulate components can be separated (or alternatively the particles can be extracted). In that case, the conditions should also be specified.
- the non-particulate components cannot be separated (or the feasibility is unknown)

An example of the categorisation of a sunscreen containing coated titanium dioxide particles is presented in Figure 18, assuming that no further solid phase is dispersed in the sunscreen. The non-particulate components can be removed and the type of matrix is a gel/liquid. The particulate material is a composite of the core-shell type, has three small dimensions and the exact shape is unknown. Due to the latter condition, the option 'unknown' has been chosen instead of '3 (spherical, equiaxial)'. Further, it is expected that the size range is between 20 and 120 nm and it is known that the particles are of only one type. A known specific property is size-dependent photon absorption, whereas others are also unknown, so that the corresponding boxes remain unfilled. Matching these characteristics with the technique performance table leads to EM and AFM as applicable techniques. The main limiting condition for this recommendation is the fact that the shape of the particles is not known.

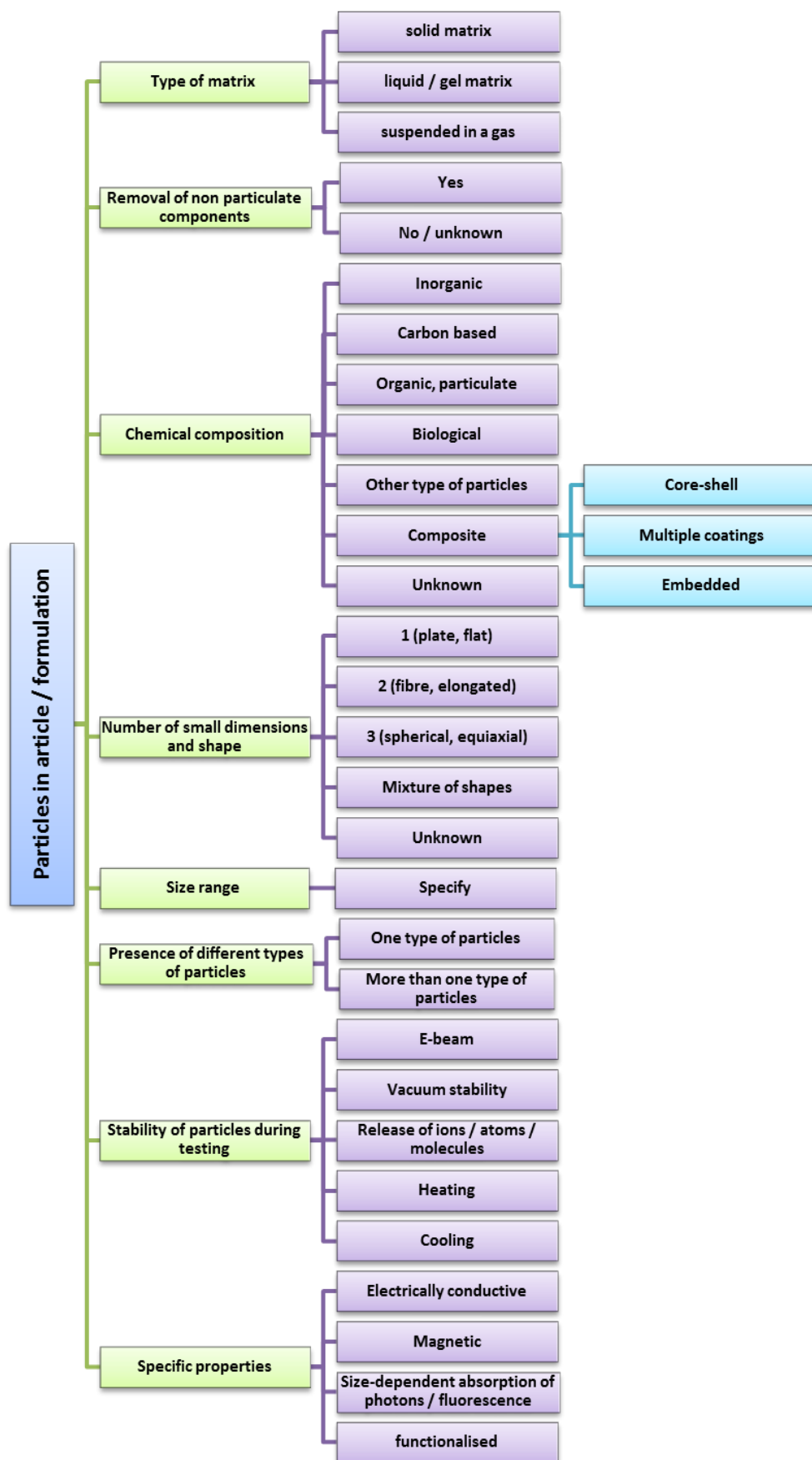


Figure 17: The Material Categorisation Scheme for an article / formulation that contains particles

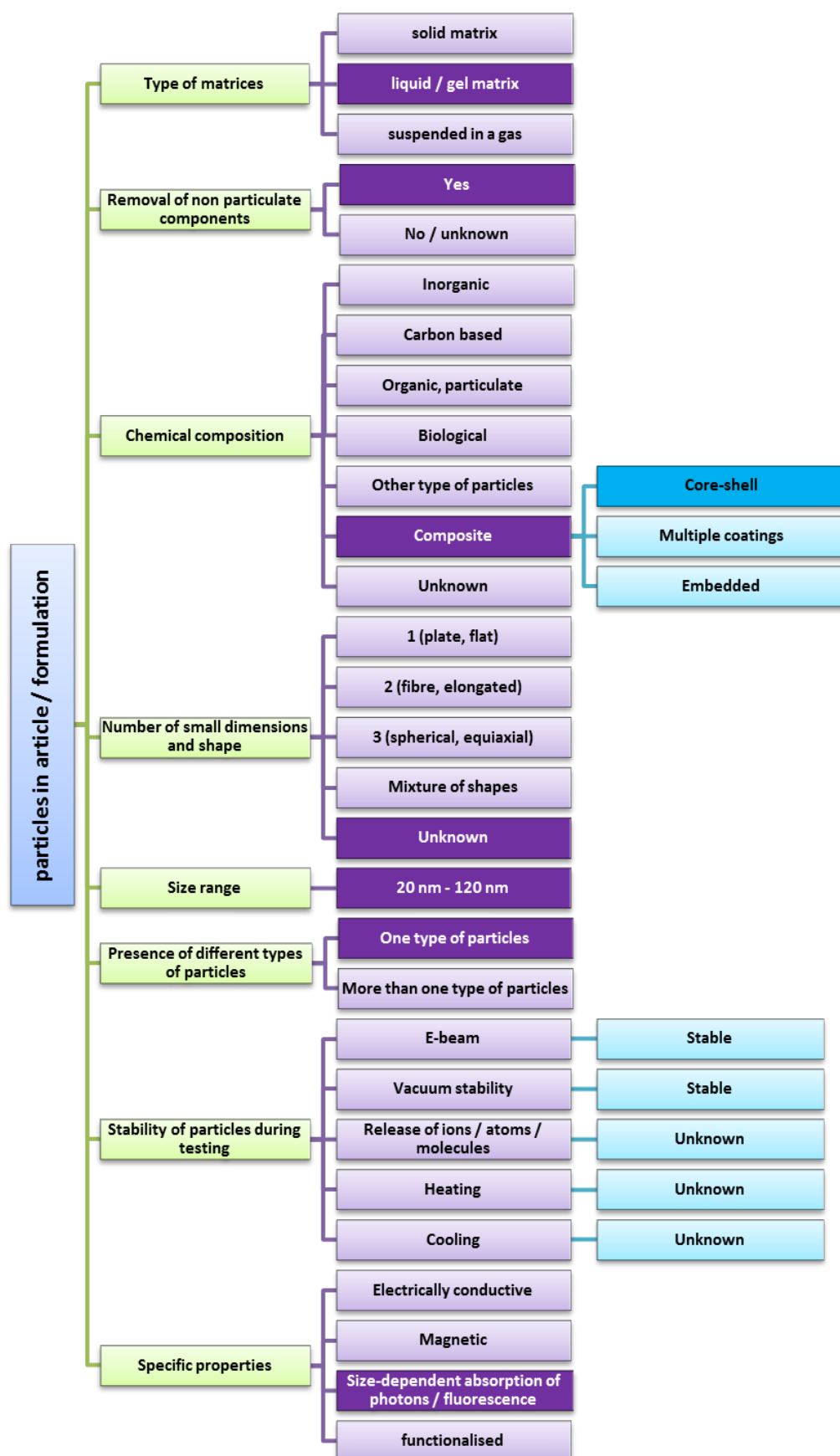


Figure 18: The Material Categorisation Scheme applied to coated titanium dioxide particles in a sunscreen formulation

Annex 2 Derivation of the quantity $dmin_{VSSA}$

In the following, the minimal particle dimension $dmin_{VSSA}$ is calculated from the materials' VSSA, reproduced from the supporting information of a NanoDefine publication.¹⁵ This quantity enables a direct comparison of the results to the EM-derived d_{50} of the smallest particle dimension, which is more straightforward than using VSSA cut-off values and leads to the same identification results.

The property $dmin_{VSSA}$ is defined as

$$dmin_{VSSA}(D) = \frac{2D}{VSSA}$$

where D is the number of small dimensions¹⁰. In this section it will be shown how it can be derived for several classes of particle shapes: Spheres, cubes, fibres and platelets (Figure 19).

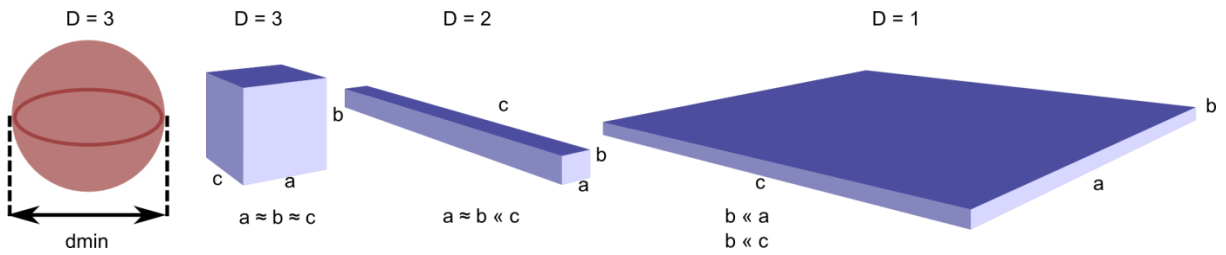


Figure 19: Scheme of prototypical particle shapes, having three, two and one small dimension

It is instructive to start with the case of a sphere (D=3) where $dmin$ is the sphere diameter. Its volume is $V = \frac{\pi}{6} dmin^3$ and its surface $S = \pi dmin^2$. Consequently, its VSSA is

$$VSSA = \frac{S}{V} = \frac{6}{dmin}$$

Therefore, a sphere with a diameter of 100 nm has a VSSA of 60 m^2/cm^3 . When the VSSA is known from a measurement, it is possible to calculate $dmin$ by

$$dmin = \frac{6}{VSSA}$$

For general cuboids, the volume V is $V = a \times b \times c$, the surface $S = 2(ab + bc + ac)$ and the VSSA:

$$VSSA = \frac{S}{V} = \frac{2}{a} + \frac{2}{b} + \frac{2}{c}$$

A cube (D=3) with equal sides ($a = b = c = dmin$) therefore has a VSSA of:

$$D = 3: \quad VSSA = \frac{S}{V} = \frac{6}{dmin}$$

which is identical to the case of the sphere. For elongated particles (D=2), the relation between the sides is $c \gg a = b = dmin$. In the limit of $c \rightarrow \infty$, the VSSA becomes

$$D = 2: \quad \lim_{c \rightarrow \infty} VSSA = \frac{2}{a} + \frac{2}{b} = \frac{4}{dmin}$$

Analogously, the side relation for platelets is $dmin = b \ll c$ and $b \ll a$. In the limit of $a, c \rightarrow \infty$, the VSSA becomes

$$D = 1: \quad \lim_{a, c \rightarrow \infty} VSSA = \frac{2}{b} = \frac{2}{dmin}$$

When generalizing the three cases for $D = 1$, $D = 2$ and $D = 3$, the following formula is obtained:

$$VSSA(D) = \frac{2D}{d_{min}}$$

Solving this expression for d_{min} gives:

$$d_{min_{VSSA}}(D) = \frac{2D}{VSSA}$$

(when entering $VSSA$ in units of m^2/cm^3 , $d_{min_{VSSA}}$ is obtained in μm)

When no information about the particle shape is known, the particles are assumed to be spherical ($D=3$). In this case, it cannot be expected that the formula derived above still yields the minimum particle dimension, but instead a spherical equivalent diameter is calculated, which will be called d_{VSSA} :

$$d_{VSSA} = \frac{6}{VSSA}$$

(when entering $VSSA$ in units of m^2/cm^3 , d_{VSSA} is obtained in μm)

It is important to keep in mind that the expressions derived here are strictly only valid for single particles or perfectly monodisperse particle size distributions.

Annex 3 Assessment of the uncertainty introduced by the aspect-ratio cut-off values

The shapes considered in the previous annex for $D = 1, 2, 3$, are convenient for calculating $dmin_{VSSA}$, but can only be seen as an approximation for the real particle shape. Therefore, it is necessary to define the properties a particle needs to have in order to be attributed a certain D value and to assess its potential influence on the results.

It is proposed to select D for a given material according to the average aspect ratio (AR) of the particles.

$D = 3$ for particles with $AR < 3:1$

$D = 2$ for particles with $AR > 3:1:1$

$D = 1$ for particles with $AR > 3:3:1$

In the following, the largest possible influence of these cut-offs on the derived $dmin_{VSSA}$ is evaluated.

For $D = 3$, the maximum possible deviation from the equal sided cube occurs when the smallest side has a length of $dmin$ and the other two sides a length of $3 \times dmin$. In this case the VSSA is:

$$VSSA = \frac{2}{dmin} + \frac{2}{3dmin} + \frac{2}{3dmin} = \frac{5}{3} \frac{2}{dmin} \approx 0.56 \frac{6}{dmin}$$

$$\Rightarrow dmin = \frac{5}{9} \frac{6}{VSSA} \approx 0.56 \frac{6}{VSSA}$$

For $D = 2$, the maximum possible deviation as compared to the case of one dimension going to infinity is to have two sides with a length of $dmin$ and the third with a length of $3 \times dmin$, hence:

$$VSSA = \frac{2}{dmin} + \frac{2}{dmin} + \frac{2}{3dmin} = \frac{7}{3} \frac{2}{dmin} \approx 1.167 \frac{4}{dmin}$$

$$\Rightarrow dmin = \frac{7}{6} \frac{4}{VSSA} \approx 1.167 \frac{4}{VSSA}$$

For $D = 1$, the maximum deviation of the shape to the case of two dimensions going to infinity is to have one side with a length of $dmin$ and two sides with a length of $3 \times dmin$:

$$VSSA = \frac{2}{dmin} + \frac{2}{3dmin} + \frac{2}{3dmin} = \frac{5}{3} \frac{2}{dmin} \approx 1.67 \frac{2}{dmin}$$

$$\Rightarrow dmin = \frac{5}{3} \frac{2}{VSSA} \approx 1.67 \frac{2}{VSSA}$$

In Table 8 below, the maximum deviations calculated here induced by the AR cut-offs are compared to the value obtained by the simple approach when characterizing the particles only by $D = 1, 2, 3$. In all cases, the largest possible relative deviation is below 70%.

Table 8: Maximum deviation of $dmin_{VSSA}$ induced by the aspect ratio cut-offs.

D	$dmin_{VSSA}(D)$	$dmin_{VSSA}$ (max deviation)	Relative deviation
1	$\frac{2}{VSSA}$	$1.67 \frac{2}{VSSA}$	+67%
2	$\frac{4}{VSSA}$	$1.167 \frac{4}{VSSA}$	+17%
3	$\frac{6}{VSSA}$	$0.56 \frac{6}{VSSA}$	-44%

Importantly, the here derived numbers are only the largest possible deviations due to the AR cut-offs. Other sources of uncertainty on the $dmin_{VSSA}$ are not considered in this evaluation.

Annex 4 NanoDefiner e-tool guide for version 1.0.0

The NanoDefiner e-tool is accompanied by a guidance document which assists the user in the practical application of the software. The sections below contain this document as included in the e-tool.

An additional video tutorial on how to use the NanoDefiner e-tool is publically available at: https://labs.inf.fh-dortmund.de/NanoDefiner/static/downloads/screencast_20171024.mkv and NanoDefine project website www.nanodefine.eu.

a. Overview

The NanoDefiner e-tool^o is a decision support tool for the identification of potential nanomaterials, according to the EC NM Definition (2011/696/EU). It is one of the products delivered by the NanoDefine project^p.

The main focus of the NanoDefiner e-tool is the recommendation: i) of suitable MTs, based on a material description, taking into account custom user lab settings regarding availability, cost, and default method uncertainty of configured MTs and ii) of the decision, based on the data input whether the analysed material is a nanomaterial or not according to the EC NM Definition.

The report generated by the NanoDefiner is intended to be used as supplementary information in a material registration process. The NanoDefiner e-tool helps to document the material identification process according to the following workflow:

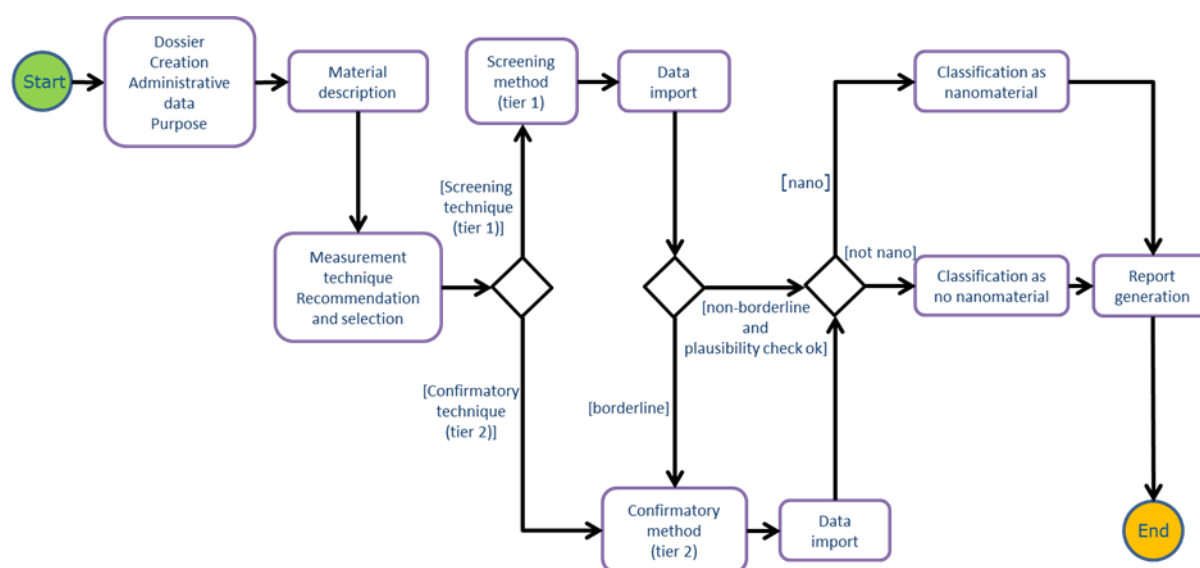


Figure 20: Workflow within the e-tool

o <https://labs.inf.fh-dortmund.de/NanoDefiner/> (accessed 2019-08-21)

p <http://www.nanodefine.eu/> (accessed 2019-08-21)

This document guides the user through this workflow as implemented in the e-tool, which consists of the following steps (see also the succeeding sections):

- Dossier creation, where the dossier name, purpose, and sample type is defined
- Material description, where each particulate component (PC) of the material is described
- Method description, application, and result upload, where MTs are chosen and analysis results are uploaded
- Report generation, where one or several applied methods are selected for inclusion into the final Portable Document Format (PDF) report

Note: The NanoDefiner e-tool has been tested with current versions of Mozilla Firefox, Google Chrome/Chromium, and Safari (versions Chrome/Chromium 62; Firefox 57 and Safari 11, and above). It may not work as expected on other browsers (e.g. Internet Explorer). If problems arise while using the e-tool, one should consider using one of the listed browsers. On touch interfaces, some form elements (e.g. sliders) may not work as expected, in these cases one should use the alternative form elements provided (for sliders, manually insert the desired values).

It is assumed here that the user already has an account. Please see section f how to apply for an account and what to do if the password is lost. After logging in, the user will see a personal *dashboard* (see Figure 21) which shows – currently empty – lists of the user's dossiers, particulate components and methods described in dossiers as well as generated dossier reports.

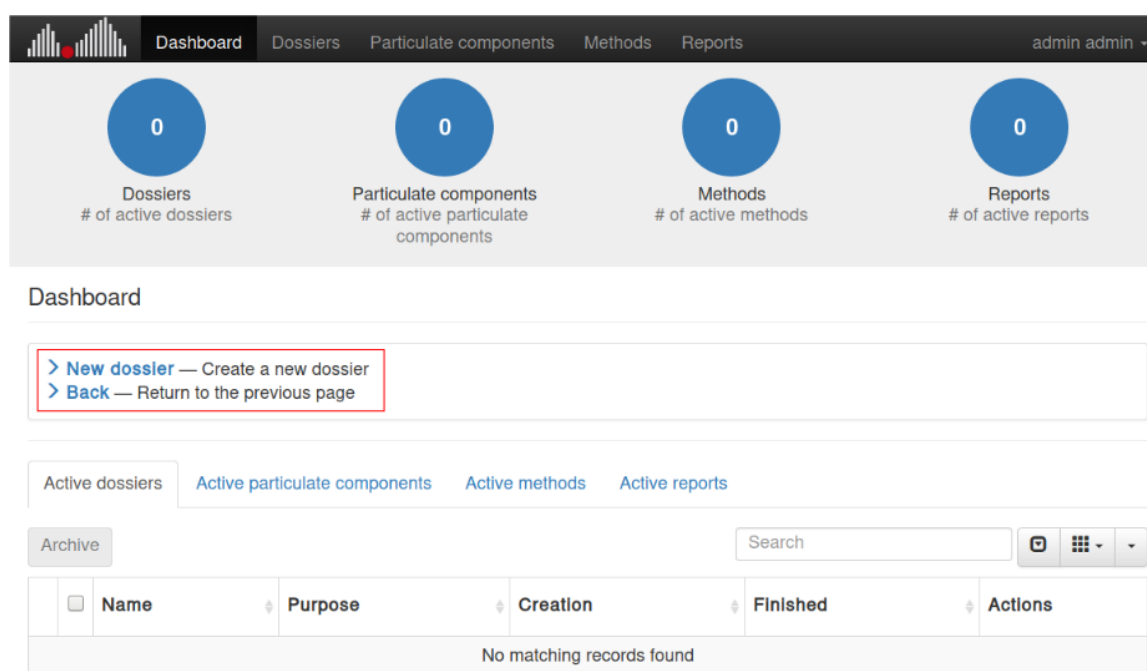


Figure 21: Initial dashboard view with highlighted action box

The highlighted area is the *action box*, which can be seen on most pages within the e-tool and contains all actions available in the context of the current page. From the dashboard, the only available action is 'New dossier', one should click on it to proceed to dossier creation.

Note: There are three types of actions: available, unavailable, and recommended actions. Unavailable actions will be displayed but can't be chosen; they may become available at a later stage of the identification process. Recommended actions are highlighted in green and denote the most likely next action depending on the current progress within the identification workflow.

b. Dossier creation

A dossier contains all entities associated with one material categorisation process: the PCs a material is composed of, the methods performed during materials analysis, and the report.

On the first page of the dossier creation form (see Figure 22), one can choose a name for the dossier, add a comment (which will be included in the report) and an internal comment (which will not be included in the report), as well as select a dossier *purpose*. The purpose is meant to allow the generation of different report types (i.e. including different information or having a different report layout) depending on the target registration authority. At the moment, the purpose influences only the availability of MTs – for REACH dossiers, only MTs assessed within the NanoDefine project are considered.

Note: The MT single particle Inductively Coupled Plasma-Mass Spectrometry (spICP-MS) is currently not assessed, so one needs to select the purpose 'other' if this MT should be available for a dossier.

Creation of a new dossier

New / Create dossier

In this step you need to describe the dossier itself. In the next step, you need to describe the dossier's sample.

Dossier definition Next

What is the purpose of the dossier?

- REACH / REACH nanoform ?
- Cosmetics regulation ?
- Food regulation ?
- Biocide regulation ?
- Other ?

Dossier name*

The dossier's name, displayed in the report

Dossier comment

Optional comment on the dossier, displayed in the report

Internal comment

Optional internal comment on the dossier, not displayed in the report

Next

Figure 22: First page of the dossier creation form

The second page of the dossier creation form (see Figure 23) allows you to describe the sample by setting its name and choosing the sample type, either mono-type or multi-type. Mono-type samples contain only one PC, while multi-type samples can contain more than one PC. Additionally, for

multi-type samples, MTs that don't support such samples are automatically excluded during recommendation – so make sure to only select multi-type if you really have a multi-type sample.

Creation of a new dossier
First dossier / Create dossier
In this step you need to describe the sample you want to analyse.

Sample definition Back Save and proceed

Which type of sample would you like to assess?
 Mono-type – The sample consists of one particulate component to be analysed
 Multi-type – The sample consists of two or more particulate components to be analysed

Sample name*
First dossier mono sample Back Save and proceed

Figure 23: Second page of the dossier creation form

Proceed to the creation of the first particulate component by clicking 'Save and proceed' when you're done.

Note: Clicking on a button labelled 'Save' within the application indicates that all changes that have been made to the dossier or current entity have been saved, so feel free to interrupt the material classification process at any point; it can always be resumed exactly from where you left off.

c. Material description

The next form is the MCS in which you can describe the PC of your sample using more than 20 attributes. Before getting to the actual attributes, you can provide some basic information about the PC (see Figure 24) like its name, whether you want to derive it from a pre- or self-defined PC template and if it belongs to a certain material group or type. Selecting a PC template will load all the associated values into the MCS. Initially, the list of PC template consists of pre-defined reference materials, but users can add their own templates at any point (see section h). The material type/group allows fine-tuning MT recommendation considering materials for which the MTs are known to behave differently.

Definition of a new particulate component

Screencast / Particulate component #1 based on [BAM-11] Zeolite powder / Create particulate component

Please name and describe the particulate component.

Definition of a particulate component Next

Would you like to use a template?

[BAM-11] Zeolite powder

Particulate component name*

Particulate component #1 based on [BAM-11] Zeolite powder

Particulate component comment

Optional comment on the particulate component, will be displayed in the report.

Does the particulate component match a specific material group or type?

No specific particulate component type or group Next

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Figure 24: First page of the material description form

On the next three form pages, you are able to describe the PC in detail, using single and multi-selects, yes/no questions and number ranges. All attributes can be set to unknown (and by default, all attributes are unknown). While making changes to the PC attributes, you will notice some information changing at the top of the form, labelled *Live feedback decision making* and *Particulate component description incompleteness* (see Figure 25). The latter just shows the number of attributes set to 'unknown', a higher value means less reliable MT recommendation. For example, if the incompleteness of the initial material description by the user leads the e-tool to recommend a Tier 1 technique that is not actually suitable for this material, then the e-tool would identify false negative or false positive identification after Tier 1. This is a consequence of the DSFS, which is designed for exactly that filtering purpose, and it is entirely implemented in the e-tool. Live feedback shows a list of generally available MTs (based on the dossier purpose and sample type) along with their suitability for the material (also considering the other, previously created PCs for a multi-type sample).

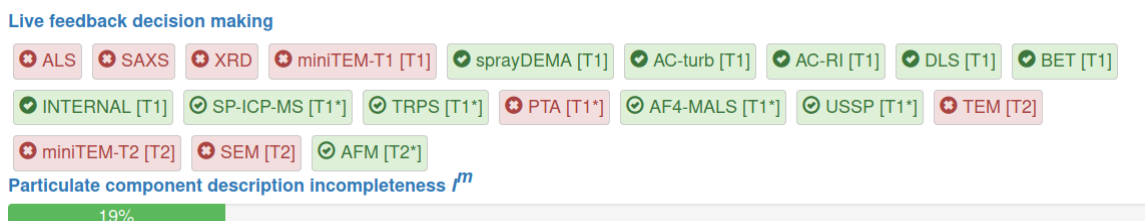


Figure 25: Live feedback and PC description incompleteness for the initial PC description form where all properties are unknown

Clicking on an MT label gives you more detailed suitability information (see Figure 26) along with the *weighted particulate component description incompleteness* (this is a method-specific version of the particulate component description incompleteness described above). In the table below that, you can compare MT support for each attribute with the current particulate component values. For multi-type samples, other particulate components can be accessed via tabs at the top of the window.

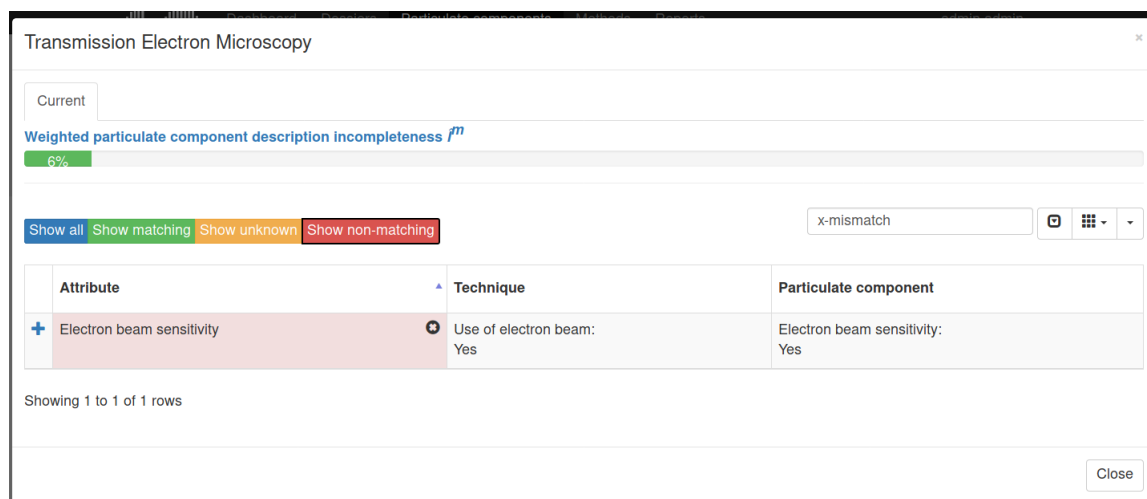


Figure 26: Method-specific suitability information

Note: All labels in the MCS (and many in other parts of the application) can be clicked to reveal further information.

When you are done describing the PC, you have the choice of continuing to method description or return to the dossier overview (from where you can create additional PCs and review the current dossier state and information). Before submitting the form, you will be asked to confirm all unknown PC properties. When you are ready, continue to method description from the particulate component description form or from the dossier overview.

d. Method description

The method description form starts with a selection of MTs to choose from (see Figure 27). You can decide to switch between tier 1 (screening) and tier 2 (confirmatory) MTs with the button above the method selection table. The methods in the table are initially sorted according to suitability and the user lab settings (availability, cost, duration). The table also contains (weighted) method incompleteness information for each MT, representing how well the MT is described in the knowledge base (KB) of the NanoDefiner.

Clicking on the icons in the 'Suitable' column will give you information similar to the live feedback in the MCS earlier. Additionally, method- and attribute-specific warnings are displayed. These warnings will be included in the report later, so make sure that you read and resolve them if possible (e.g. by specifying values for unknown PC properties).

Creation of a new Tier 1 method

Screencast / AC-RI / Create method

In this step you need to choose the method's technique. In the next step, you get to choose the method's potential preprocessing protocols.

Matching techniques Next

Switch to: Tier 2 (confirmatory) ☰

	Name	Cost (€)	Duration (h)	Warnings	Suitable	Available	TI	WTI
<input checked="" type="radio"/>	AC-RI	n/a	n/a	✔	✔	✔	<input type="text"/>	<input type="text"/>
<input type="radio"/>	AC-turb	n/a	n/a	✔	✔	✔	<input type="text"/>	<input type="text"/>
<input type="radio"/>	sprayDEMA	n/a	n/a	✔	✔	✔	<input type="text"/>	<input type="text"/>
<input type="radio"/>	BET	n/a	n/a	!	✔	✔	<input type="text"/>	<input type="text"/>
<input type="radio"/>	DLS	n/a	n/a	!	✔	✔	<input type="text"/>	<input type="text"/>
<input type="radio"/>	miniTEM-T1	n/a	n/a	!	✘	✔	<input type="text"/>	<input type="text"/>

Next

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Figure 27: First page of the method selection form, where recommended measurement techniques of tier 1 and 2 can be selected

Note: for more information on the MTs and their tiers, refer to the methods manual which can be accessed from the user menu in the top right using the link 'View manual'.

After selecting an MT (you will be forced to confirm your choice if method-specific warnings exist or the MT is not suitable for the material), you can choose from some basic pre-processing protocols and provide further pre-processing details using the textbox below. Finally, choose a name and comment for the method and submit the form when you are ready. All information, including the comments, will be present in the generated report.

Note: At this point, the lab analysis is performed. As mentioned before, the dossier is saved after you create the method, so you can close the application or switch to another dossier and resume with the upload of method results at a later time.

On the following page (see Figure 28) you can view details of the method you just entered, and, more importantly, upload analysis result data. Depending on the MT, there are several types of result data you can upload:

- for BET, you can enter the volume-specific surface area (VSSA) value based on which the result will be calculated
- for most other MTs, size distribution information as generated by the ParticleSizerq can be uploaded
- for spICP-MS, you can upload a spreadsheet generated by the Single Particle Calculation tool (SPC)r.

q http://imagej.net/ParticleSizer#Use_the_ParticleSizer_with_the_NanoDefiner_e-tool (accessed 2017-10-12)

- for all MTs you can always manually enter a x_{50} (or d_{50}) d_{50} value, please make sure to
- include supporting evidence as custom attributes

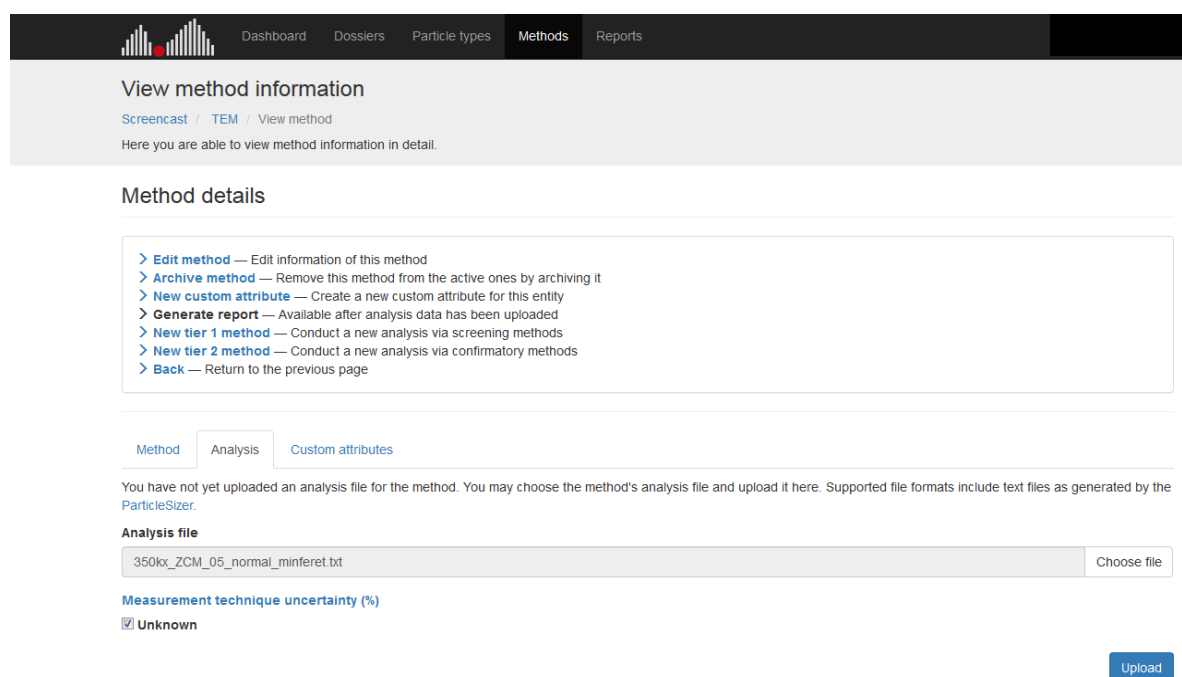


Figure 28: Page where method results can be uploaded, for the Transmission Electron Microscopy, Tier 2 settings (TEM-T2) MT in this example

For all uploaded results, you can optionally specify the measurement uncertainty (according to Guide to the Expression of Uncertainty in Measurement (GUM)), which is unknown or set to the value specified in your lab settings by default.

After uploading the method results, you can either describe additional methods or proceed to report generation via the link in the action box. Depending on the type of the uploaded data, additional details like plots are available as well.

Note: Custom attributes provide a way to attach additional information to dossiers, PCs and methods, which will be included in the generated report. Custom attributes have a name, a value, and an optional comment. The value can be a simple text, or you can upload files (e.g. supporting evidence for methods).

e. Report generation

As the last step of the NanoDefiner e-tool workflow, you will create a report which will summarize all dossier information, including a selection of methods for which results were uploaded. In the first step of the report generation (see Figure 29) you can select one or several methods to be included in the report (only methods with results can be chosen). Afterwards, only a report name has to be chosen before the report generation can be triggered. Only one report can be active per dossier at a time, meaning that upon report generation, the last generated report (if any) will be archived (see section i). However, archived reports can still be viewed and download as before.

r <https://www.wur.nl/en/show/Single-Particle-Calculation-tool.htm> (accessed 2017-10-12)

Create new report
[First dossier](#) / [New](#) / [Create report](#)
 Choose the methods to be included in the report

Select methods Next

📄
🗃️
⌵

<input type="checkbox"/>	#	Name	Technique	Result	Tier
<input checked="" type="checkbox"/>	7	AFM	AFM	316.1	Potential tier 2 (not assessed)

Showing 1 to 1 of 1 rows Next

Figure 29: First page of the report generation form. Here you can select which methods to include

After generating the report, which can take a couple of seconds, you can download the PDF version of it (which should look similar to Figure 30) or review the involved entities. With this, you have successfully completed documenting a material classification process in the NanoDefiner e-tool.



Figure 30: First page of a generated report

f. Registration, activation, and password reset

Before being able to use the NanoDefiner e-tool, you will need an account. The registration process can be started by clicking the 'Register' button in the top right. On the subsequent form, you have to choose a username and password and enter an e-mail address. Optionally, you can enter a title and your name (forename and surname can only be changed by an administrator later on).

After submitting the form, you will be informed which way of activation is currently enabled for the e-tool: either **e-mail activation** or **manual activation** by an administrator.

In case of e-mail activation, you will receive an e-mail containing an activation link and further instructions. Visiting this link will activate your account and you can then log in.

If e-mail activation is disabled, your account has to be activated by an administrator. You will receive an e-mail after your account has been activated.

In case you have forgotten your password, press the login button once to view the complete login form which contains a 'Forgot your password?' link where you can enter your username or e-mail address to issue a password reset e-mail containing further instructions. The e-mail contains a link which will bring you to a password reset form where you can choose a new password. This link is all that is needed to change your password and thus take control of your account, so make sure to perform the password reset quickly after receiving the mail or login soon if you get a password reset mail that you have not requested.

g. User profile and lab settings

After logging in, the user profile can be accessed by clicking on your name at the top right of the application and choosing 'view and edit profile' from the drop-down list. There, you can change account and profile settings (e-mail address, password, as well as your title). Some settings like your username as well as first and last name, cannot be changed after the registration (contact an administrator if you do need to change these settings).

The more interesting part of the profile are the *lab settings* (see Figure 31), where MT-specific cost, duration, availability, and default measurement uncertainty can be configured. These settings will be considered by the NanoDefiner e-tool when giving you MT recommendations.

Account settings Profile settings Lab settings

Technique settings

Name	Cost (€)	Duration (h)	Uncertainty (%)	Enabled
AC-RI	10.0	1.0	3.6	✓
AC-turb	0.0	0.0	Unknown	✓
AF4-MALS	0.0	0.0	Unknown	✓
AFM	0.0	0.0	Unknown	✓
ALS	0.0	0.0	Unknown	✓
BET	0.0	0.0	Unknown	✓
DLS	0.0	0.0	Unknown	✓
INTERNAL	0.0	0.0	Unknown	✓
miniTEM-T1	0.0	0.0	Unknown	✓
miniTEM-T2	0.0	0.0	Unknown	✓

Showing 1 to 10 of 19 rows 10 rows per page

1 2

Save

Figure 31: First page of the default lab settings with an example configuration added for AC-RI

Click on an **MT** to change the values (these will be used for your account only).

h. Material description templates

When describing several very similar materials, it can be tedious to fill out the MCS from scratch each time. For that reason, *material description templates* have been introduced, which allow you to re-use PCs. On the first page of the MCS, you may have noticed that there is already a list of templates to choose from. These are the reference and test materials. You can create your own templates from an existing PC, or from scratch.

To create a template from a pre-configured NanoDefine PC or PC template, first click on the PC from the dossier or the global PC list, and then select 'New particulate component template' from the available actions.

This will open the MCS, already filled with the properties of the original PC. Changes here will not affect the original PC, and the new template will only be created after you click 'Save and return' at the end of the MCS form. After that, the template will be available for selection in the MCS when creating new PCs.

i. Entity archiving and removal

The NanoDefiner e-tool supports archiving of dossiers, materials, methods, and reports. Archiving an entity marks it as read-only and will move it to the list of archived entities, which is an irreversible operation. Archiving a dossier will mark its sub-entities (i.e. contained materials, methods and reports) as archived. Archiving sub-entities of active dossiers will hide them from the entity lists within the dossier, but will show them in the global archived entities list. E.g. when

archiving a material within a dossier, it will not be shown in the 'Particulate component' tab of the dossier, but only in the 'Archived particulate components' when clicking 'Particulate components' in the main navigation at the top. There can only be one active report within a dossier at any time – when generating a new report, the last one will be archived. Archived entities can still be viewed and will never fully be removed from the system.

The only entities which can be removed instead of archived are particulate component templates and custom attributes. Removing these entities will delete them from the system completely, they can no longer be viewed and this step cannot be undone.

Annex 5 Case studies

Within the NanoDefine project case studies were performed for testing this decision support flow scheme for material categorisation. For this purpose the experimentally supported flow scheme presented in Figure 10 was applied and only methods that are recommended by the NanoDefine project were employed. The detailed results of the case studies can be found in a NanoDefine Technical Report³⁹.

- The case studies involved:
 - Various compositions (organic / inorganic / carbonaceous)
 - Different sizes (nano / borderline / not nano)
 - Heterogeneous evaluators (academia / industry / regulator)
 - Different shapes (compact particles / fibres / platelets)
 - Heterogeneous levels of reliability of the characterisation data (data generated in NanoDefine / pre-existing data obtained by methods that do not contradict NanoDefine guidance / data generated de novo)

The decision tree was found to be internally consistent: Case studies explored both Tier 1 (powder route) and Tier 1 (suspension route), and were benchmarked against a Tier 2 method (SEM or TEM). No case of inconsistent identification was found, but two cases remained inconclusive. The specific reasons for inconclusiveness were: the lack of reliability of pre-existing data (especially the EM sample preparation and interpretation); hesitation of the operator to identify constituent particles that are fused together within aggregates (an automated EM evaluation by the NanoDefiner software may have been a solution). One case study identified limits of the linear, hierarchical decision flow scheme for articles (sunscreen), whereas the flow scheme and e-tool are intended for substances.

In all cases consistent results were obtained both from manual assessment and from e-tool assessment. The input parameters for the case studies were implemented as default options in the e-tool: Future users of the e-tool can select an existing case study, and can then replace only the specific parameters by which their new material differs from the case study material. This will save time and enhances comparability.

The case studies show remaining challenges for the implementation of the EC NM Definition, but clearly demonstrate the consistency of the decision tree and e-tool⁴⁰. The varied chemical composition, polydispersity, different shapes, different sizes, different intended uses did not seem to compromise the application of the decision flow scheme.

Annex 6 Main EU legislation of relevance

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and restriction of Chemicals (**REACH**), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L396 (1), 1-849. 2006.

Commission Regulation (EU) 2018/1881 of 3 December 2018 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annexes I, III, VI, VII, VIII, IX, X, XI, and XII to address **nanofORMs** of substances. OJ L 308, 4.12.2018, p. 1–20.

Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures (**CLP**). OJ No. L353, 31.12.2008, p. 1.

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Commission Regulation (EC) No 450/2009 of 29 May 2009 on **active and intelligent materials and articles intended to come into contact with food**. OJ L135, p. 3-11. 2009.

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