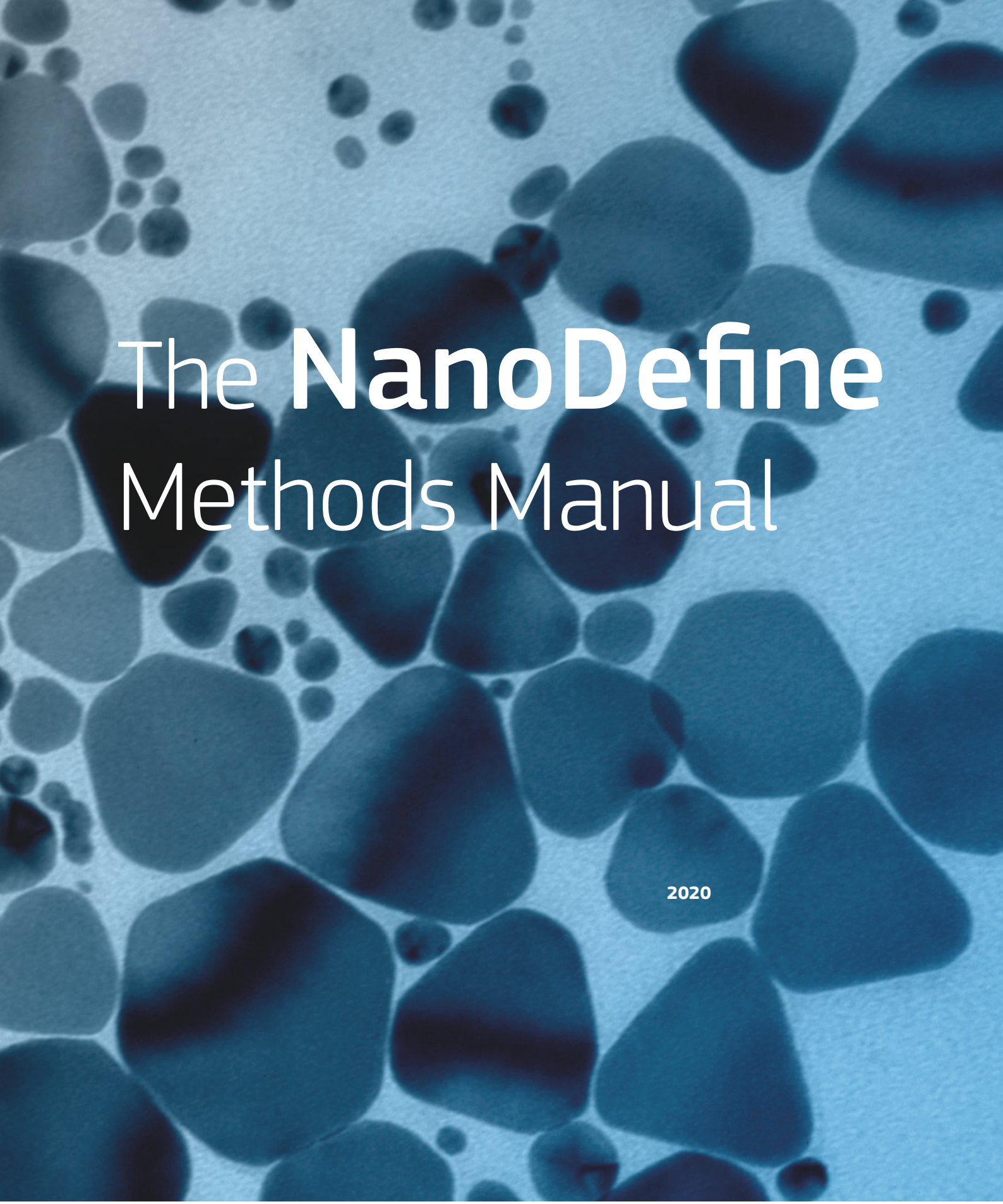

The NanoDefine Methods Manual

2020

European Commission

SEVENTH FRAMEWORK PROGRAMME

NanoDefine

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The NanoDefine Methods Manual

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Foreword

This document is **a collection of three JRC Technical Reports** that together form the "NanoDefine Methods Manual", which 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.

The overall goal of the NanoDefine project was to support the implementation of the European Commission Recommendation on the definition of nanomaterial (2011/696/EU). The project has developed an integrated empirical approach, which allows identifying a material as a nano- or not a nanomaterial according to the EC Recommendation.

The NanoDefine Methods Manual consists of three parts:

- **Part 1: The NanoDefiner Framework and Tools**, which 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: Evaluation of Methods**, which discusses the outcome of the evaluation of the nanomaterials characterisation methods for measuring size.
- **Part 3: Standard Operating Procedures (SOPs)**, which presents the 23 Standard Operating Procedures developed within the NanoDefine project

In this combined document, these three parts are included as **stand-alone reports**, each having its own abstract, table of contents, page, table and figure numbering, and references. Each of the reports **should be cited as follows**:

Part 1: Mech et al., *The NanoDefine Methods Manual. Part 1: The NanoDefiner Framework and Tools*, EUR 29876 EN, Publications Office of the European Union, Luxembourg, 2019, ISBN 978-92-76-11950-0, doi:10.2760/55181, JRC117501

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PART 1

European Commission

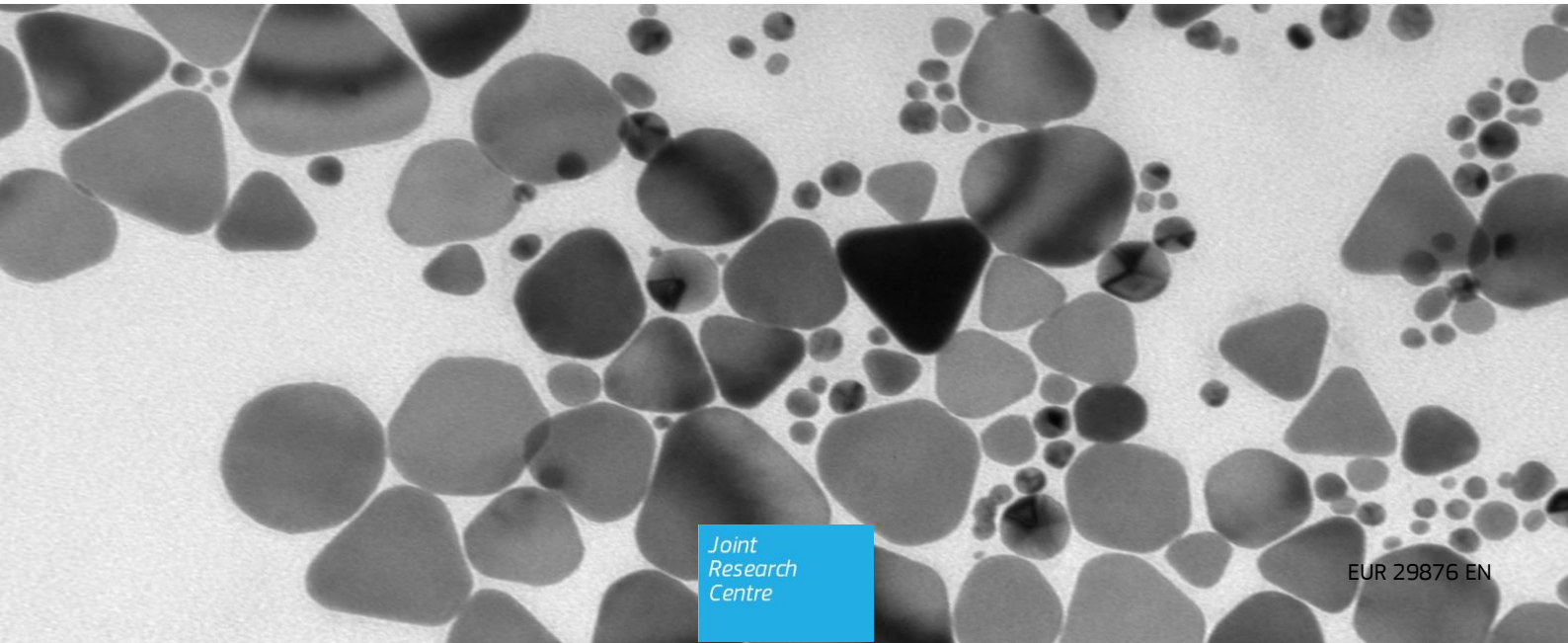
JRC TECHNICAL REPORTS

The NanoDefine Methods Manual

Part 1: The NanoDefiner Framework and Tools

Agnieszka Mech, Hubert Rauscher, Frank Babick, Vasile-Dan Hodoroaba, Antoine Ghanem, Wendel Wohlleben, Hans Marvin, Stefan Weigel, Raphael Brüngel, Christoph M. Friedrich

2019



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EUR 29876 EN

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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.

Disclaimer

Certain commercial equipment, instrument brand names and materials are identified in this document as examples or to specify adequately an experimental procedure. In no case does such identification imply recommendation or endorsement by the NanoDefine Consortium, nor does it imply that the material or equipment is necessarily the best available for the purpose and for the material under consideration.

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
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Website: http://www.nanodefine.eu/
Project co-ordinator: Wageningen Food Safety Research (WFSR), NL

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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 purposes[1]. 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	*Engineering System International* 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 nanomaterial[1], 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 m^2/cm^3. 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 m^2/cm^3.

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.

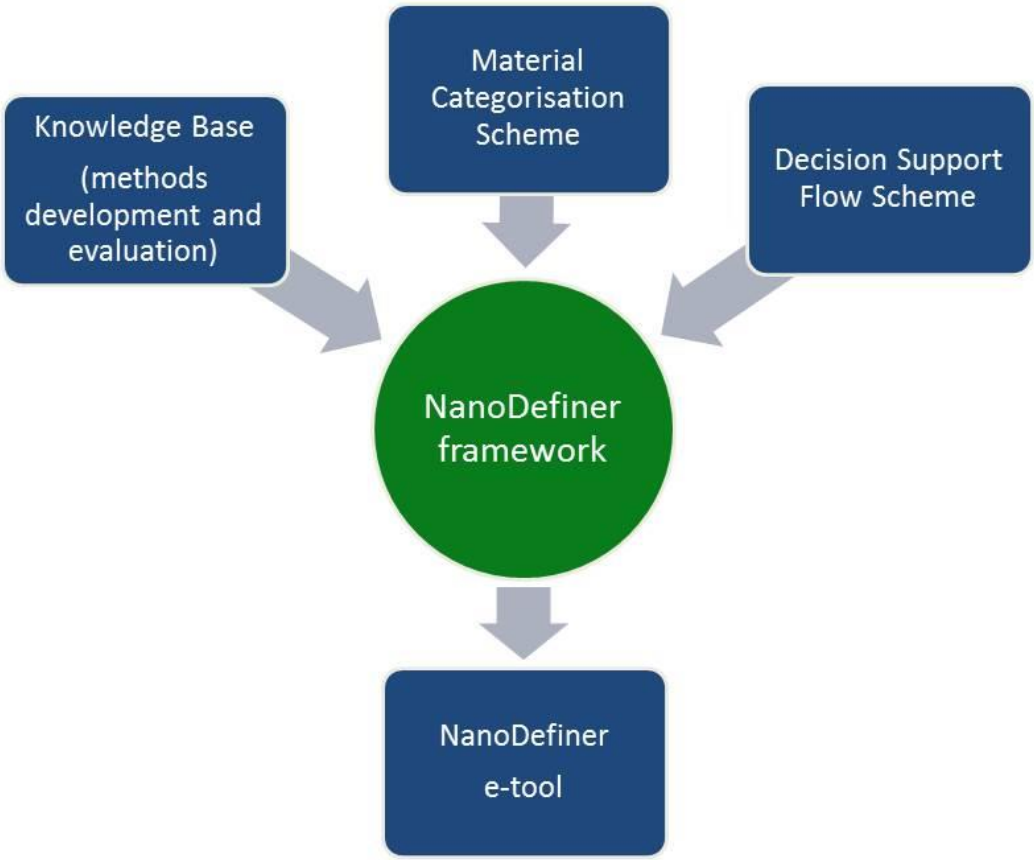

Figure 1: NanoDefiner Framework. Pillars and implementation in the e-tool.

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 interchangeable.

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 Scheme**[2] (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.

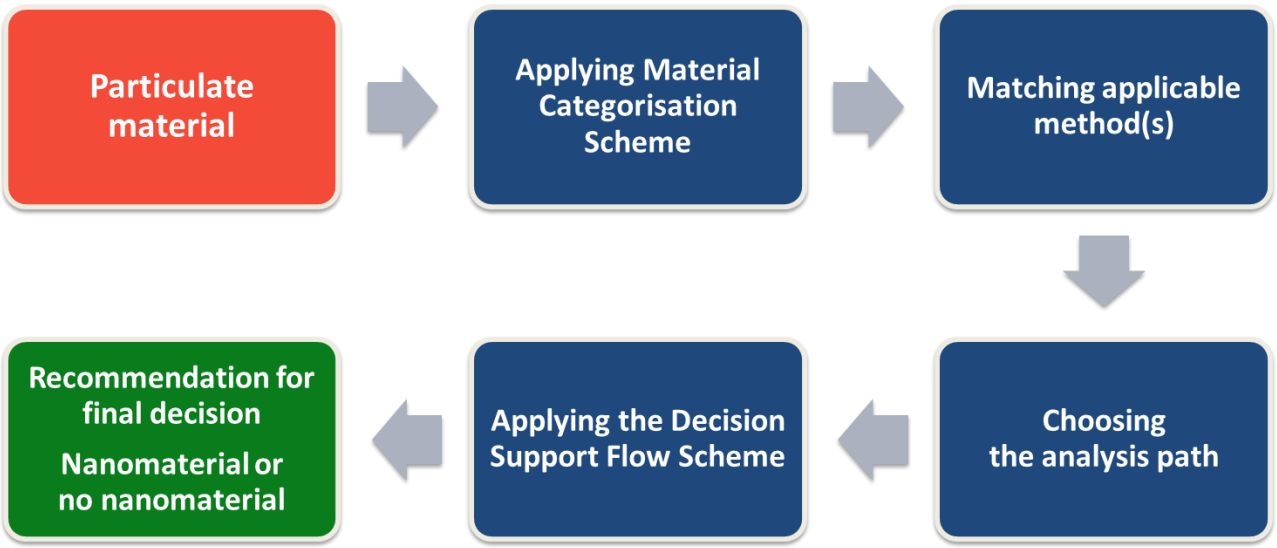

Figure 2: Logical sequence within the NanoDefiner Framework

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						
		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 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						
		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 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						(+ EDX)	
		TSEM						(+EDX)	
		TEM						(+EDX)	
	SFM/AFM								
	PTA								
	TRPS								
	spICP-MS								
Ensemble	DLS								
	SAXS								
	USSP								
	ALS								
Fractionating	FFF							(+Detector)	
	AC								
	DEMA								
Integral	BET								

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 materials[2]. 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 'nanoform' (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. SiO_2) or (ii) different elements or compounds, but with the same internal structure. In the latter case, the particles are composite[4] 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.

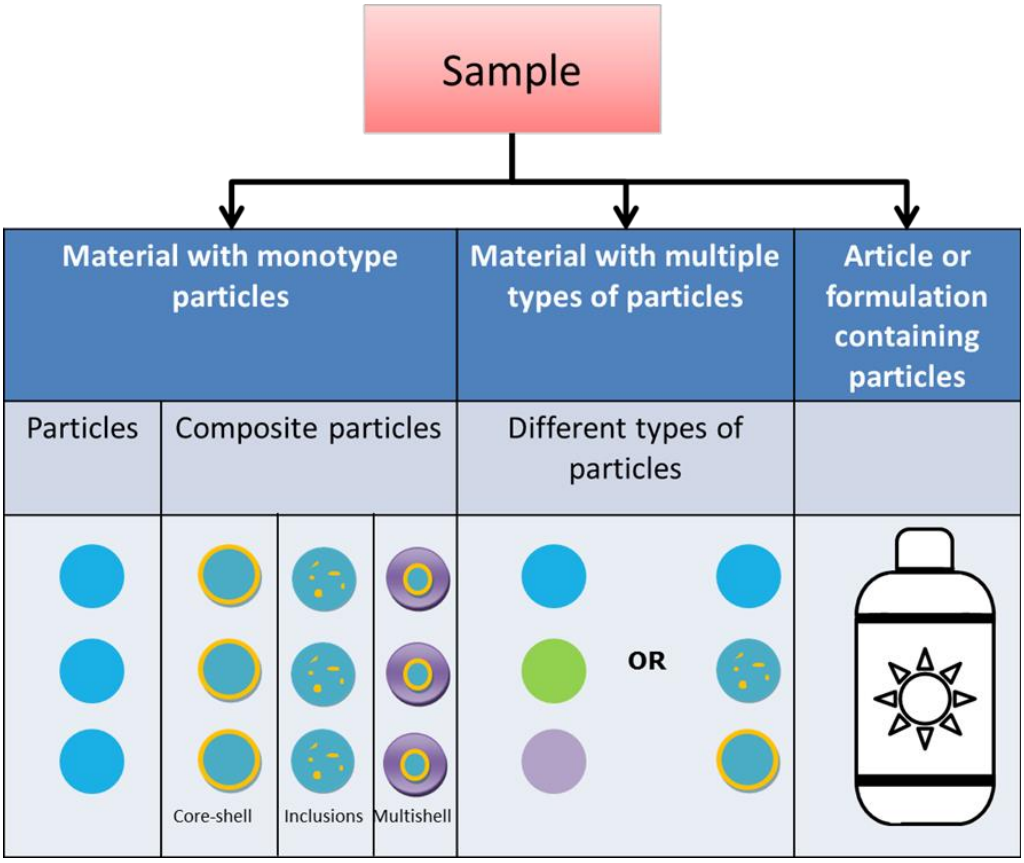

Figure 3: Different types of particles and particulate materials are considered in the categorisation scheme. 'Sample' is the generic term for the material to be analysed (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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.

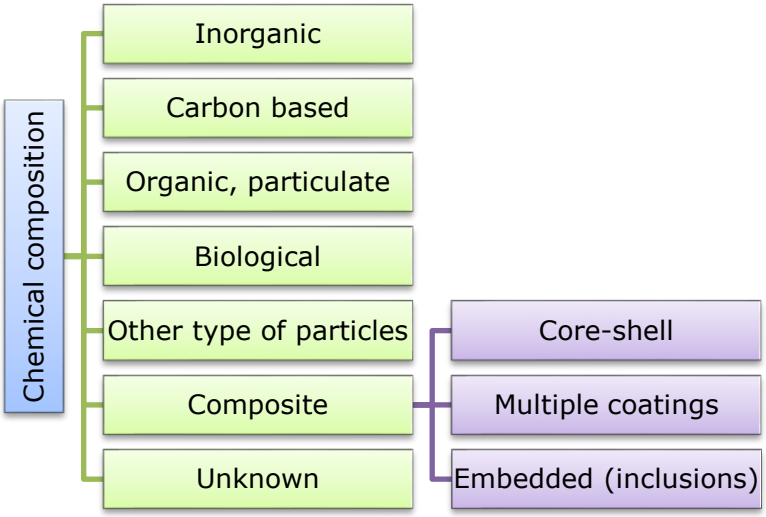

Figure 4: Chemical composition (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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.

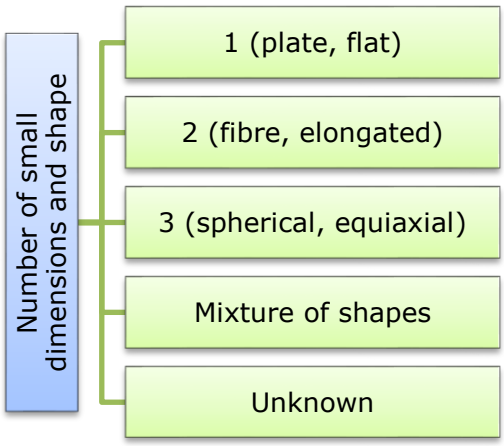

Figure 5: Number of small dimensions and shape (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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.

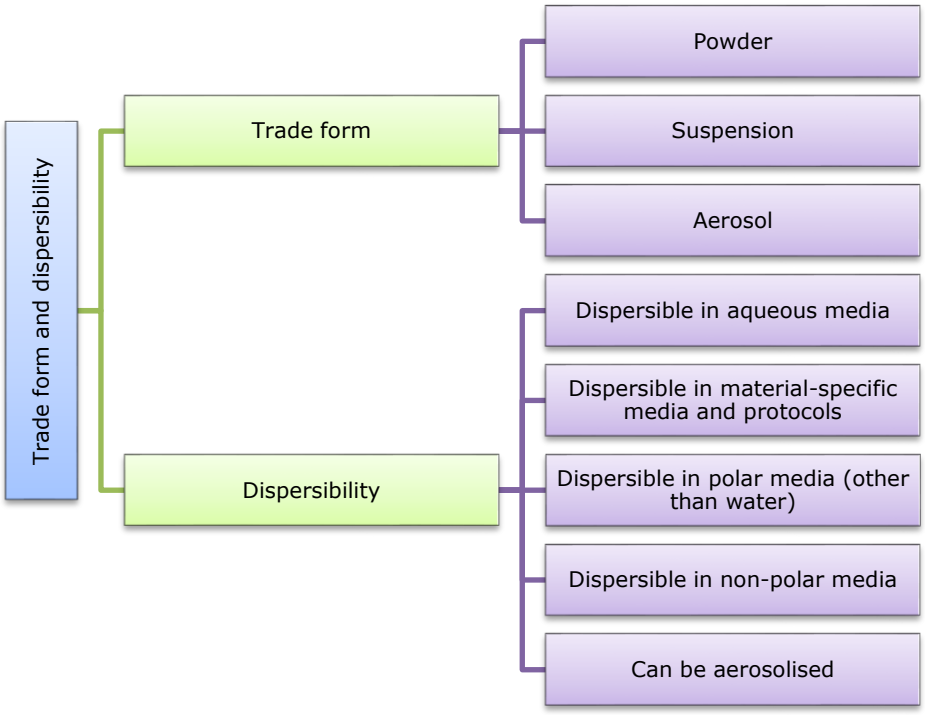

Figure 6: Trade form and dispersibility. (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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.

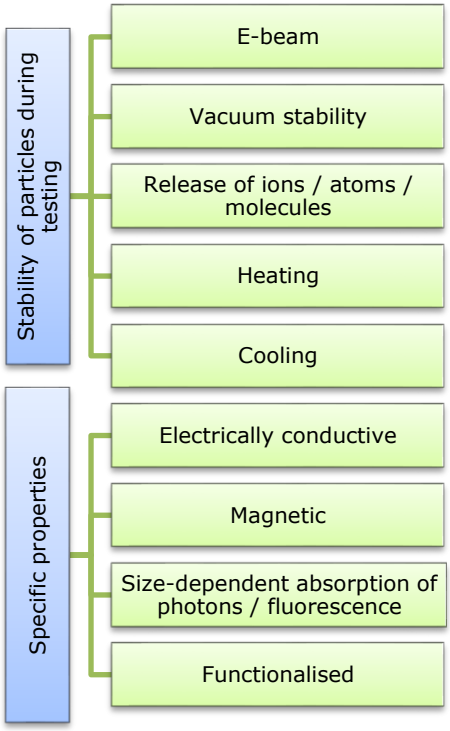

Figure 7: Stability of particles during the testing and specific properties (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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.

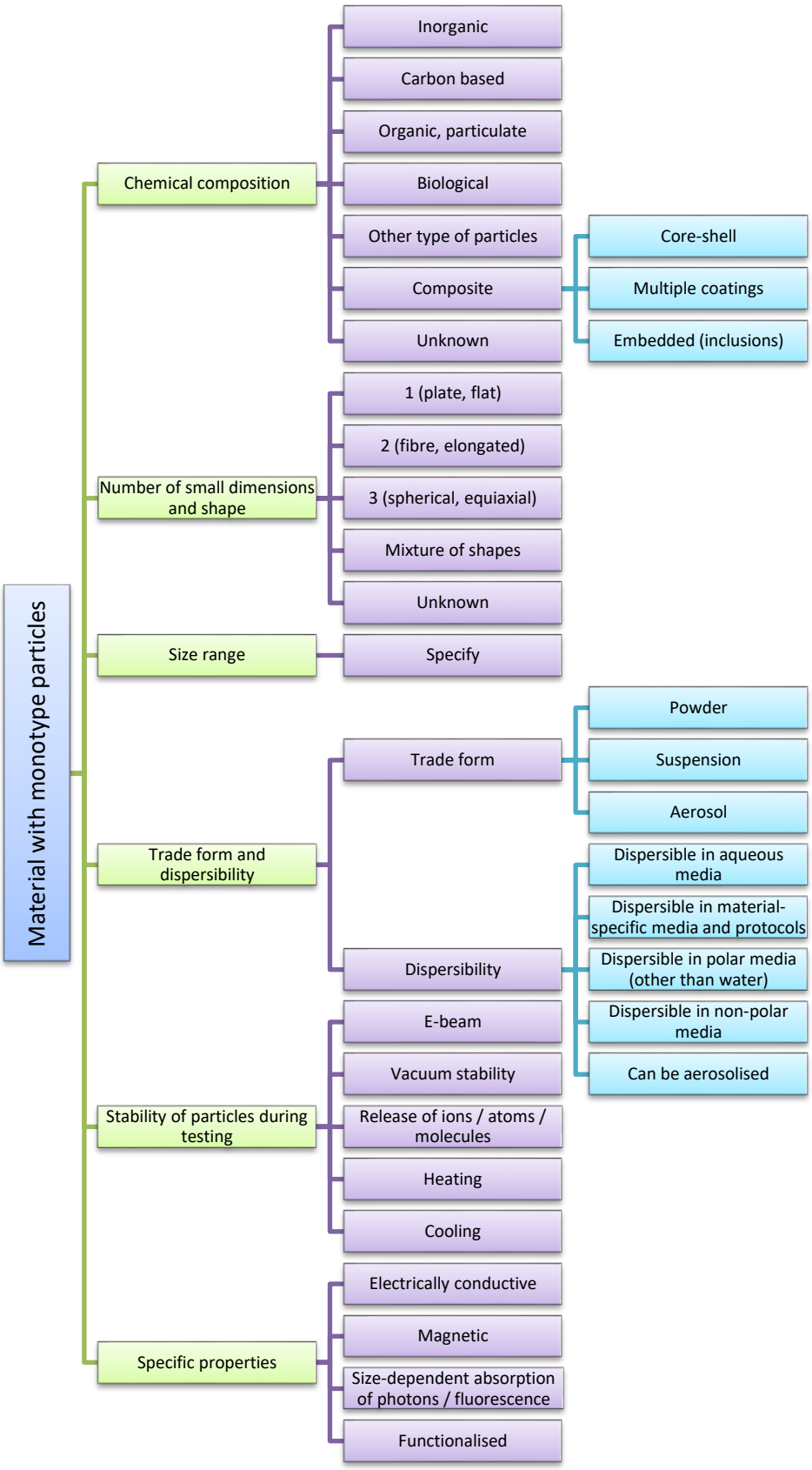

Figure 8: Material Categorisation Scheme (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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 MCS[8] within the NanoDefine[9] 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.

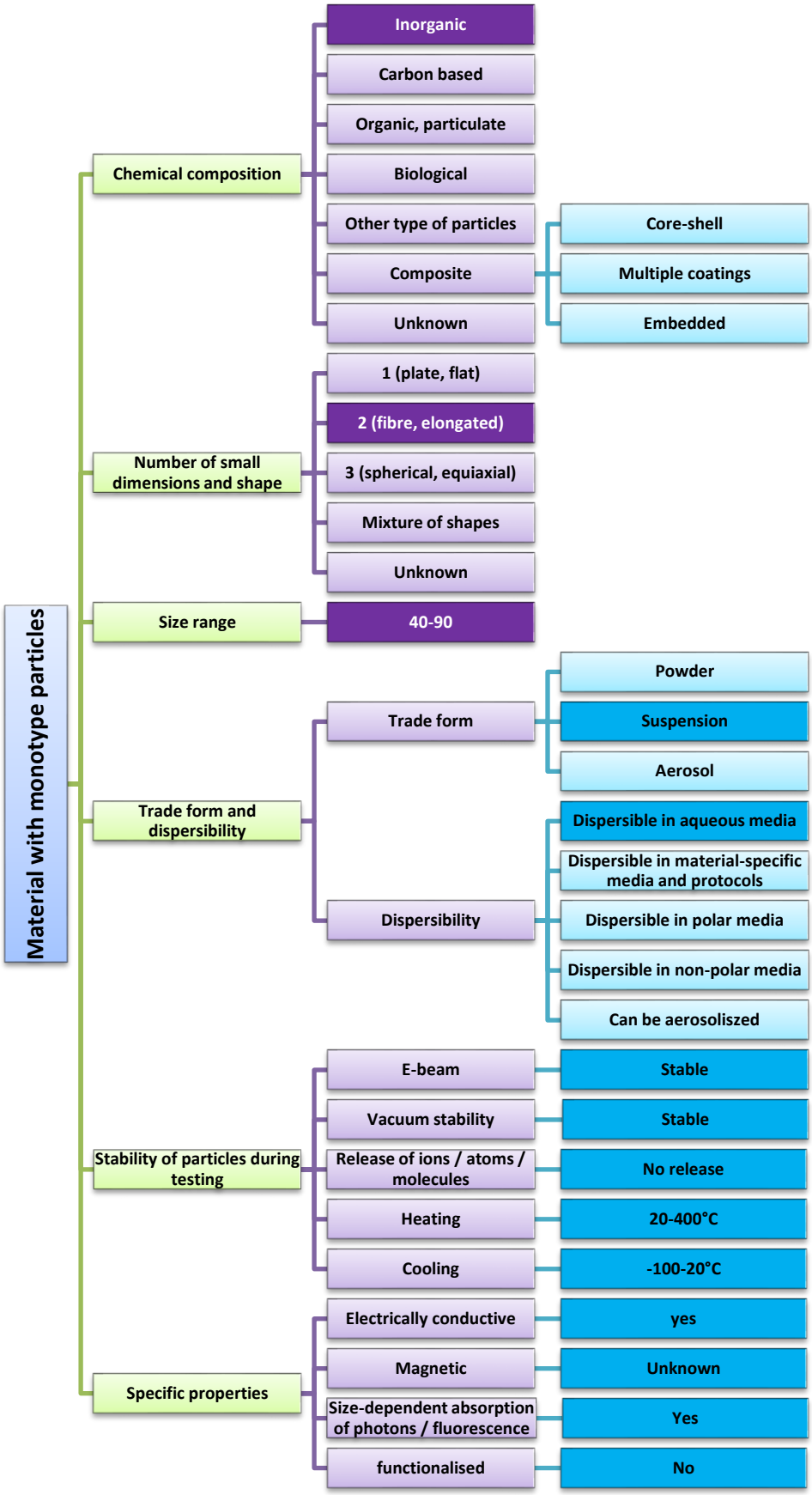

Figure 9: The material categorisation scheme applied to gold rods (Reproduced from Ref. [2] with permission from the Royal Society of Chemistry).

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
	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

[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 mm	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	X[d]	X[d]		X	X	X	X	X	X	X	X	X	X	X	X[d]
	Polar	X[d]	X[d]		X	X	X	X		X	X	X	X	X		X[d]
	Non polar															
	Specific media	X[d]	X[d]			X	X					X	X	X	X	X[d]
	Can be aero-solised												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[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 manual[3]). 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

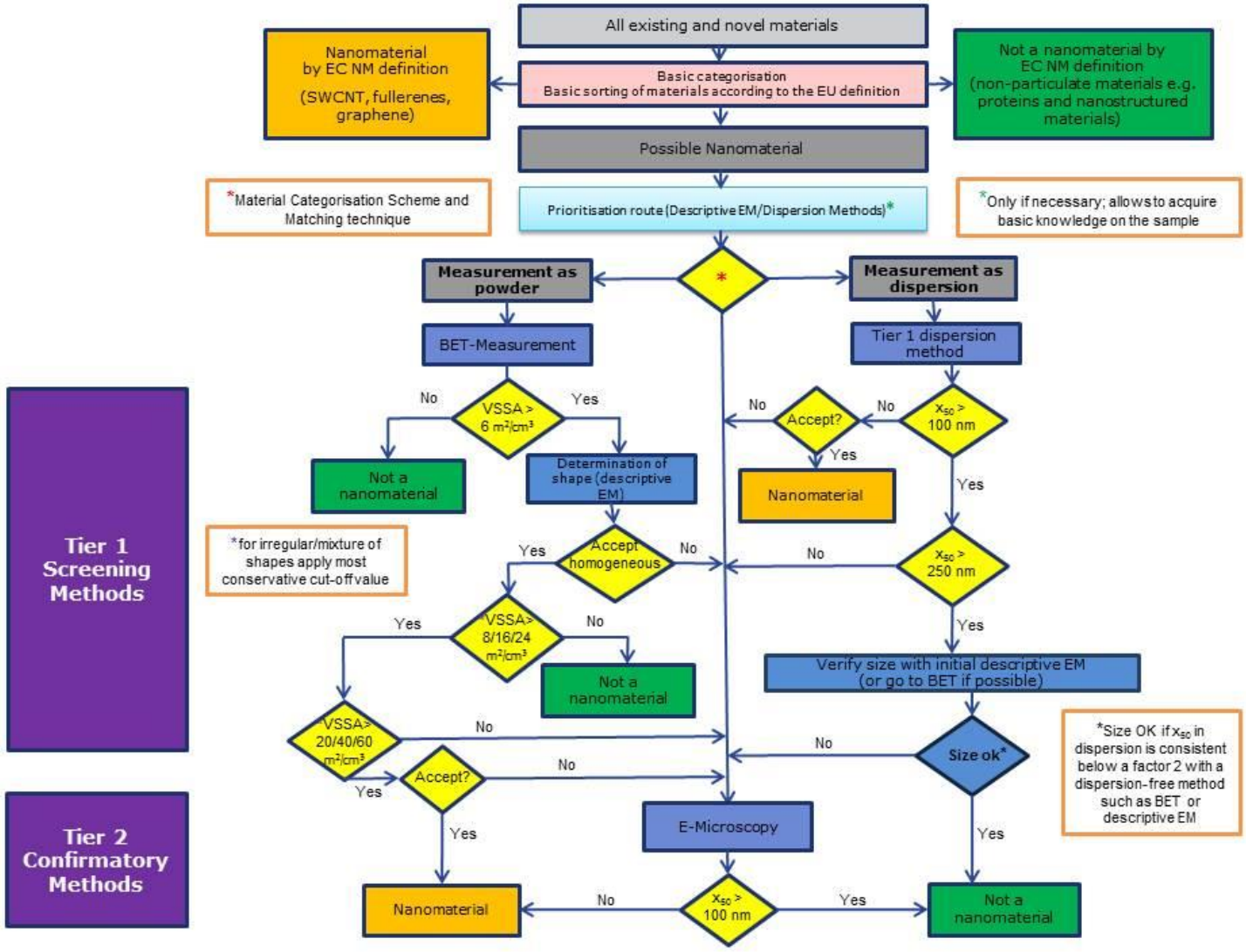

Figure 10: Decision support flow scheme for identification of nanomaterials. Units have been omitted for clarity. x_{50} designates the number based median size of particles.

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 paper[11] 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.

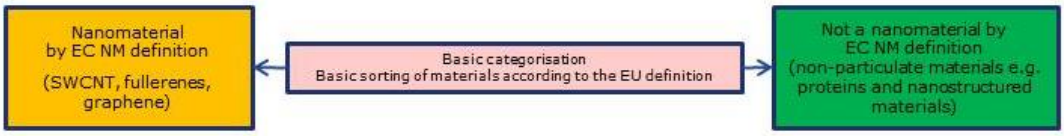

Figure 11: Basic categorisation of nanomaterials

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 discussed[2] ('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 website[9].

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 materials[14].

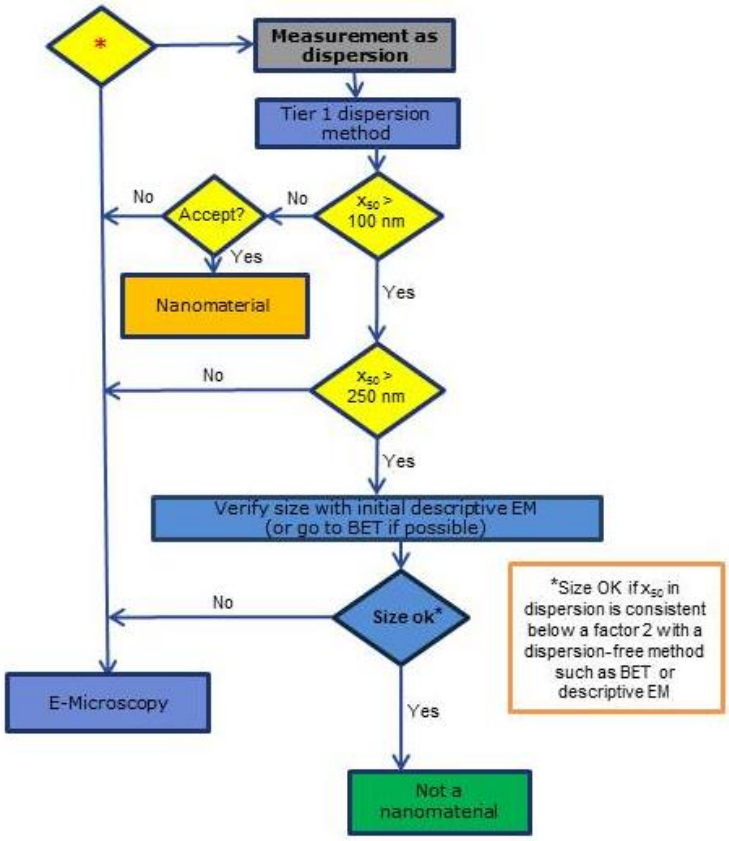

Figure 12: Tier 1 dispersion route flow scheme.

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 materials[10] 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 2.5[10] 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 analysis[10]. 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 analysis[17] by ρ (skeletal density[18]) 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):

$$dmin_{VSSA}(D) = \frac{2D}{VSSA} \quad (1)$$

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

A detailed description of the quantity $dmin_{VSSA}$ 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.

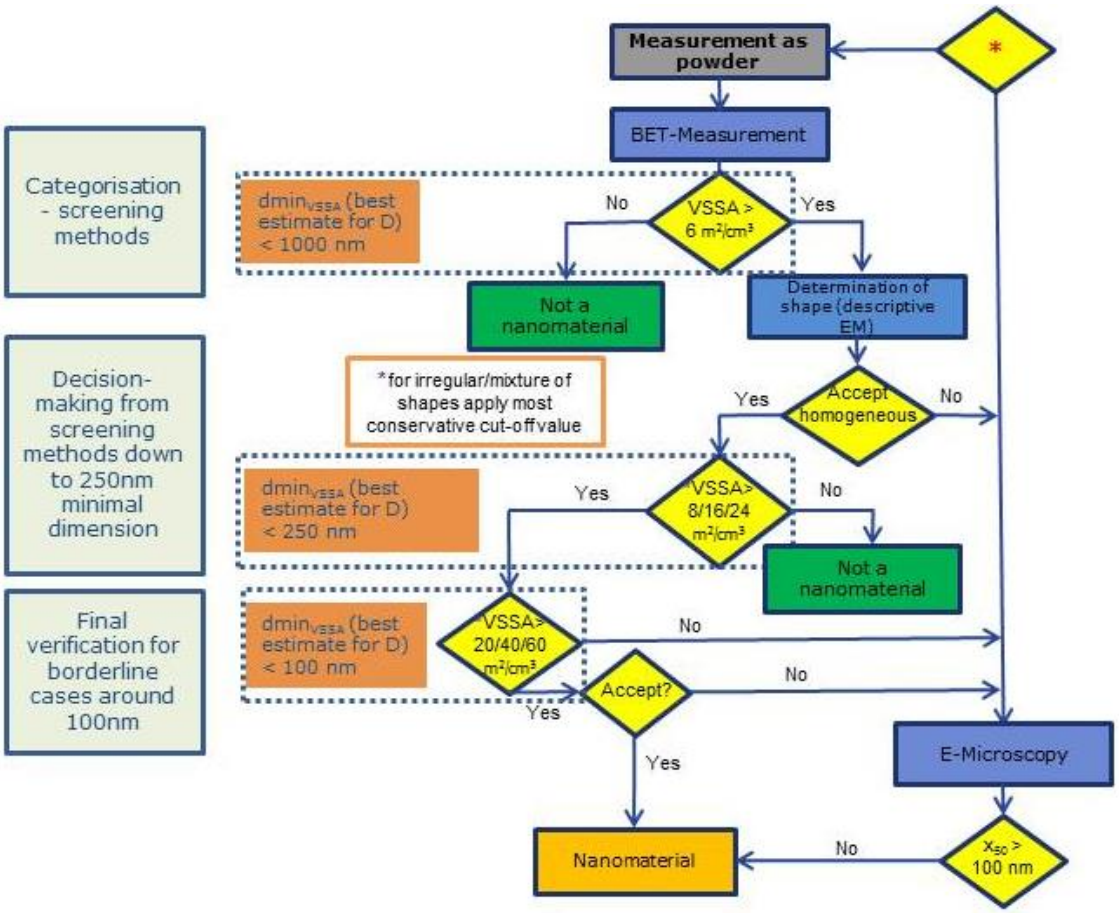

Figure 13: Tier 1 powder route decision tree involving BET measurements for nanomaterial identification. Homogenous: same shape, same composition of the particles

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 m^2/cm^3 (equivalent to a d_{VSSA} =1000 nm for spherical particles) the material likely is not a nanomaterial.

The 6 m^2/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 m^2/cm^3. The maximum deviation between d_{VSSA} and $d_{minVSSA}$ due to the unknown particle shape is a factor of 3 (60 m^2/cm^3 vs. 20 m^2/cm^3). Furthermore, a mismatch between $dmin_{VSSA}$ 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 × 100 nm = 1000 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 m^2/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 $dmin_{VSSA}$ is 250 nm. As previously explained 250 nm originates from the maximum mismatch between $dmin_{VSSA}$ 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 m^2/cm^3
- Elongated, fibre-like (aspect ratio >3:1:1, D=2) the threshold for VSSA = 16 m^2/cm^3
- Flat, platelet (aspect ratio >3:3:1, D=1) the threshold for VSSA = 8 m^2/cm^3

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

- VSSA < 24 m^2/cm^3 for spherical particles
- VSSA < 16 m^2/cm^3 for elongated, fibre-like particles
- VSSA < 8 m^2/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 m^2/cm^3 for spherical particle (aspect ratio <3:1) D=3
- VSSA > 40 m^2/cm^3 for elongated, fibre-like particles (aspect ratio >3:1:1) D=2
- VSSA > 20 m^2/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 < VSSA < 60 m^2/cm^3 or
- Elongated, fibre-like particles (aspect ratio >3:1:1), D=2, and 16 < VSSA < 40 m^2/cm^3 or

- Flat, platelet-like particles (aspect ratio >3:3:1), D=1, and 8 < VSSA < 20 m^2/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.

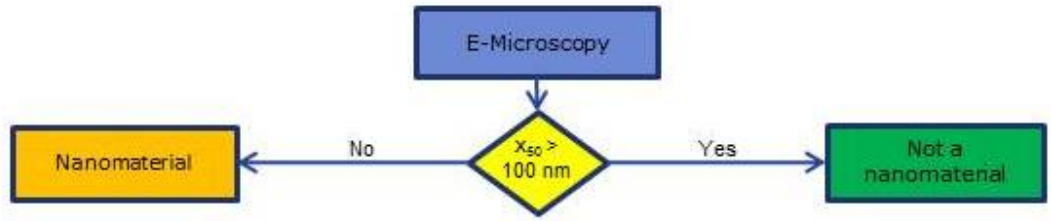

Figure 14: Tier 2 classification

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 project[20], 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 scheme[2], the method (or technique) performance tables[3], the results obtained on the NanoDefine training set materials[10] and results regarding the use of VSSA measurements[15]. 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 scheme[23]. 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 ParticleSizer[21] or Single Particle Calculator tool[i] (spICP-MS)) is fed into the e-tool. The scheme of the logical workflow of the e-tool is presented in Figure 16.

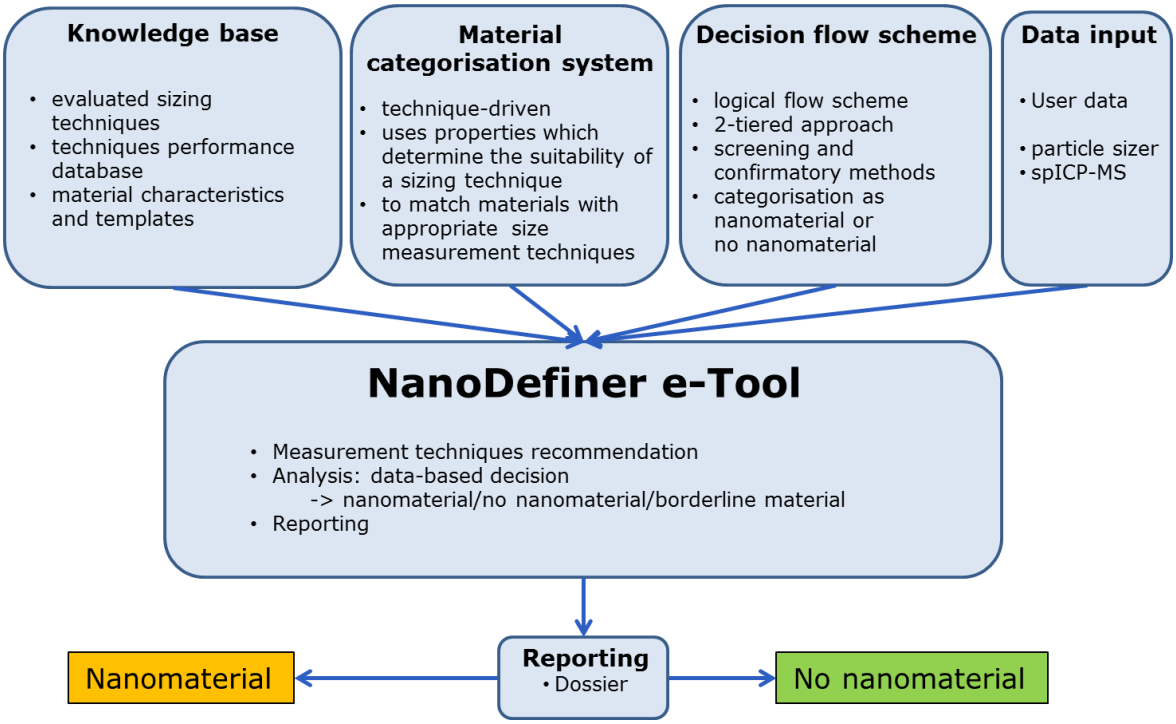

Figure 15: Implementation of the NanoDefiner framework in the NanoDefiner e-tool

[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.

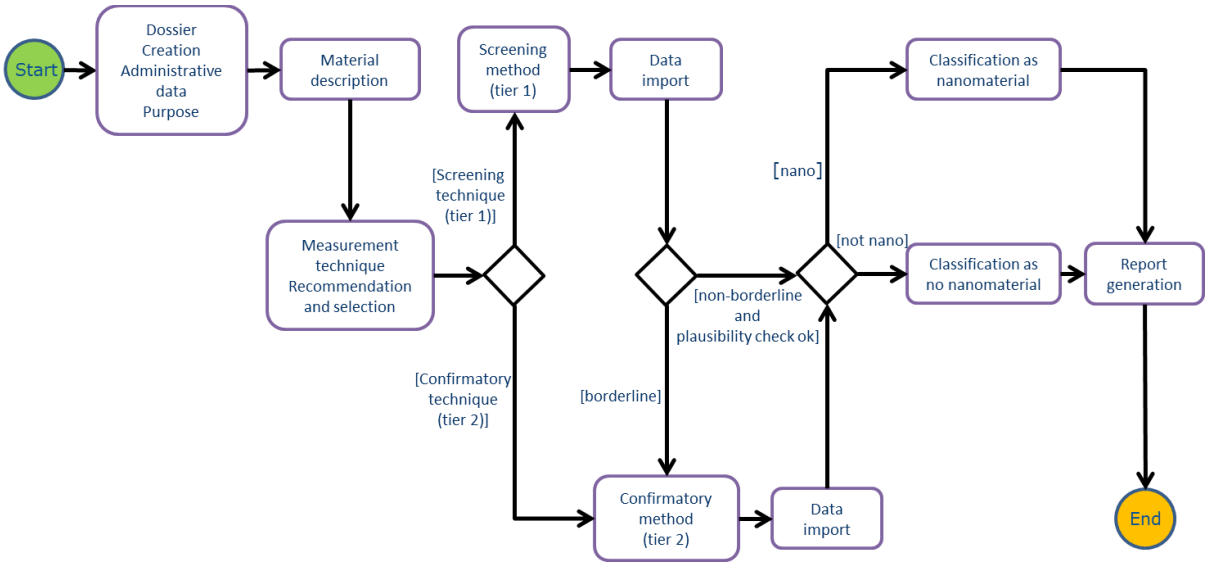

Figure 16: Workflow within the NanoDefiner e-tool (adapted from [23])

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 <u>does</u> 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? **<u>It does not</u>**

- 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 homogenously 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.

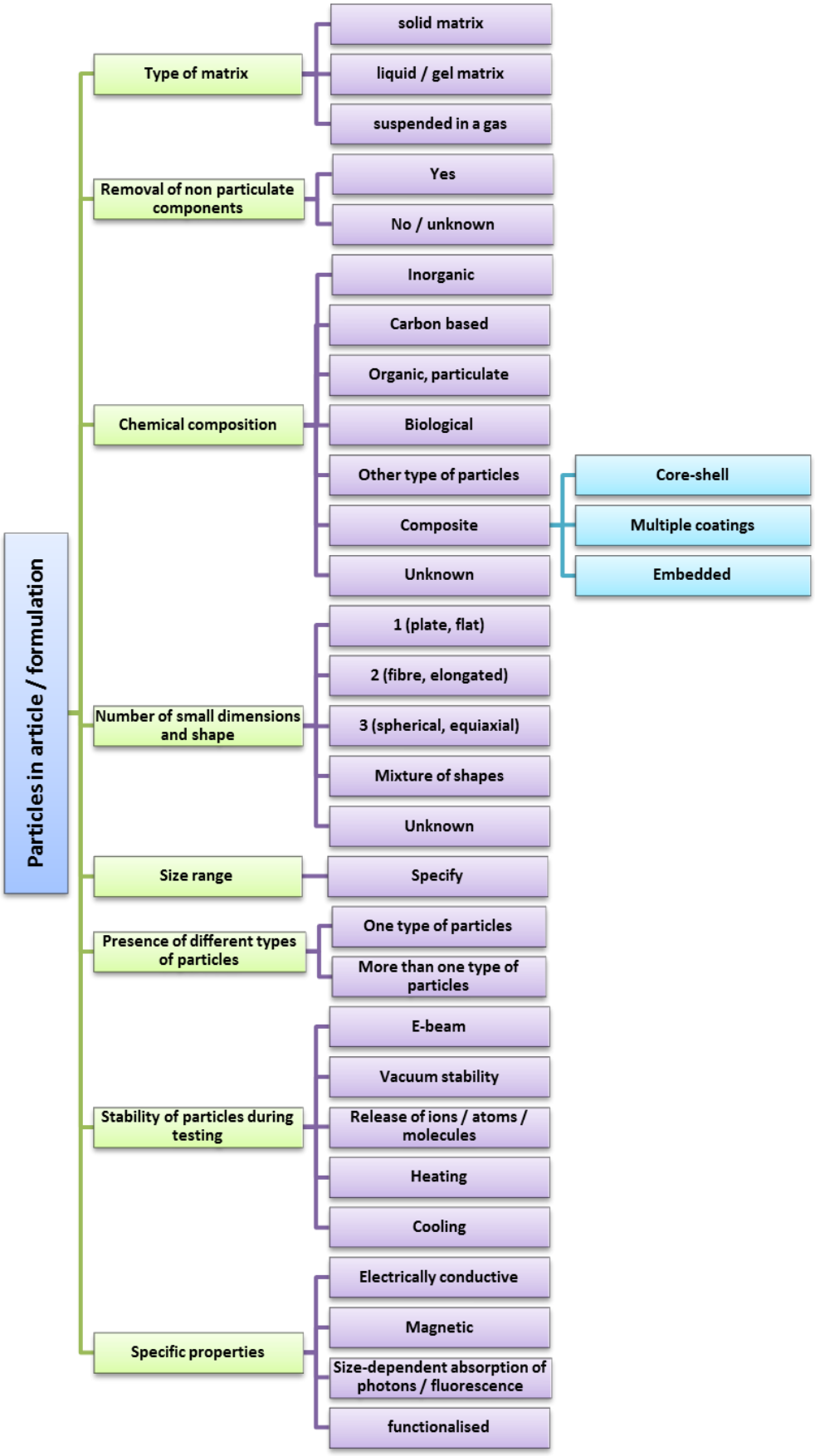

Figure 17: The Material Categorisation Scheme for an article / formulation that contains particles

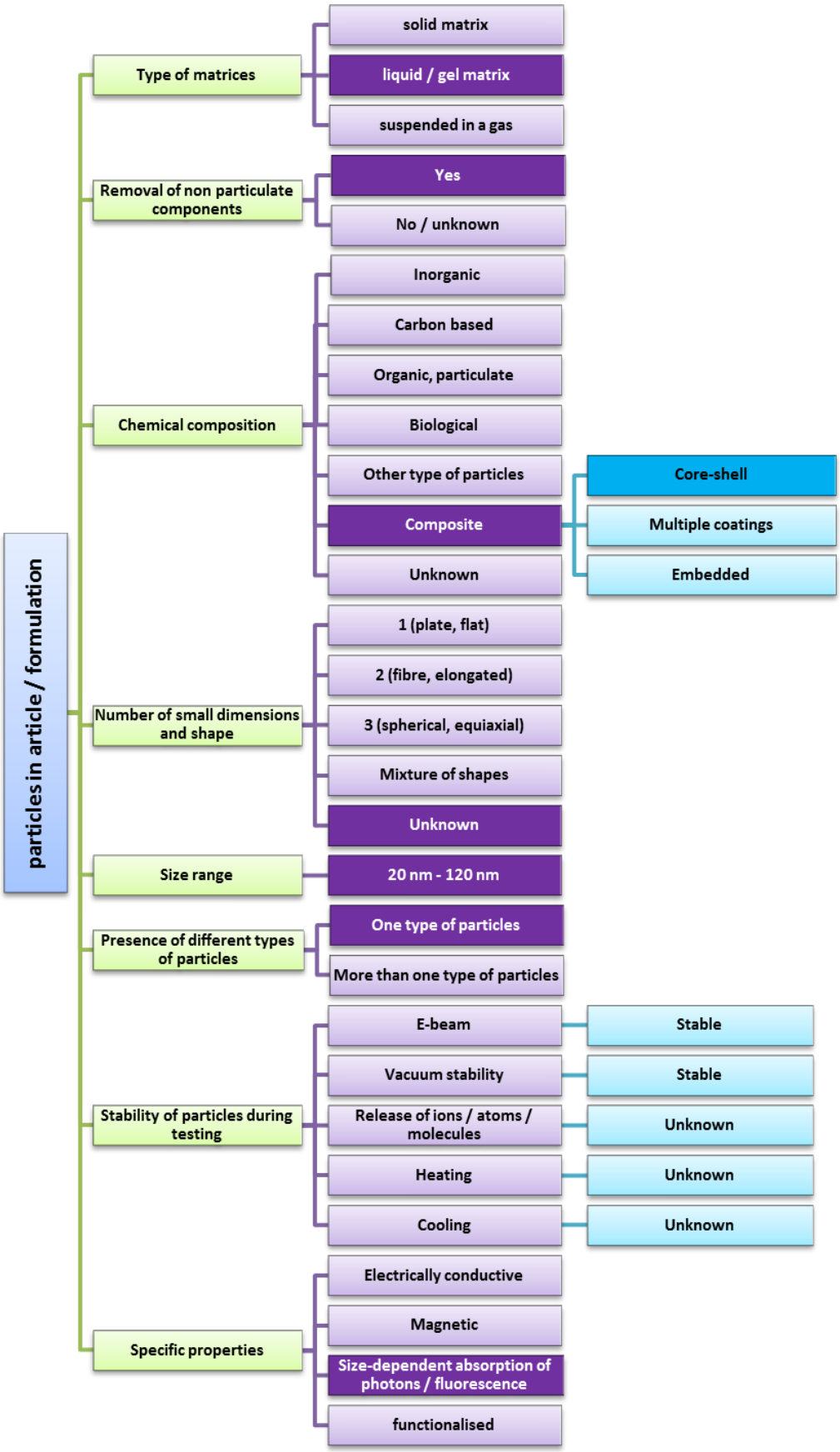

Figure 18: The Material Categorisation Scheme applied to coated titanium dioxide particles in a sunscreen formulation

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 dimensions[10]. 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).

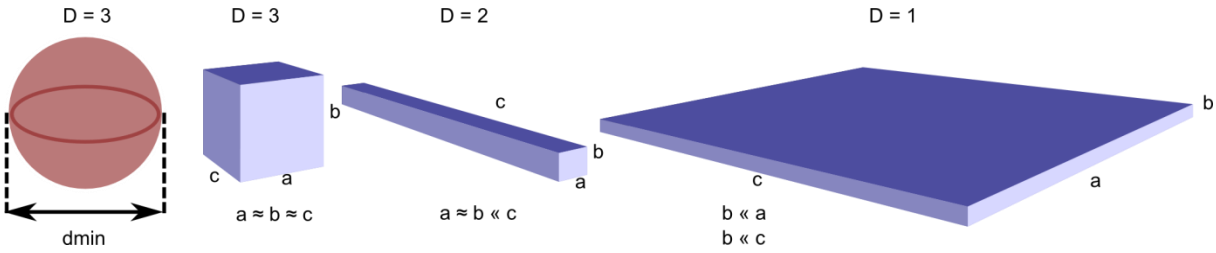

Figure 19: Scheme of prototypical particle shapes, having three, two and one small dimension

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:

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

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 $c \to \infty$, the VSSA becomes

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

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

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

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

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

Solving this expression for *dmin* gives:

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

(when entering *VSSA* in units of m^2/cm^3, $dmin_{VSSA}$ 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:

$$VSSA = \frac{2}{dmin} + \frac{2}{3dmin} + \frac{2}{3dmin} = \frac{5}{9}\frac{6}{dmin} \approx 0.56\frac{6}{dmin}$$

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

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:

$$VSSA = \frac{2}{dmin} + \frac{2}{dmin} + \frac{2}{3dmin} = \frac{7}{6}\frac{4}{dmin} \approx 1.167\frac{4}{dmin}$$

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

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 $3\times dmin$:

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

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

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:

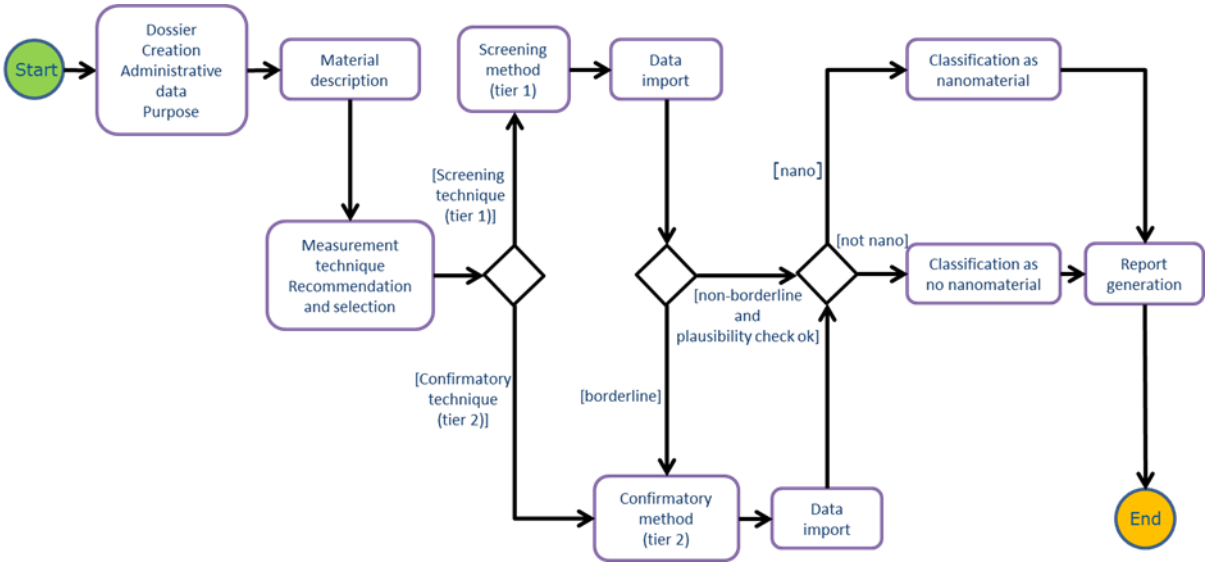

Figure 20: Workflow within the e-tool

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.

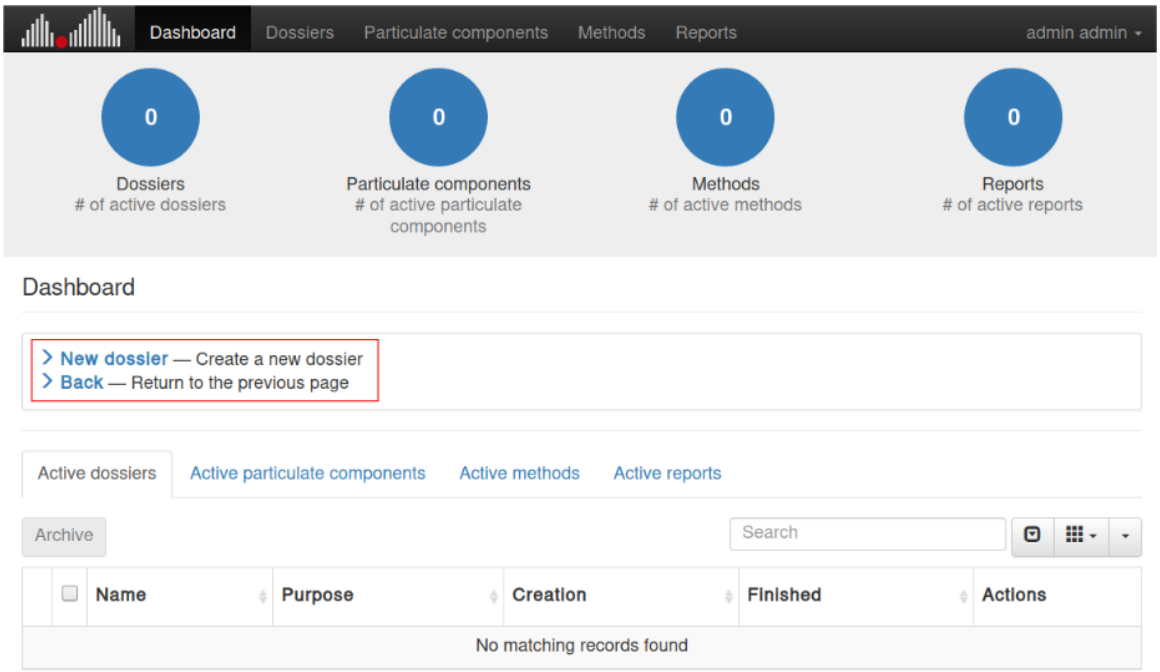

Figure 21: Initial dashboard view with highlighted action box

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.

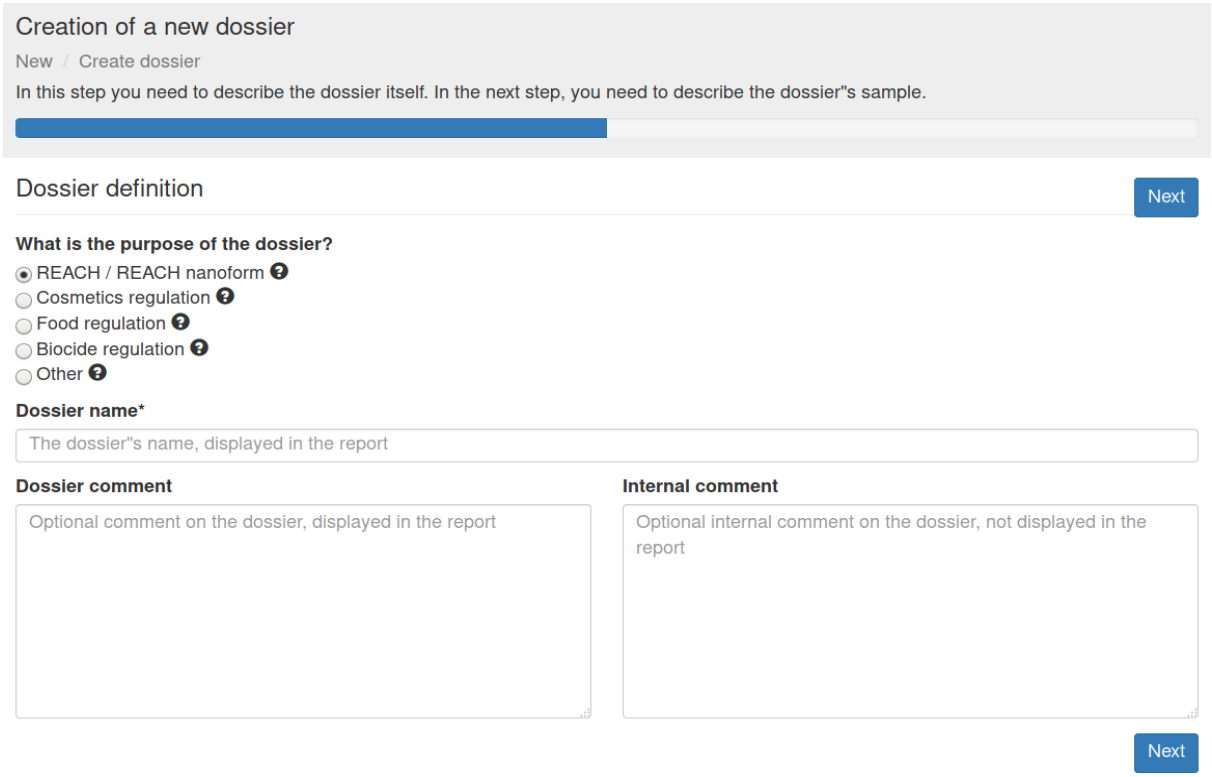

Figure 22: First page of the dossier creation form

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.

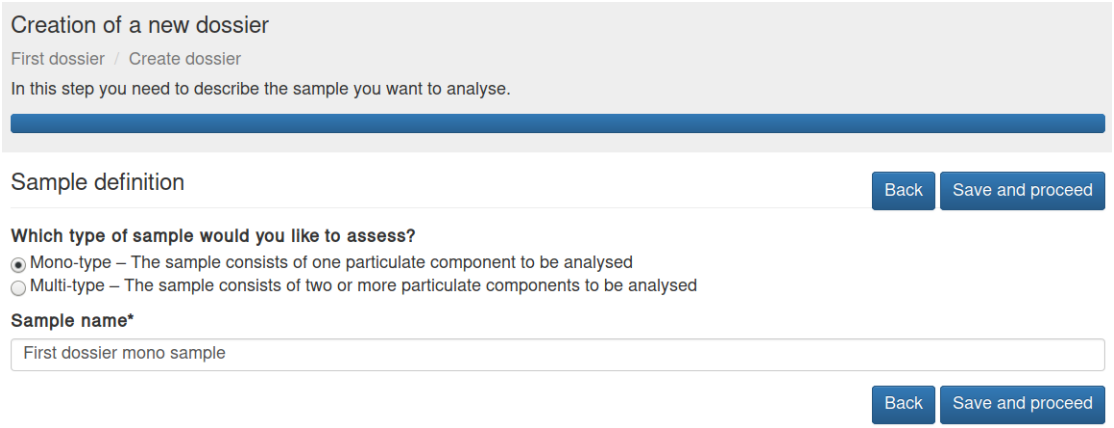

Figure 23: Second page of the dossier creation form

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.

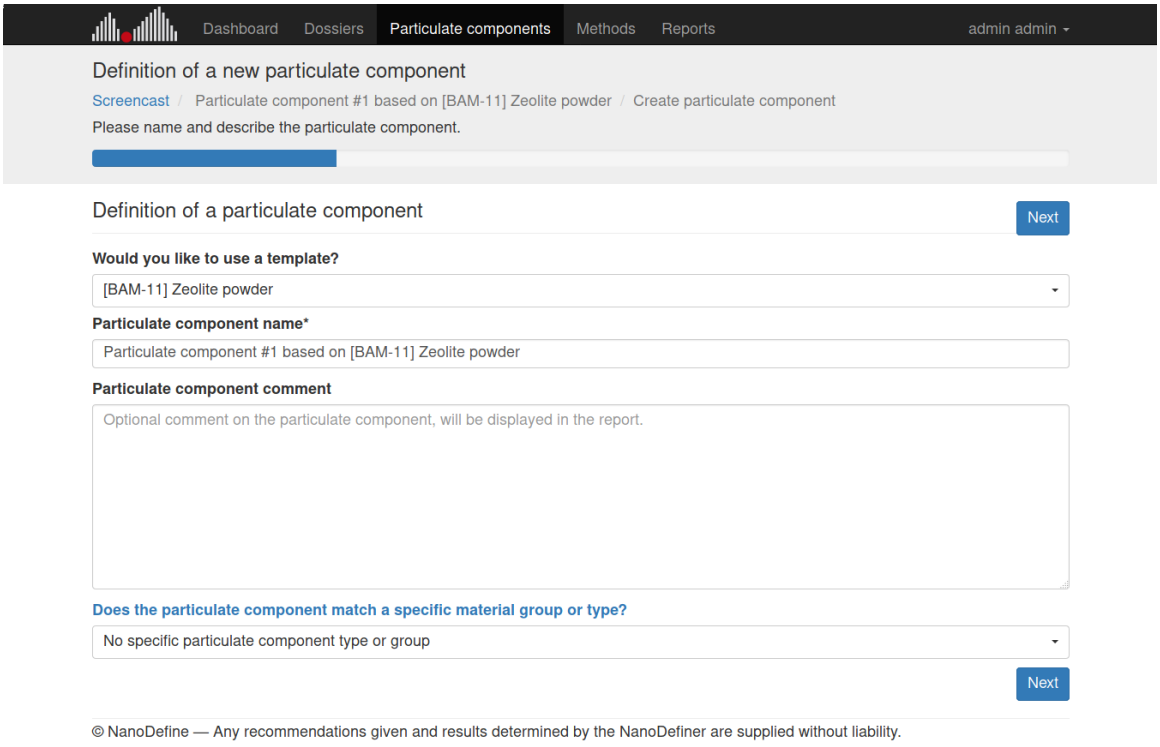

Figure 24: First page of the material description form

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).

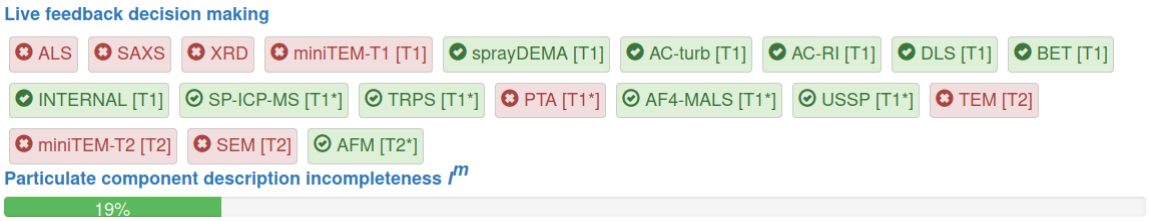

Figure 25: Live feedback and PC description incompleteness for the initial PC description form where all properties are unknown

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.

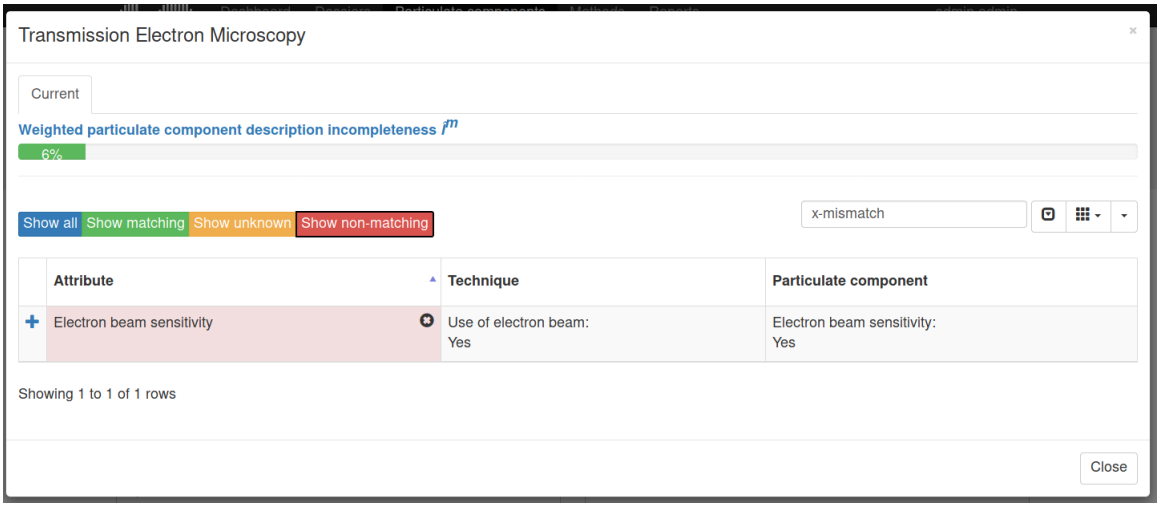

Figure 26: Method-specific suitability information

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).

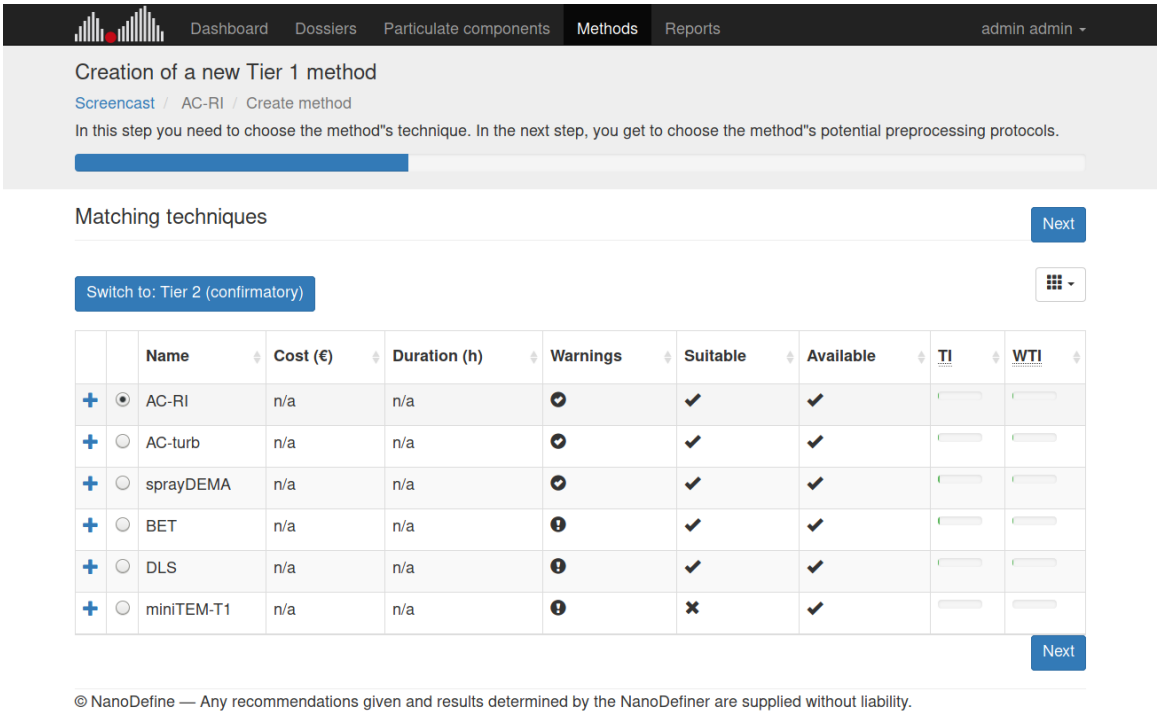

Figure 27: First page of the method selection form, where recommended measurement techniques of tier 1 and 2 can be selected

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

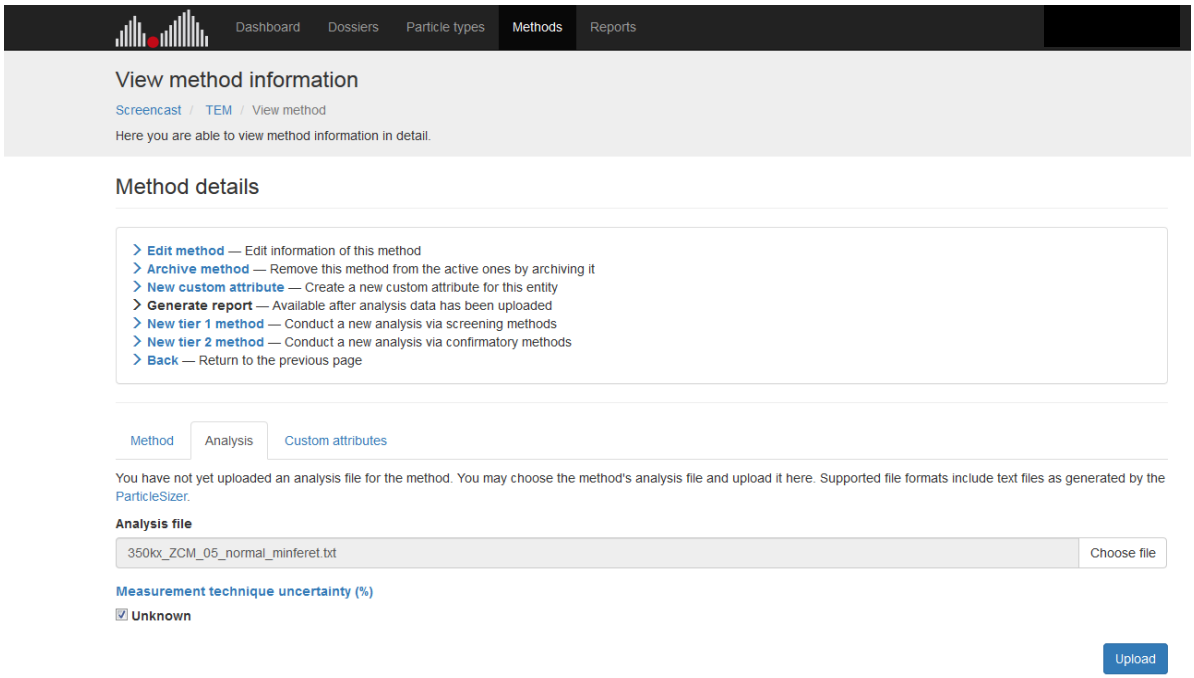

Figure 28: Page where method results can be uploaded, for the Transmission Electron Microscopy, Tier 2 settings (TEM-T2) MT in this example

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)

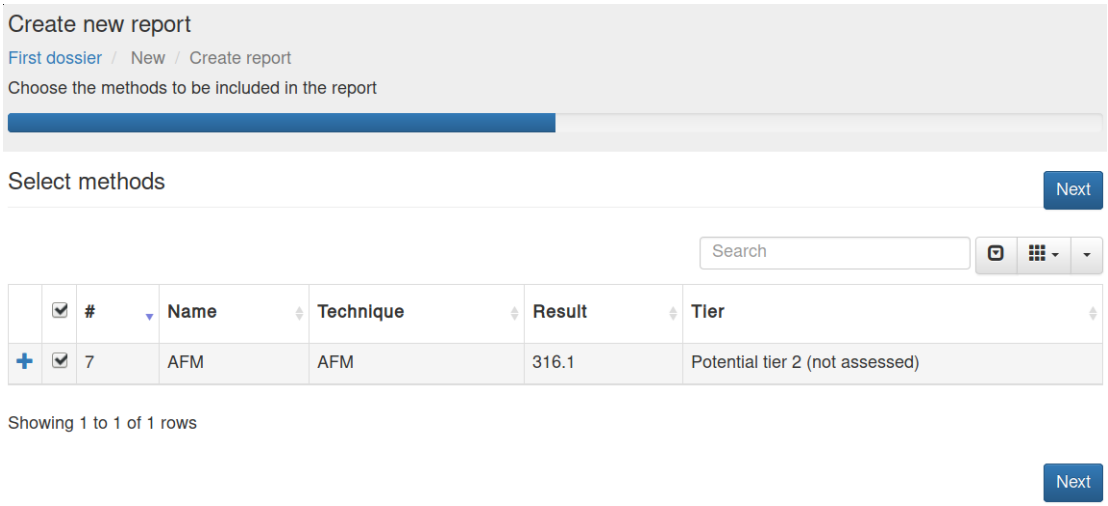

Figure 29: First page of the report generation form. Here you can select which methods to include

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.

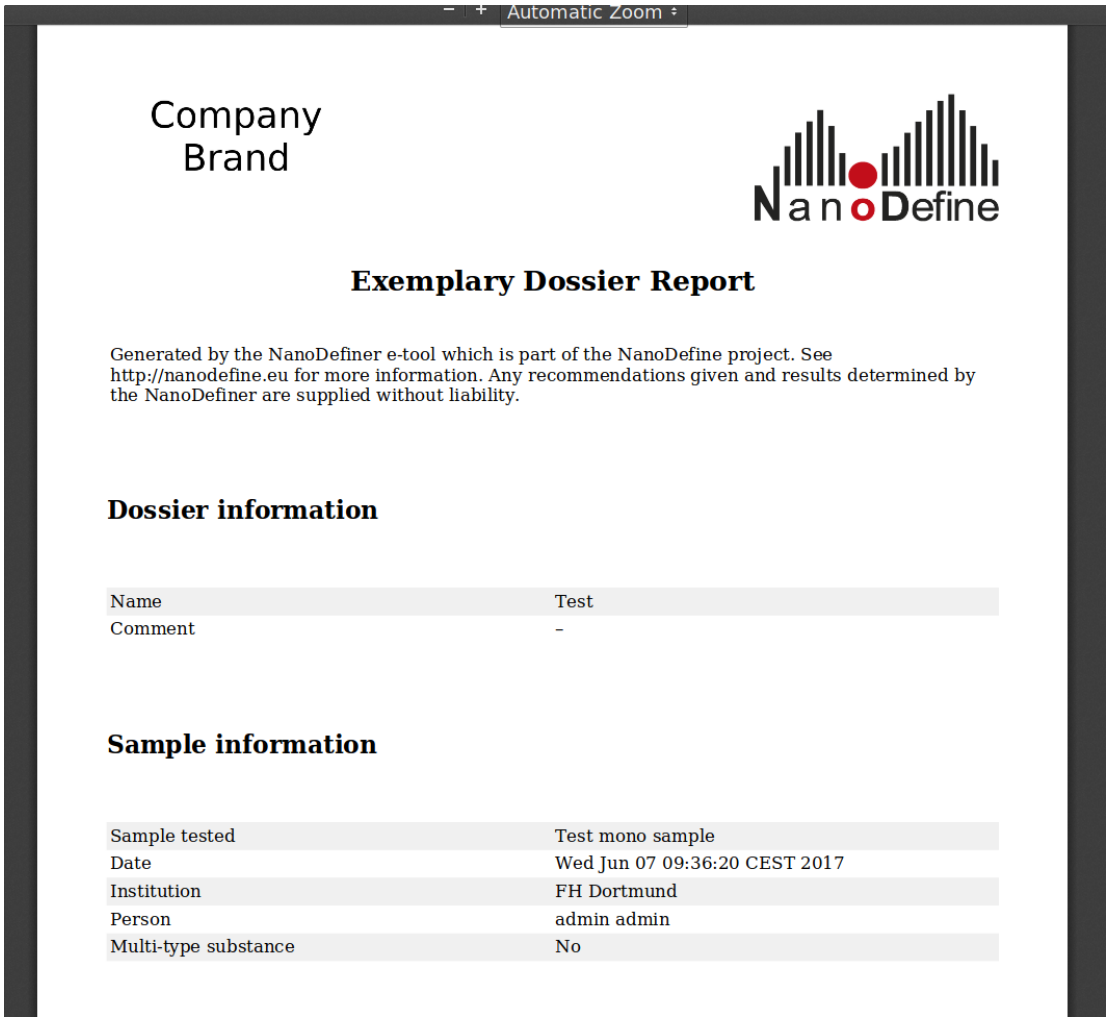

Figure 30: First page of a generated report

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.

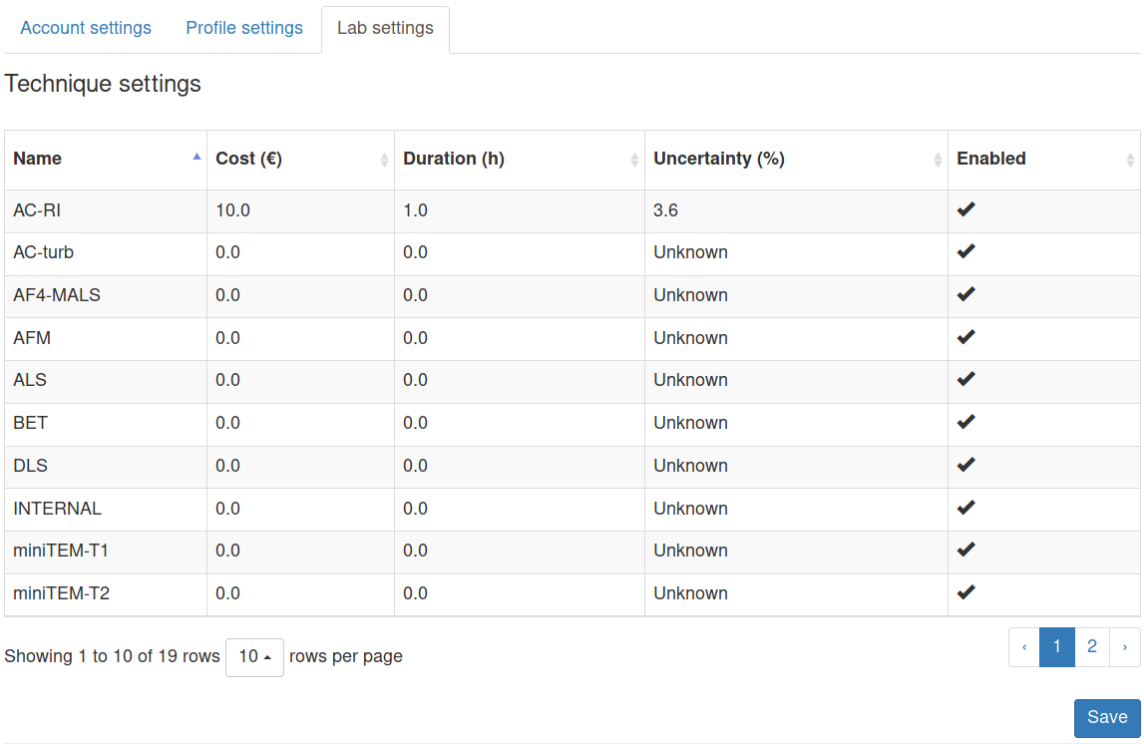

Figure 31: First page of the default lab settings with an example configuration added for AC-RI

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 Report[39].

- 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-tool[40]. 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.

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of **biocidal products**. OJ L 167, p. 1-123. 2012.

Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on **medical devices**, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC. OJ L 117, P.1-175. 2017.

Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on **Cosmetic Products**, O. J., L342, 59. 2009.

Regulation (EU) No 2015/2283 of the European Parliament and of the Council on **novel foods**, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L327, p. 1-22. 2015.

Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of **food information to consumers**. OJ L 304, p. 18-63. 2011.

EU, 2011b. Commission Regulation (EU) No 10/2011 of 14 January 2011 on **plastic materials and articles intended to come into contact with food**. OJ L328, p. 20-29. 2011.

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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European Commission

JRC TECHNICAL REPORTS

The NanoDefine Methods Manual

Part 2: Evaluation of methods

Agnieszka Mech, Hubert Rauscher, Kirsten Rasmussen, Frank Babick, Vasile-Dan Hodoroaba, Antoine Ghanem, Wendel Wohlleben, Hans Marvin, Raphael Brüngel, Christoph M. Friedrich

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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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Certain commercial equipment, instrument brand names and materials are identified in this document as examples or to specify adequately an experimental procedure. In no case does such identification imply recommendation or endorsement by the NanoDefine Consortium, nor does it imply that the material or equipment is necessarily the best available for the purpose and for the material under consideration.

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 2: Evaluation of methods

The research leading to these results has received funding from the
European Community's Seventh Framework Programme (FP7/2007-2013)
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Website: http://www.nanodefine.eu/
Project co-ordinator: Wageningen Food Safety Research (WFSR), NL

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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 purposes[1]. 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 2.

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
CFFF	Centrifugal Field-Flow-Fractionation
CLS	Centrifugal Liquid Sedimentation
CPC	Condensation Particle Counter
DEMA	Differential Electrical Mobility Analysis (also spray-DEMA)
DMA	Differential Mobility Analyser
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	Engineering System International 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
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry
KB	Knowledge Base
LD	Laser Diffraction
LoD	Limit of Detection
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

Executive summary

This document is Part 2 ("Evaluation of methods") of the NanoDefine Methods Manual. It is based on the results of a comprehensive study performed within the NanoDefine project ('NanoDefine') of the available measurement techniques, which are candidates for performing a reliable analysis of the number-based size distribution of a particulate material, with the goal to identify nanomaterials according to the European Commission recommendation on the definition of nanomaterial[1]. NanoDefine was executed under the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement n° 604347.

Based on the performance criteria established in NanoDefine the potential measurement techniques were evaluated (i) according to studies available in the literature, (ii) in a comparative interlaboratory study with selected real world materials ("NanoDefine priority materials"), as well as (iii) through the expertise of the NanoDefine consortium partners. The detailed information on the evaluation process is presented in NanoDefine Technical Reports which can be found on the project website at http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-technical-reports.

This document discusses most of the available size measurement techniques for nanomaterials. The different types of the methods that allow the determination of size and size distributions are explained and an overview of techniques and their capabilities is presented in user-friendly overview tables. These tables provide the reader with a possibility for quick selection of the method(s) which may be appropriate for the characterisation of given material. For the final selection of the method(s) to be employed it is highly recommended to use the detailed performance tables. These are described in the four main sections of the report. Each section introduces one of the four types of methods: counting, fractioning, spectroscopic ensemble and integral methods and explains the basic principles of that type of method as well as its performance, advantages and disadvantages.

This report presents the outcomes of the evaluatyion in two types of tables per method: one table that presents general performance information highlighting also advantages and disadvantages of the technique. The second table provides additional and very detailed information on the capabilities of the method.

1 Introduction

This report is Part 2 of the NanoDefine Methods Manual and provides the 'Evaluation of methods'. It reflects the outcomes of a comprehensive study performed within the NanoDefine project on the available measurement techniques, which are candidates for performing a reliable analysis of the number-based size distribution of a particulate material, with the goal to identify nanomaterials according to the European Commission recommendation on the definition of nanomaterial[1] the EC NM Definition. Based on the performance criteria already established in NanoDefine and the NanoDefine Technical Reports, which all can be found on the project website[2], these measurement techniques were evaluated (i) according to studies available in the literature, (ii) in a comparative interlaboratory study with selected real world materials ("NanoDefine priority materials", see Annex 1 for a list of these materials), and (iii) through the expertise of the NanoDefine consortium partners. The detailed information about the evaluation process is presented in the NanoDefine Technical Reports.

This document discusses most of the available size measurement techniques for nanomaterials, see Figure 1. The methods described are divided into four main groups, which are counting, spectroscopic ensemble, fractioning and integral methods. For each evaluated measurement technique, the basic principles are briefly explained and described in a dedicated section.

An overview of the techniques and their capabilities is presented in the form of three types of user-friendly tables. The first table type of table is presented in section 0, which presents overview tables of the evaluation of all the methods (see Table 3 to Table 6), providing the reader with the possibility for a quick selection of the method(s) which may be appropriate for the characterisation of given material. As the purpose of this report is to present the outcomes of evaluation the size measurement methods these overview tables are also the conclusions of this report.

The second and third types of tables, general performance tables and technical characteristics tables, give additional and much more detailed information on the capabilities of each method. The performance tables give an overview of main features, main advantages and main disadvantages for each measurement technique. The technical characteristics tables first present the material-related technical characteristics of the criteria, and then the metrological aspects of the technique, and the content of the technical characteristics tables is explained in section 2.2.1. The chapters 3 to 6 of the report have 11 sections that present one general performance table and one technical characteristics table for each measurement technique. In particular, these tables highlight any further information that should be considered for the choice of the most appropriate method with a yellow warning sign ⚠ to draw attention of the reader. Furthermore, the column “notes” lists additional relevant information that may be of particular importance for specific cases.

It is highly recommended to use the technical characteristics tables, which are described in this report, for final selection of the method(s) to be employed.

2 Measurement techniques

This chapter presents the summary of the results of a comprehensive study performed within the NanoDefine project. NanoDefine studied, among others, the available measurement techniques, which 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 Defintion.

The following chapters explain the basic principles of each evaluated method and general performance information is presented in a dedicated table per technique, which also highlight main advantages and disadvantages of the technique. Furthermore, additional detailed information on the performance and suitability of each method is presented as a table which allows the user a quick and simple identification of the question (criterion) and clear answer. Additional information to take into consideration when choosing the most appropriate method is highlighted with the sign ⚠. It is also strongly suggested to pay attention to the information included in the column "notes" as it may be important for specific cases.

As already stated in chapter 1 different sets of tables help the reader to identify and select the (most) appropriate technique for given material. A non-exhaustive list of relevant international standards (ISO) on particle sizing is included in Annex 2 of this document.

2.1 General introduction to the methods

There are several means and physical phenomena that allow for the determination of size and size distributions (e.g. imaging, sedimentation, light extinction). A more general distinction of particle sizing techniques is based on how the weights of the individual size fractions are determined.

- counting techniques (measuring particle properties at the level of individual particles)
- ensemble techniques (measuring the spectral or parametric response of a representative particle ensemble of the total particle system)
- fractionating techniques (measuring the amount or concentration of size/property classes after fractionating the particle system)
- integral methods (measuring effective properties at the level of a whole particle system, without being able to provide a size distribution)

Figure 1 gives an overview of measurement techniques that can be used for each of the four types of methods, and a description of each type of method is given below.

Screening methods give an initial indication of whether a given material is a nanomaterial according to the EC NM Defintion or not and may under favourable conditions allow identifying nanomaterials. These methods are called tier 1 methods. The tier 2 methods (currently only EM methods are recommended by NanoDefine) are confirmatory methods that allow to unambiguously determine if a given material is a nanomaterial according to the EC NM Defintion or not.

2.1.1 Counting methods

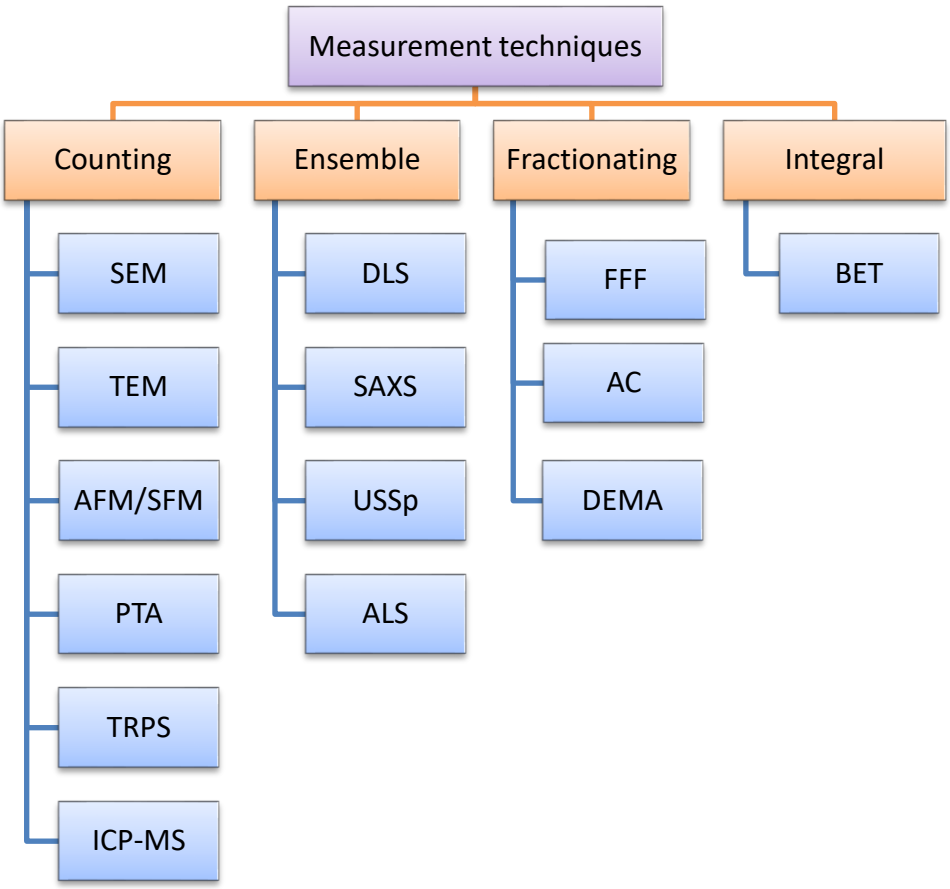
In the counting methods individual particles are measured and counts of similar-sized particles are placed into a "size bin" or size class to construct a size distribution.

Counting methods inherently yield particle number weighted distributions (Q_0) of a certain particle property or of a physical quantity that is related to a certain particle property (e.g. particle size, or the average displacement as a measure of the diffusion coefficient). They rely on the individualisation of the

particle sample, which can be either achieved by analysing microscopy images (e.g. from electron microscopes), by sufficient sample dilution or by reduction of the sample or measurement volume. The probed particle property may be either geometric (in particular for image analysis), optical (e.g. scattering cross section), or related to mobility (diffusion coefficient).

2.1.2 Ensemble methods

In the ensemble methods (Spectroscopic) all particles in the sample are measured at the same time and the size distribution is extracted from a combined signal from all particles. The immediate result of this type of method is the variation of the measured signal g over the spectral parameter s (time, space or frequency). Each size fraction x has a characteristic spectrum $k_r(s,x)$, which in general covers the whole range. Assuming that each size fraction contributes independently and linearly to the measured signal, the determination of the size distribution requires the inversion of a linear integral equation (Fredholm type). The intrinsic type of quantity is not necessarily obvious; it refers to the impact of a single particle to the integrated signal. The probed particle property of an ensemble method frequently relates to the particle mobility (diffusion) or to its interaction with external fields (scattering, extinction).



ABBREVIATIONS: COUNTING: EM: Electron microscopy. SFM: Scanning force microscopy (SFM) / AFM: atomic force microscopy. PTA: Particle tracking analysis / DUM: Dynamic ultramicroscopy. TRPS: Tunable Resistive Pulse Sensing / ESZ: Electrical sensing zone / nano Coulter counter. spICP-MS: Single particle ICP-MS. ENSEMBLE: DLS: Dynamic light scattering. SAXS: Small-angle X-ray scattering. USSp: Ultrasonic spectroscopy. ALS: Angular light scattering, including LD: Laser diffraction (LD). FRACTIONING: DEMA: Differential electrical mobility analysis. FFF: Field-Flow-Fractionation. AC: Analytical centrifugation. INTEGRAL METHODS: BET for determination of volume specific surface area (VSSA).

Figure 1: Measurement techniques for particle size distribution described in this report

2.1.3 Fractionating methods

In the fractioning methods an external force/process is used to separate particles according to their size; subsequently the quantities of the separated different sizes or size fractions are determined to construct a size distribution.

Fractionating methods include the two steps of fractionation and detection. The former can either result in a physical separation of the different size classes or in the depletion of coarse or fine particles in the measurement zone. In the case of colloidal suspensions, the fractionating effect is usually related to the mobility of the particles (e.g. settling velocity). The detection system monitors the fractionation process and thus serves for evaluating the class frequencies. Detection frequently employs phase shift, extinction or scattering of some radiation (e.g. X-rays). The applied detection system determines the type of quantity in which the size fractions are intrinsically weighted (e.g. extinction of X-rays is mass proportional – Q_3).

2.1.4 Integral methods

In additional to the methods that resolve the distribution of particle sizes, there are a few methods which solely measure an integral (effective/mean) property of a particle system such as the specific surface area (S_V or S_m) or the turbidity of a suspension. These properties can be directly converted into mean values of a PSD (e.g. $S_V \rightarrow$ harmonic mean of the volume weighted PSD). Note that ensemble methods, in principle, also yield such integral properties (e.g. the mean decay of signal fluctuation in DLS gives x_{cum}, i.e. the harmonic mean of the intensity weighted size distribution).

2.2 Performance criteria

For the purpose of the NanoDefiner framework performance criteria of each measurement method were elaborated in detail; they include: applicability to different groups of substances (chemical scope of the method), applicability to polydisperse samples, capability to measure aggregates, agglomerates, constituent particles (in agglomerates/aggregates) and/or non-spherical particles, accuracy of the results determined with the measurement technique, standardisation status (traceability of the measured values / availability of CRMs).

Each measurement technique was evaluated against these criteria depending on the substance to analyse (if the measurement technique is suitable for this type of substance) and on the technical factors. The applied criteria, which are discussed below, are not listed according to their priority. The presented tables include more information than eventually was included into the final Material Categorisation Scheme and NanoDefiner e-tool, but as these detailed data are available they are included in the report for information and possible use.

2.2.1 Applicability to different groups of substances

This section discusses criteria which affect the suitability and performance of particles sizing methods. Particle sizing methods were assessed against these criteria, and the detailed results can be found in the NanoDefine methods performance tables in chapters 3 to 6 of this report.

2.2.1.1 Nanoparticles in powder, or liquid suspensions or embedded in a matrix

Certain measurement techniques are only applicable to nanoparticles dispersed in a liquid phase; others are for aerosols or for powders and granulates. For the characterisation of nanomaterials e.g. in articles or formulations it is necessary to know if a method can characterise nanoparticles embedded in a matrix. One should also take into consideration that some nanoparticles may change significantly during sample

preparation, which restricts the possible dispersion medium and thus excludes certain measurement techniques. As the physical state of the sample has a major impact on the choice of the measurement technique the primary grouping was done based on the type of the physical state of the sample:

- Dispersed in liquids
- Solid particulate form (powder…)
- Dispersed or embedded in different types of matrices (paste, resin, elastomer…)

2.2.1.2 Dispersibility according to dispersion protocols

Some measurement techniques may be applied only to dispersed materials (in liquid and gas). Therefore it is essential to know if the substance can be dispersed by standardised protocols that specify both a dispersion medium and a dispersion protocol.

Measured size distributions can be severely affected by sample preparation protocols, for instance by the choice of the dispersion media and the particle concentration but also by the selected dispersant, which controls the state of aggregation and agglomeration. It should be underlined that the surface chemistry of particles strongly affects their dispersibility behaviour hence they are divided in five major groups:

- Dispersible in aqueous media (by generalised protocols)
- Dispersible in non-polar liquids (by generalised protocols)
- Dispersible in polar liquids other than water (by generalised protocols)
- Dispersible in material-specific media (by specific protocols)
- Can be aerosolized

2.2.1.3 Nature of the substance

One of the most important criteria is the nature of the substance considering that the measurement technique has to be chosen and/or adapted accordingly. For instance, the lower size limit of spICP-MS is directly related to the elemental composition of the material, and organic particles cannot be detected (in the nanorange).

Consequently materials can be grouped as follows:

- 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[3]…)
- Carbon-based (CNT, nanodiamond, carbon black…)
- Organic, particulate (polymers, dyes, etc., nanonized, precipitated)
- Organic, non-particulate (dendrimers, liposomes, supramolecular assemblies…)
- Biological (nucleic acid, peptide, protein)
- Composite particle
- Other

A **composite** is a solid substance where each particle consists of two or more phase-separated constituents[4]. Depending on the internal structure, **composite particles** can be divided into three types, see Table 1. As the structure of the composite particle may influence the result of the analysis it is

necessary to acquire detailed knowledge on the composite particles structure in order to apply a suitable measurement technique including the correct data evaluation.

Table 1: Types of composite particles (Reproduced from Ref. [5] with permission from the Royal Society of Chemistry).

Type of composite particle	**Visualisation**
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.	

2.2.1.4 Particle shape and number of small dimensions

Many of the currently employed measurement techniques implicitly assume that the particles are spherical or yield an equivalent spherical size, which severely limits their applicability to particles with non-spherical shape. Furthermore, plate- or fibre-like particles requires specific methods, which allow to measure the smallest dimensions of the particles as often this is the dimension which should be assessed to determine if the material meets the EC NM Definition requirements. An additional difficulty occurs if the analysed sample consists of a mixture of particles of different shapes. In such case, only EM and possibly scanning probe microscopy could 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 of shape and number of nanoscale dimensions of nanoparticles can assume the following values:

- 1, 2 or 3 (number of nanoscale dimensions)
- Mixture of nanoparticles with different shapes

In principle, indication of the number of small dimensions is sufficient, 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) (ratio thickness:laterial extension ≤ 0.25)
- Two small dimensions: fibres (elongated shapes such as tubes, fibres, rods) (length:diameter ≥ 3)
- Three small dimensions: Spherical, equiaxial or similar (e.g. prismatic, cubic, tetrahedral)
- Mixture of nanoparticles with different shapes
- Other (incl. unknown)

2.2.1.5 Thermal degradation sensitivity

Some measurement techniques may lead to a thermal load on the sample and consequently alter their chemical or physical properties. Therefore it is important to know if a given measurement technique can cause damages to heat sensitive samples, which would hamper the validity of such measurements. Subsequently substances may be categorised as below:

- Sensitive above 0 °C
- Sensitive above 25 °C (room temperature)
- Sensitive above 37 °C (body temperature)
- Sensitive above 50 °C
- Sensitive above 100 °C
- Sensitive above 150 °C
- Sensitive above 500 °C
- Sensitive above 1000 °C

The entry 'yes' in the performance table means that the method can be used to measure a material with the stated sensitivity.

2.2.1.6 Cooling degradation sensitivity

It is important to know if the measurement technique may cause damage to temperature sensitive samples during a cooling process, hence jeopardising the validity of such measurements. Consequently substances may be divided in six general groups:

- Sensitive below 25 °C
- Sensitive below 0 °C
- Sensitive below - 18 °C (freezer)
- Sensitive below - 35 °C (deep freezer)
- Sensitive below - 78 °C (dry ice)
- Sensitive below – 195 °C (liquid nitrogen)

The entry 'yes' in the performance table means that the method can be used to measure a material with the stated sensitivity.

2.2.1.7 Electron beam sensitivity

This criterion takes into account that some substances are sensitive to electron irradiation and therefore cannot be characterised reliably with EM, or require more sophisticated EM techniques, such as cryo-EM or low-dose techniques:

- Sensitive to electron beam
- Not sensitive to electron beam

The entry 'yes' in the performance table means that the method can be used to measure a material with the stated sensitivity.

2.2.1.8 Particle size dispersity and modality

All samples have, to a certain degree, a polydisperse particle size distribution. Therefore it is important to recognize if a specific measurement technique can be used to analyse polydisperse samples without obtaining false results. Hence dispersity and modality of the sample has to be considered as an important factor in the performance of the method. The following general groups of particle size dispersity have been identified:

- (Quasi-) Monodisperse
- Polydisperse
- Multimodal
- Monomodal

It is also important to define at which degree of polydispersity a sample may not be considered monodisperse anymore.

2.2.1.9 Optional criteria

Electrical conductivity

Another important criterion to be considered is electrical conductivity of the sample as certain measurement techniques are more appropriate for electrically conductive substances.

- Conductive
- Semiconductive
- Insulator

Magnetic properties

Magnetic particles may be characterised with some specific measurement techniques such as magnetic force microscopy (MFM)[6] or magnetic particle spectrometer[7, 8]. Thermomagnetic[9], direct current (dc) magnetisation[10] and alternating current (ac) susceptibility[11] measurements enable also to determine the size distribution of magnetic particles. In this regard, nanoparticles can be classified as:

- Magnetic
- Non magnetic

Functionalisation / no functionalisation of the surface

One important criterion is to know if the nanoparticles are surface functionalised or not. Measurement techniques have to be adapted according to this characteristic.

- Functionalised
- Not functionalised

It is also important to determine the difference between functionalisation of the surface and the presence of a shell layer. For the purpose of the NanoDefiner e-tool it was established that nanoparticles completely covered with a uniform layer belong to the category composite materials.

Most of the measurement techniques give can measure the size of the functionalised nanoparticles, but do not give any information on the type of functionalisation.

Agglomeration/ aggregation state

Nanoparticles can be aggregated or have a tendency to agglomerate. This specific feature of a material needs to be accounted for in the performed data analysis; nanoparticles can be categorised as:

- Nanoparticles are aggregated
- Nanoparticles are not aggregated
- Nanoparticles are agglomerated
- Nanoparticles are not agglomerated

2.2.2 Capabilities of the measurement techniques

2.2.2.1 What type of measurement technique is it? (Counting, ensemble technique or fractionating)

- Single particle counting
- Calculate number or concentration from ensemble methods
- Method combination (hyphenated methods)

2.2.2.2 Working range

The working range is the range in which the method provides reliable results. The working range may be dependent also on the material and its preparation and on the instrument type.

- Size range
- Concentration range
- Minimum sample intake (How much material is needed?)
- Linearity/proportionality
- Limits of detection/quantification
- Sensitivity (Counting or detection efficiency as a function of size)

2.2.2.3 Trueness

Trueness is defined as a difference between the averages of several measurements on the same sample or material and the true value of the measured property (associated quantitative term "bias").

Trueness may be expressed either in terms of size or in terms of amount of particles.

2.2.2.4 Robustness

Robustness is defined as an influence of slight variations in the test protocol on the outcome of the test.

2.2.2.5 Precision

Precision is a test result variation within one test series (repeatability) or several test series (intermediate precision), either in terms of size or in terms of amount of particles.

2.2.2.6 Resolution

Resolution means to which degree a certain size fraction can be distinguished from another (e.g. minimum distance or size ratio needed between different modes so that they can be identified in a mixture of monodisperse samples).

2.2.2.7 Size distribution

The following criterion was considered: Does the method provide a size distribution[12] or a certain average value?

2.2.2.8 Selectivity

Selectivity is how well the measurement technique can distinguish between:

- nanoparticles and non-nanoparticles of the same composition
- nanoparticles and non-nanoparticles of another composition (e.g. mixtures of powders)
- nanoparticles of another composition (mixtures of powders)

Moreover, if the substance to analyse is not pure, it is essential to know if and how the results are affected by impurities, including dissolved ionic species from the same substance. "Yes" in the performance table means that the measurement technique can distinguish between the nanomaterial and the impurity, whereas a "No" mean that the impurities will be detected but the measurement technique cannot distinguish between the nanoparticles and impurity particles.

2.2.2.9 Capability to measure aggregation

Can the method identify and/or measure agglomerates or aggregates of particles?

2.2.2.10 Capability to measure single particles

Can the method measure the size and number of individual particles?

2.2.2.11 Counting constituent particles in aggregates

Is the method able to count constituent particles in aggregates?

2.2.2.12 Chemical composition

Does the method provide information on the chemical composition?

2.2.2.13 Specification of the measurand (diameter)

Size measurement is method dependent.[13] Different methods address different measurands (equivalent diameters) which need to be specified (hydrodynamic diameter, Stokes diameter, projected area diameter, etc.).

2.2.2.14 Non-destructive / destructive

This criterion indicates whether the method is destructive or not.

2.2.3 Template for technical characteristics

An overview of the outcomes of the evaluation of each measurement technique is presented in a self-explanatory performance table with standardised information content, giving an over view of the main features of each method, and main advantages and disadvantages.

The outcomes of the evaluation of each measurement technique are presented in a dedicated technical characteristics table with standardised information content; an empty table is shown as Table 2. For clarity it is divided into two sections. The first (blue rows) is related to the type of substance being analysed, and indicating whether the method is suitable for characterising materials with specific properties. The second section (orange rows) is related to the technique, indicating the characteristics of

each measurement technique according to the specified criteria. This report presents detailed performance tables filled in for each of the measurement techniques investigated in NanoDefine.

Table 2: Technical characteristics 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		
	Multishell particles		
	Particles with inclusions		
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?		

2.3 Evaluation and performance of the techniques: overview

A general overview of the recommended particle size measurement techniques described in this chapter is shown in Table 3 to Table 6. The criteria selected are of two representative natures: direct relation to the EC NM Definition and analytical one. For the sake of clarity, only the scores 'fair', 'good' and 'very good' were highlighted in the tables. Please note that these tables only aim to give a general overview of the recommended methods and for selecting an appropriate method the detailed performance tables given in chapters 3, 5, 4 and 6 in this report should be used.

The overview tables include the measurement technique scanning electron microscopy working in transmission mode (TSEM), which does not have a detailed performance table associated. TSEM is mentioned in the EM section.

Table 3 provides an overview of the performance of the measurement techniques with regard to measuring size. Table 4 gives an overview and an evaluation of the measurement techniques' capacities when measuring different types of materials. The overview in Table 5 is for the evaluation of the measurement techniques with regard to their capacities for measuring single particles, agglomerates and aggregates in the samples. Table 6 collects an overview of how the measurement techniques perform with regard to "Additional information", i.e. if it is a direct counting technique, how easily the result of the measurement is convertible to the number-weighted PSD (including quantitative and size accuracy), how well the technique can measure the smallest dimension of each particle, and if it gives access to measuring constituent particles, the chemical selectivity of the method, if it can measure the material as it is and availability of ISO standards.

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						
		TSEM						
		TEM						
	SFM/AFM							
	PTA							
	TRPS							
	spICP-MS							
Ensemble	DLS							
	SAXS							
	USSp							
	ALS							
Fractionating	FFF							
	AC							
	DEMA							
Integral	BET							
Legend			Fair	Good	Very good	Not applicable		

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	Multishell particles	Inclusion	Sphere	Equiaxial	Tubes, fibres, rods	Flakes discs
Counting	EM	SEM														
		TSEM														
		TEM														
	SFM															
	PTA															
	TRPS															
	spICP-MS															
Ensemble	DLS															
	SAXS															
	USSp															
	ALS															
Fractionating	FFF															
	AC															
	DEMA															
Integral	BET															

Legend	Fair	Good	Very good	Not applicable

Table 5: Evaluation of the methods: single particles, agglomerates and aggregates

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						
		TSEM						
		TEM						
	SFM							
	PTA							
	TRPS							
	ICP-MS							
Ensemble	DLS							
	SAXS							
	USSp							
	ALS							
Fractionating	FFF							
	AC							
	DEMA							
Integral	BET							

Legend	Fair	Good	Very good	Not applicable

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						(+EDX)	
		TSEM						(+EDX)	
		TEM						(+EDX)	
	SFM								
	PTA								
	TRPS								
	spICP-MS								
Ensemble	DLS								
	SAXS								
	USSp								
	ALS								
Fractionating	FFF							(+Detector)	
	AC								
	DEMA								
Integral	BET								

Legend	Fair	Good	Very good	Not applicable

3 Counting methods

3.1 Electron microscopy

3.1.1 Measurement principle

One analytical method widely used for sample visualization down to the nm scale is electron microscopy (EM), which uses an electron beam for visualization.

The preparation of the nanoparticle sample to be investigated by EM is key to successful analysis of the particle size distribution. Ideally, particles, which are well separated, free of preparation artefacts, and are distributed on the proper support not too far away from each other, shall be accurately imaged. Care must be taken of possible beam or vacuum influence on the size of NPs. Once such an accurate image is taken, the post-measurement of the particle size can be performed with appropriate software packages. Decisive steps in the evaluation of the accurate particle size determined with an electron microscope are the calibration of the image magnification, i.e. of the pixel size including its re-calibration in the processing software, and the setting of the threshold in the image histogram corresponding to the real position of the particle boundaries.

Size analysis with EM relies on counting individual particles. Depending on the number of particles acquired in an image, most often several images are necessary to achieve good counting statistics. The automation tools such as motorized stage and sequential image acquisition should be available in order to speed up the whole measurement process. Also automatic image processing of batch images speeds up the determination of the size distribution.

In scanning electron microscopy (SEM), images are constructed based on electrons coming from the sample surface[14]. In case of transmission electron microscopy (TEM), images are constructed based on electrons passing through the sample. Both SEM and TEM give 2-dimensional projections of 3-dimensional particles. EM images facilitate the determination of number-weighted size distributions by analysing each identifiable particle individually[14]. EM also allows an assessment of the morphology of particles. The analysis of flattened particles (e.g. disks, flakes) could be problematic because the smallest axis of the particle could be hidden. This could lead to a measured size that is overestimated compared to the size relevant for the EC NM Definition. Most EM measurements are performed in high vacuum chambers. Therefore, the use of EM is limited to the analysis of particles which are not sensitive to the combined action of high vacuum and electron beams.

In SEM, the sample to be examined is bombarded with a finely (i.e. in nm range) focused electron beam which is scanned over a defined field. Low-energy secondary electrons are released after inelastic collisions with the atoms in the specimen, and high-energy backscattered electrons after elastic collisions with the atoms in the specimen. Depending on their kinetic energy, the information range carried by the released electrons varies from micrometre (typical for backscattered electrons) down to nanometre (typical for secondary electrons). Hence, the secondary electrons are suited for high-resolution morphological characterization of the specimen surface at nanometre scale and even individual nanoparticles may be visualized and lateral dimensions measured with SEM. Depending on the instrument used, but also strongly on the challenging sample preparation onto typical sample substrates/holders, accurate size characterization of nanoparticles is possible with sizes down to about 7 nm[13].

Whereas in the case of an SEM typical beam voltages up to 30 kV may be applied and secondary electrons/ backscattered electrons are collected by various detectors, in the case of TEM the beam voltage is up to 300 kV. The samples to be analysed must be in the range of the electron

transparency so that the electrons transmitted through the thin sample can be collected. The highly energetic electron beam is even finer than in the SEM case (more sophisticated aberration correctors being often also available), so that spatial resolution well below 1 nm can be attained. TEM has similar requirements as SEM for NPs regarding sample preparation, suitable substrate (typically the so-called TEM grids), vacuum suitability[15], calibration of the pixel size and automation of both acquisition and image processing. By combining TEM imaging and semi-automatic image analysis accurate characterization of the size, shape, and surface morphology of colloidal nanomaterials,[16] aggregated nanomaterials[17,18], and primary particles in aggregates (or, in terms of the EC NM Definition, constituent particles)[19] is enabled. A characterization methodology which includes a systematic selection procedure for unbiased random image collection, semi-automatic image analysis and data processing has been validated for size, shape and surface morphology measurements of silica nanoparticles. The expanded uncertainty of size measurements of two colloidal silica certified reference materials was estimated to be about 3 %.

TEM can be operated in the scanning mode, i.e. STEM, when the electron beam is focused into a narrow spot which is scanned over the sample. According to the range of angles by which they are scattered in the sample, the transmitted electrons can be differentiated as: bright-field electrons are those electrons slightly or not at all scattered and dark-field electrons are those collected concentrically to the optical microscope axis. The so-called HAADF-STEM (High-Angle Annular Dark-Field STEM) imaging mode results when only the strongly scattered electrons are collected with an annular dark-field detector. The contrast of this type of imaging with atomic resolution is directly related to the atomic number of the elements constituting the sample (Z-contrast image).

One hybrid type of electron microscopy is constituted by the SEM able to work in the transmission mode, i.e. TSEM, T-SEM or STEM in SEM etc. This means that by using TEM grids as support for NPs, the transmitted electrons (of lower energies than in the TEM case) are more or less absorbed by the NPs and a so-called STEM detector placed under the sample holder detects them. The alternative is to use a dedicated transmission sample holder, which enables performing TSEM with the available secondary electrons/backscattered electrons detector without the need to use an additional STEM detector. It was recently demonstrated that both types of transmission modes with SEM are well suited for metrological measurement of NP size and size distribution down to about 10 nm[20, 21, 22].

3.1.2 Performance

Current limitations in application include:

- Strong dependency on sample preparation (incl. suitable substrate)
- Possibility to use vacuum for certain types of material (organic, coated etc.)
- SEM is not able to measure accurately the size of NPs below about 7 nm (depending on instrument)
- Automation (batch image acquisition and batch image processing)
- Time-consumption that depends on the complexity of sample preparation and degree of automation
- High cost, but still in the same order of magnitude as e.g. ICP-MS
- Accuracy in identification of the NPs in the image processing software
- The size resolution of EM is good with the following analytical lower limits:
- SEM minimal NP size to be measured accurately: about 7 nm depending on the instrument employed.

- TEM minimal NP size to be measured accurately: below 1 nm depending on the applied contrast and on the instrument employed.
- TSEM minimal NP size to be measured accurately: about 10 nm depending on the instrument employed.

Systematic results of metrological measurement of NP size and size distribution by SEM, TEM and TSEM were carried out recently in the frame of various, specifically dedicated round robin tests[11]. According to these reported results, EM provides traceable results, which are also consistent, i.e. comparable, with those obtained by AFM, SAXS and SMPS, but not with DLS.

Table 7 and Table 8 below give the general performance of electron microscopy and the detailed performance table for this method, respectively.

Table 7: General performance of electron microscopy (EM)

Main features	
Type of samples	particles properly deposited onto substrates or particles embedded in an electron-transparent medium
Type of sizing	counting technique (by identifying individual objects in images)
Particle property measured	selected properties of the 2D particle image, e.g. Feret diameter, area equivalent circle diameter (ECD), wide range of 1D and 2D size, shape and surface measurands
Type of quantity	particle number
Size range	• SEM: 7 nm - 1000 µm • TEM (incl. HAADF-STEM): <1 nm - 1000 µm • TSEM: 10 nm - 100 µm
Concentration range	"0" (individual particles) ... monolayer (immobilised particles)
Information content	• very high, i.e. can well resolve details of the size distribution • good in x-y direction (i.e. parallel to the substrate)
Main advantages	
• intrinsically yields number-weighted size distributions • facilitates determination of particle size and shape as well as aggregate structure and surface measurands • in principle can distinguish aggregates from their constituent particles and allows for size measurement of the constituents • size, shape and surface measurands can be measured on 2D images • sub-nm resolution for TEM, nm resolution for SEM • access to smallest dimension of particles in X-Y plane • high resolution of particle size distribution • capable of chemical specificity of single particles by the attached EDX • crystallographic information is available by electron diffraction • significant instrumental developments (spatial resolution, automation, EDX detector sensitivity, table-top instruments, etc.) • automated image processing: available and in further development	

Main disadvantages
• strongly dependent on sample preparation (immobilised particles on substrate need to be representative for the material) • needs vacuum and expensive instrumentation • limited dynamic range (highest size/lowest size < 40) based on one image only

Table 8: Technical characteristics for electron microscopy (EM)

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	No	Possible if a cryo-stage is available[23] Yes ⚠ successful deposition on substrates required
	Solid particulate form	Yes	
	Dispersed or embedded in matrices	Yes ⚠	Only at the surface, in thin films or in ultramicrotomed sections
Dispersibility by dispersion protocols	Dispersible in aqueous media	No	Yes ⚠ successful deposition on substrates required
	Dispersible in non-polar liquids	No	
	Dispersible in polar liquids other than water	No	
	Dispersible in material-specific media	No	
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	Yes	
	Composite particles	Yes	
	Other	Yes	

Composite particles (see Section 2.2.1.3)	Core/shell	Yes	Can detect the internal structure in some cases
	Multishell particles	Yes	Difficult
	Particles with inclusions	Yes	Only outer size. Can detect the internal structure in some cases
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No (⚠ Yes)	Typically ⚠ For specialised solutions (EELS, EFTEM)
	2 (e.g. diameter of nanofibres)	Yes	
	3	Yes	Measurement of Z (height) is difficult in SEM'. In TEM it is possible (not routine)
Shape of particles	Sphere or similar	Yes	
	Equiaxial	Yes	
	Tubes, fibres, rods (length: diameter ≥ 3)	Yes	
	Flakes and discs (thickness: lateral extension ≤ 0.25)	No (⚠ Yes)	Typically for specialised solutions (EELS, EFTEM)
	Other	No (⚠ Yes)	Typically for specialised solutions (EELS, EFTEM)
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-25 °C)	Above 0 °C	No	⚠ Yes, with cryo stage
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-25 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	

Electron beam sensitivity	Electron beam sensitive	No	Low-dose measurement may work for particles with weak electron beam sensitivity
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	With altered image quality
	Insulator	Yes	SEM: low-voltage option or conductive high resolution sputter-coating needed
Magnetic properties	Magnetic	Yes	Depending on the strength of the magnetic field the performance of the technique may worsen
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes	
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Yes only for sophisticated sample preparation or fairly small agglomerates
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	Yes	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble measurement techniques	No	
	Integral technique	No	
	Used in hyphenated methods	Yes	E.g. with EDS or EELS

Working range	Size range	7 nm to 1000 µm (SEM) 1 nm to 1000 µm (TEM)	Lower range depends on instrument type, sample type and preparation, ⚠ full size range requires more than one image (at different magnifications)
	Concentration range	N/A	Accurate measurements only for single particles deposited on a substrate → optimum concentration depends on size and deposition procedure. For example, a droplet of 0.1-1 µL at 0.1 %-vol. conc. is typically sufficient
	Minimum needed sample amount	0.1 µL	Minimum 500 NPs for a monodisperse/ monomodal sample[13]
	Linearity/proportionality	Yes	When differentiating between linearity in size and linearity in quantity then: Size: yes Quantity: N/A or (Yes), as the correct quantification is mainly determined by sample preparation (representative and homogeneous deposition on substrate) and measurement procedures (image acquisition)
	Limits of detection/quantification	1 nm to 10 nm	Depending on instrument, sample type and preparation, etc.
	Sensitivity (counting efficiency) as a function of size	good	
Trueness	Indicate the trueness of this measurement technique in measuring the particle size	good	Based on size traceability of reference materials; critical for NPs below 10 to 20 nm with SEM
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	good	To be evaluated for specific cases
Robustness	Specify the robustness of this measurement technique	average	Strong dependency on sample preparation
Precision	Specify the precision of the measurement technique	1 nm to 10 nm	Depending on many parameters, mainly preparation; better for TEM and poorer for SEM
Resolution	Specify the resolution of this	1 nm to	

	measurement technique	10 nm	
Size distribution	Is it possible to measure particle size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes	Depending on the size of the big particles and the width of the size distribution
	Discrimination between NPs and non-NPs of another chemical composition	Yes	
	Discrimination from NPs of another chemical composition	Yes ⚠	In cases when image contrast between the particulate species is high enough
	Impurities	Yes ⚠	Depends on the nature of the impurity
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	Yes ⚠	Difficult and possible only after sampling on substrates and measurements performed in vacuum
Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	Yes ⚠	Depending on contrast and size of aggregate
Chemical composition	Does this measurement technique analyse chemical composition?	No Yes	Typically Modern instruments with EDS and EELS (hyphenation)
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	"Diameter" "Feret diameter"	All size and shape parameters available that can be deduced by 2D image analysis
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	Sample must be prepared on substrates or as thin films, etc.
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	Yes	
Sample support	Does this measurement technique need preparation on suitable supports?	Yes	

3.2 Scanning force microscopy (SFM), or atomic force microscopy (AFM)

3.2.1 Measurement principle

Atomic force microscopy (AFM) or scanning force microscopy (SFM) is a technique in which a sharp tip, a needle, is fixed on a cantilever and moved along the surface being analysed. Different ways to measure exist: the cantilever can tap the surface, touch the object constantly, and not be in contact with it. The shape of the tip as well as the substrate can influence the AFM images.

Particles need to be fixed to the surface in order to be characterised and not to be moved by the tip. AFM is an imaging method and can be used to measure the size of polydisperse and polyshaped particles. Organic particles can also be analysed with this technique.

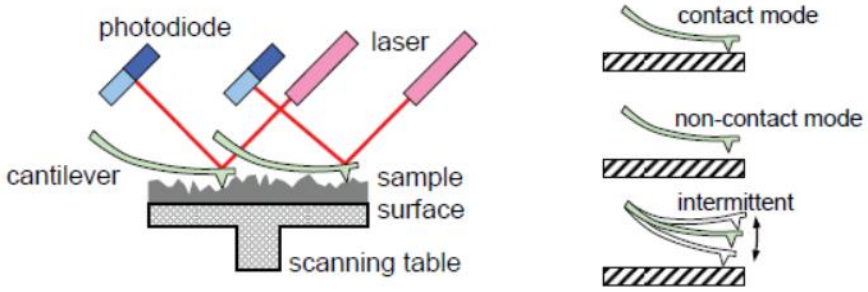


Figure 2: Measurement principle of SFM

This technique, able to image colloidal particles or powders, was developed by Binnig and his co-workers in the 1980s.[24] AFM (or SFM) is the most used type of scanning probe microscopy (SPM). The AFM and its related techniques are based on the interaction between a very fine probe tip with the atoms or molecules at the surface of the sample (see Figure 2)[25, 26]. This can be used to resolve surface morphologies or particles on a substrate with vertical/out-of-plane resolution of 0.1 nm to 10 nm (lateral/in-plane resolution: 10 nm). The AFM is usually employed for the characterisation of films and surfaces (e.g. roughness), whereas the morphological characterisation of particles is a less frequent application. Its real strength is the sensitivity to the forces between probe and sample, which allows an evaluation of surface chemistry (e.g. functional groups, hydrophobicity) and the quantification of particle interactions, or interactions between particles and surfaces (e.g. adhesion, friction[27, 28]). Depending on the situation, forces that are measured in AFM include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces, etc. As well as force, additional quantities may simultaneously be measured through the use of specialised types of probe. Samples in air or in liquid can be analysed, but the sample must adhere to a substrate and be rigid and well dispersed on it. The roughness of the substrate must be significantly smaller than the size of the nanoparticles being measured. The AFM can be operated in several modes. In general, imaging modes are divided into static (also called contact) modes and a variety of dynamic (or non-contact) modes where the cantilever is vibrated (Figure 2). The use of the AFM in biology, biochemistry and bionanotechnology, also for the characterization of nanomaterials (size, shape), are reviewed in an article by Kada et al.[29]

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

confidence level increases with polydispersity. The accuracy of the measured particle properties depends on a variety of factors (e.g. magnification or spatial resolution of the scanning mode, or image processing). Most crucial, however, is the representativeness of the imaged particles for the whole particle system. That requires that the particle deposition on the substrate is neither size-selective nor inhomogeneous.[30] In general, sample preparation is a key issue for imaging methods.

3.2.2 Performance

The AFM technique is applicable to both powders and suspensions, and the particles need to be immobilised and be well dispersed on a support surface. Almost any material can be measured. The technique can only qualitatively distinguish between individual particles and agglomerates/aggregates and thus the size of constituent particles inside aggregates/agglomerates cannot be measured reliably. AFM measures external dimensions and the best resolution is achieved in the direction perpendicular to the surface on which the particles are immobilised. This technique provides an access to the minimum dimension of a particle, which is especially important for platelets-like shaped particles (thickness). The roughness of the substrate may introduce errors thus it must be significantly smaller than the size of the nanoparticles being measured. AFM can directly provide number-based particle size distributions.

Table 9 and Table 10 below give the general performance of scanning force microscopy (SFM) / atomic force microscopy (AFM) and the detailed performance table for this method, respectively.

Table 9: General performance of scanning force microscopy (SFM)/ atomic force microscopy (AFM)

Main features	
Type of samples	particles of (almost) any material, in vacuum, air or liquid immobilised on a substrate
Type of sizing	counting technique (by identifying individual objects in images)
Particle property measured	dimensions of the 2D particle image and particle height above the level of a substrate
Type of quantity	particle number
Size range	• 1 nm to <10 µm (z-size, "height", most reliable), • 10 nm to 100 µm (lateral size, depends on tip geometry)
Concentration range	"0" (individual particles) ... monolayer (immobilised particles)
Information content	• relatively high, i.e. can well resolve details of the size distribution • good in z-direction (i.e. in "height") • not reliable in x-y direction (laterally, parallel to the substrate)
Main advantages	
• intrinsically yields number-weighted size distributions, Q0 • facilitates determination of particle size and shape as well as surface properties • access to the minimum dimension of a particle • measures a wide range of materials • instruments are widely available and not expensive	

Main disadvantages
• strongly dependent on sample preparation (immobilised particles on substrate need to be representative for the material) • lateral size measurements are less reliable than altitude measurements (dependent on tip conditions) • low sample throughput, slow • limited dynamic range

Table 10: Technical characteristics for scanning force microscopy (SFM) / atomic force microscopy (AFM)

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	No	⚠ Yes after successful immobilization on substrates
	Solid particulate form	Yes ⚠	After successful immobilization on substrates, generally from a liquid dispersion
	Dispersed or embedded in different kinds of matrices	Yes ⚠	Only at the surface or in thin films or in ultramicrotomed sections, as long as the surface is smooth enough with respect to the particle size and there is enough contrast with the matrix; possible bias due to random sectioning at non-controlled distance from the diameter plane (for spheres)
Dispersibility by dispersion protocols	Dispersible in aqueous media	No	⚠ Yes after successful immobilization on substrates
	Dispersible in non-polar liquids	No	
	Dispersible in polar liquids other than water	No	
	Dispersible in material-specific media	No	
	Can be aerosolized	No	

Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	Yes	
	Composite particles	Yes	
	Other	Yes	
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Only external size
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	Yes	
	2 (e.g. diameter of nanofibres)	Yes	
	3	Yes	
Shape of nanoparticles	Sphere or similar	Yes	
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	Yes	
	Flakes and discs (thickness:laterial extension ≤ 0.25)	Yes	
	Other	Yes	
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-40°C)	Above 0 °C	No	
	Sensitivity above 25 °C	Yes	
	Sensitivity above 37 °C	Yes	
	Sensitivity above 50 °C	Yes	
	Sensitivity above 100 °C	Yes	
	Sensitivity above 150 °C	Yes	
	Sensitivity above 500 °C	Yes	
	Sensitivity above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	

Technique working range: 15-40 °C)	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes	Only aggregate size, with limitations (size and roughness)
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Only for small agglomerates or sophisticated sample preparation
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	Yes	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	No	
	Used in hyphenated methods	No	

Working range	Size range	> about 1 nm for height; >10 nm for lateral size; up to 10 µm	Range varies in dependence on instrument type, sample type and preparation
	Concentration range	N/A	Accurate measurements only for single particles deposited on a substrate → optimum concentration depends on size and deposition procedure, e.g. a droplet of 0.1-1 µL at 0.1%-vol. conc. is typically sufficient
	Minimum needed sample amount	0.1 µL for liquid suspension 1 mg for powder	Minimum sample size is about 500 particles, i.e. 0.1 – 1 µL at 0.1%-vol. conc.
	Linearity/proportionality	Yes	
	Limits of detection/quantification	1 nm / several tens of nm	About 1 nm for height and several tens of nm for lateral dimensions (depending on tip convolution)
	Sensitivity (counting efficiency) as a function of size	good	Low throughput
Trueness	Indicate the trueness of this measurement technique in measuring the particle size	very good	Only for height, as convolution with the tip geometry leads to a bias in lateral dimensions
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	good	Depending on polydispersity and sample preparation; to be evaluated for specific cases
Robustness	Specify the robustness of this measurement technique	average	Strong dependency on sample preparation; to be evaluated for specific cases
Precision	Specify the precision of the measurement technique	< 1 nm for height	Depending on many parameters; precision on lateral size depends, among others, on tip convolution. Depends also on the type of material.

Resolution	Specify the resolution of this measurement technique	1 nm to 5 nm (height)	Depending on dimension (height versus lateral), instrument type and imaging conditions and material type
Size distribution	Is it possible to measure size distribution?	Yes ⚠	If the size difference is not too big
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes ⚠	If the size difference is not too big
	Discrimination between NPs and non-NPs of another chemical composition	Yes ⚠	If the size difference is not too big. In cases when image contrast is high enough
	Discrimination from NPs of another chemical composition	average	In cases when hardness properties are significantly different (in intermittent contact mode); modes based on other properties (electrical, magnetic, thermal…) could be helpful, but need to be evaluated
	Impurities	No	Particulate impurities (in the appropriate size range and with sufficient contrast) can be detected and counted. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	Although coupling with Raman or FTIR does exist, but remains limited (in resolution, among others) and not widespread
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	height / lateral size	Bias on lateral size due to tip convolution

Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	Sample must be immobilised on substrates or as thin films, etc.
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	Also possible, but not required.
Sample support	Does this measurement technique need preparation on suitable supports?	Yes	

3.3 Particle tracking analysis (PTA), dynamic ultramicroscopy (DUM)

3.3.1 Measurement principle

Fine colloidal particles are usually smaller than the spatial resolution of an ordinary light microscope, which means that they are invisible with regard to an affine projection. However, when they are laterally illuminated by very intense light against a dark background (dark field microscopy), it is possible to see the scattering patterns with an optical microscope. Such an instrument is named ultramicroscope. When ultramicroscopy is used for particle sizing, one evaluates the Brownian motion of the scattering centres (i.e. particles), because of which this type of sizing is called particle tracking analysis (PTA) or dynamic ultramicroscopy (DUM).

In contrast to the majority of sizing techniques for NP suspensions PTA is in principle capable of measuring the particle number concentration. However, the reliability of such a measurement depends very much on material properties and particle size distribution width. The current state-of-the-art concentration measurement is not very reliable in the general case and needs further investigation[31].

3.3.2 Performance

The application limits of ultramicroscopy result from the requirements that the inter-particle distances should be much larger than the optical resolution limit of the microscope, which can be achieved by appropriate dilution, and that the scattered light of all individual particles is sufficiently strong for detection. This requirement is not fulfilled for particles below a material-specific size limit. Additionally, there are principal difficulties in detecting weak scatters in the presence of strong scatters. That means, though providing number weighted PSD (similar to ordinary microscopy), PTA has a bias to strongly scattering particles (similar to dynamic light scattering[32]). This concerns very broad size distributions as well as multi-component particle systems.

Table 11 and Table 12 give the general performance of the method particle tracking analysis (PTA) / dynamic ultramicroscopy (DUM) and the detailed performance criteria for this method, respectively.

Table 11: General performance of particle tracking analysis (PTA) / dynamic ultramicroscopy (DUM)

Main features	
Type of samples	particles (solid, liquid, gaseous) in liquid dispersion medium
Type of sizing	counting technique (by identifying individual objects in video images)
Particle property measured	diffusion coefficient equivalent diameter; translational hydrodynamic diameter
Type of quantity	particle number
Size range	10 nm to 1 µm (depending on the scattering properties of the material) e.g. Au, Ag 10 nm to 1 µm, polystyrene latex (PSL) 50 nm to 1 µm, SiO2 70 nm to 1 µm
Concentration range	<< 1 vol.-%
Information content	relatively high, i.e. can well resolve details of the size distribution
Main advantages	
• intrinsically yields number-weighted size distributions, Q0 • fairly good resolution of particle size distribution • provides particle number concentration of the analysed sample	
Main disadvantages	
• does not resolve particle shape, but measures a hydrodynamic equivalent diameter • measures aggregate size rather than size of the constituent particles • lower size limit is >10 nm and depends on material properties • poor sensitivity in the fine particle range • limited dynamic range	

Table 12: Technical characteristics for particle tracking analysis (PTA) / dynamic ultramicroscopy (DUM)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes	
	Dispersible in polar liquids other than water	Yes	

	Dispersible in material-specific media	Yes ⚠	Dispersion medium must be compatible with the instrument
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes ⚠	Requires that scattering is sufficiently strong
	Organic, non-particulate	Yes ⚠	Requires that scattering is sufficiently strong (typically if size >> 100 nm)
	Biological	Yes ⚠	Requires that the particle is stationary (i.e. shall not move itself, as e.g. bacteria may do)
	Composite particles	No	
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Only outer size
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	Does not resolve shape
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	
Shape of particles	Sphere or similar	Yes	
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 10-40 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	

	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 10-40 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes ⚠	Insensitive to fine NPs for very broad PSDs
	Monomodal	Yes	
	Multimodal	Yes ⚠	Insensitive to fine NPs for very broad PSDs
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Measurement technique measures only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Measurement technique measures only agglomerate size
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	Yes	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number	No	

	concentration from spectroscopic ensemble techniques		
	Integral technique	No	
	Used in hyphenated methods[1]	Yes	
Working range	Size range	10 nm – 1 µm	Lower limit is material dependent
	Concentration range	10^{7} to 9 particles/ mL	i.e. << 0.1 vol-%
	Minimum needed sample amount	10 mL	
	Linearity/proportionality	Yes ⚠ No	Yes with regard to diffusion velocity, i.e. size Not really, with regard to concentration
	Limits of detection/quantification		Depend on optical contrast and size, e.g. 10 nm for Au, 70 nm for SiO_2
	Sensitivity (counting efficiency) as a function of particle size	good ⚠	Poor for very fine NPs in the presence of large particles
Trueness of particle size measurement	Indicate the trueness of this measurement technique in measuring the particle size	good	Size: "falseness" if wrong calibration of microscope and wrong model parameters (e.g. viscosity)
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions by this measurement technique	good	Number/frequency: falseness if inappropriate illumination and image analysis or if too a high particle concentration; measurement technique has a bias to strongly scattering particles

[1] There are 2 independent signals: average track length and spot size, i.e. diffusion coefficient and scattering intensity; a 2-dimensional plot of a monoconstituent sample would therefore show the functional relationship between size and scattering cross section (i.e. a strong, non-linear correlation); for multiconstituent samples we would expect different groups of such functional dependencies, which offers a possibility to couple size analysis with material or shape analysis

Robustness	Specify the robustness of this measurement technique	poor	
Precision	Specify the precision of the measurement technique	5-10%	PSD parameters to be defined (PSD mean, width, etc.)
Resolution	Specify the size resolution of this measurement technique	5-7%	
Size distribution	Is it possible to measure particle size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes	
	Discrimination between NPs and non-NPs of another chemical composition	No	
	Discrimination from NPs of another chemical composition	No	
	Impurities	No	Particulate impurities (in the appropriate size range and with sufficient contrast) will be detected and counted. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes	
Counting constituent particles in aggregates	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	Translational hydrodynamic diameter	(long time self-) diffusion coefficient of particles
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	Usually, because of dilution

Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suitable supports?	No	

3.4 Tunable resistive pulse sensing (TRPS)[2] / electrical sensing zone (ESZ) / nano Coulter counter

3.4.1 Measurement principle

A Coulter counter is an apparatus for counting and sizing particles suspended in electrolytes[33]; the measuring technique is also called electrical sensing zone. It is used for cells, bacteria, prokaryotic cells and virus particles and more recently for fine particles. A typical Coulter counter has one or more microchannels that separate two chambers containing electrolyte solutions. As fluid containing particles or cells is drawn through each micro channel, each particle causes a brief change to the electrical resistance of the liquid. The counter detects these changes in electrical resistance. The Coulter principle relies on the fact that particles moving in an electric field cause measurable disturbances in that field. The magnitudes of these disturbances are proportional to the volume of the particles in the field[34, 35, 36]. For accurate measurement a few prerequisites should be fulfilled. Firstly, the particles should be suspended in a conducting liquid. Secondly, the electrical field should be physically constricted so that the movement of particles in the field causes detectable changes in the current. Finally, the sample should be sufficiently dilute so that only one particle at a time passes through the physical constriction, preventing an artefact known as coincidence.

3.4.2 Performance

The Coulter counter needs calibration (usually with spherical polymer latex). This calibration holds true for any other non-conducting material with particles that do not deviate considerably from spherical shape. The calibration constants should be changed for non-spherical particles (even though the signal is still proportional to the particle volume). Conducting particles require a defined adjustment of the applied voltage. Porous particles or aggregates need appropriate models or calibration, which means that the morphology of such particles has to be known.

The electrical sensing zone technique principally allows the measurement of number-weighted size distributions with high resolution. In addition, it can be used to measure the particle number concentration and their volume concentration (because the signal is volume proportional).

[2] TRPS (tunable resistive pulse sensing) is the preferred designation of the manufacturer (izon) for their newly introduced instrument (qnano): particles dispersed in water with dissolved salt move through the single pore of an elastic separator (hence the 'tunable' detection interval) which separates two electrodes that detect the ion current. Whenever a single particle blocks the pore, the current reduces, and the duration and depth of this 'pulse' provide information on size. The sequential detection of blockade events constitutes a size distribution in number metrics without further conversion. This detection principle is related, but not identical to the conventional ESZ (electrical sensing zone), and hence the designation 'Nano Coulter counter' for TRPS is not preferred.

Table 13 and Table 14 below give the general performance of the method Tuneable Resistive Pulse Sensing and the performance table for this method, respectively.

Table 13: General performance of Tunable Resistive Pulse Sensing (TRPS) / Electrical sensing zone (ESZ) / nano Coulter counter

Main features	
Type of samples	particles (solid, liquid, gaseous) in liquid dispersion medium
Type of sizing	counting technique (by identifying individual objects in continuous signals)
Particle property measured	particle volume equivalent sphere diameter: volume equivalent diameter
Type of quantity	particle number
size range	• minimum size ≥50 nm • maximum is in the range 1-10 µm
concentration range	10^5 - 10^{12} particles / mL
information content	very high, i.e. can well resolve details of the size distribution
Main advantages	
• intrinsically yields number-weighted size distributions, Q0 • good resolution of particle size distribution • provides number concentration of analysed sample	
Main disadvantages	
• does not resolve particle shape, but measures a volume equivalent diameter, • measures aggregate size rather than size of the constituent particles • lower size limit is clearly above 10 nm • limited dynamic range because of clogging	

Table 14: Technical characteristics for tunable resistive pulse sensing (TRPS)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar	No	

	liquids		
	Dispersible in polar liquids other than water	Yes	
	Dispersible in material-specific media	Yes	
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	No	
	Biological	Yes	
	Composite particles	Yes ⚠	Only outer size
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Only outer size
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes	Possible in certain cases
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	Measurement technique does not resolve shape
	2 (e.g. diameter of nanofibres)	No	Volume correct, if aspect ratio length/diameter<3
	3	Yes	Measures particle volume replacing liquid volume
Shape of nanoparticles	Sphere or similar	Yes ⚠	Measurement technique can measure objects of different shapes, but only provides an equivalent diameter
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other	No	

Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-25 °C)	Above 0 °C	No	
	Sensitivity above 25 °C	Yes	
	Sensitivity above 37 °C	Yes	
	Sensitivity above 50 °C	Yes	
	Sensitivity above 100 °C	Yes	
	Sensitivity above 150 °C	Yes	
	Sensitivity above 500 °C	Yes	
	Sensitivity above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-25 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	

Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Measurement technique measures only aggregate size
	Nanoparticles are not aggregated	Yes	Provided that they fit to the measurement range of the instrument
	Nanoparticles are agglomerated	Yes ⚠	Measurement technique measures only agglomerate size, and only if they are small enough to avoid capillary clogging
	Nanoparticles are not agglomerated	Yes	Provided that they fit to the measurement range of the instrument
Counting, fractionating or ensemble technique	Single particle counting	Yes	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	No	
	Used in hyphenated methods	No	
Working range	Size range	50 nm - 10 µm ⚠	Lower size limit depends on sensing pore size, which may be blocked. 50 nm - 10 µm for flow through membrane; 0.4µm – 1200 µm classical for capillary flow
	Concentration range	10^5 - 10^{12} particles/mL for porous membrane; 10^1-10^9 mL^{-1}: capillary flow	
	Minimum needed sample amount	500 µL	
	Linearity/proportionality	Yes	Pulse amplitude linear to displaced electrolyte volume
	Limits of detection/quantification	50 nm	For membrane flow technique

	Sensitivity (counting efficiency) as a function of size	good	
Trueness	Indicate the trueness of this measurement technique in measuring the particle size	good	
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	good	
Robustness	Specify the robustness of this measurement technique	average	
Precision	Specify the precision of the measurement technique		Not examined in NanoDefine; in general it depends on the number of particle counts
Resolution	Specify the resolution of this measurement technique	>= 2%	
Size distribution	Is it possible to measure size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes	
	Discrimination between NPs and non-NPs of another chemical composition	No	
	Discrimination from NPs of another chemical composition	No	Limited discrimination reported to be possible
	Impurities	No	Particulate impurities (in the right size range and with sufficient contrast) are detected. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	

Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	Volume equivalent diameter	
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suitable supports?	No	

3.5 Single particle ICP-MS (spICP-MS)

3.5.1 Measurement principle

Single particle inductively coupled plasma - mass spectrometry (spICP-MS) is based on the measurement of highly diluted nanoparticle dispersions by ICP-MS operated in time-resolved mode for a pre-selected mass-to-charge ratio (m/z) value[37, 38, 39, 40, 41]. Ideally, individual particles enter the ion source and are atomised and ionised in the plasma torch to produce a plume of element ions that is transferred to the mass spectrometric detector. The discrete measurement intervals (dwell times) of the MS are set to a value (≤ 10 ms) that allows the registration of the signal of the ion plume from only one particle. A prerequisite to operate in the single particle modus is (besides the short dwell times) that the concentration of particles is small enough to avoid simultaneous ionisation of more than one particle or the generation of overlapping ion plumes per dwell time. When these requirements are met, the signal intensity is proportional to the mass of the respective elements in the particle. The diameter of spherical particles can then be calculated from the measured mass based on the known or assumed stoichiometry and density of the target analyte. The number concentration of the particles in the measured dispersion can be inferred from the number of signals, the infusion rate, nebulisation efficiency and the acquisition time.

3.5.2 Performance

This measurement technique has a number of unique features. It is a relatively robust technique and can be run on conventional ICP-MS instruments that are widely available in both commercial and official control laboratories. Sample preparation is simple (often only dilution) and the measurement time per sample very short (1 min) which allows high throughput analysis. Furthermore, it is chemically specific and provides actual number-based size distributions.

Current application limits include:

- The detection limits in terms of size are constrained by (i) the sensitivity of the detector for the target element, and (ii) isobaric interferences/background for the target isotope. In general, sensitivity is higher for heavier elements.
- The correct size determination is limited to spherical particles of known density.
- Current instruments only allow mono-isotopic detection, i.e. different particles that carry the same target element cannot be distinguished (e.g. Ag NP from Ag/Au NP). New instruments are on the edge to allow bi-isotopic detection (at the cost of compromising correct quantification and thus size determination).
- Constituent particles in aggregates are not resolved, in agglomerates only by appropriate dispersion in the sample preparation step (not in the instrument).

Table 15 and Table 16 below give the general performance of the method single particle Inductively Coupled Plasma – Mass Spectrometry and the performance table for this method, respectively.

Table 15: General performance of single particle inductively coupled plasma – mass spectrometry (spICP-MS)

Main features	
Type of samples	particles (typically solid) in liquid dispersion medium
Type of sizing	counting technique (by identifying individual objects in continuous signals)
Particle property measured	mass of specified elements equivalent diameter: mass or volume equivalent diameter
Type of quantity	particle number
Size range	depending on target element, e.g.: Au 15 – 1000 nm, Ag 20 - 1000 nm, TiO_2 50 – 1000 nm, SiO_2 200 – 1000 nm
Concentration range	depending on element, particle size, instrument (e.g. Ag 60 nm: 5 – 500 ng/L)
Information content	• very high with respect to size distribution, i.e. can well resolve details of the size distribution • good (particle size, particle number concentration, mass concentration)

Main advantages
• intrinsically yields number-weighted size distributions, Q0 • good resolution of particle size distribution • provides number concentration of the analysed sample • chemically specific • rapid and cost-efficient
Main disadvantages
• does not resolve particle shape, but measures a mass or volume equivalent diameter and needs the particle density • measures aggregate size rather than size of the constituent particles • lower size limit is >10 nm and depends on material properties • cannot be applied to all kinds of material • accurate size determination limited to spherical particles

Table 16: Technical characteristics for single particle inductively coupled plasma – mass spectrometry (spICP-MS)

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	Yes ⚠	Only aqueous liquids
	Solid particulate form	No	
	Dispersed or embedded in matrices	No	
Dispersibility by dispersion protocols (instrument requires that test material is in liquid dispersion)	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	No	
	Dispersible in polar liquids other than water	No	
	Dispersible in material-specific media	⚠ No	Only aqueous media (which can be modified with e.g. dispersants, buffers, low percentage of organic solvents)
	Can be aerosolized	No	

Substance Nature	Inorganic	Yes ⚠	Sensitivity and interferences depending on element
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	No	
	Organic, particulate	No	
	Organic, non-particulate	No	
	Biological	No	
	Composite particles	Yes ⚠	Requires that particle contains detectable elements (of inorganic nature)
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	If particle contains detectable elements and the composition of core and shell is known
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	Measurement technique does not resolve shape
Shape of particles	Sphere or similar	Yes ⚠	Measurement technique can measure objects of different shapes, but only provides an equivalent diameter
	Equiaxial	Yes ⚠	
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other	No	
Thermal degradation sensitivity (Must be compatible with measurement technique working range: 15-40 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	

(Must be compatible with measurement technique working range: 15-40 °C)	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron-beam sensitivity	Electron-beam sensitive	Yes	
	Not electron-beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Measurement technique measures only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Measurement technique measures only agglomerate size
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	Yes	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	No	
	Used in hyphenated methods	Yes	E.g. with FFF
Working range	Size range	15 to 1000 nm	Depending on element (e.g. Ag: 15 – 1000 nm, TiO_2 50 to 1000 nm)

	Concentration range	N/A	Depending on element, particle size, instrument (e.g. Ag 60 nm: 5 – 500 ng/L). Result is mass
	Minimum needed sample amount	5 mL of injection dispersion	Usually not an issue due to the high dilution factors (1000 – 100 000) of the original sample
	Linearity/proportionality	size: no quantity: yes	Linear range depending on element, particle size, instrument
	Limits of detection (LoD)/quantification	N/A	Depending on element it is between 10 and 30 nm
	Sensitivity (counting efficiency) as a function of size	N/A	
Trueness	Indicate the trueness of the results of this measurement technique in measuring the particle size	good	Depending on analyte, matrix, laboratory
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	good	Starting from 2 x LoD
Robustness	Specify the robustness of this measurement technique	good	
Precision	Specify the precision of the measurement technique	0.1% to 2%	for $x_{50,0}$ [53]
Resolution	Specify the resolution of this measurement technique	1%	
Size distribution	Is it possible to measure size distribution?	Yes	Size distribution will be measured as a mass distribution
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes	
	Discrimination between NPs and non-NPs of another chemical composition	No	The technique is tuned to a single m/z value during measurement
	Discrimination from NPs of another chemical composition	No	The technique is tuned to a single m/z value during measurement
	Impurities	Yes ⚠	Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	

Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	Yes	At the moment only one m/z
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	mass equivalent diameter	Can be used to calculate diameter for spherical, non-porous particles by using a density
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suitable supports?	No	

4 Ensemble methods (spectroscopic)

4.1 Dynamic light scattering (DLS)

4.1.1 Measurement principle

Dynamic Light Scattering (DLS), also called Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a technique for characterisation of colloidal systems based on the scattering of visible light resulting from the difference in refractive index between the dispersed colloids and the dispersion medium. Fluctuations of scattered light may be caused by any changes in the microstructure of the suspension, e.g. by particle motion or vibrations of particle networks. For this reason, there are manifold applications for DLS, e.g. particle sizing, molecular weight determination, studying particle aggregation, monitoring phase transition in colloidal suspensions, or measuring the strength of colloidal gels.

The principle in DLS is measurement of fluctuations in laser light scattered by vibrating particles suspended in a liquid as function of time (Figure 3). The vibration is due to Brownian motion caused by collision with solvent molecules of the liquid. The Brownian motion varies as a function of particle size and causes variation in the intensity of scattered light as function of time. A correlator compares the signal measured at a time t_0 with different very short time delays Δt (autocorrelation). As the particles move, the correlation between t_0 and subsequent Δt signals decreases with time, from a perfect correlation (1) at t_0, to a complete decorrelation (0) at infinite time (order of milliseconds). In the case of large particles, the signal changes slowly and the correlation persists for a long time, whereas small particles have fast Brownian movement causing rapid decorrelation.

Thus, a DLS instrument measures the velocity of Brownian motion, defined by the translational diffusion coefficient D of the particles. The particle size, or more precisely its hydrodynamic diameter d_h, is then estimated using the Stokes-Einstein equation assuming spherical shape:

$$d_h = \frac{kT}{3\pi\eta D}$$

k: Boltzmann's constant

D: translational diffusion coefficient

T: absolute temperature

η: viscosity

It should be noted that even if a particle is truly spherical, the spherical DLS size is fundamentally different from the physical spherical size. The hydrodynamic size includes the double-layer of highly polarized water molecules around the physical particle. When the particle morphology is highly non-spherical, the hydrodynamic size should be understood as the equivalent hydrodynamic spherical size. Establishment of mean hydrodynamic size and size distributions (intensity, number, volume) is reached software algorithms, by fitting the correlation function in the data treatment.

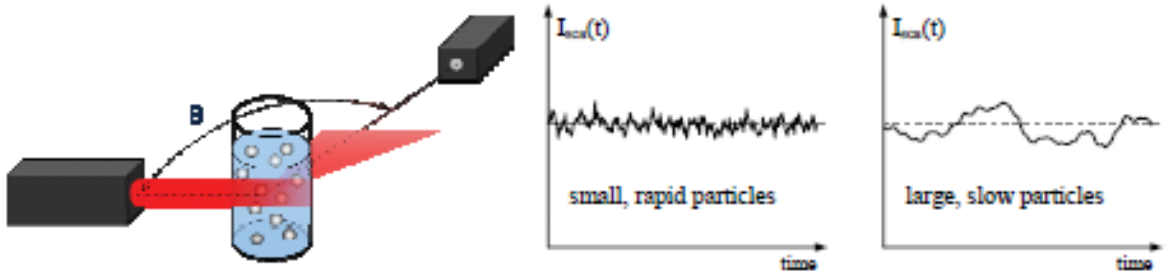


Figure 3: Measurement principle of DLS

The intensity fluctuations of DLS experiments can be analysed in terms of (ISO 22412 and [43]):

- a frequency spectrum (frequency analysis, FA), or
- a time correlation function (photon correlation spectroscopy, PCS).

PCS requires a different hardware than FA, but it can be shown that the results of both techniques are equivalent[42, 43]. Today, a large variety of commercial or "self-made" DLS instruments are used. Apart from data processing (FA or PCS), they can be distinguished with regard to laser optics and signal modulation.

In quiescent, dilute suspensions, the light fluctuations result essentially from the Brownian displacement of the single particles and thus reflect the particles' translational diffusion coefficient D_t. For spherical particles, this parameter (D_t) is inversely proportional to the sphere diameter (Stokes-Einstein equation).

4.1.2 Performance

Limits for the applicability of DLS are mainly set by size and concentration of particles. First of all, the concentration should be low enough to avoid strong multiple scattering. For particle sizing the concentration should be low enough to ensure measurement of short time self-diffusion of particles. As a rule of thumb, this may be achieved for concentrations below 0.01 to 0.1 vol.-%, but lower concentration values may be necessary for very fine nanoparticles.

Additionally to the upper concentration limits, there is also one at the lower range, where the intensity fluctuations start to become affected by the statistic variation of particle number in the measurement zone[44], a minimum of 100 particles should stay in the measurement zone. This is fulfilled for most colloidal suspensions; problems may arise for micrometre particles (i.e. ≥1 µm).

Sedimentation sets a limit to the detection of large particles because the particle displacement of micrometre particles is governed by sedimentation rather than by diffusion. This is of particular importance for polydisperse particle systems, where diffusion and sedimentation are coupled[45]. Moreover, sedimentation may affect the size distribution in the measurement zone. However, in the colloidal size range (x ≤1 µm) there is virtually no impact of sedimentation on DLS results[46]. A lower size limit exists only as much as the scattering intensity of the particles should considerably exceed that of the fluid molecules. Xu[43] proposes a minimum factor of 2.5; however, for highly reliable DLS this value should be multiplied by 10.

During the last two decades, dynamic light scattering has evolved into a major characterisation technique for colloidal suspensions. A recent interlaboratory study of the characterisation of a monomodal colloidal silica[47] showed that state-of-the-art DLS instrumentation facilitates a highly

reproducible and very reliable acquisition of correlation function and corresponding mean particle size x_{cum}. The study involved 17 participants from EU and USA, which provided 19 independent data sets from 6 different commercial instruments covering sideward scattering (90°) and backscattering. An earlier study with a different test material already indicated the high interlaboratory comparability[48].

Table 17 and Table 18 below give the general performance of the method dynamic light scattering and the detailed performance table for this method, respectively.

Table 17: General performance for dynamic light scattering (DLS)

Main features	
Type of samples	particles (solid, liquid, gaseous) in liquid dispersion medium
Type of sizing	spectroscopic ensemble technique
Particle property measured	diffusion coefficient, equivalent diameter: (apparent) hydrodynamic diameter (apparent because signal is affected by Brownian rotation)
Type of quantity	intrinsically: intensity of scattered light; for NPs: $I_{sca} \propto x^6$
Size range	1 nm to 1 µm
Concentration range	≤1 vol.-% (depends on the material)
Information content	relatively low, only few details of the PSD can be resolved
Main advantages	
• lower size limit <10 nm • relatively wide dynamic range • when DLS detects NPs then there are certainly NPs, • fast and robust • a minimum amount of information about the sample is needed to run the analysis • testing is non-destructive (sample may be recovered) • small amount of sample is needed to run a test	
Main disadvantages	
• number-weighted size distributions Q0 cannot directly be measured • does not resolve particle shape, but measures a hydrodynamic/mobility equivalent diameter, non-spherical particles will introduce errors • measures aggregate size rather than size of the constituent particles • size distribution obtained by inverting a signal spectrum → principally limits resolution • low resolution	

Table 18: Technical characteristics for dynamic light scattering (DLS)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes	
	Dispersible in polar liquids other than water	Yes	
	Dispersible in material-specific media	No	
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes ⚠	See size criteria
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	Yes	
	Composite particles	Yes ⚠	Outer particle size
	Other	No	
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Outer particle size
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	
Shape of nanoparticles	Sphere or similar	Yes	
	Equiaxial	Yes ⚠	Conventional DLS measurement and analysis do not resolve shape
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral	No	

	extension ≤ 0.25)		
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 5-60 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (Must be compatible with Measurement Technique working range: 5-60 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	Insensitive to fine NPs for (very) broad PSDs
	Monomodal	Yes	
	Multimodal	Yes	Insensitive to fine NPs for (very) broad PSDs
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Only agglomerate size
	Nanoparticles are not agglomerated	Yes	

Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	Yes	Intrinsically: intensity-weighted size distribution
	Integral technique	No	
	Used in hyphenated methods	Yes	E.g. in FFF for measuring hydrodynamic diameter at narrow size fractions
Working range	Size range	3 nm – 5 µm	
	Concentration range	0.00001 to 0.3 vol.-%	Depending on optical contrast, particle size
	Minimum needed sample amount	0.1 mL to 2 mL	Depending on instrumentation
	Linearity/proportionality	No Yes	No, with regard to size (decay of ACF [autocorrelation function] or spectral shift) Yes, with regard to concentration (total intensity) for diluted systems (where linear dependence on concentration)
	Limits of detection/quantification	1 nm to 20 nm	Depending on optical contrast and size
	Sensitivity (counting efficiency) as a function of size	good	Insensitive to very fine particles (weak scattering signal) and very coarse particles (do not contribute to signal fluctuation and disappear from measurement zone)
Trueness of particle size measurement	Indicate the trueness of this measurement technique	good	Size: “falseness” if wrong model parameters (e.g. viscosity, wavelength) or if too a high concentration (multiple scattering, hydrodyn. interaction)

Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	moderate	Intensity weights: "falseness" if multiple scattering or if too high laser intensities
Robustness	Specify the robustness of this measurement technique	good	
Precision	Specify the precision of the measurement technique	0.2% to 20% 0.4% to 5%	For $x_{50,0}$ (NanoDefine) For $x_{50,int}$[3] (NanoDefine)
Resolution	Specify the resolution of this measurement technique	20% to 50%	
Size distribution	Is it possible to measure size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes ⚠	If NP dominate
	Discrimination between NPs and non-NPs of another chemical composition	No	
	Discrimination from NPs of another chemical composition	No	
	Impurities	No	Particulate impurities (in the right size range and with sufficient contrast) are detected. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size	Specify: for example hydrodynamic…	apparent hydrodyn	Short time self-diffusion: translation affected by

[3] $x_{50,int}$ is a median of (scattering) intensity-weighted size distribution Q_{int}, which is intrinsically measured by DLS; the term Q_{int} is given in ISO 22412:2017

(diameter)		amic diameter	rotation for non-spherical objects
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	If dilution is required; otherwise not
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suited supports?	No	

4.2 Small-angle X-ray scattering (SAXS)

4.2.1 Measurement principle

Small-angle X-ray scattering (SAXS) is a technique based on the interaction between X-rays and matter to probe the structure of materials. X-rays passing through a medium that is not completely homogenous is forced to deviate from the straight direction. The scattering angle, i.e. the deviation from the straight line (see

Figure **4**), depends on the wavelength of X-rays and the size of the particles on which they are scattered. The processed data are the intensity, *I*, of X-ray scattered by a sample as a function of angular position of a detector.

The intensity can be expressed in relative units or in absolute scale. The absolute scale is independent from test parameters such as X-ray wavelength, experimental background, time of acquisition and sample thickness. In both cases, 2D raw data images are converted into diffractograms displaying the scattered intensity *I* as a function of scattering vector **q** defined by:

$$|\mathbf{q}| = \frac{4\pi \sin\theta}{\lambda}$$

with λ: X-ray wavelength.

The intensity pattern of the scattered radiation can give information about the particle size, size distribution, as well as the shape of the particles and their nanostructure. Size and shape of the particles are obtained by fitting of the measured scattering curves (intensity vs angle).

Two main applications of SAXS are characterization of nanoparticles and determination of large surface areas. The SAXS theory is based on fundamental physical processes[49], and it is very mature. A study of six European metrology institutes proved recently that SAXS allows traceable size determination of monomodal, spherical nanoparticles[11].

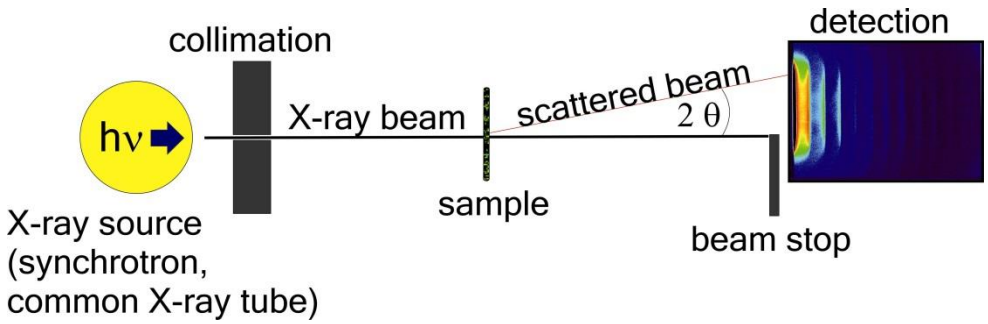


Figure 4: Measurement principle of SAXS

4.2.2 Performance

The method is accurate, non-destructive and requires only a minimum of sample preparation. SAXS covers only the lower range of interest from 1 to 100 nm. The experimental devices at synchrotrons and common laboratories are well developed. Numerous technical improvements have been made during the last five years. High throughput instruments were realized by using sample changing robots[50] and new X-ray detectors for low noise data recording are available[51] for elesynchrotron and laboratory SAXS. Currently, numerous SAXS manufacturers in Europe have improved SAXS instruments. These instruments are suitable for routine and standardized measurements in accordance with ISO standards.

Table 19 and Table 20 below give the general performance of the method small-angle X-ray scattering and the detailed performance table for this method, respectively.

Table 19: General performance for small-angle X-ray scattering (SAXS)

Main features	
Type of samples	particles (solid, liquid) dispersed in liquid media or in solid matrices; or solid particles in powder form (any two-phase systems where the phases have different density)
Type of sizing	spectroscopic ensemble technique
Particle property measured	scattering pattern (which reflects particle size, shape and orientation as well as state of aggregation) a) size analysis for particle with defined shape: external dimensions of the particles (e.g. diameter and length of rod-like particle) b) size analysis for particles of arbitrary (irregular) shape → scattering equivalent diameter
Type of quantity	intrinsically: particle surface (for typical size analysis)
Size range	1 to 100 nm (upper limit depends on specific instrument)
Concentration range	0.01 vol.-% to 100 vol.-% (lower limit depends strongly on size and density of particles, as well as on the quality of the instrument)

Information content	relatively low with respect to size distribution, i.e. only few details of the PSD can be resolved
Main advantages	
• can, in principle, resolve particle shape and aggregate structure • facilitates distinction between aggregates and their constituent particles • particles can be detected with minimal sample preparation. No or little danger exists for sample preparation artefacts • can be employed to determine the volume specific surface area (VSSA) • advanced instrumentation allows for determination of particle number concentration	
Main disadvantages	
• number-weighted size distribution cannot directly be measured • typically limited to particles ≤100 nm (even with advanced instrumentation ≤400 nm) • size distribution obtained by inverting a signal spectrum → principally limits resolution • low resolution • shape cannot be resolved for highly polydisperse materials	

Table 20: Technical characteristics for small-angle X-ray scattering (SAXS)

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	Yes	
	Solid particulate form	No	Determination of VSSA may be possible
	Dispersed or embedded in matrices	Yes ⚠	As long as the X-rays transmit through the material
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes ⚠	In-situ measurement
	Dispersible in non-polar liquids	Yes ⚠	
	Dispersible in polar liquids other than water	Yes ⚠	
	Dispersible in material-specific media	Yes ⚠	
	Can be aerosolized	No	Aerosols can be measured with special setups[52]
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes ⚠	Size dependent absorption or fluorescence in the UV-Vis range does not affect the SAXS results
	Carbon based	Yes	

	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	Yes	
	Composite particles	Yes	
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	No Yes ⚠	For unknown materials with increased polydispersity it is rather difficult to predict the measurement result of SAXS. Thickness of individual coatings can be determined if core and shell have different electron densities
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	Yes ⚠	Size and size distribution can be measured after knowledge of shape (from EM)
	2 (e.g. diameter of nanofibres)	Yes ⚠	
	3	Yes	
Shape of particles	Sphere or similar	Yes	
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	Yes	Yes for low polydispersity
	Flakes and discs (thickness:lateral extension ≤ 0.25)	Yes	Yes for low polydispersity
	Other	No ⚠	If different shapes are mixed in the same sample SAXS analysis is possible only with additional information such as shape information from imaging methods
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: -10-60 °C)	Above 0 °C	No	
	Sensitivity above 25 °C	Yes	
	Sensitivity above 37 °C	Yes	
	Sensitivity above 50 °C	Yes	
	Sensitivity above 100 °C	Yes	
	Sensitivity above 150 °C	Yes	
	Sensitivity above 500 °C	Yes	
	Sensitivity above 1000 °C	Yes	

Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: -10-60 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes ⚠	If polydispersity is above ~20%, data evaluation may become ambiguous without further information
	Monomodal	Yes	
	Multimodal	Yes ⚠	If modes are too close to each other, distinction of the modes is difficult
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes	
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes	
	Nanoparticles are not agglomerated	Yes	

Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	Yes	Intrinsically: surface-weighted size distribution
	Integral technique	No	
	Used in hyphenated methods[4]	No	
Working range	Size range	1 nm – 100 nm	Typically 1 to 100 nm, but size range depends on specific instrument. Specialized instruments work up to 10 micrometres.
	Concentration range	<= 1 vol%	The concentration range depends strongly on the electron density of the particles. The higher the electron density is, the more sensitive SAXS is
	Minimum needed sample amount	1 to 100 µL	Depending on the instrument and sample holder used
	Linearity/proportionality	Yes	
	Limits of detection/quantification	1 nm	
	Sensitivity (counting efficiency) as a function of particle size	good	Sensitivity increases with size
Trueness of particle size measurement	Indicate the trueness of this measurement technique in measuring the particle size	good	SAXS is a metrologically traceable method[11]
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions by this measurement technique	average	Can be quantified if uncertainties are considered properly[51]
Robustness	Specify the robustness of this measurement technique	good	Results are robust for defined SOPs of data processing
Precision	Specify the precision of the measurement technique	1% to 5%	For $x_{50,0}$ [53]

[4] There are 2 independent signals: average track length and spot size, i.e. diffusion coefficient and scattering intensity; a 2-dimensional plot of a monoconstituent sample would therefore show the functional relationship between size and scattering cross section (i.e. a strong, non-linear correlation); for multiconstituent samples we would expect different groups of such functional dependencies, which offers a possibility to couple size analysis with material or shape analysis

Resolution	Specify the size resolution of this measurement technique	5% to 20%	
Size distribution	Is it possible to measure particle size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	No	Size range is typically limited to <= 100 nm
	Discrimination between NPs and non-NPs of another chemical composition	No	
	Discrimination from NPs of another chemical composition	Yes	With contrast variation techniques
	Impurities	Yes	
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	Yes	Restricted to small agglomerates/aggregates (typically <100 nm)
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregates	Is the measurement technique able to characterise single constituent particles in aggregates?	No	SAXS is able to measure the (average size) of particles within aggregates, but not by counting as it is a spectroscopic method
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	physical length	Based on density differences
Destructive measurement technique or not	Is it a destructive measurement technique?	No	(high-flux) synchrotron radiation may induce damage
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	Yes, if required ⚠
Sample support	Does this measurement technique need preparation on suitable supports?	No	

4.3 Ultrasonic spectroscopy (USSp)

4.3.1 Measurement principle

Ultrasonic spectroscopy is the generic term for all particle sizing techniques that are based on the frequency-dependent measurement of sound velocity or attenuation in the ultrasonic domain (mostly within 100 kHz to 200 MHz). While velocity spectroscopy is mainly used for the study of inter- and intramolecular processes, attenuation spectroscopy has found its major application in particle sizing[54,55]. The most promising feature of acoustic characterisation techniques is their applicability to highly concentrated particle systems (up to 70 vol.-%) under non-equilibrium conditions[56]. That means it offers the opportunity to monitor the state of dispersion of dense product streams, to control the de-agglomeration of suspensions or the homogenisation of emulsions, and to study polymerisation or crystallisation processes.

As mentioned before, the attenuation spectroscopy has found application in particle sizing[55]. The principle of this technique is that plane sound waves moving through a particle suspension are attenuated in a predictable manner according to size and concentration of the particles in suspension.

Attenuation of an ultrasonic wave passing through a suspension may be modelled given a set of mechanical, thermodynamic and transport properties describing both the continuous and particulate media. The relationship between spectral data and particle size is illustrated by the attenuation curves. Each curve shows the attenuation of sound waves of a particular frequency as a function of the size of a monosize population of fixed volume concentration. Due to the measurement noise along with modelling errors, for the reliable analysis, measurements should be performed for a greater number of frequencies.

4.3.2 Performance

In principle, ultrasonic spectroscopy can be used for the characterisation of particles in the colloidal and micrometre size range, provided that the particle concentration is sufficiently high (at least 1 vol.-%) and that the signal contribution by the particles is significant compared to those by the continuous phase (may be a problem for attenuation measurements in highly viscous solvents).

A major difficulty for the particle sizing by USSp is that the acoustic behaviour depends on a variety of material parameters. This is of particular relevance for emulsions (14 properties), whereas for aqueous suspensions only the viscosity and sound speed of liquid and the density contrast have to be known[57].

In colloidal suspensions, the sound propagation is typically governed by the acoustophoretic motion of particles. For monodisperse spheroids that are not extremely flat or stretched (1/10 < aspect ratio < 10/1) the attenuation spectrum essentially reflects the volume specific surface area of the particles[58]. Similar results would probably be obtained for any convex particle shape. For particle aggregates, the inner structure is decisive. Regarding the type of quantity, acoustically measured size distributions are ideally volume-weighted distributions.

It could be shown that the results of ultrasonic spectroscopy agree fairly well with those of other characterization methods and are hardly affected by the extent of sample dilution[59]. Interlaboratory comparisons of ultrasonic spectroscopy measurements on suspensions of inorganic particles also showed good agreement[60]. That is why ultrasonic spectroscopy is considered as a powerful tool for

monitoring colloidal processes. However, the method does not allow for a very sharp resolution of size distributions in the colloidal size range[61].

Table 21 and Table 22 below give the general performance of Ultrasonic spectroscopy and the detailed performance table for this method, respectively.

Table 21: General performance of ultrasonic spectroscopy (USSp)

Main features	
Type of samples	particles (solid, liquid) in liquid dispersion medium
Type of sizing	spectroscopic ensemble technique
Particle property measured	depends on size range and kind of material • e.g. coarse particles ≥10 µm: acoustic scattering • e.g. fine particles ≤1 µm with high density contrast: acoustophoretic motion
Type of quantity	depends on way of signal processing; typically: particle volume
Size range	10 nm - 100 µm
Concentration range	> 1 vol %
Information content	• relatively low for x ≤1 µm, i.e. only few details of the PSD can be resolved • relatively high for x ≥10 µm, i.e. can well resolve details of the size distribution
Main advantages	
• for aggregates of NPs USSp detects the internal aggregate structure rather than the outer proportions, • lower size limit: 10 nm • wide dynamic range • does not require dilution for dense, turbid suspensions (which may affect the state of dispersion) • fast and robust, technique relatively easy to implement	
Main disadvantages	
• number-weighted size distributions cannot directly be measured • does not resolve particle shape, but measures an acoustical equivalent diameter • size distribution obtained by inverting a signal spectrum → principally limits resolution • low resolution of size distribution in the range ≤10 µm • does not allow the characterisation of dilute suspensions, i.e. requires a lot of substance • needs intense data evaluation based on mathematical modelling	

Table 22: Technical characteristics for ultrasonic spectroscopy (USSp)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes ⚠	If not too viscous
	Dispersible in polar liquids other than water	Yes ⚠	If not too viscous
	Dispersible in material-specific media	No	
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes ⚠	But not always, e.g. not feasible when density contrast NP/medium is not large enough
	Organic, non-particulate	No	
	Biological	No	
	Composite particles	Yes ⚠	Particle outer size, provided that effective material properties are known
	Other	No	
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Particle outer size
	Multishell particles	Yes ⚠	Particle outer size
	Particles with inclusions	No	Yes, particle outer size, if mixture composition is known ⚠
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	

Shape of nanoparticles	Sphere or similar	Yes	Measurement technique can measure objects of different shapes, but provides an equivalent diameter
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: -10-60 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity(of test material) (Must be compatible with Measurement Technique working range: -10-60 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	Difficult for NPs with broad PSD
	Monomodal	Yes	
	Multimodal	Yes	Difficult for NPs with broad PSD
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization /	Functionalised	Yes	

no functionalisation	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Reflects internal lengths (e.g. pore size, inter-particle distance)
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Reflects internal lengths (e.g. pore size, inter-particle distance)
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	Yes	Intrinsically: volume-weighted size distribution
	Integral technique	No	
	Used in hyphenated methods	No	
Working range	Size range	1 nm to 100 µm	
	Concentration range	2 to 70 vol.-%	
	Minimum needed sample amount	1 mL to 100 mL	Depends on instrumentation
	Linearity/proportionality	No Yes	No, with regard to size Yes, with regard to quantity (volume) for dilute suspensions
	Limits of detection/quantification	1 nm to 50 nm	Depends on density/ thermoacoustic contrast and size
	Sensitivity (counting efficiency) as a function of size	N/A	
Trueness of particle size measurement	Indicate the trueness of this measurement technique	average	Size: "falseness" if wrong model parameters (e.g. viscosity, sound speed) or if too a high particle concentration (>> 10 vol.-%)

Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	average	Volume weights: "falseness" if frequency range too narrow or PSD too broad (low information content below 5 µm)
Robustness	Specify the robustness of this measurement technique	good	
Precision	Specify the precision of the measurement technique	1% to 5%	Estimate based on Dukhin et al. 2012[60]
Resolution	Specify the resolution of this measurement technique	10% to 50% 5% to 20%	For x<10 µm For x>10 µm
Size distribution	Is it possible to measure size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same composition	Yes	
	Discrimination between NPs and non-NPs of another composition	Yes ⚠	No, if similar acoustic properties
	Discrimination from NPs of another chemical composition	No	
	Impurities	No	Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	⚠ However for known suspension/emulsion it is possible to recognise agglomeration from signals
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	Yes ⚠	Reflects internal lengths (e.g. pore size, inter-particle distance)
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	"acoustophoretic diameter"	For inorganic particles in water (i.e. high-frequency hydrodynamic diameter) which is approx. the specific surface area
Destructive measurement technique or not	Is it a destructive measurement technique?	No	

Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suited supports?	No	

4.4 Angular light scattering (ALS), including laser diffraction (LD)

4.4.1 Measurement principle

Angular light scattering (ALS) techniques measure the spatial distribution of scattered light. Historically, this has been realised by two different concepts of instrumentation which cover distinct size ranges. These are the *static light scattering* (SLS), which is conventionally employed for fine colloids, and the *laser diffraction*, which was originally used for micrometre particles only. Even though the measurement ranges of both techniques have actually converged in the recent past, there still remain qualitative differences in the sensor set-up and in data analysis, which justify their separate treatment.

SLS is operated for a wide range of scattering angles (typically of 10° to 150°). The time averaged angular distribution of scattered light is then commonly employed for the characterisation of macromolecules (molecular weight, radius of gyration, the second virial coefficients), but can be used to study suspensions of inorganic colloids as well (e.g. [62]). However, the angular distribution of scattered light is insensitive to particle size for nanoparticles, in which case only an average particle size can be determined. For very fine nanoparticles (<10 nm) the size information may be even completely lost. It could be shown that above this critical size, SLS measures the average particle size with fairly good reproducibility[63].

The term *laser diffraction* (LD) spectroscopy comprises angular light scattering techniques, which are primarily designed to resolve the scattering pattern at small scattering angles. Historically, LD instruments and software were restricted to the characterisation of micrometre objects for which the scattering pattern is mainly caused by diffraction and can be explained by Fraunhofer's theory from 1821. In the micrometre range, which is diffraction dominated, size distribution can be determined with high accuracy and good resolution[64, 65]. In order to extend the instrument applicability to colloidal particle systems, several modifications have been realised, e.g.: variation of wavelength and polarisation or inclusion of wide angle scattering ([43](p. 111-181); ISO 13320). These modifications have evidently enhanced the sensitivity to colloidal particles far below 1 µm, but not in a uniform, reproducible way as interlaboratory comparisons prove (e.g. [66]). Even so, laser diffraction may serve as a useful tool for the characterisation of colloidal suspensions, in particular for monitoring dispersion procedures or for evaluating the coarse particle content (≥ 1 µm).

4.4.2 Performance

'Optical' measurement techniques, including ALS and LS, are prone to underestimate the amount of NPs and, thus, to overestimate the number-weighted median $x_{50,0}$, ALS also has a lower detection limit well above 1 nm, which restricts its applicability and reduces its general reliability regarding the quantification of nanoparticle fractions[53, 66]. ALS was shown[53] to have deficiencies in measuring

the number-weighted median size ($x_{50,0}$) over the particularly interesting size range of 10–1000 nm, which is an important restriction for the applicability to identifying NMs.

Table 23 and Table 24 below give the general performance of angular light scattering and the detailed performance table for this method, respectively.

Table 23: General performance of angular light scattering (ALS)

Main features	
Type of samples	particles (solid, liquid, gaseous) in liquid media or particles (solid, liquid) in gas phase
Type of sizing	spectroscopic ensemble technique
Particle property measured	in general: scattering equivalent diameter for x>>1 µm (diffraction regime): dimensions of the projected image
Type of quantity	particle volume
Size range	100 nm - 1000 µm
Concentration range	<0.1 vol.-%
Information content	• relatively low for x ≤1 µm • relatively high for x ≥1 µm
Main advantages	
• fast and reliable particle sizing technique • high resolution of PSDs in the range x> 1 µm • small amount of sample is needed to run a test	
Main disadvantages	
• does not yield number-weighted size distributions • weak performance in the colloidal size range, in particular <100 nm • typically requires dilution of samples • needs data evaluation based on mathematical modelling • does not resolve particle shape, but measures an equivalent diameter	

Table 24: Technical characteristics for angular light scattering (ALS)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes	
	Dispersible in polar liquids other than water	Yes	
	Dispersible in material-specific media	Yes	
	Can be aerosolized	Yes	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	Optical properties should be known
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	No	
	Biological	No Yes	No for nanosized viruses Yes for cells ≥100 nm
	Composite particles	Yes	
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	No Yes ⚠	Yes if optical contrast of shell is not much smaller than that of core and structure is known OR in diffraction domain
	Multishell particles	No Yes ⚠	Yes if optical contrast of coatings is similar to that of core OR in diffraction domain
	Particles with inclusions	No	

Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	<100 nm not really accessible
	2 (e.g. diameter of nanofibres)	No	
	3	No Yes ⚠	<100 nm not really accessible, yet monodisperse equiaxial particles >70 nm may be measurable
Shape of particles	Sphere or similar	No Yes ⚠	<100 nm not really accessible, yet monodisperse equiaxial particles > 0 nm may be measurable. Measurement technique can measure objects of different shapes, but provides an equivalent diameter
	Equiaxial	No Yes ⚠	<100 nm not really accessible, yet monodisperse equiaxial particles >70 nm may be measurable
	Tubes, fibres, rods (length:diameter ≥ 3)	No	<100 nm not really accessible
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other	No	
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: -40 - 100 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: -40 - 100 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	

Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes ⚠	Difficult for sub-µm
	Monomodal	Yes	
	Multimodal	Yes ⚠	Difficult for sub-µm
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Only aggregate size, and <100 nm not really accessible
	Nanoparticles are not aggregated	No	<100 nm not really accessible
	Nanoparticles are agglomerated	Yes ⚠	Only agglomerate size, and <100 nm not really accessible
	Nanoparticles are not agglomerated	No	<100 nm not really accessible
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	Yes	Intrinsically: surface or volume-weighted size distribution
	Integral technique	No	
	Used in hyphenated methods[5]	Yes	E.g. in FFF for measuring scattering intensity and radius of gyration (there named as MALLS)

[5] There are 2 independent signals: average track length and spot size, i.e. diffusion coefficient and scattering intensity; a 2 dimensional plot of a monoconstituent sample would therefore show the functional relationship between size and scattering cross section (i.e. a strong, non-linear correlation); for multiconstituent samples we would expect different groups of such functional dependencies, which offers a possibility to couple size analysis with material or shape analysis

Working range	Size range	70 nm to 10 mm	Range dependent on instrument type, <100 nm only mean size
	Concentration range	0.0001 to 0.1 vol.-%	The concentration should be adjusted so that „obscuration“ lies within 2% to 20%
	Minimum needed sample amount	10 µL to 50 mL	Depends on instrumentation
	Linearity/proportionality	No Yes	No, with regard to size Yes with regard to concentration (total intensity)
	Limits of detection/quantification	50 nm to 100 nm	Depends on optical contrast and size
	Sensitivity (counting efficiency) as a function of particle size	good	Insensitive to very fine particles (weak scattering signal) and very coarse particles (which mainly scatter in forward direction)
Trueness of particle size measurement	Indicate the trueness of this measurement technique in measuring the particle size	average	Size: “falseness” if wrong model parameters (e.g. refractive index, wavelength) or if too a high concentration (multiple scattering)
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions by this measurement technique	average	Intensity weights: “falseness” if multiple scattering or if too high laser intensities
Robustness	Specify the robustness of this measurement technique	good	
Precision	Specify the precision of the measurement technique	0.1% to 2%	For $x_{50,3}$
Resolution	Specify the size resolution of this measurement technique	10% to 50% 10% to 20%	For x <1 µm For x >1 µm
Size distribution	Is it possible to measure particle size distribution?	Yes	

Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes ⚠	<100 nm not really accessible (depends on polydispersity)
	Discrimination between NPs and non-NPs of another chemical composition	No	<100 nm not really accessible
	Discrimination from NPs of another chemical composition	No	
	Impurities	No	Particulate impurities (in the right size range and with sufficient contrast) are detected. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregates	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	lengths of main axes	Needs shape model to be known, otherwise "equivalent ALS diameter"
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes ⚠	If dilution is required; otherwise not
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suitable supports?	No	

5 Fractionating methods

5.1 Field-flow-fractionation (FFF)

5.1.1 Measurement principle

FFF is a fractionation technique that separates particles, e.g. macromolecules or particles in colloidal suspensions, according to their hydrodynamic size. The sample is pumped through a channel in a laminar flow, which coincides with a parabolic velocity profile. Fluid elements close to the wall have a considerably higher residence time than fluid elements at the channel centre.[67] Perpendicularly to this channel flow, a field is applied. In most of the cases, it is a second flow but the field can be electric, magnetic or centrifugal. The field causes a separation of particles along the lateral axis with respect to their size, which means that they leave the channel after different residence times. Quantification of the amount of particles depends on the sort of detector used and its calibration. FFF is performed in liquids and it is limited to the analysis of the particles which can to be dispersed in liquids[67].

Figure 5 visualises the FFF technique principles; Figure 5a is a schematic diagram of a FFF-system including commonly used detection systems, and

Figure 5 shows a cross-section of the channel illustrating the separation mechanism.

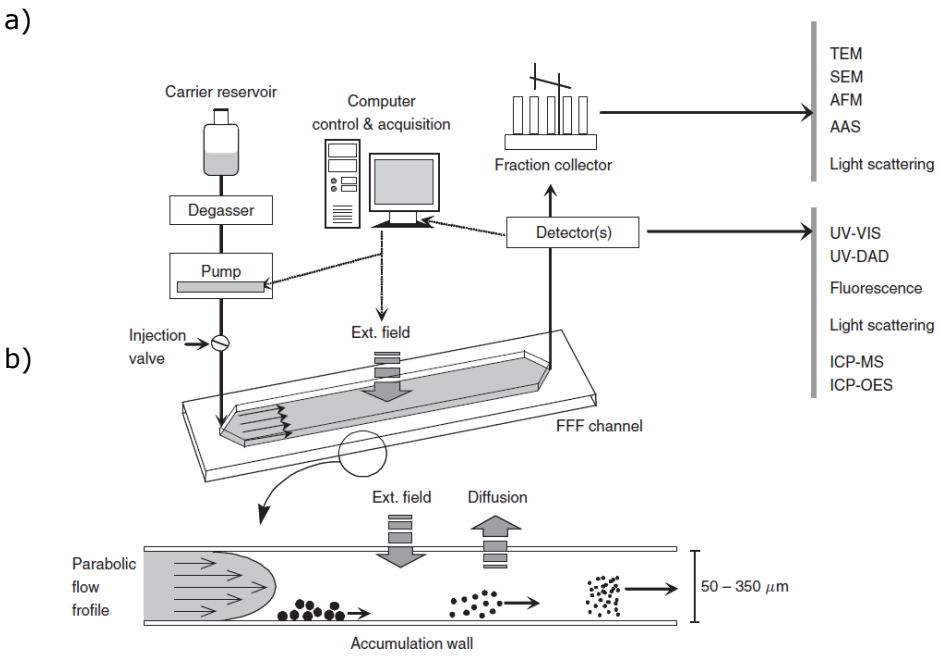


Figure 5: Measurement principle of FFF
a) schematic diagram of a FFF-system including commonly used detection systems; b) cross-section of the channel illustrating the separation mechanism (adapted from v. d. Kammer 2005)[67]

Several FFF techniques have been developed, based on various separation principles such as particle diffusion (e.g. asymmetric flow field-flow-fractionation, AF4, which is based on perpendicular flow field) or buoyant mass (e.g. sedimentation field-flow-fractionation, SedFFF, which is based on centrifugal force). In combination with a suitable detection system, FFF

techniques enable us to derive particle size or molecular mass from sample specific properties, such as particle diffusion. The separation concept of all FFF techniques is similar, as described above.

NanoDefine analysed in particular AF4 separation, during which a force drives the sample (i.e. dissolved and particulate components) towards the accumulation wall that is covered by an ultrafiltration membrane which is permeable for components smaller than the cut-off of the membrane. Components which are retained in the channel, will be distributed in the channel profile according to their diffusional properties (i.e. size), and as described above, depending on these properties the particles will experience different laminar flows resulting in a separation according to their hydrodynamic size. The separated sample is detected online by a suitable detector; the combination studied in NanoDefine was AF4-LS, asymmetric flow field flow fractionation –light scattering.

5.1.2 Performance

FFF separation methods provide a robust technique to separate particles according to their size or molecular mass. Together with a subsequent detection technique, FFF can provide distributions of both physical and chemical properties.

Current limits of application include:

- particle size determined by FFF is always an equivalent spherical particle diameter. Thus, values obtained for non-spherical particles from different sub-techniques will differ from each other,
- in case of ideal conditions in AF4 only diffusional properties of the sample affect the separation. However, in practice, completely ideal conditions cannot be achieved. Therefore, particle sizing might be affected by a number of potentially interfering instrumental factors. It has to be emphasized that optimization of the run conditions for both size standard and sample have to be performed rigorously until behaviour close to ideal elution is achieved,
- for SedFFF no size calibration is required. For size determination the knowledge of buoyant mass of the particle sample is required.

Table **25** and

Table 26 below give the general performance of field-flow-fractionation and the detailed performance table for this method, respectively.

Table 25: General performance of field-flow-fractionation (FFF)

Main features	
Type of samples	particles (solid, liquid) in liquid dispersion medium
Type of sizing	fractionating technique
Particle property measured	a) FFF with concentration detectors dependent on applied field, e.g. FlowFFF: particle diffusion coefficient (equivalent diameter : hydrodynamic / mobility diameter) b) FFF with concentration and size detectors dependent on size measurement behind FFF unit e.g. MALLS: radius of gyration, DLS: apparent hydrodynamic diameter
Type of quantity	detector dependent: UV/VIS: extinction (turbidity), for transparent nanoparticles ~ squared particle volume, for light-absorbing nanoparticles ~ particle volume fluorescence: particle mass concentration if particle is fluorescing, MALLS→ intensity of scattered light; ICP-MS → element mass concentration (element must be constituent of the particle and stoichiometry known); if counting detectors are used, particle number concentrations can be determined (e.g. spICP-MS)
Size range	in general 1-1000 nm. Instrumental settings need to be tuned for the size range of interest, dynamic range (ratio of upper to lower measurable diameter) is typically 20 – 40
Concentration range	adjustable, case-specific, the minimum and maximum acceptable concentration depends strongly on the sample characteristics, the size range and applied detector
Information content	relatively high, i.e. can well resolve details of the size distribution • for AF4: diffusion coefficient, hydrodynamic radius; • for Sedimentation FFF: volumetric radius • further information depending on coupled detector, e.g. - light scattering (SLS): root-mean-square (rms)-radius and geometric radius, intensity-weighted size distribution - ICP-MS: chemical composition, mass concentration, mass-based size distribution) - UV/DAD: indicator for chemical composition (element specific wavelength absorption and surface plasmon resonance)
Main advantages	

- physical separation of particles: can operate on complex mixtures and matrices
- element specific: can operate on complex mixtures and matrices
- coupling of FFF with arbitrary techniques for size and concentration measurement principally allows a comprehensive analysis with respect to sample chemical composition and particle shape

Main disadvantages

- typically: number-weighted size distribution Q0 cannot directly be measured.
- does not resolve particle shape, but measures a hydrodynamic/mobility equivalent diameter,
- measures aggregate size rather than size of constituent particles
- lower size limit depends on detectors, design of the FFF unit and parameters of operation
- limited dynamic range (different elution regimes for different particle sizes)
- high efforts for preparing FFF unit and sample
- operators require high level of proficiency

Table 26: Technical characteristics for field-flow-fractionation (FFF)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes ⚠	Channels for organic liquids available. Not routinely used
	Dispersible in polar liquids other than water	Yes	
	Dispersible in material-specific media	Yes ⚠	Case by case decision
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	Macromolecules and similar are possible
	Biological	Yes	
	Composite particles	Yes	
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Only outer size. Depends on the type of fractionation and the detector
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	
Shape of nanoparticles	Sphere or similar	Yes	Measurement technique can measure objects of different shapes, but typically provides an equivalent diameter
	Equiaxial	Yes	

	Tubes, fibres, rods (length:diameter ≥ 3)	No	⚠ Yes in certain cases with highly specialised instrumentation (impractical)
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 10-40 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (Must be compatible with Measurement Technique working range: 10-40 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	Only outer size
	Not functionalised	Yes	

Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes	⚠ Only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes	⚠ Only agglomerate size
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	Yes	⚠ Yes in special FFF-setups with a detection system that counts particles of narrow fractions provided by FFF
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	No	
	Used in hyphenated methods	Yes	Intrinsically hyphenated as fractionation and detection are locally separated, e.g. FFF+UV/VIS+MALS+spICP-MS
Working range	Size range	1 - 1000 nm	Depending on the detector and type of field, e.g. AF4-MALS: 3 nm to 1000 nm
	Concentration range	20 µg/L – 500 mg/L	Depending on the detector
	Minimum needed sample amount	10 µL	
	Linearity/proportionality	Size: No (for diffusion, volume, settling velocity or MALS) Quantity: Yes for light scattering. No for extinction - since measured via transmission	Depending on detector

	Limits of detection/quantification	1-10 nm	Depending on detector
	Sensitivity (counting efficiency) as a function of size	good	Detector dependent
Trueness of particle size measurement	Indicate the trueness of this measurement technique	average	If reference materials available
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	excellent	Mass quantification if performed by mass specific detector
Robustness	Specify the robustness of this measurement technique	good	Important parameter is the membrane quality
Precision	Specify the precision of the measurement technique	1 nm to 10 nm	
Resolution	Specify the resolution of this measurement technique	1 nm to 10 nm	
Size distribution	Is it possible to measure size distribution?	Yes	
Selectivity	Discrimination between NPs and non-NPs of the same chemical composition	Yes	⚠ Pre-treatment of the sample is necessary[6]
	Discrimination between NPs and non-NPs of another chemical composition	No Yes ⚠	Pre-treatment of the sample is necessary[6]. Depends on detection technique
	Discrimination from NPs of another chemical composition	Yes	Depends on detection technique
	Impurities	⚠	Depends on the detector. Dissolved impurities are ignored
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	

[6] Pretreatment of the sample is necessary because of an steric effect combined with a hydrodynamic lift effect; both phenomena push coarse particles back in the channel centre and cause them to leave the channel together with fine, rapidly diffusing particles; 'coarse' depends on the specific conditions of operation, but typically we talk about particles > 0.5 µm

Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	N/A	For chemical analysis the FFF needs to be coupled to ICP-MS or ICP-OES
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	Hydrodynamic diameter	Diffusion coefficient (FlowFFF) hydrodynamic diameter can be derived, volumetric diameter (SedFFF or CFFF). In few cases when MALS is applicable also root-mean-square and geometrical diameter, respectively
Destructive measurement technique or not	Is it a destructive measurement technique?	No	Fractions can be collected for the case that sample was not diluted
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suited supports?	No	

5.2 Analytical centrifugation (AC)

5.2.1 Measurement principle

Analytical centrifugation (AC) techniques employ the principle of particle migration in a viscous fluid under influence of a centrifugal force. This leads to variations in the local particle concentration when measured along the settling path and/or over time. Settling distance and settling time correspond to the terminal settling velocity, which in the case of isolated particles solely depends on their individual size, shape, and density. Hence, concentration profiles or time curves reflect the size distribution of the particle system.

Analytical centrifugation is realised in disc-centrifuges, cuvette centrifuges and analytical ultracentrifuge (i.e. cuvette centrifuges with extremely high centrifugal accelerations up to 10^6 g). The sedimentation process can be monitored by means of (spectral) light and X-ray extinction or via the interferometric measurement of the refractive index (RI). Cuvette and ultracentrifuges are operated in the "homogenous" mode, where the particles are homogenously distributed in the cuvette before measurement. Disc centrifuges also allow this mode of operation, yet are frequently operated in the "line-start" mode, where a thin strap of suspension is added on particle-free solvent with density gradient. This variety of centrifuges, detection systems and modes of operations makes it difficult to provide general answers on the specification. This report is restricted to disc

and cuvette centrifuges with light extinction measurement (discAc-turb, cuvAC-turb) and to ultracentrifuges with refractive index measurement (AUC-RI).

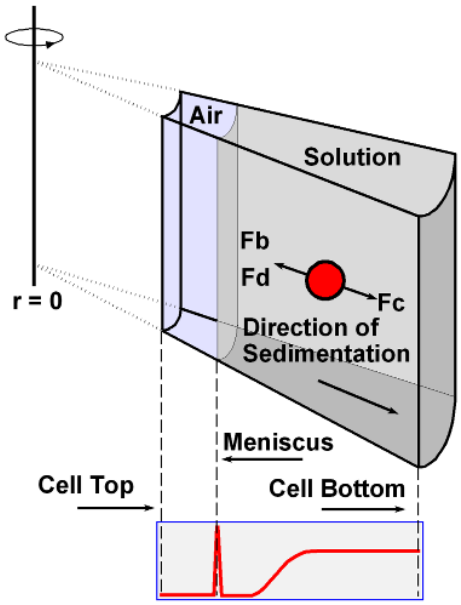


Figure 6: Schematic diagram of an AC cuvette centrifuge system

Disc centrifuges consist of a hollow disc which contains the suspension medium. Upon rotation, the liquid forms a stagnant layer on which a thin layer of the particle system is injected (line-start technique). The particles migrate according to their settling velocity to the outer diameter of the disc. All particles of a certain size (or settling velocity) move in a narrow band with growing distance from the initial position. In the case of multidisperse particle systems, one can observe several of such bands in analogy to chromatographic techniques. The radial concentration profile is, hence, a distorted projection of the density function of the size distribution (q(xStokes)). The line-start technique requires a density gradient in the suspension medium (e.g. by sugar) before the particles are injected; otherwise, there would be a convective transport of particles within strands of the (heavy) suspension layer. The density gradient sets some practical limits to the measurement (e.g. duration) and has to be calibrated before conducting size measurements.

A different set-up and mode of operation is found in cuvette centrifuges, where the particle sedimentation is observed in small cuvettes that are fixed on a rotating table. In this case, the particles are homogeneously suspended in the continuous phase before the centrifugation starts (homogeneous technique which is schematically presented, see Figure 6). During the centrifugation, all particles migrate towards the bottom of the cuvette, which results in the formation of a sediment, in a continuous decline of local particle concentrations above the sediment, and in a monotonous decrease of particle concentration in the direction from the sediment to the meniscus. The two types of variation in particle concentration, the temporal evolution, and the radial profile, can be considered as distorted projections of the cumulative function of the particle size distribution ($Q(x_{Stokes})$). The cuvette centrifuge was introduced by Svedberg and co-workers[68,69]. They called the instrument "ultra-centrifuge". Nowadays the term “Analytical Ultracentrifugation” (AUC) is only used for centrifugal accelerations above 100,000×g.

Apart from the centrifuge type, instruments differ with regard to the measurement of particle concentration. The most important principles are:

- optical extinction (photocentrifuge), which yields volume-weighted size distributions (q3) for light-absorbing nanoparticles and intensity-weighted size distribution for non-absorbing nanoparticles (q6);
- X-ray absorption (X-ray centrifuge), which always yields volume weighted size distributions (q3);
- refractive index determination by interferometry, which (approximately) yields volume-weighted size distributions for nanoparticles (q3).

Last but not least it should be mentioned that the AUC usually employs a set of different detection systems.

5.2.2 Performance

The AC technique is applicable to both suspensions and powders that can be suspended in liquids. All type pf materials (organic, inorganic) can be measured, however the effective density of particles must be different from the density of the dispersing liquid. Most instruments require that the particle density is higher than the density of water, which can make the measurement of organic particles difficult (note that some instruments also allow measurement of particles with a lower density than the surrounding liquid). The effective density of all particles must be homogenous and known before measurements. Sample preparation and the stability of the suspension are of key importance for the reliability of the measurements. The technique can measure particles below 10 nm but it measures the size of aggregates/agglomerates rather than constituent particles. The technique does not measure actual external dimensions but gives the diameter of an equivalent spheres, i.e. spheres that sediment as fast as the particles measured. It does not provide number-based particle size distribution within acceptable limits of uncertainty.

Table 27 and Table 28 below give the general performance of analytical centrifugation and the detailed performance table for this method, respectively.

Table 27: General Performance of analytical centrifugation (AC)

Main features	
Type of samples	particles (solid, liquid, gaseous) in liquid dispersion medium
Type of sizing	fractionating technique
Particle property measured	• settling velocity • equivalent diameter: • Stokes diameter determined from settling velocity and skeletal density, or • hydrodynamic diameter from settling velocity and the effective particle density (used for compact aggregates)
Type of quantity	• dependent on detector • solid volume by X-ray, refractive index or turbidity measurement of light-absorbing nano-particles (q3) • squared solid volume by turbidity measurement non-absorbing nano-particles (q6) • for non-nanoparticles the physical quantity should be stated (e.g. extinction)

Size range	< 5 nm - 10 µm with minimum depending on particle density
Concentration range	≤ 0.1 wt.%
Information content	relatively high, i.e. can well resolve details of the size distribution
Main advantages	
• lower size limit < 10 nm • good resolution of particle size distribution (for discAC even high resolution) • in principle wide dynamic range • rapid screening	
Main disadvantages	
• number-weighted size distribution Q0 cannot directly be measured • does not resolve particle shape, but measures a settling velocity equivalent diameter (i.e. Stokes diameter), non-spherical particle geometry will introduce errors • measures aggregate size rather than size of constituent particles • only discAC: limited dynamic range - needs a balance between sedimentation rate (depending on particle size), density of gradient and centrifugal speed.	

Table 28: Technical characteristics of analytical centrifugation (AC)

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	Yes	
	Solid particulate form	No	
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes	
	Dispersible in non-polar liquids	Yes ⚠	Depending on compatibility of liquid with instrument
	Dispersible in polar liquids other than water	Yes ⚠	
	Dispersible in material-specific media	Yes ⚠	Depending on compatibility of liquid with instrument
	Can be aerosolized	No	

Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes ⚠	Size-dependent scattering relevant to algorithm converting detector signal to mass value
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	No Yes ⚠	Only for AUC-RI
	Biological	Yes ⚠	Need for material density, which is not always known for biological samples
	Composite particles	No Yes ⚠	The composition needs to be known in some detail and e.g. just TiO_2 core with SiO_2 shell would be not enough
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	No Yes ⚠	The composition needs to be known in some detail and e.g. just TiO_2 core with SiO_2 shell would be not enough
	Multishell particles	No	⚠ Theoretically possible but in practice very complex
	Particles with inclusions	No Yes ⚠	Not possible with standard AC instrument (single-detector operation) Possible by combining different optical (turbidity, RI, UV) and X-ray detectors
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	Does not resolve shape
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	
Shape of nanoparticles	Sphere or similar	Yes	Measurement technique can measure objects of different shapes, but provides
	Equiaxial	Yes ⚠	

			an equivalent diameter
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	No	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 5-60 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 5-60 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes ⚠	Functionalisation can affect the detected size, if it significantly changes size and density of the particles
	Not functionalised	Yes	

Agglomeration / aggregation state	Nanoparticles are aggregated	Yes ⚠	Measurement technique measures only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Measurement technique measures only agglomerate size
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	Yes	
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	No	
	Used in hyphenated methods	No	However AUC can employ multiple detectors to acquire differently weighted size distributions during the same fractionation or to measure UV-vis-spectra of sedimenting samples
Working range	Size range	1 nm-10 µm	Range depends on type of instrument, sample, preparation. Maximum and minimum may not be possible in a single run. 1 nm needs AUC
	Concentration range	0.0001 to 0.3 vol.%	Depending on material, size and detector type
	Minimum needed sample amount	0.1 to 1 mL	
	Linearity/proportionality	No	Non-linear linearity in size: No linearity in quantity: No normally, Yes for AC-RI
	Limits of detection/quantification	1 to 50 nm	Strongly depending on material, size and detector type
	Sensitivity (counting efficiency) as a function of size	good	Decreasing sensitivity with size for photometric detection

Trueness of particle size measurement	Indicate the trueness of this measurement technique	average to good	
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	average to excellent	Depends on material and algorithms used to convert measured signal to weight-% and then to number-%. if "weights" refers to the intrinsic type of quantity (e.g. turbidity/extinction or volume) it is 'excellent' for AC, if it refers to "number" then it is "average to good"
Robustness	Specify the robustness of this measurement technique	good	Particle density must be reliably known
Precision	Specify the precision of the measurement technique	0.1% to 4% (17%) 0.1 to 2% (8%)	For $x_{50,0}$[70, 53] For $x_{50,ext}$[71, 53]
Resolution	Specify the resolution of this measurement technique	2%	For non-aggregated materials
Size distribution	Is it possible to measure size distribution?	Yes	
Selectivity	Discrimination between nanoparticles and non-NPs of the same composition	Yes	
	Discrimination between nanoparticles and non-NPs nanoparticles of another composition	No	AC probes the settling velocity of particles; particles of different composition (density) may settle equally fast although they differ in size; moreover, a different composition means a different detection sensitivity ➔ interpretation of measured size distributions is hardly possible
	Discrimination from nanoparticles of another chemical composition	No	
	Impurities	No	Dissolved impurities are ignored

Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	Stokes diameter	i.e. equivalent diameter for settling velocity based on the particles' skeleton density
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suited supports?	No	

5.3 Differential electrical mobility analysis (DEMA)

5.3.1 Measurement principle

The differential electrical mobility analysis is a technique for sizing submicron aerosol particles based on their electrical mobility. For this purpose, the particles needs to be electrically charged in a defined manner, classified according to their electrical mobility and finally quantified for each mobility fraction. There are different ways to realise the DEMA principle in technical instruments, for which reasons there is a number of varying terms: MPSS for mobility particle size spectrometer, SMPS for scanning mobility particle sizer, DMPS for differential mobility particle sizer and DMAS for differential mobility analysing system.

DMAS combines a particle classifier (differential mobility analyser DMA or DEMC for differential electrical mobility classifier) that transmits particles within a narrow interval of sizes from an initially polydisperse aerosol, and a detector (for example, a condensation particle counter CPC) that counts the particles within that differential size interval. First, the aerosol passes through an inertial impactor to avoid that particles larger than 1 micrometre enter the DMA column, then the aerosol enters a particle charge conditioner like a charge neutraliser to be conditioned, so, particles that carry several charges lose their charge excess. Once the aerosol is well conditioned, particles are selected using electrical classification inside DMA column: an electric field is created and the

airborne particles drift along the DMA according to their electrical mobility Z_d. It is related to the particle diameter d_p via the expression:

$$Z_d(p, d_p) = \frac{peC_c(d_p)}{3\pi\mu_g d_p}$$

where e is the charge of the electron, μ_g the dynamic gas viscosity and C_c the slip correction factor defined as:

$$C_c(d_p) = 1 + K_n(d_p)\left[\alpha + \beta \exp\left(\frac{\gamma}{K_n(d_p)}\right)\right]$$

$$K_n(d_p) = \frac{2\lambda_m}{d_p}$$

where K_n is the Knudsen number and λ_m is the mean free path of a particle. (α; β; γ) are taken from experiments.

Figure 7 presents the fundamental components of DMAS (ISO 15900:2009). The pre-conditioner indicated in this figure serves generally two goals: removing the large particles with impactor the most used and, if necessary, reducing the sample humidity using a dryer. Concerning the aerosol detector, there are two types of detector: a CPC and an aerosol electrometer. Concerning the particle charge conditioner, a bipolar diffusion particle charger (also called aerosol neutralizer) is often used in SMPS. This is often done using a radioactive source like ^{85}Kr or a bipolar ion generator. These chargers establish the equilibrium charge distribution on the aerosol particles. Unipolar Corona chargers may also be used in a DMAS. The DMAS is operated by software controlling the sheath air flow, reading the aerosol flow, reading other system parameters such as T, p, setting the voltage, and reading the CPC output.

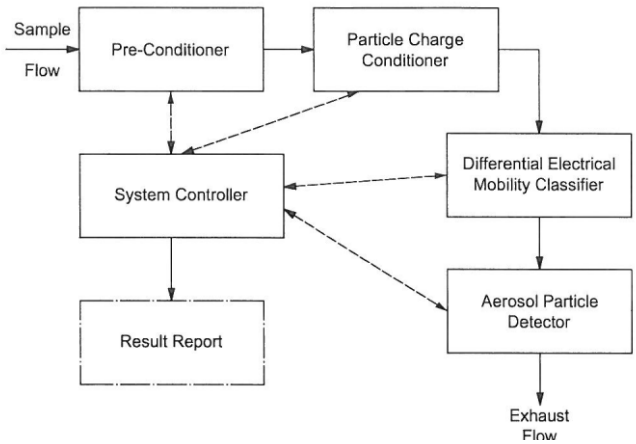


Figure 7: Fundamental components of the differential mobility analysing system (ISO 15900:2009)

5.3.2 Performance

Current limitations of this method include:

Stability of the aerosol:

The system (DMA + CPC) can only be used when the aerosol is stable during the time of scan. If the aerosol (number size distribution) is unstable below the specified scan time, other systems must be used (DMA + Electrometers detectors like commercial instrument DMS 500, FMPS, EEPS). Nevertheless, it has to be noted that the size resolution for such system is lower compared to the coupled system DMA and CPC.

- Strongly dependent on the physical model used to retrieve the size distribution (charge distribution function, transfer function, etc.)
- When using the system (DMA + CPC), the estimation of the size distribution strongly depends on the type of inversion being implemented in the commercial software.
- System is optimized for particles with spherical shape.

Data analysis, resolution of particle size distribution: The measured electrical mobility distribution is converted to a particle number size distribution employing the charge distribution (see also ISO 15900) and the DMA-transfer probability. Additional corrections could be done for internal particle losses due to diffusion and the size-dependent CPC counting efficiency.

The performances of four SMPS were evaluated by Fissan et al.[72] under the same conditions for flow rates, flow ratio, input monodisperse aerosols, and transport-line lengths in the 6–50 nm size range. Their results provide a quantitative comparison of the mobility resolution and diffusion loss of the nanometre aerosols in such systems. Moreover, the performance assessment of Fast MPS (FMPS) and ultrafine water-based condensation particle counter (UWCPC) equipped SMPS was performed by Jeong and Evans[73] under various conditions on urban ambient particles, urban indoor particles, rural ambient particles, and laboratory-generated particles. Asbach et al.[74] tested four different mobility particle sizers on NaCl and diesel soot particles measurements. Recently, Wiedensohler et al.[75] report on harmonization of measurement procedures to facilitate high quality long-term observations of atmospheric particle size number distributions obtained by SMPS. Some results of metrological measurement of NP size and size distribution by SMPS have been carried out recently in the frame of various interlaboratory comparisons specially dedicated to this purpose[13]. Electrospray-differential mobility analysis (ES-DMA), a technique that exerts electrical and drag forces on clusters, can be used to determine the size and packing of colloidal small clusters (and aggregates more generally) of nanoparticles[76]. NanoDefine studied especially spray-DEMA.

Table 29 and Table 30 below give the general performance of differential electrical mobility analysis and the detailed performance table for this method, respectively.

Table 29: General performance of differential electrical mobility analysis (DEMA)

Main features	
Type of samples	airborne submicrometre particles; either prepared by dispersing powders in a gas phase or by spray-drying of dilute suspensions, or taken from environment
Type of sizing	fractionating technique
Particle property measured	electrical mobility equivalent diameter: mobility diameter
Type of quantity	particle number
Size range	2.5 nm to 1 µm (range varies in dependence on instrument type like DMA and CPC and the parameter used (flow rate,...))

Concentration range	1 to 10^8 particles/cm^3 (10^8 can be achieved by counting with electrometers, whereas CPC maximum concentration counting is 10^7 particles/cm^3)
Information content	relatively high, i.e. can well resolve details of the size distribution
Main advantages	
• intrinsically measures number-weighted size distributions • applicable to polydisperse population between 3 nm to 1 µm • good resolution of particle size distributions • provides number concentration of the analysed sample • quick measurement (a few min)	
Main disadvantages	
• does not resolve particle shape, but measures a mobility equivalent diameter, • measures aggregate size rather than size of the constituent particles • particles > 1 µm are physically removed from aerosol sample; they are thus not measured • spray-dried aerosol samples may contain nanosized contaminant particles generated from previously dissolved species	

Table 30: Technical characteristics for differential electrical mobility analysis (DEMA)

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	Yes ⚠	spray-DEMA: Measurement technique measures only aerosolized NPs
	Solid particulate form	No ⚠ Yes ⚠	spray-DEMA: No as a liquid suspension is needed DEMA: Yes; DEMA is intended to measure aerosols, including ultrafine dust or aerosolised powders
	Dispersed or embedded in different kinds of matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	Yes ⚠	Measurement technique measures only aerosolized NPs
	Dispersible in non-polar liquids	Yes ⚠	
	Dispersible in polar liquids other than water	Yes ⚠	
	Dispersible in material-specific media	No	
	Can be aerosolized	Yes	Measurement technique measures only aerosolized particles

Substance Nature	Inorganic	Yes	
	Size-dependent absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	Yes	
	Composite particles	Yes	
	Other	Yes	
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Only outer size
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	No	Does not resolve shape
	2 (e.g. diameter of nanofibres)	No	
	3	Yes	
Shape of nanoparticles	Sphere or similar	Yes	Measurement technique can measure objects of different shapes, but only provides an equivalent diameter
	Equiaxial	Yes ⚠	
	Tubes, fibres, rods (length:diameter ≥ 3)	No	
	Flakes and discs (thickness: lateral extension ≤ 0.25)	No	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 10-40 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes	
	Sensitive above 37 °C	Yes	
	Sensitive above 50 °C	Yes	
	Sensitive above 100 °C	Yes	
	Sensitive above 150 °C	Yes	
	Sensitive above 500 °C	Yes	
	Sensitive above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	

range: 10-40 °C)	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	
Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Only aggregate size
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes ⚠	Only agglomerate size. Spray-DEMA: only if agglomerates are small enough to avoid capillary clogging
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	Yes	Measurement technique measures the number concentration of very narrow particle size fractions
	Calculates number or number concentration from spectroscopic ensemble methods	No	
	Integral technique	No	
	Used in hyphenated methods	Yes	E.g. by coupling DEMA with aerosol centrifuges (not standard)

Working range	Size range	2 nm to 1 µm	Range varies in dependence on instrument type like DMA and CPC and the parameter used (flow rate,…)
	Concentration range	10^4 to10^6 particles/cm^3	For aerosols and dispersed powders and for CPC For sprayed suspensions: 10^4 to 10^7 particles/cm^3 (suspension)
	Minimum needed sample amount	⚠	For aerosols: approx. 30 L For powders: <10 g For sprayed suspensions: 10 to 100 mL
	Linearity/proportionality	Yes No	With regard to concentration With regard to particle size (for small particle diameters the fraction of charged particles is very low)
	Limits of detection/quantification	2 nm to 10 nm	Depending on instrument type and operation
	Sensitivity (counting efficiency) as a function of size	good	There is a strong impact of the size particle <10 nm for the counting efficiency (CPC)
Trueness of particle size measurement	Indicate the trueness of this measurement technique	good	
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions of this measurement technique	good	
Robustness	Specify the robustness of this measurement technique	good	
Precision	Specify the precision of the measurement technique	0.2 to 5%	For $x_{50,0}$ (NanoDefine)
Resolution	Specify the resolution of this measurement technique	3%	
Size distribution	Is it possible to measure size distribution?	Yes	

Selectivity	Discrimination between nanoparticles and non-NPs of the same composition	Yes	
	Discrimination between nanoparticles and non-NPs of another composition	Yes	
	Discrimination from nanoparticles of another chemical composition	No	
	Impurities	N/A	"impurities" only refer to residual particles (salt, polymers) after aerosolisation from suspension; this entry is irrelevant for aerosol characterisation
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	Yes ⚠ No ⚠	Yes for typical DEMA setup and low aerosol concentration, No otherwise (including spray-DEMA)
Counting constituent particles in aggregations	Is the measurement technique able to characterise single constituent particles in aggregates?	No	
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	Electrical mobility diameter	equivalency with respect to the electrical mobility, based on the particle's electric charge (which is assumed to be known)
Destructive measurement technique or not	Is it a destructive measurement technique?	Yes	
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	No	
Sample support	Does this measurement technique need preparation on suited supports?	No	

6 Integral methods

6.1 BET for determination of volume specific surface area (VSSA)

6.1.1 Measurement principle

The Brunauer-Emmett-Teller (BET) theory was derived in 1938 to explain the physical adsorption of gas molecules on a solid surface[77]. BET serves as the most often applied technique for the measurement of the specific surface of powders. BET explains mono- and multilayer adsorption of gas molecules on a solid and dry material. Nitrogen and argon gas are widely used for measurements. BET is based on three hypotheses:

1.) gas molecules physically adsorb in infinite layers,

2.) no interactions exist between adsorbed layers, and

3.) the Langmuir theory is applicable for each layer of gas molecules.

The resulting BET equation is applied for fitting experimental gas adsorption isotherms and gives the adsorbed monolayer gas quantity. Knowledge of gas quantity, adsorption cross section of the adsorbing gas and the molar gas volume allows calculation of the specific surface area of the material[78].

The BET equation is

$$\frac{1}{v[(p_0/p)-1]} = \frac{c-1}{v_m c}\left(\frac{p}{p_0}\right) + \frac{1}{v_m c}$$

where p and p_0 are the equilibrium and the saturation pressure of adsorbates at the temperature of adsorption, v is the adsorbed gas quantity (for example, in volume units), and v_{mono} is the monolayer adsorbed gas quantity, c is the BET constant.

$$c = exp(\frac{E_1 - E_{L1}}{RT})$$

where E_1 is the heat of adsorption for the first layer, and E_L is that for the second and higher layers and is equal to the heat of liquefaction.

The equation is an adsorption isotherm and can be plotted as a straight line with the y-axis showing $1/v[(p0/p)-1]$ and $\varphi = p/p0$ on the x-axis according to experimental results (BET plot). p is the equilibrium pressure and p_0 is the saturation pressure. The value of the slope, A, and the y-intercept, I, of the line are used to calculate the monolayer adsorbed gas quantity v_{mono} and the BET constant c. The following equations are used:

$$v_m = \frac{1}{A+I} \quad and \quad c = 1 + \frac{A}{I}$$

A total surface area S_{BET}, total and a specific surface area S_{BET} are estimated by the following equations:

$$S_{BET,Total} = \frac{v_m N S}{V} \quad and \quad S_{BET} = \frac{S_{Total}}{a}$$

where v_m is in units of volume which are also the units of the molar volume of the adsorbate gas, N_A is Avogadro's number, S is the adsorption cross section of the adsorbing species, V is the molar volume of adsorbate gas, a is the mass of adsorbent (in g).

6.1.2 Performance

The BET method is widely used and accepted in industry, academia and (governmental and regulatory) research institutes. For example, the National Institute of Standards and Technology (NIST, USA) and BAM provide a practical guide for its application which is available without charge from NIST[79]. BET can be applied easily and is also standardized by ISO (ISO 9277:2010). The BET theory is based on expansive assumptions (see above), and therefore, the results obtained by BET are method-defined[80]. Also different values for the same material can be obtained if different gases are used. As a consequence, the specific surface area values should be named BET surface area and must often be considered as apparent. Nevertheless, Round-Robin tests for the development of reference materials for BET as performed by BAM proved good accuracy of the BET method[81].

Table 31 and Table 32 below give the general performance of BET for determination of specific surface area and the detailed performance table for this method, respectively.

Table 31: General performance table for BET for determination of specific surface area

Main features	
Type of samples	dry powder
Type of sizing	integral sizing technique (yielding a mean size of the particle system)
Particle property measured	• mass specific surface area, • mean size for non-porous particles of unknown shape: surface-weighted mean of VSSA equivalent diameter • mean size for non-porous particles of known dimensionality: surface-weighted mean of VSSA equivalent smallest external dimension
Type of quantity	surface-weighted
size range	all size ranges
concentration range	not applicable (measurements at dry powders)
information content	one piece of information
Main advantages	
• measures size of constituent particles within aggregates • lower size limit < 10 nm • wide dynamic range • certified reference materials are available for a wide range of SSA up to 1300 m2/g,	
Main disadvantages	
• cannot resolve size distribution • mean size is surface-weighted • does not resolve particle shape, application to material classification according to the EC NM Definition requires knowledge of dimensionality • Materials must be free of any volatile compounds, for example, water-free • Measurement times can be in the range of hours and increase with increasing surface area	

Table 32: Technical characteristics for BET for determination of specific surface area

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	No	
	Solid particulate form	Yes	
	Dispersed or embedded in matrices	No	
Dispersibility by dispersion protocols	Dispersible in aqueous media	No	
	Dispersible in non-polar liquids	No	
	Dispersible in polar liquids other than water	No	
	Dispersible in material-specific media	No	
	Can be aerosolized	No	
Substance Nature	Inorganic	Yes	
	Size-dependent light absorption / fluorescence	Yes	
	Carbon based	Yes	
	Organic, particulate	Yes	
	Organic, non-particulate	Yes	
	Biological	No	
	Composite particles[7]	Yes	
	Other		
Composite particles (see Section 2.2.1.3)	Core/shell	Yes ⚠	Measures only surface accessible by the probe gas
	Multishell particles	Yes ⚠	
	Particles with inclusions	Yes ⚠	
Number of nanoscaled dimensions	1 (e.g. thickness of nanoplates)	Yes	Size derived from measured surfaces as available for gas sorption with pre-knowledge on particle shape (from a descriptive SEM micrograph)
	2 (e.g. diameter of nanofibres)	Yes	
	3	Yes	Size derived from measured surfaces as available for gas sorption

[7] See NanoDefine Manual, part 1 for definition

Shape of particles	Sphere or similar	Yes	Measurement technique can measure objects of different shapes, but provides a mean equivalent thickness
	Equiaxial	Yes	
	Tubes, fibres, rods (length:diameter ≥ 3)	Yes	
	Flakes and discs (thickness:lateral extension ≤ 0.25)	Yes	
	Other		
Thermal degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-40 °C)	Above 0 °C	No	
	Sensitive above 25 °C	Yes ⚠	If the sample preparation includes outgassing by heating, (often at 150 °C), and the sample is sensitive to that temperature, the answer is No
	Sensitive above 37 °C	Yes ⚠	
	Sensitive above 50 °C	Yes ⚠	
	Sensitive above 100 °C	Yes ⚠	
	Sensitive above 150 °C	Yes ⚠	
	Sensitivity above 500 °C	Yes	
	Sensitivity above 1000 °C	Yes	
Cooling degradation sensitivity (of test material) (Must be compatible with Measurement Technique working range: 15-40 °C)	Sensitive below 25 °C	Yes	
	Sensitive below 0 °C	Yes	
	Sensitive below -18 °C	Yes	
	Sensitive below -35 °C	Yes	
	Sensitive below -78 °C	Yes	
	Sensitive below -195 °C	Yes	
Electron beam sensitivity	Electron beam sensitive	Yes	
	Not electron beam sensitive	Yes	
Particle size dispersity and modality	Monodisperse	Yes	
	Polydisperse	Yes	
	Monomodal	Yes	
	Multimodal	Yes	
Conductivity properties (electrical)	Conductive	Yes	
	Semiconductive	Yes	
	Insulator	Yes	
Magnetic properties	Magnetic	Yes	
	Non magnetic	Yes	
Functionalization / no functionalisation	Functionalised	Yes	
	Not functionalised	Yes	

Agglomeration/ aggregation state	Nanoparticles are aggregated	Yes ⚠	Resulting surface will be smaller than the sum of the surfaces of the individual particles
	Nanoparticles are not aggregated	Yes	
	Nanoparticles are agglomerated	Yes	
	Nanoparticles are not agglomerated	Yes	
Counting, fractionating or ensemble technique	Single particle counting	No	
	Measures or calculates number or number concentration from fractionating techniques	No	
	Calculates number or number concentration from spectroscopic ensemble techniques	No	
	Integral technique	Yes	
	Used in hyphenated methods[8]	No	
Working range	Size range	1 nm to ~10 µm	
	Concentration range	N/A	Measurements on dry powders
	Minimum needed sample amount	~100 mg	
	Linearity/proportionality	Yes	
	Limits of detection/quantification	1 nm	
	Sensitivity (counting efficiency) as a function of particle size	good	
Trueness of particle size measurement	Indicate the trueness of this measurement technique in measuring the particle size	average	The values are often apparent BET surfaces. For large specific surfaces these are not real. The measured surface depends often on the type of gas used for measurement
Trueness in weighting the size fractions	Specify the trueness in weighting the size fractions by this measurement technique	average	
Robustness	Specify the robustness of this	good	For a defined SOP or the ISO

[8] There are 2 independent signals: average track length and spot size, i.e. diffusion coefficient and scattering intensity; a 2-dimensional plot of a monoconstituent sample would therefore show the functional relationship between size and scattering cross section (i.e. a strong, non-linear correlation); for multiconstituent samples we would expect different groups of such functional dependencies, which offers a possibility to couple size analysis with material or shape analysis.

	measurement technique		standard ISO 9277:2010
Precision	Specify the precision of the measurement technique	0.1% to 5%	
Resolution	Specify the size resolution of this measurement technique	N/A	Does not measure size distribution
Size distribution	Is it possible to measure particle size distribution?	No	
Selectivity	Discrimination between nanoparticles and non-NPs of the same chemical composition	No	
	Discrimination between nanoparticles and non-NPs of another chemical composition	No	
	Discrimination from nanoparticles of another chemical composition	No	
	Impurities	No	
Identifies state of aggregation	Does the measurement technique reveal whether the measured particles are aggregated or agglomerated?	No	
Measurement of individual particles	Does this measurement technique characterise individual particles?	No	
Counting constituent particles in aggregates	Is the measurement technique able to characterise single constituent particles in aggregates?	No	Constituents are not counted, yet their average size is measured, i.e. BET does not reveal $x_{50,0}$ of constituents
Chemical composition	Does this measurement technique analyse chemical composition?	No	
Specification of the type of size (diameter)	Specify: for example hydrodynamic…	VSSA equivalent	Surface-weighted mean of VSSA equivalent diameter
Destructive measurement technique or not	Is it a destructive measurement technique?	No	As long as the particles are stable in high vacuum
Other Specificity			
Vacuum	Does the measurement technique operate under vacuum?	Yes	
Sample support	Does this measurement technique need preparation on suitable supports?	No	

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[70] $x_{50,r}$ is the median value of type "r"-weighted size distribution (ISO 9276-1&2; however, both standards only refer to geometric types of quantities like volume i.e. r=3)

[71] $x_{50,ext}$ is median of extinction-weighted size distribution Q_ext, which is intrinsically measured by AC-turb; the term is not standardised, yet commonly used (e.g. https://doi.org/10.1007/978-3-319-30663-6_2)

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Annex 1 NanoDefine priority materials

Material	Description
IRMM-380 Pigment yellow 83 (transparent grade)	Organic diarylide dye with hydrophobic character. Sub-100 nm particulates
IRMM-381 $BaSO_4$ (fine grade)	Inorganic hydrophilic metal salt with low water solubility
IRMM-382 MWCNT	Highly tangled fibrous carbonaceous materials which are strongly hydrophobic in nature
IRMM-383 Nano steel	Highly anisotropic (platelets) particulates with negatively charged surface at neutral pH
IRMM-384 $CaCO_3$ (fine grade)	Inorganic hydrophilic metal salt with low but non-negligible water solubility
IRMM-385 Kaolin	Highly anisotropic ceramic/mineral (platelets) particulates with negatively charged metal-oxide surface at neutral pH
IRMM-386 Pigment yellow 83 (opaque grade)	Organic diarylide dye with hydrophobic character. Mainly non-nano (>100 nm) particulates
IRMM-387 $BaSO_4$ (ultrafine grade)	Inorganic salt/mineral with low water solubility
IRMM-388 Coated TiO_2 (pigment grade)	Inorganic metal oxide with thin hydrophilic coating, code Kronos 2360
IRMM-389 Basic methacrylate copolymer particles (BMC)	Hydrophilic organic particles, insoluble in water and highly soluble in most organic liquids
BAM-11 Zeolite powder	Nanoporous ceramic/mineral particulates of irregular shape with negatively charged metal-oxide surface at neutral pH

Annex 2 International standards on particle sizing

This Annex includes a non-exhaustive list of international standards available for particle sizing techniques.

Sizing

ISO 9276-1:1998, Representation of results of particle size analysis – Part 1: Graphical representation

ISO 9276-1:1998/Cor 1:2004

ISO 9276-2:2014, Representation of results of particle size analysis - Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions

ISO 9276-3:2008, Representation of results of particle size analysis - Part 3: Adjustment of an experimental curve to a reference model

ISO 9276-6:2008, Representation of results of particle size analysis – Part 6: Descriptive and quantitative representation of particle shape and morphology

ISO 26824:2013, Particle characterization of particulate systems – Vocabulary

ISO/TS 11888:2011, Nanotechnologies – Characterization of multiwall carbon nanotubes – Mesoscopic shape factors

Sampling and sample preparation

ISO 14887:2000, Sample preparation – Dispersing procedures for powders in liquids

ISO 8780-1:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 1: Introduction

ISO 8780-2:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 2: Dispersion using an oscillatory shaking machine

ISO 8780-3:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 3: Dispersion using a high-speed impeller mill

ISO 8780-4:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 4: Dispersion using a bead mill

ISO 8780-5:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 5: Dispersion using an automatic muller

ISO 8780-6:1990, Pigments and extenders – Methods of dispersion for assessment of dispersion characteristics – Part 6: Dispersion using a triple-roll mill

ISO 23900-1:2015, Pigments and extenders – Methods of dispersion and assessment of dispersibility in plastics – Part 1: General introduction

ISO/TS 16176:2011, Rubber compounding ingredients – Carbon black – Determination of the aggregate-size distribution at ultimate dispersion

Electron microscopy

ISO 13322-1:2014, Particle size analysis – Image analysis methods – Part 1: Static image analysis methods

ISO/TS 10797:2012, Nanotechnologies – Characterization of single-wall carbon nanotubes using transmission electron microscopy

ISO/TS 10798:2011, Nanotechnologies – Characterization of single-wall carbon nanotubes using scanning electron microscopy and energy dispersive X-ray spectrometry analysis

ISO 22493:2014, Microbeam analysis – Scanning electron microscopy – Vocabulary

ISO 15932:2013, Microbeam analysis – Analytical electron microscopy – Vocabulary

Particle tracking analysis/ Dynamic ultramicroscopy

ISO/DIS 19430, Determination of particle size distribution – Particle tracking analysis

Tunable resistive pulse sensing / electrical sensing zone / nano-Coulter-counter

ISO 13319:2007, Determination of particle size distributions – Electrical sensing zone method

Inductively coupled plasma – mass spectrometry

ISO/TS 19590:2017, Nanotechnologies - Size distribution and concentration of inorganic nanoparticles in aqueous media via single particle inductively coupled plasma mass spectrometry

ISO/TS 16965:2013, Soil quality - Determination of trace elements using inductively coupled plasma mass spectrometry (ICP-MS)

ISO 17294-1:2004, Water quality - Application of inductively coupled plasma mass spectrometry (ICP-MS) – Part 1: General guidelines

ISO 17294-2:2003, Water quality: 'Application of inductively coupled plasma mass spectroscopy (ICP-MS) – Part 2: Determination of 62 elements

ISO 30011:2010, Workplace air – Determination of metals and metalloids in airborne particulate matter by inductively coupled plasma mass spectrometry

ISO/TR 17276:2014, Cosmetics – Analytical approach for screening and quantification methods for heavy metals in cosmetics

Differential mobility analysing system / Differential electrical mobility analysis

ISO 15900:2009, Determination of particle size distribution – Differential electrical mobility analysis for aerosol particles

ISO 27891:2015, Aerosol particle number concentration – Calibration of condensation particle counters

ISO 12025:2012, Nanomaterials -- Quantification of nano-object release from powders by generation of aerosols

Analytical centrifugation

ISO 13318-1:2001, Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines

ISO 13318-2:2007, Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method

ISO 13318-3:2004, Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 3: Centrifugal X-ray method

ISO 15825:2017, Rubber compounding ingredients – Carbon black – Determination of aggregate size distribution by disc centrifuge photosedimentometry

Dynamic light scattering

ISO 13321:1996, Particle Size Analysis – Photon Correlation Spectroscopy

ISO 22412:2017, Particle size analysis – Dynamic light scattering (DLS)

Angular light scattering – laser diffraction

ISO 13320:2009, *Particle size analysis - Laser diffraction methods.*

ISO 24235:2007, Fine ceramics (advanced ceramics, advanced technical ceramics) – Determination of particle size distribution of ceramic powders by laser diffraction method

ISO 8130-13:2001, Coating powders – Part 13: Particle size analysis by laser diffraction

Small angle light scattering

ISO 17867:2015, Particle size analysis – Small-angle X-ray scattering

Ultrasonic spectroscopy

ISO 20998-1:2006, Measurement and characterization of particles by acoustic methods — Part 1: Concepts and procedures in ultrasonic attenuation spectroscopy

ISO 20998-2:2013, Measurement and characterization of particles by acoustic methods — Part 2: Guidelines for linear theory

ISO/CD 20998-3, Measurement and characterization of particles by acoustic methods — Part 3: Guidelines for non-linear theory

BET, Brunauer-Emmett-Teller

ISO 9277:2010, Determination of the specific surface area of solids by gas adsorption – BET method

ISO 18757:2003, Fine ceramics (advanced ceramics, advanced technical ceramics) – Determination of specific surface area of ceramic powders by gas adsorption using the BET method

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PART 3

European Commission

JRC TECHNICAL REPORTS

The NanoDefine Methods Manual

Part 3: Standard Operating Procedures (SOPs)

Agnieszka Mech, Hubert Rauscher, Kirsten Rasmussen, Frank Babick, Vasile-Dan Hodoroaba, Antoine Ghanem, Wendel Wohlleben, Hans Marvin, Raphael Brüngel, Christoph M. Friedrich, Katrin Löschner, Douglas Gilliland

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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 3: Standard Operating Procedures

The research leading to these results has received funding from the
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Website: http://www.nanodefine.eu/
Project co-ordinator: Wageningen Food Safety Research (WFSR), NL

Table of Contents

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'[1] funded by the European Union's 7th Framework Programme, under grant agreement 604347.

In 2011 the European Commission (EC) published the recommendation (2011/696/EU) for a definition of the term 'nanomaterial'[1], the EC NM Definition, as a reference to determine whether an unknown material can be considered as a 'nanomaterial' for regulatory purposes. 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 non-nano material 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 D_{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 3.

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
CFFF	Centrifugal Field-Flow-Fractionation
CLS	Centrifugal Liquid Sedimentation
CPC	Condensation Particle Counter
DEMA	Differential Electrical Mobility Analysis (also spray-DEMA)
DMA	Differential Mobility Analyser
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	Engineering System International 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
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry
KB	Knowledge Base
LD	Laser Diffraction
LoD	Limit of Detection
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

Executive summary

In the NanoDefine project ('NanoDefine') approach a characterisation method includes both sample preparation and analysis with a defined measurement technique. To ensure repeatability, the sample preparation should be done according to Standard Operation Procedures (SOPs). The aim of this report, part 3 of the NanoDefine Manual, is to present the twenty-three SOPs developed within NanoDefine to facilitate and harmonise the particle size distribution measurements. These SOPs have been developed for different purposes, leading to different the types of SOPs that are gathered in this document.

SOPs were developed for the purposes listed below:

- dispersing powders in a liquid phase and ensuring maximum de-agglomeration and disintegration of aggregates and stability (i.e. minimum re-agglomeration)
- ensuring comparability of dispersion procedures
- conducting measurements of particle size distribution (and other properties of the particles) with specific measurement techniques
- extracting particles (or a specific particulate species) from a complex dispersion
- entire methods covering the steps from sample preparation to particles size analysis

Eleven of the SOPs are presented as detailed, material specific dispersion protocols designed to produce liquid (aqueous) dispersions of the NanoDefine priority materials, which are: IRMM-380 (Pigment yellow 83, transparent grade), IRMM-381 ($BaSO_4$, fine grade), IRMM-382 (MWCNT), IRMM-383 (Nano steel), IRMM-384 ($CaCO_3$, fine grade), IRMM-385 (Kaolin), IRMM-386 (Pigment yellow 83, opaque grade), IRMM-387 ($BaSO_4$, ultrafine grade), IRMM-388 (Coated TiO_2, pigment grade), IRMM-389 (Basic methacrylate copolymer particles, BMC) and BAM-11 (Zeolite powder). The SOPs for these materials are based on the high intensity energy input methods of probe and vial sonication. To ensure maximum harmonisation across the project, all materials have an associated dispersion protocol describing probe sonication, as this type of sonicator was available to all partners, while for selected materials (IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 ($CaCO_3$), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO_2)) also a protocol for vial-sonication was developed. The sonication conditions used were chosen to ensure that the two sonicator systems were supplying a similar volume-specific energy input.

One SOP was developed because sonication, although effective for dispersing the test material, introduces a significant variable in the dispersing process as a wide variety of different sonication instruments exists with different nominal power and probe size. To reduce the variability that this may introduce a SOP 'Generic SOP for calorimetric calibration of an ultrasonic probe sonication' was developed. The use of this SOP ensures (better) harmonisation of the power output when using significantly different sonicator types or probe types/sizes for dispersion compared to those used in the development of in the optimised protocols.

The remainder of the SOPs concerns procedures for combinations of size measurement techniques and materials, and materials in products.

Additionally, the report notes that the use of sonication probes, although the most commonly available, should be done with caution as probe material may be released due to wear of the probe, and above all the onset of this may start after only a few hours of use, and that this wear may not be easy to detect.

1 Introduction to the Standard Operating Procedures

1.1 About the Standard Operating Procedures

The NMs in powder form can be analysed via two routes: as dry powders (which is not further discussed in this report) or in liquid dispersion. The Standard Operating Procedures (SOPs) presented here were developed for dispersing powders for analysis as dispersions. Part 1 of the NanoDefine Methods Manual describes how these dispersions, once available, are analysed with regard to particle size. The SOPs were developed with the purpose of minimising the variability of the dispersion procedure on the measured size, and this is described in section 1.3.

NanoDefine developed eleven SOPs for generating an aqueous dispersion the priority materials based on probe sonication for all the priority materials, see chapters 2 to 12. For four of the materials (IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 ($CaCO_3$), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO_2)), these SOPs included also vial sonication.

Additionally the following twelve SOPs were developed:

1) A generic SOP for calorimetric calibration, see chapter 13.

2) The DLS method, which was developed for four NanoDefine priority materials (IRMM-381 ($BaSO_4$, fine grade), IRMM-384 ($CaCO_3$, fine grade), IRMM-385 (Kaolin), IRMM-388 (Coated TiO_2, pigment grade) but which can also be applied to comparable types of materials, considering that adaptions might be needed, see chapter 15.

3) The Cuvette-AC method which was developed for two of the NanoDefine priority materials (IRMM-381 ($BaSO_4$, fine grade), IRMM-387 ($BaSO_4$, ultrafine grade) but which can also be applied to comparable types of materials, considering that adaptions might be needed, see chapter 16.

4) A method for the analysis of Fe_2O_3 in polyethylene matrix with electron microscopy, which illustrates protocols for preparation of products for microscopy methods and covers sample preparation and fully automatic particle size distribution (PSD) analysis of Fe_2O_3 nanoparticles in high density polyethylene, see chapter 17.

5) A method for the analysis of TiO_2 in sunscreen with electron microscopy, see chapter 18.

6) A method for size characterisation of suspended particles by AUC-RI with speed ramp option.

7) Particle size distribution measurement of $BaSO_4$ using Line-Start Disc Centrifuge with Optical Detection.

8) Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software.

9) Analysis of TiO_2 particles from sunscreen by AF4-MALS-ICP-MS.

10) Sample preparation and spICP-MS analysis of TiO_2 nanoparticles in sunscreen products.

11) Sample preparation and spICP-MS analysis of TiO_2 nanoparticles in suspensions.

12) Sample preparation and spICP-MS analysis of Al_2O_3 nanoparticles in toothpaste.

All SOPs are presented in the document as stand-alone, self-explanatory documents which can be easily extracted from the report; thus repetitions of some text and images occur. All NanoDefine

technical reports, including the ones for the SOPs, can be found on the project website at http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-technical-reports[a].

1.2 Priority materials

The materials which have been chosen as priority substances in NanoDefine (Table 1) provide examples of the major classes of nanomaterials: metals, metal oxides, metal salts, polymers, carbonaceous materials (MWCNT) and ceramics. Such a wide range of materials provided a challenge for the development of dispersion protocols as, firstly, each material potentially may require a different type of stabiliser and secondly, the binding strength between materials may be widely different making careful optimization of sonication time and power critical to maximize disaggregation without inducing undesirable fusion of particulates, see e.g. Babick et al. (2016)[2].

Table 1: NanoDefine priority materials

Material	Description
IRMM-380 Pigment yellow 83 (transparent grade)	Organic diarylide dye with hydrophobic character. Sub-100 nm particulates
IRMM-381 $BaSO_4$ (fine grade)	Inorganic hydrophilic metal salt with low water solubility
IRMM-382 MWCNT	Highly tangled fibrous carbonaceous materials which are strongly hydrophobic in nature
IRMM-383 Nano steel	Highly anisotropic (platelets) particulates with negatively charged surface at neutral pH
IRMM-384 $CaCO_3$ (fine grade)	Inorganic hydrophilic metal salt with low but non-negligible water solubility
IRMM-385 Kaolin	Highly anisotropic ceramic/mineral (platelets) particulates with negatively charged metal-oxide surface at neutral pH
IRMM-386 Pigment yellow 83 (opaque grade)	Organic diarylide dye with hydrophobic character. Mainly non-nano (>100 nm) particulates
IRMM-387 $BaSO_4$ (ultrafine grade)	Inorganic salt/mineral with low water solubility
IRMM-388 Coated TiO_2 (pigment grade)	Inorganic metal oxide with thin hydrophilic coating, code Kronos 2360
IRMM-389 Basic methacrylate copolymer particles (BMC)	Hydrophilic organic particles, insoluble in water and highly soluble in most organic liquids
BAM-11 Zeolite powder	Nanoporous ceramic/mineral particulates of irregular shape with negatively charged metal-oxide surface at neutral pH

[a] NanoDefine technical reports are available at the site http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-technical-reports

1.3 General considerations for dispersion

1.3.1 Introduction

The issue of dispersion is particularly important in the evaluation of nanoparticle size as many nanomaterials are normally found in the form of dried powders which need to be brought into stable dispersions in liquid before they can be measured by many of the most common particle size measuring methods such as dynamic light scattering (DLS), laser scattering (LS), centrifugal liquid sedimentation (CLS) and analytical ultracentrifuge (AUC). The dispersion procedure is a pivotal step in the process of measuring the particle size distribution and the dispersion procedures must be effective, efficient, reproducible, and the final dispersion should have a particle size distribution that is as close as possible to the true size distribution of constituent particles.

The first step in developing a dispersion procedure is the choice of media, pre-dispersion and wetting. Primary considerations concerning the media are possible limitations imposed by the instrumental method to be used later and the need to ensure that the particles are inert towards the media, i.e. do not dissolve or swell.

The second step is choosing the method to use for the mechanical de-agglomeration of the particles. Here, the primary concern is the input energy (both in terms of local stress intensity and energy density) and whether it is sufficient to disaggregate without causing damage to the particles or their coating, if relevant. For the reasons previously discussed, the usual choice for most materials will be ultrasonic using either probe or vial sonicators. The lower power of bath sonication will, in practical terms, probably be insufficient for most materials but may be the only choice when evaluating certain 'soft' nanomaterials such liposomes or drug and food supplement carriers.

The final step in the process, stabilisation, is by far the most complex area to advice on due to the very large number of possible variables and the following issues need to be considered. The first consideration is always compatibility of the stabilisation method with the measurement method. The second consideration will be the effectiveness of the stabilisation and it includes consideration of the time scale for which stability must be guaranteed. Simplicity should also be considered and in the cases that adequate performance can be achieved with commonly available simple stabilisers these should be used in preference to high performance proprietary products.

1.3.2 Sonication type

The scientific literature, e.g. Hartmann et al. (2015)[3], regarding dispersion shows a common theme that, in the vast majority of cases effective dispersion of dry nano-powders into a liquid, requires high energy sonication; low energy methods such as bath sonication are not effective. Thus, in NanoDefine development of protocols has concentrated on the use of probe sonication as the primary method with additional data being provided for the use of vial-sonicator where the developing laboratory had this instrumentation available.

For general use in the dispersion of nanopowders it strongly recommended when using a probe sonicator to carefully consider the maximum input power level. Successful sonication can be obtained with a nominal maximum input power level of 100-200 W, which does not affect the size distribution of the constituent particles [3]. For the relatively small volumes (1-10 mL) considered in the protocols a lower value of 50 W may be acceptable subject to verification. Ultrasonic probe devices having a nominal power above 200 W may still be used, as this may not affect the local

stress intensities by imploding cavitation bubbles nor the relationship between particle size and energy density. The important issue is the calorimetric calibration for a combination of given instruments, probes, beakers, sample volumes, immersion depth of the probe and temperature.

Sonication may be done in constant mode or in pulsed mode. The principal advantage of constant mode sonication is that it is possible with most models of probe sonicators, while a more limited number of instruments may have the option of also using a pulsed mode. Where pulsed mode is available there may be a number of advantages. Firstly, the use of pulsed mode allows the use of higher acoustic intensity but with reduced temperature increase as heat may transfer to the environment during the off-cycles. This is of particular relevance in vial sonication, which relies on heat transfer through the metal of the probe, as active cooling of the sample (by e.g. immersion in an ice bath) is not normally possible. This issue may be important where temperature sensitive samples are treated. Secondly, the off-cycle in pulse-mode allows the dissolution of bubbles, which can create acoustic shielding, and thus it may overall improve efficiency of the process.

1.3.3 Sonication power and energy requirements

It appears likely that once a certain minimum intensity of sonication (amplitude) is achieved then further increases in the power settings have a limited influence on the final minimum particle size which can be achieved but influences more the time required to achieve the minimum particle size. In the work of Guillemin et al. (2012)[4] on the de-agglomeration of ZnO nanopowders there is a theoretical and experimental examination of the effect of sonication power which shows that the breakage frequency during sonication increases with the square root of the thermal power confirming the limited advantages of adding additional power. In other studies, e.g. Taurozzi et al. (2011)[5] it has been noted that the use of higher power levels may actually be detrimental to efficient re-dispersion as the excess energy may result in fusion of de-agglomerated particles with the irreversible formation of aggregates.

Another factor which may influence the sonication efficiency is the temperature, as it affects properties of the liquid, thus cavitation, critical bubble size and viscous dissipation of micro-jets as well as properties of the particulate phase, e.g. strength of interparticle bonds. In many of the studies for nanotoxicology cooling in an ice-bath is recommended but in practice the use of such low temperatures may be important mainly to avoid thermal degradation the protein molecule (albumin) which is added as a surfactant. In the work of Raman and Abbas (2008)[6] studies of the sonication of Al_2O_3 show that the *energy transfer* from the probe sonicator to the liquid peaked at low temperature decreasing by 10-15 % when the temperature was increased from 10 °C to 50 °C. The actual efficiency of *particle breakage* showed a maximum around 25 °C with only a moderate decrease of 10-15 % occurring as the temperature increased to 50 °C. More significantly it was found that reducing the liquid temperature from 25 °C to 10 °C produced a decrease of around 80 % in the efficiency of particle breakage compared to the maximum value achieved. From this it would seem that the use of cooling during sonication should be done with caution as excessive reduction in temperature may be detrimental to the overall efficiency of the process.

From these considerations it is evident that the determination of the sonication conditions must firstly verify that the minimum intensity value is achieved and thereafter verify from that value what is an appropriate power level to reach the minimum size in a experimentally reasonable time without significant formation of fresh agglomerates due re-fusion of particles by the ultrasonic energy. For the work in NanoDefine it has been considered realistic that the sonication time should not exceed 1 hour and ideally should be less than 30 minutes.

1.3.4 Towards harmonisation of sonication

The final and possibly most important point is that there is a clear need to develop methods that standardise the sonication in a manner which can reduce the variability introduced by the wide variety of different types of sonicators used in different laboratories. Some sonication description reports consider the use of electrical power input as a means to define the sonication power but, in general, this is likely to be unsuccessful due to the potential for variations in the efficiency of converting electrical energy into ultrasonic energy applied to the probe, and, more importantly, the variation in transferring that energy from the probe into the sample solution.

This problem has led to the proposal by a number of groups that the sonication process should be in some way harmonised by measuring the energy effectively transferred to the sample. The possibility of using calorimetric methods to evaluate the relative power of different sonicators has proved to be a simple and attractive strategy as a first step towards this harmonisation. These methods are based on the assumption that the acoustic energy absorbed by a liquid sample of known mass is converted into thermal energy. By monitoring the temporal increase in temperature during sonication it is possible to obtain a relative measure of the power output of a sonicator. It is possible that the efficiency of energy transfer will be influenced by many factors, such as liquid viscosity, volume, shape, and container materials. However, by standardising on a relatively large volume, but still smaller than 100 mL, in a similarly shaped vessel the influence from such variables may be sufficiently minimised to make the method suitable for inter-comparison of different models of sonicators.

These methods, often based on variations of the basic methods described by Taurozzi et al.[7], show a promising route to determine the power output of different sonicators and more importantly provide a means to define sonication conditions based on measurable independent physical parameters rather than a specific setting on an instrument.

The situation of the vial sonicator system is in some ways simpler as, to the authors' knowledge, currently only one manufacturer exists and thus there is no need to harmonise between such instruments. The problem remains how to compare vial sonicator performance with that of probe sonicators. While it was not possible to make a direct comparison through a standard method (chapter 13) it was possible to measure the rate of temperature increase when operating with a single full (2 mL) vial so that an approximate measure of power output could also be determined.

The remaining sonication system, cup-horn, has not been considered in NanoDefine as this instrumentation was not available amongst the method developers.

1.3.5 Probe sonicator issues

While there is no doubt that high intensity sonication is a necessary step in producing dispersions of nanomaterials starting from dry powders there is another potential issue which should be considered when deciding exactly which method is the best to be applied. As discussed previously, high intensity sonication can be achieved by 3 main methods which are (a) standard probe sonication, (b) vial sonication and (c) cup-horn sonication. Of these three methods the first is by far the most common due to the greater flexibility of use (variable probes size and processing volume), ease of liquid cooling and general availability. The major disadvantage of this method is that the sonicator probe is placed in direct contact with the liquid containing the sample creating a risk that the sample would be contaminated should there be either ionic or mechanical release of material

from the probe[b] during operation. The onset of this effect cannot be predicted and may in many cases be difficult to detect other than by regular verification of probe integrity. It was noted that this problem can be serious, as can be seen in the examples shown below which were observed after 30 minutes of sonication of IRMM-380 (6 mm diameter probe) and IRMM-386 (3 mm diameter probe) samples (see Figure 1).

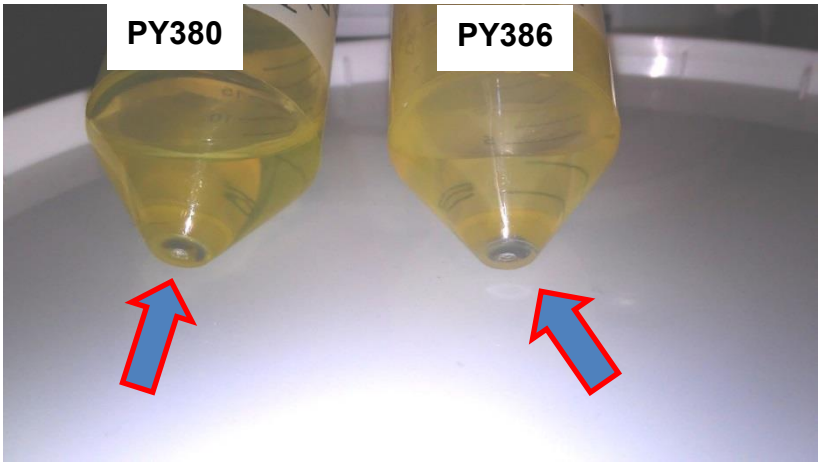

Figure 1: Photograph of pigment yellow 83 samples after probe sonication showing sediment of residue

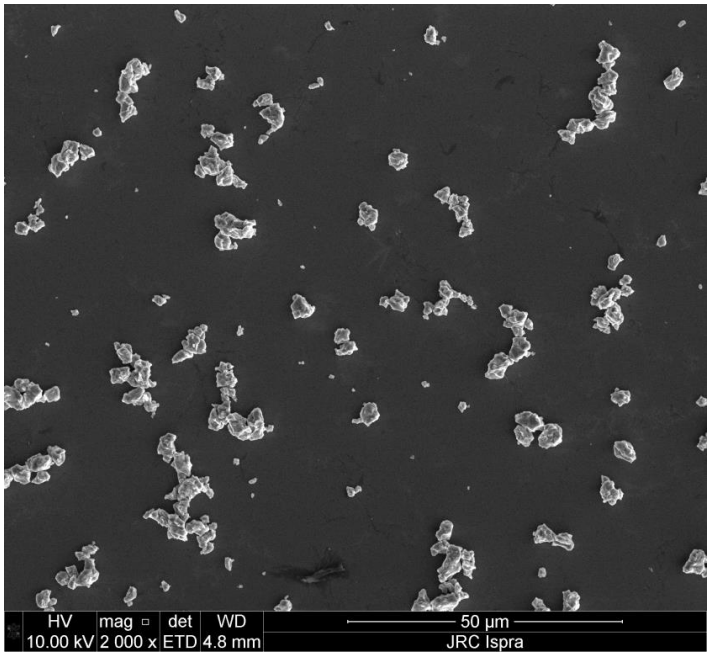

Figure 2: SEM Image of residue produced by probe sonicator

In this particular case the probe heads had been operated over 4-6 weeks and used only for the sonication of IRMM-380 and 386 (pigment yellow 83, transparent and opaque grades respectively), IRMM-384 ($CaCO_3$ (fine grade)) and IRMM-388 (Coated TiO_2). The deterioration was noted on two different probes with different diameters (3 mm and 6 mm). One of the probes was operated in pure water for a further 50 minutes period and the residue collected for analysis by SEM. An

[b] Probes are often made from titanium based materials

example of the residues can been seen in the SEM image shown in Figure 2. In additional studies of these samples, elemental analysis of these fragments by SEM-EDX confirmed them to be composed of mainly titanium as would be expected of debris from the type of probe used. In this example it should be noted that the use of the probes with the hard and abrasive TiO_2 (IRMM-388) may have contributed to a more rapid than normal degradation of the tip surface; with other, softer priority materials this may not be observed.

Given the experience of probe degradation it is advisable that, when appropriate equipment is available and sample volumes and temperature sensitivity permit, non-contact methods of sonication should be adopted to avoid any risk of this problem. In cases where direct contact probe sonication is to be used then it may be preferable to use a probe sonicator which has an exchangeable tip so that this may be easily inspected and if necessary replaced whenever necessary. In this way regular substitution may be undertaken with a lower cost than in the case of substituting mono-block probes.

1.4 Specific considerations of dispersion in NanoDefine

1.4.1 Requirements of the dispersions

Besides aiming at dispersions with a maximum number of unbound single particles and a minimum of agglomerates/aggregates, the requirements of the analytical techniques (Table 2) were taken into consideration for the development of the protocols. In particular, several methods, such as AF4 and CLS, made it desirable, although not essential, that the dispersion medium would be water-based. Required particle mass concentrations were in the range of ≤ 1 to 10 $mgmL^{-1}$ and sample volumes in the range of 100 µL to 2 mL. With regard to the stability of the dispersion and the need for chemical stabilisers, it was concluded that for most instrumental methods a temporal stability of 30 minutes was sufficient and as such placed less stringent requirements on the performance and therefore the choice of surfactants. Thus, while it is it is possible that the most effective stabilisation could be realised by using highly optimised proprietary surfactants or surfactant mixtures, the limited temporal stability required means that in most cases relatively simple surfactants could be used. Furthermore, since the protocols needed to be transferred to numerous laboratories it was desirable that they were as simple, safe and widely applicable as possible thus also favouring the choice of relatively simple and commonly available molecular surfactants rather than more complex or less commonly available proprietary commercial surfactants, polymers or surfactant mixtures.

In the scientific literature, many methods can be found for dispersing specific types of particles in specific liquids but there is little information on more generically applicable protocols. In fact, the development of true dispersion protocols in the recent literature has been driven almost exclusively by the needs of the nanotoxicology testing community and their need to produce short-term stability dispersions which, apart from the nanoparticles, contain only compounds which are compatible (non-toxic) with biological systems. Thus, it is clear that the range of possible stabilising agents available to researchers is severely restricted as the stabilising agent must not contribute to any observed toxicity to cells and in most cases the stabilising agent must also work within the pH conditions, which may not be suited to some materials. Given these limitations, it is quite possible that the quality of dispersion achieved with these protocols is sub-optimal for many materials.

Table 2: Minimum requirements for various measurement techniques identified in NanoDefine

Instrument method	Measurement volume (approx.)	Stability time required (minimum)	Preferred media	Final concentrations required
CLS	100 µL	<30 min	Aq.	<1 $mgmL^{-1}$
AUC	100 µL	<30 min	Aq./Org	<10 $mgmL^{-1}$
Cuvette Centrifuge	1 mL	<30 min	Aq./Org	<10 $mgmL^{-1}$
DLS/LS/MALS	1 mL	<30 min	Aq./Org	<10 $mgmL^{-1}$
AF4	100 µL	<30 min	Aq./Org*	<10 $mgmL^{-1}$
TEM	<100 µL	<30 min by drop	Aq./Org	≤10 $mgmL^{-1}$
spICP-MS	1-2 mL (to dilute)	<30 min prior to dilution	Aq.	≤1 $mgmL^{-1}$
PTA	1- 2 mL	<30 min prior to dilution	Aq./Org*	≤1 $mgmL^{-1}$
ESI-SMPS	1-2 mL	<30 min	Aq./Org*	≤ 1 $mgmL^{-1}$
SAXS	2 mL	30 min	Aq./Org	≤10 $mgmL^{-1}$

***Only selected organic media may be acceptable**

Another issue to consider is the final application of the dispersion, which in NanoDefine is to produce a sample which is appropriate to specific particle size measuring techniques. In the case of nanotoxicity testing the use of a protein stabiliser, such the commonly used BSA, is easily justified as cell culture media is very often rich in albumin or blood serum and consequently the testing of particles pre-coated with protein is scientifically valid. However when considering the requirements for dispersion for measurement purposes, then the fact that the generic toxicity testing protocols are actually coating the particle with a fairly large protein (BSA) should be taken into account as this coating may have an influence on the measurement result obtained. For example, dispersion stabilisation with a protein may be a quite acceptable and effective method for preparing inorganic materials for EM based analysis, where the low relative contrast of the organic materials means it is not a strong source of interference in the accurate measurement of particle size. In contrast, the use of protein-coated samples with DLS or CLS/AC methods is likely to produce results which would be influenced by the presence of protein but in a way which would be difficult to quantify accurately. In the case of DLS, the presence of the protein as a coating on the surface of the particles would result in an apparent increase in the hydrodynamic diameter of the particle. Since the protein has a size of a few nanometres in its natural folded state it would be reasonable to assume that this would not have a great effect on large particles; however, when coating small particles of a few nanometres, the potential error is very large. In addition, any remaining free protein, having a size of a few nanometres in monomer form but being able to form larger dimer and trimers, may be a source of confusion for techniques with non-specific particle detectors. If the same situation is considered for centrifugal sedimentation based methods such as CLS and AC the problem becomes more complicated as the coating not only changes the apparent hydrodynamic diameter of the particle but also changes its mean density, which is critical to the size determination. In addition, depending on the quantity of protein used, there is a possible influence

on dispersion medium viscosity and optical properties which may introduce additional uncertainties to some measurement methods such as DLS, AC, PTA etc. Finally, the ability of albumin or similar proteins to effectively coat many types of particles means that it also can coat the inside surface of the measurement instrument and consequently render the surface prone to non-specific bonding of particles. This phenomenon can lead to problems of instrument operation, loss of material, cross contamination, memory effects and in certain circumstance may produce aggregates.

1.4.2 Evaluation of the dispersion quality

The aim of the dispersion protocols developed in NanoDefine was to disperse the materials in such a way that the resulting dispersions of (nano)particles are stable and contain only or mainly constituent particles. In the development of the dispersion protocols, optimisation of the procedures has been done by identifying likely combinations of sonication and stabilisers. These have then been developed into working protocols by systematic variation and optimisation of parameters based on the resultant mean particles size using dynamic light scattering (DLS) or centrifugal liquid sedimentation (CLS). This approach is suitable for the development of protocols but it is based on ensemble / fractionation techniques, which cannot distinguish between single particles and agglomerates/aggregates and is thus poorly suited to evaluate the true extent to which the dry powder has been dispersed into constituent particles. To evaluate the final state of the dispersed material in a more definitive and unambiguous manner, the dispersion was evaluated by qualitative and quantitative TEM analysis. Table 3 gives an overview of the qualitative evaluation of dispersions achieved using the NanoDefine optimised protocols and evaluated by TEM.

Table 3 Qualitative evaluation, based on TEM, of dispersions achieved using optimised protocols

Material Code	**Description**	**Qualitative assessment of the effectiveness of dispersion protocol based on TEM analysis**
IRMM-380	Pigment Yellow 83 (transparent)	Results in a combination of single constituent particles, and aggregates/agglomerates.
IRMM-381	$BaSO_4$ (fine)	The protocol for IRMM-381 – $BaSO_4$ (fine grade) allows dispersion of the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles).
IRMM-382	MWCNT	Both dispersion protocols result in a combination of single constituent particles, and smaller and larger agglomerates.
IRMM-383	Nano steel	Allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 particles). Due to the layered structure of the material, it remains debatable if some of the apparent intensity fluctuations in the TEM images of the particles are caused by different grains making up one platelet, or by even smaller constituent particles attached to the dispersed platelets. Visual inspection of the liquid dispersion after sonication suggested that the material is not completely dispersed in the medium.
IRMM-384	$CaCO_3$	Allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).

IRMM-385	Kaolin	Allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)
IRMM-386	Pigment Yellow 83 (opaque)	Allows dispersion of the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)
IRMM-387	$BaSO_4$ (Ultrafine)	The 'Protocol for IRMM-387 – $BaSO_4$ (ultrafine grade) ' allows dispersing the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).
IRMM-388	Coated TiO_2	Allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-20 constituent particles)
IRMM-389	Basic Methacrylate Copolymer, BMA	The dispersion protocol of BMA by TUD allows dispersing the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).
BAM-11	Zeolite	The protocol for BAM-11 – zeolite results in a combination of single constituent particles, and aggregates/agglomerates.

1.5 References

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Dispersion SOPs: Production of an aqueous based dispersion of the NanoDefine priority materials

2 SOP for production of an aqueous based dispersion of IRMM-380 (Pigment Yellow 83, Fine grade)

2.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-380 starting from dry powder form.

2.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based, surfactant stabilised colloidal suspension of IRMM-380, fine grade Pigment Yellow 83. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

2.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NM	Nanomaterial
MALS	Multi-angle Light Scattering
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS.
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
SOP	Standard Operating Procedure
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

2.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 $mgmL^{-1}$ of IRMM-380)

of IRMM-380, Pigment Yellow 83 (Transparent grade). The procedure foresees starting from a dry powder sample of the IRMM-380 materials and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the commercial surfactant NEKAL BX. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL, while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

2.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

2.4.1.1 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

2.4.2 Material supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

2.4.3 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- Pigment Yellow 83 transparent grade distributed by IRMM with project ID no. IRMM-380
- High purity methanol (analytical grade)
- Ice-water mixture for cooling the sample during sonication.
- Surfactant: 30 wt% aqueous solution of NEKAL-BX (Sodium Butyl naphthalene sulphonate (CAS No. 25638-17-9)

2.4.4 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional is information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

2.4.4.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielsche UPS200S instrument whose power output characteristics were determined as described in chapter 13and the results shown in Figure 1.

In this procedure for dispersion of IRMM-380 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

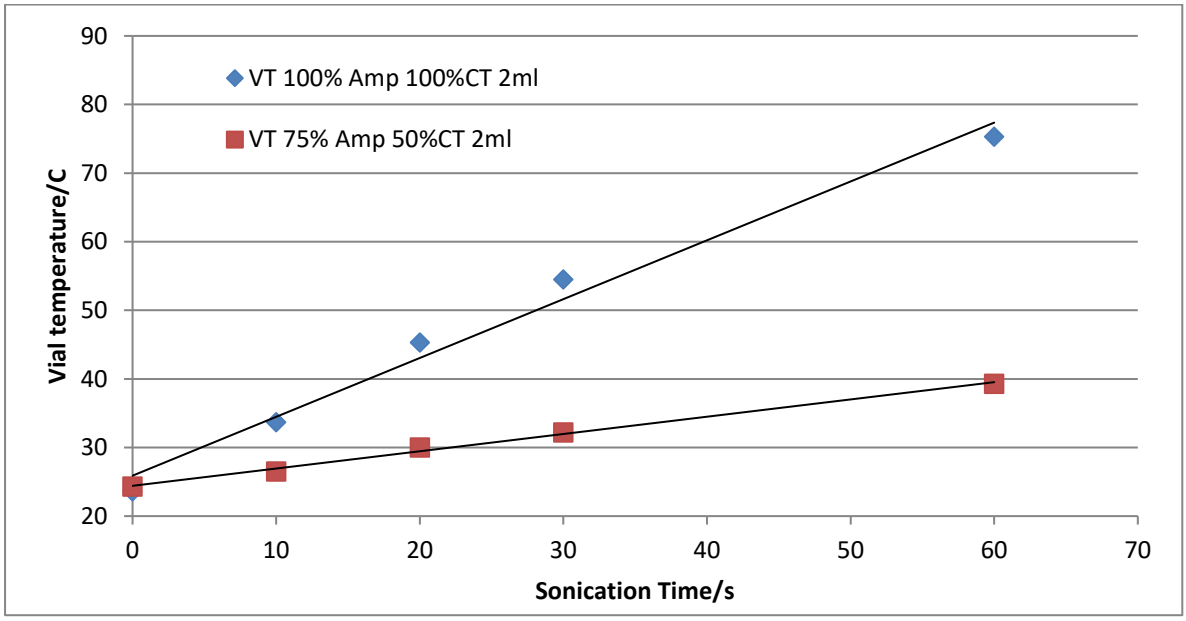


Figure 1: Temperature increase of 2 mL water in vial sonicator at (a) 100 % Amplitude and 100 % cycle-time and (b) 75 % Amplitude and 50 % cycle time.
Specific power absorbed is (a) 3.8 WmL^{-1} and (b) 1.1 WmL^{-1} respectively

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

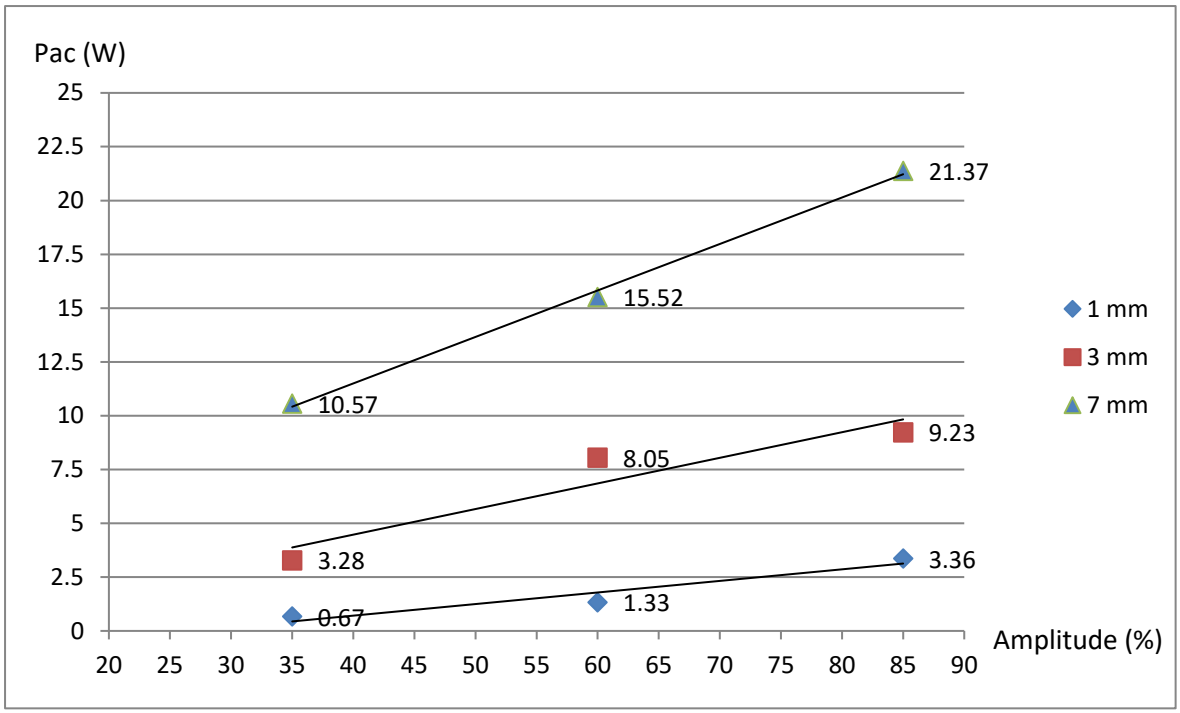


Figure 2: Acoustic power absorbed when using different probe diameters and amplitude settings

2.4.4.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 2.4.4.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

2.4.4.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 15 µL of Nekal BX solution (30 wt%) using a pipette. Reweigh the vial and calculate by difference the amount of NEKAL BX before adding sufficient pure methanol to give a mass concentration of 0.5 $mgmL^{-1}$: This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-380 into a 22 mL glass vial and add sufficient pure methanol to give a concentration of 1 $mgmL^{-1}$. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): Add solution A to solution B in a ratio of 10 μLmL^{-1}.

Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): This will hereafter be referred to as solution B.

Prepare a heated water bath under a chemical safety hood and heat to 40-50 °C. Suspend the lower half of vial in the water bath until the MeOH evaporates leaving a layer of surfactant coated particles on the bottom of the vial. Add sufficient MilliQ water to get 10 $mgmL^{-1}$ solids in water and seal the vial with a suitable lid. Re-disperse the solids into the water by immersing the bottom half of the vial in a USB and sonicating for 2 minutes or until the solids appear uniformly distributed in the water. This will hereafter be referred to as solution C

Take an empty 22 mL vial and add 5.94 ml of pure water followed by 60 µL of solution C to give a final concentration of 0.1mg/mL IRMM-380 in water. This will hereafter be referred to as solution D

Sonication using probe sonicator: Take the 22 mL glass vial containing solution D and mount the probe sonicator head inside the vial as shown in Figure 3. The probe head should be immersed in the solution to a depth of approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is fully immersed in the cooling water. In this procedure the sonicator used was a Hielscher UPS200S and this was operated in pulsed mode with an amplitude of 75 % and a cycle time of 50 % which, as described in the Section 2.4.4.1, produces a mean adsorbed power of 7.8 W (50 % cycle time) corresponding to 1.3 WmL^{-1} when normalised to the specified volume of 6 mL. A sonication time of 20 minutes was determined to be the optimum treatment time for this material under the described conditions.

Figure 3: Photograph showing recommended positioning of probe sonicator in sample

When attempting to use a probe sonicator which is different from the model used in the development of this method users must firstly determine the power output characteristics of their

own instrument using the method described in chapter 13. From this data, instrument settings should be determined which can approximate the power output values detailed above and in section 2.4.4.1.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Alternative method using vial sonicator: Place 2 mL of solution D in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 4).The vial should sonicated for 15 min at 75 % amplitude with the cycle time being set at 50 %. As cooling cannot be applied in the case of vial sonicator the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

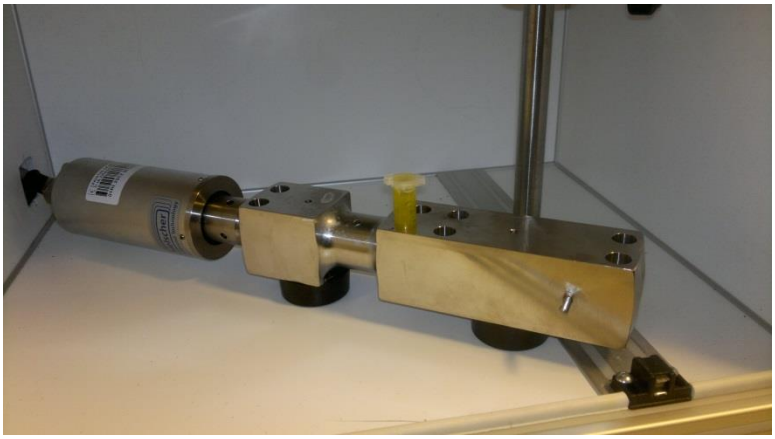


Figure 4: Positioning of sample in vial-sonicator

2.4.5 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 2.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 2.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease in mean size.

2.4.6 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepare in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of

30 minutes following completion of the primary dispersion procedure outlined in the previous section 2.4.4. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of brief vortexing followed by a single additional step of bath sonication. In the case of IRMM-380, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and the treated for 15 minutes in a laboratory scale bath sonicator at room temperature.

2.4.7 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

2.5 Validation Status

This method has not yet been subjected to validation

2.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

2.7 Information on expected particle size distribution

Table 1 Values of mean particle size as determined by CLS and DLS

Dispersion approaches	**Methanol+nekalBX method + 15' VS**
Mean particle size (IRMM-380) by CLS (weight-size distribution)	**50 nm**
Mean particle size (IRMM-380) by DLS (Z-average)	**47 nm**

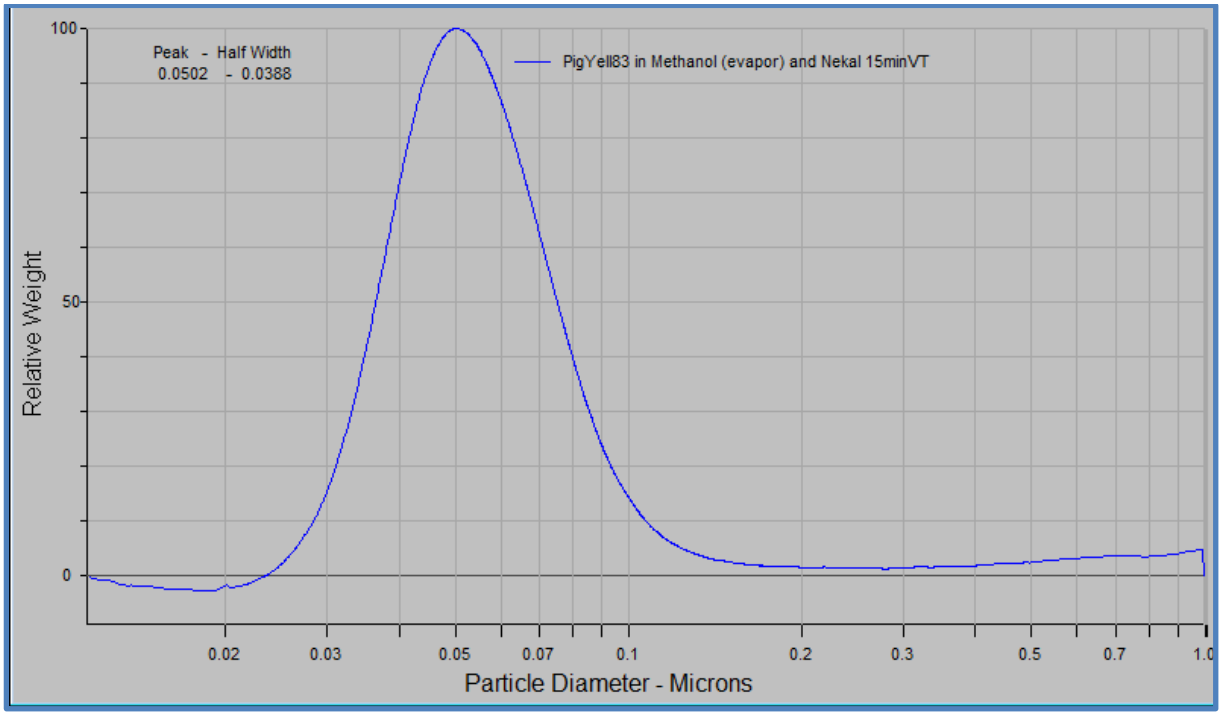


Figure 4: Particle size distribution as determined by CLS

3 SOP for production of an aqueous based dispersion of IRMM-381 ($BaSO_4$ (fine grade))

3.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-381 starting from a dry powder form.

3.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-381, fine grade $BaSO_4$. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS, does not significantly change (according to DLS measurements) over a time period of at least 60 minutes from completion of the dispersion procedure.

3.3 Abbreviations

DLS	Dynamic Light Scattering
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

3.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 $mgmL^{-1}$) of IRMM-381, $BaSO_4$ (fine grade). The procedure foresees starting from a dry powder sample of IRMM-381 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing the commercial stabilising agent sodium hexametaphosphate (SHMP). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and allows to produce a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particle size values while longer treatment times will either degrade the quality (re-formation of larger aggregates/agglomerates) or will not provide a significant further reduction in the mean size. The amount of SHMP used for dispersion can have an effect on the dispersion quality and obtained particle size distribution. This procedure has been developed to produce the lowest mean particle size distribution for dispersion in 2 $mgmL^{-1}$ SHMP. If required, the concentration of SHMP can be lowered down to 0.2 mg^{-1}. The particle size distributions obtained upon dispersion in 2 and 0.2 $mgmL^{-1}$ SHMP have been evaluated by DLS and were found to be comparable.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 60 minutes (aged, after re-dispersion by vortexing) with the results showing no major variation in the means size distribution. Sedimentation may be observed if the dispersion stands during some minutes after preparation. In this case re-dispersion is possible by vortexing.

3.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W
- Ultrasonic bath sonicator
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

3.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

3.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

3.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm^{-1}, 0.2 µm in-line filtration)
- $BaSO_4$ (fine grade) distributed by IRMM with project ID no. IRMM-381
- Ice-water mixture for cooling the sample during sonication
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity ≥ 96 %, e.g. 305553 Aldrich)

3.4.5 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

3.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W. The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 µm).

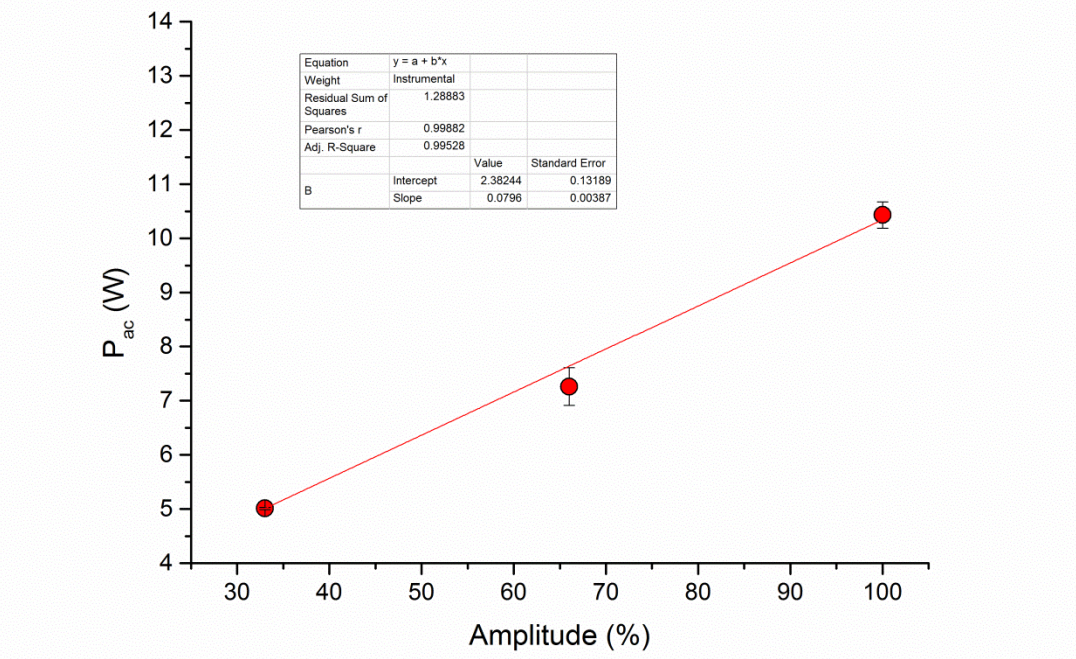


Figure 1: Calculated delivered output power P_{ac} of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to Pac = 10.3 W (in this example: amplitude of 100 %)

The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1. In this procedure for the dispersion of IRMM-381 the sonicator was operated at a set amplitude value of 100 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 10.3 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 10.3 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

3.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 3.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

3.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare stabilising agent solution (2 $mgmL^{-1}$ SHMP) by dissolving the appropriate amount of SHMP powder into MilliQ water. Shake vigorously to ensure that all powder is solubilized. Subsequently, filter the prepared SHMP solution using a 0.2 µm filter to ensure that no large particulates are present.

Weigh approximately 15.6 mg of IRMM-381 into a 20 mL glass vial. It is recommended that an ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of SHMP solution to give an IRMM-381 concentration of 2.6 $mgmL^{-1}$ (6 mL for exactly 15.6 mg of IRMM-381) adjusting the volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Take the 20 mL glass vial containing the 2.6 $mgmL^{-1}$ IRMM-381 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant power 10.3 W for 20

minutes. The correct power setting should be determine from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

3.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 3.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 3.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

3.4.7 Recovery of dispersions after aging beyond verified period of stability.

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 h following completion of the primary dispersion procedure outlined in section 3.4.5.3. Sedimentation may be observed if the dispersion stands during some minutes after preparation. In this case re-dispersion is possible by vortexing.

3.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

3.5 Validation status

This method has not yet been subjected to validation

3.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

3.7 Information on expected particle size distribution

Table 1 Summary of the DLS results obtained for IRMM-381 – $BaSO_4$ (fine grade) suspensions in MilliQ water (N=1) and 2 $mgmL^{-1}$ hexametaphosphate (N=2) prepared at different probe sonication times

Sonication Time	**0 min**	**2.5 min**	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**
Intensity-weighted mean diameter: by DLS(Z_{ave}, cumulants method)	657.6 ± 17.1	493.8 ± 11.3	466.2 ± 1.0	415.6 ± 1.7	399.0 ± 1.6	**377.4 ± 1.3**	363.9 ± 2.0	365.9 ± 13.2

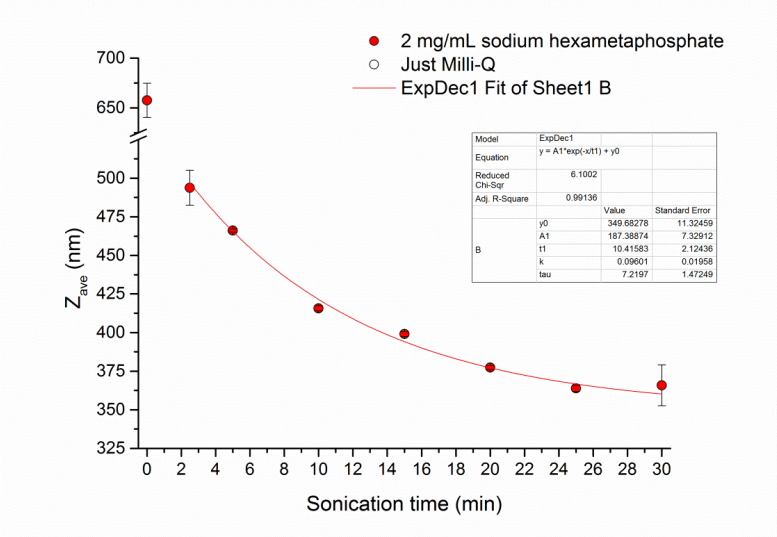


Figure 3: Z_{ave} values obtained by DLS for IRMM-381 – $BaSO_4$ (fine grade) suspensions in MilliQ water (N=1) and 2 $mgmL^{-1}$ hexametaphosphate (N=2) prepared at different probe sonication times

Table 2 Mean diameter corresponding to the major peak of the intensity-weighted size distribution obtained by the NNLS method (DLS) for dispersed IRMM-381– $BaSO_4$ (fine grade)

Sonication Time	**0 min**	**2.5 min**	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**
Peak mean (nm)	661.8 ± 10.4	506.0 ± 7.3	489.0 ± 7.5	442.0 ± 4.5	429.1 ± 6.7	**408.2 ± 5.1**	392.2 ± 2.3	387.2 ± 3.0

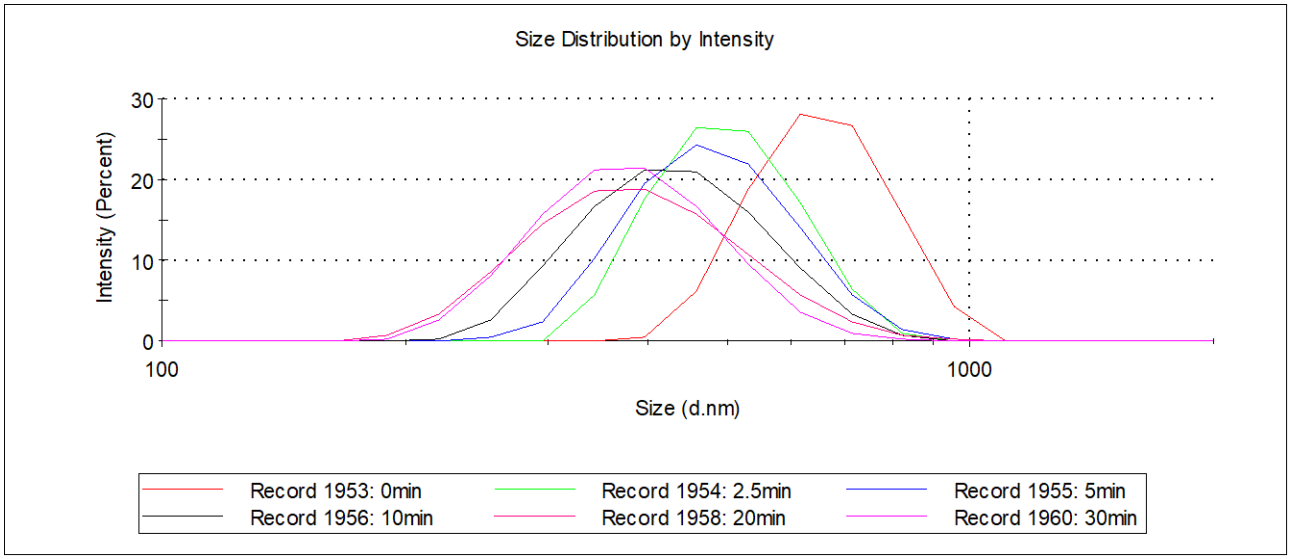


Figure 4: Intensity-weighted size distribution obtained by DLS for IRMM-381 – $BaSO_4$ (ultrafine grade) suspensions in 2 $mgmL^{-1}$ hexametaphosphate (N=2) prepared at different probe sonication times

4 SOP for production of an aqueous based dispersion of IRMM-382 (Multi-wall Carbon nanotubes)

4.1 Aim

The aim of the procedure is to describe a laboratory scale methods to produce a colloidally stable water-based dispersion of the multi-walled carbon nanotubes starting from IRMM-382 in dry powder form.

4.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-382, Multi-wall Carbon nanotubes. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose absorbance, as determined by UV-Vis measurements, does not significantly change over a time period of at least 30 minutes from completion of the dispersion procedure.

4.3 Abbreviations

MWCNT	Multi-wall Carbon nanotubes
NM	Nano/Material
SOP	Standard Operating Procedure
Tannic acid	Natural extract of plant material (CAS No. 1401-55-4)
TEM	Transmission Electron Microscopy
Triton X-100	Commercial surfactant (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, CAS No. 9002-93-1)
USP	Ultrasonic probe sonicator
UV-Vis	Ultraviolet-Visible absorption spectroscopy

4.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of a surfactant stabilised aqueous suspension (1 $mgmL^{-1}$ or 0.2 $mgmL^{-1}$ of IRMM-382) of IRMM-382, Multi-wall Carbon nanotubes. The procedure foresees starting from a dry powder sample of IRMM-382 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the stabilisers/surfactants Tannic Acid or Triton X-100. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The sonication time and power values detailed in the procedure have been determined to produce the most stable dispersion where minimum precipitation of MWCNT is observed in an experimentally relevant time (<1 hour). The use of a higher power may produce froth (depending on the dispersant) and the carbon nanotubes may attach to the generated bubbles, hence leading to a less effective dispersion of the MWCNT, while longer treatment times may potentially either degrade the quality by breaking MWCNT.

To evaluate the stability of the dispersions, UV-Vis absorbance has been measured immediately after sonication (pristine) and again after a rest periods of up 21 days (aged) with the results showing no major variation in the UV-Vis absorbance confirming the stability of the dispersion.

4.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W. Alternatively a 200 W vial sonicator may be used
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication

4.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Ultraviolet-visible spectrometer (UV-Vis)
- Transmission Electron Microscope (TEM)

4.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of dispersant
- Disposable plastic weighing boats or similar for weighing of Tannic acid and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

4.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm^{-1}, 0.2 µm in-line filtration)
- Multi-wall Carbon nanotubes distributed by IRMM with project ID no. IRMM-382.
- Ice-water mixture for cooling the sample during sonication
- 1 wt% aqueous solution of Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, CAS No. 9002-93-1)
- A 300 mgL^{-1} aqueous solution of Tannic Acid (CAS No. 1401-55-4)

4.4.5 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

4.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonictor used was Hielscher UPS200S with nominal maximum power of 200 W. This sonicator could be fitted with probe heads with diameters of 1mm, 3 mm or 7 mm diameter. The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1. In this procedure for the dispersion of IRMM-382 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of either 35 % or 50 %. From the calibration curve it was be determined that under these operating conditions the instrument was producing a measured power output of 10.6 W or 13.7 W respectively.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce the output power specified in the materials and method section of this SOP. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

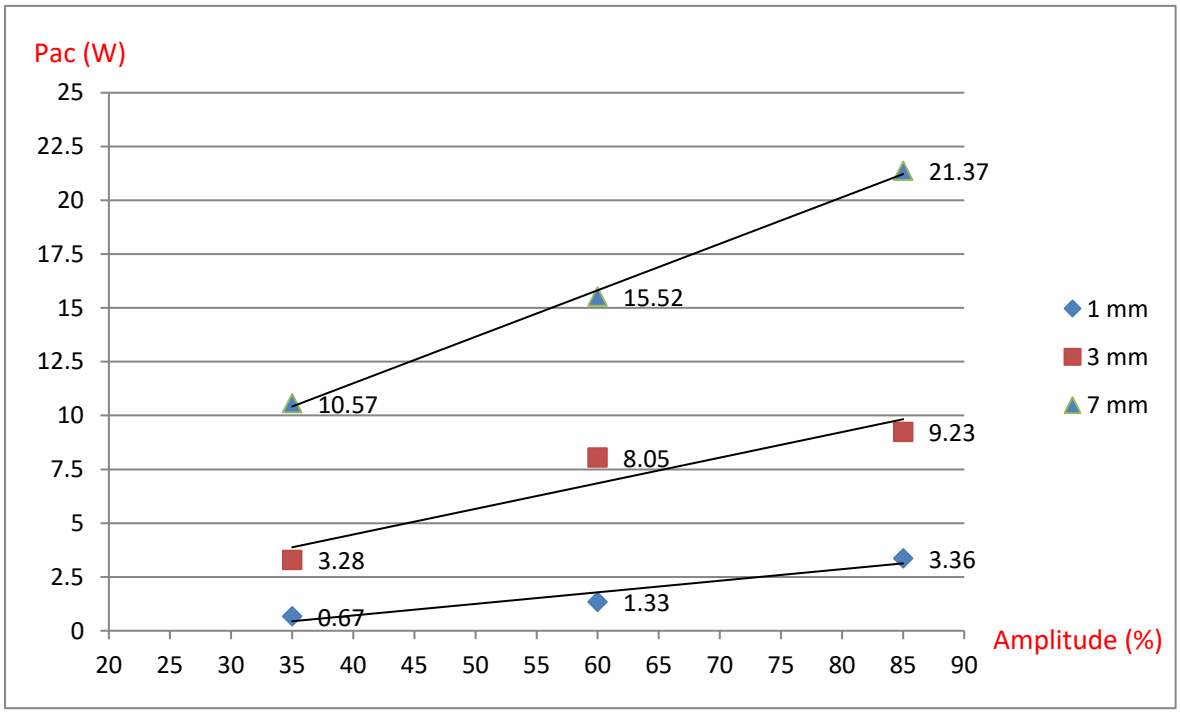


Figure 1: Acoustic power absorption characteristics of model UPS200S Heilscher ultrasonic processor

4.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 4.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

4.4.5.3 Detailed dispersion procedure for IRMM-382-MWCNT

This SOP describes two methods of dispersing the priority material IRMM-382 (MWCNT) using different stabilising agents. The first method, based on the use of Triton X100 surfactant, is able to produce dispersions with a higher mass of nanomaterials (1 $mgmL^{-1}$) but requires a relatively high concentration of surfactant (10 $mgmL^{-1}$). The second method using tannic acid can disperse a lower quantity of solids (0.2 $mgmL^{-1}$) but uses a much lower relative mass of stabiliser (0.3 $mgmL^{-1}$).

Pre-dispersion of IRMM-382-MWCNT in 1 wt% aqueous Triton X100: Weigh accurately 6 mg of IRMM-382 into a 22 mL glass vial and add sufficient of Triton X-100 (1 % wt) aqueous solution using a pipette to give a concentration of 1 $mgmL^{-1}$. Treat solution for 10 minutes in an ultrasonic bath to ensure wetting of the nanomaterial

Pre-dispersion of IRMM-382-MWCNT in (300 mgL^{-1}) aqueous tannic acid: Weigh accurately 2 mg of IRMM-382 into a 22 mL glass vial and add sufficient aqueous Tannic acid (300 mgL^{-1}) solution using a pipette to give a concentration of 0.2 $mgmL^{-1}$. Treat the solution for 10 minutes in an ultrasonic bath to ensure wetting of the nanomaterial.

Sonication using probe sonicator: The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning steps noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Take the 22 mL glass vial containing 6 mL of either solution A or B and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is full immersed in the cooling water.

Ultrasonic dispersion of IRMM-382-MWCNT in Triton X100: A 6 mL sample of solution A should be sonicated for 60 minutes with power setting adjusted to give constant power of 10.6 W (35 % amplitude on Heilscher UPS200S sonicator))

Ultrasonic dispersion of IRMM-382-MWCNT in aqueous tannic acid: A 6 mL sample of solution B should be sonicated for 30 minutes with power setting adjusted to give constant power of 13.7 W (50 % amplitude on Heilscher UPS200S sonicator)

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. The correct power settings for probe sonicators should be determined from calibration

curves measured by the method described in chapter 13. The resulting colloidal solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

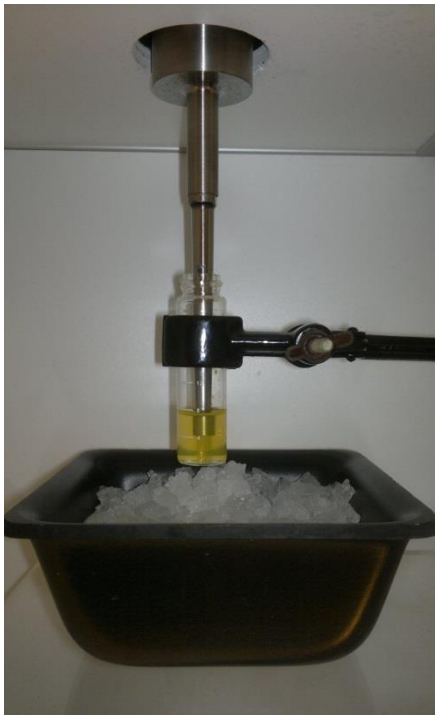


Figure 2: Photograph showing recommended positioning of probe sonicator in sample

4.4.6 Optional verification of dispersion quality

The physical form of the MWCNT particles means that light scattering and sedimentation methods are not reliable for assessing the size of the conventional. The only suitable method is by EM and where the operator has access to TEM instrumentation it is strongly recommended that the dispersion quality and stability be evaluated by TEM.

4.4.7 Recovery of dispersions after aging beyond verified period of stability

In the work of Rastogi et al. (Journal of Colloid and Interface Science 328 (2008) 421–428) it was reported that UV-Visible absorption spectroscopy was a suitable method for evaluating the dispersion quality of MWCNT and a similar approach was adopted to check the temporal stability of IRMM-382 dispersed by these procedures. To do this the UV-Visible spectra were periodically acquired from dispersed samples and the absorption values at 500 nm determined. Measurement of the UV-Visible absorption of the dispersed solutions of MWCNT (both Triton X100 and tannic acid stabilised) shows a near constant level of absorption (500 nm) over a period of 21 days confirming that little or no tendency to agglomeration and sedimentation occurs with the materials dispersed following this procedure. It is therefore not considered necessary to evaluate any procedure for recovering aged samples.

4.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

4.5 Validation status

This method has not yet been subjected to validation.

4.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

4.7 Information on expected particle size distribution

No examples of size distributions can be reported here as dispersions of MWCNT cannot be reliably measured using DLS or CLS methods, and TEM analysis was only able to provide qualitative information.

5 SOP for production of an aqueous based dispersion of IRMM-383 (Nano steel)

5.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a water-based dispersion of IRMM-383 (Nano steel) starting from dry powder form. The presented protocol allows dispersing the material in a highly dispersed state.

5.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based suspension of IRMM-383, Nano steel. The method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution.

The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

This instability is not compatible with several of the particle size measurement methods adopted in the NanoDefine project. The protocol remains useful for methods such as AFM and EM, where the sample is transferred to a solid carrier by sedimentation. Such sedimentation results in a preferential orientation of the platelets composing this material, which biases the measurements of conventional 2D EM-based methods. Because the Z-dimension is not measureable, the minimal external dimension referred to in the EC NM definition, cannot be estimated by EM.

5.3 Abbreviations

AFM	Atomic Force Microscopy
EM	Electron Microscopy
NM	Nanomaterial
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

5.4 Description

The following SOP describes a method for the preparation of small volumes (10 mL) of an aqueous dispersion of IRMM-383 at a concentration of 2.56 $mgmL^{-1}$. The procedure foresees starting from a dry powder sample of IRMM-383 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a

shorter time may produce measurably larger mean particles size, while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine). The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

5.5 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 20 kHz and equipped with a probe with a tip diameter of approximately 13 mm (e.g. Vibracell 75041 ultrasonifier, Fisher Bioblock Scientific, Aalst, Belgium)
- Adjustable volume pipettes of 100 µL, with disposable tips.
- Pipettes of 10 mL
- Box for ice-cooling of samples during sonication.
- Vortex mixer

5.5.1 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument

5.5.2 Material Supplies

- 20 mL glass vial (e.g. 10560503-X500, Wheaton Science Products, Millville, New Jersey, distributed by Fisher Scientific) with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of EM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs
- Parafilm M
- Flask rings (e.g. Heathrow scientific lead rings)
- Vial holder

5.5.3 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- Nano steel distributed by IRMM with project ID no. IRMM-383
- Ice-water mixture for cooling the sample during sonication

5.5.4 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

For laboratories equipped with a TEM, additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

5.5.4.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium). This sonicator is fitted with a probe head with a diameter of 13 mm diameter. The power characteristics of this sonicator probe have been experimentally determined. The resulting calibration curves can be seen in Figure 1. The output energy in function of the sonication time and the output power in function of the selected amplitude are shown in Figure 1A and Figure 1B, respectively.

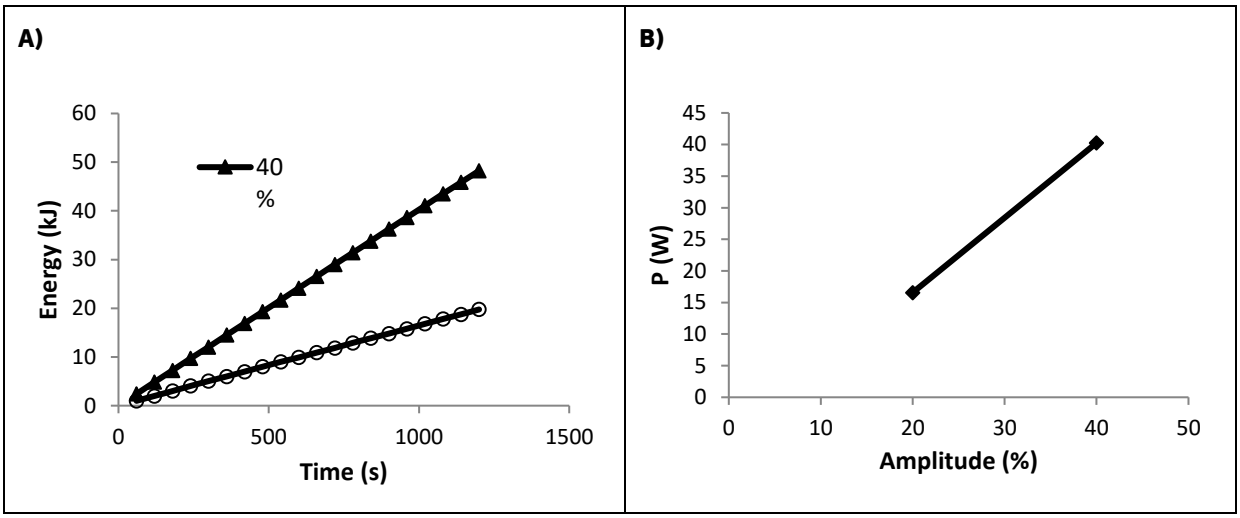


Figure 1: Calibration curves for the probe sonicator Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium) fitted with a probe head with a diameter of 13 mm, showing (A) the output energy in function of the sonication time and (B) the output power in function of the selected amplitude

In this procedure for the dispersion of IRMM-383, the 13 mm probe head (CV33) is positioned in the bottom half of the dispersion and the sonicator is operated at a set amplitude value of 40 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 40 W. Sonication is stopped after 10 minutes, when an added specific energy of 25 ± 2 kJ is read out from the sonicator apparatus.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. Once the calibration procedure has been completed, an examination of the resulting

amplitude-power curve must be done in order to determine the correct amplitude setting required to produce an output of 40 W. The amplitude setting of the sonicator should be adjusted to this value before proceeding with the dispersion procedure.

5.5.4.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 5.5.4.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

5.5.4.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the appropriate cleaning steps noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

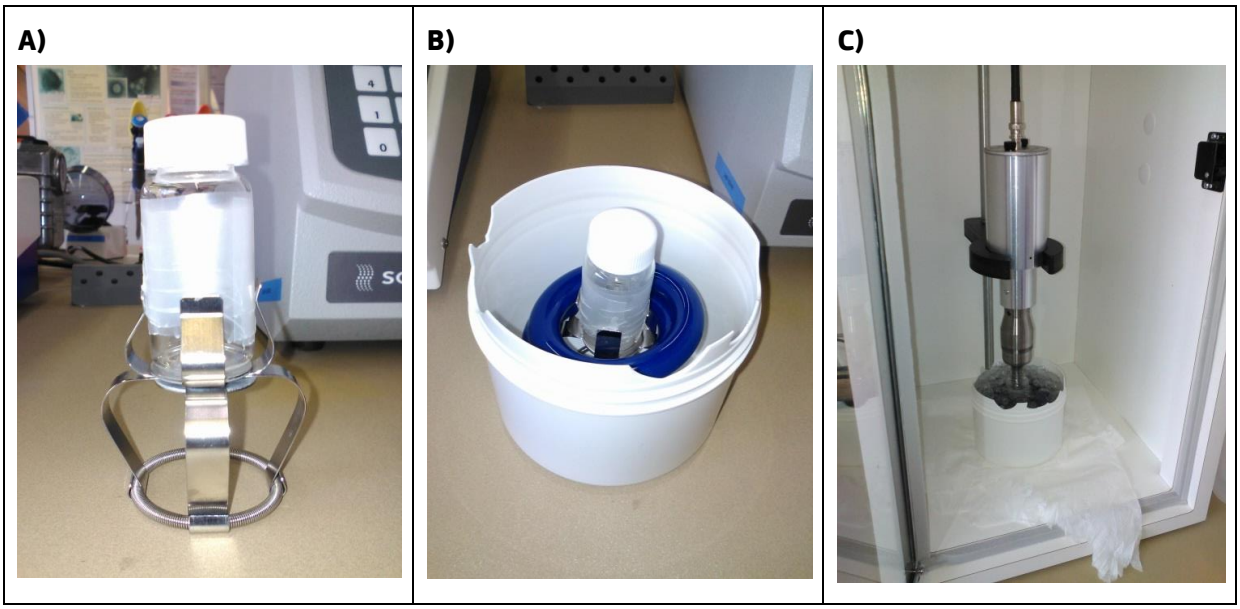


Figure 2: Setup for Sonication using the probe sonicator

Weigh approximately 25.6 mg of IRMM-383 into a 20 mL glass vial and add 10 mL of pure water to give a concentration of 2.56 $mgmL^{-1}$. It is recommended to use an Ionizer to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Wrap parafilm M around the 20 mL glass vial to avoid movement during sonication, and place the vial in the vial holder (Figure 2A). Place the vial holder in the box for ice-cooling using the flask rings (Figure 2B). Add a mixture of crushed ice and water in the box to cool the dispersion during sonication. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. Mount the probe sonicator head inside the vial

(Figure 2C). The probe head should be immersed in the dispersion to a depth of at least 1 cm. The sample should then be sonicated at a constant power of 40 W for 10 minutes. The correct power setting should be determined from the calibration curve which was previously determined.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (20 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Figure 2 (A) shows the 20 mL glass vial containing the dispersion is placed in the vial holder. Parafilm M is wrapped around the vial to avoid sliding during sonication. (B) The vial and vial holder are placed in the box for ice-cooling using the flask rings. (C) A mixture of crushed ice and water is added in the box to cool the dispersion during sonication. The vial is fully immersed in the cooling water. The probe sonicator head is immersed in the dispersion to a depth of at least 1 cm.

5.5.5 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that in section 5.8.

To become suitable for TEM analysis, the dispersion has to be diluted 10 times after sonication to obtain a concentration of 0.256 $mgmL^{-1}$. TEM specimens can be prepared following the drop-on-grid method[1]. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. The grid is then placed on 15 µL of dispersion during 10 minutes, and is rinsed 2 times afterwards with distilled water.

The described laboratory scale method produces a water-based dispersion of IRMM-383 starting from dry powder. The presented protocol disperses the material in a highly dispersed state. The material's physico-chemical properties do not allow obtaining a stable dispersion. The protocol remains useful for methods such as AFM and EM where specimens can be prepared by transferring a fraction of the sample to a solid carrier by sedimentation. TEM evaluation of the dispersion quality shows the smallest dispersible particles (referred to a single constituent particles) and some small agglomerates thereof (consisting of 2-10 particles) (Figures 3 and 4). Note that conventional TEM is not able to measure the smallest dimension (platelet thickness) of the particles due to preferential orientation of particles on the grid. The size distribution (Figure 4) should be interpreted as the distribution of the Feret min values of the particles' 2D projections on the EM grid.

If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 5.8 of this SOP, the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

5.5.6 Recovery of dispersions after aging beyond verified period of stability

The material's physico-chemical properties do not allow producing a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

5.5.7 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

5.6 Validation status

This method has not yet been subjected to validation

5.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

5.8 Information on expected particle size distribution

The qualitative TEM analysis describes the physico-chemical characteristics of the particles, such as the aggregation/agglomeration state, and the size and shape of the free single particulates, aggregates and agglomerates. Table 1 summarizes the qualitative TEM analysis of IRMM-383.

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.[2-4]. Figure 3 shows representative TEM images of IRMM-383. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Table 1 Summary of the qualitative TEM analysis of IRMM-383 Nano steel

Examined property	Description
Distribution of particles on the grid?	evenly distributed
Concentration of particles on the grid?	OK
Aggregation/agglomeration state?	single constituent particles and agglomerates
Sub-fraction	a sub-fraction of smaller particles is present in the sample (size 10-50 nm). It remains uncertain whether these small particles are nano steel or a contaminant.
Manually measured size of the constituent particles?	the size ranges from 60 nm to 1.5 µm
Manually measured size of the aggregates/agglomerates?	the size ranges from 100 nm to 2.5 µm
2D shape of the PP?	irregular polygonal
2D shape of the aggregates/agglomerates?	complex
Surface structure of the constituent particles?	rough
Surface structure of the aggregates/agglomerates?	rough
Diffraction contrast?	diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles
Efficiency of the dispersion protocol?	allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles). Visual inspection suggested however that the material is not completely dispersed in the medium. The nano steel flakes start to sink down before and immediately after sonication.

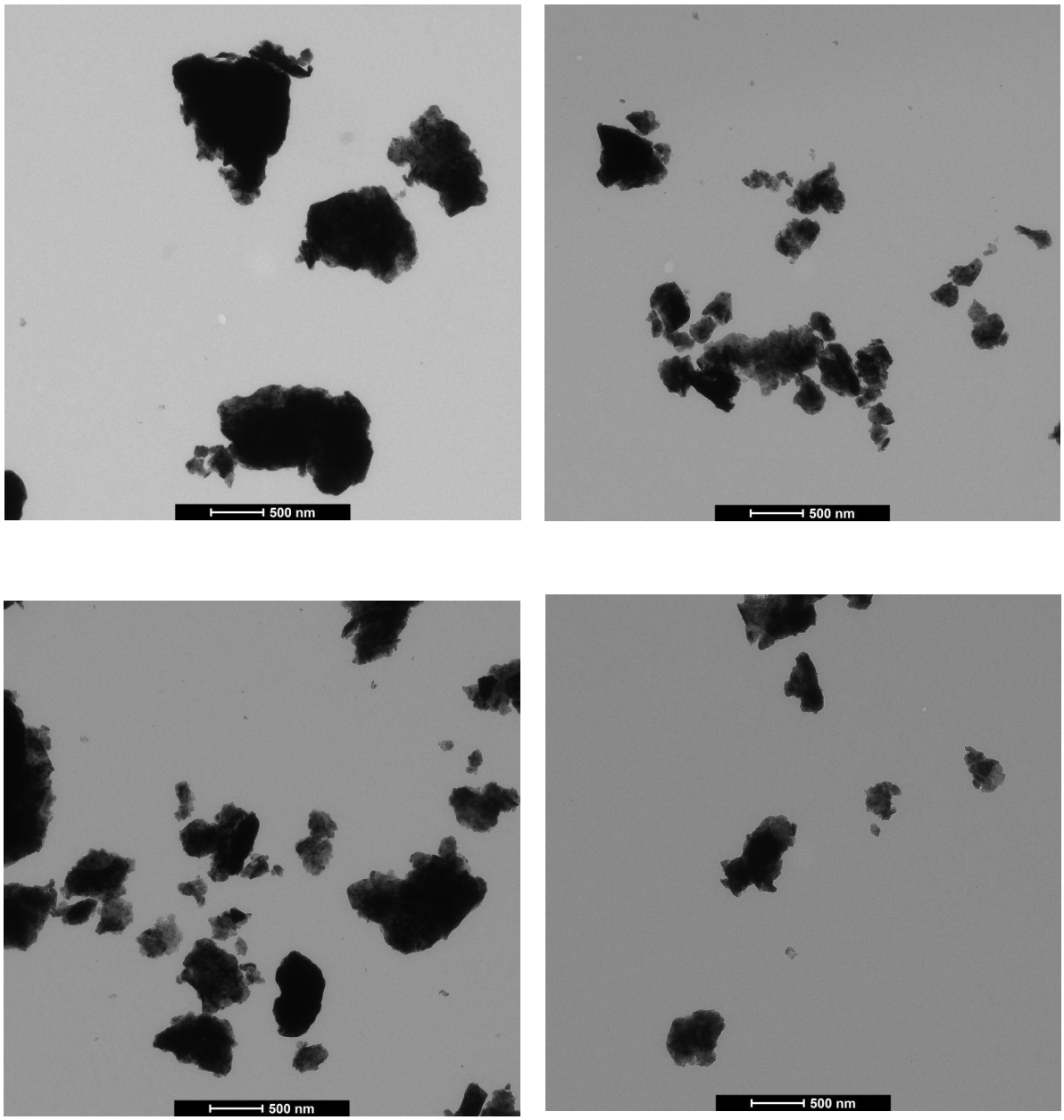


Figure 3: Representative TEM images of IRMM-383 Nano steel particles dispersed using the presented SOP

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 4). It should be noted that TEM is probably not able to measure the smallest dimension (platelet thickness) of all particles due to preferential orientation of platelet-like particles on the grid.

A sub-fraction of smaller particles is present in the sample (size 10-50 nm). It remains uncertain whether these small particles are nano steel or a contaminant.

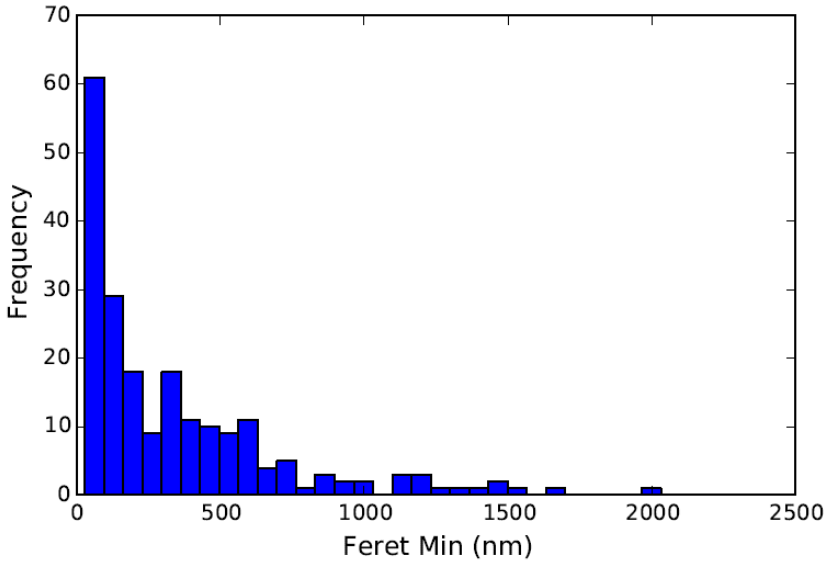


Figure 4: Representative size distribution (Feret min) of the single particulates of IRMM-383 Nano steel obtained by quantitative TEM. Note that the Feret min parameter is measured in the X-Y plane and is not suitable to estimate of the minimal external dimensions of platelet-like particles situated in the Z-plane

6 SOP for production of an aqueous based dispersion of IRMM-384 ($CaCO_3$)

6.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-384 starting from dry powder form.

6.2 Scope

The scope of this SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-384, calcium carbonate CaCO3. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

6.3 Abbreviations

Calgon	Sodium hexametaphosphate (CAS No. 10124-56-8).
CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
MALS	Multi-angle Light Scattering
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS.
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

6.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2m L or 6 mL) of a surfactant stabilised aqueous suspension (50 mgmL^{-1} of IRMM-384) of IRMM-384, Calcium carbonate ($CaCO_3$). The procedure foresees starting from a dry powder sample of IRMM-384 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a the commercial surfactant Sodium hexametaphosphate (Calgon). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjusting the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

6.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

6.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

6.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM and any chemicals in powder form
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

6.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- Calcium carbonate (CaCO3) distributed by IRMM with project ID no. IRMM-384
- Ice-water mixture for cooling the sample during sonication
- Surfactant: aqueous solution of Sodium hexametaphosphate (Calgon)

6.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

6.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

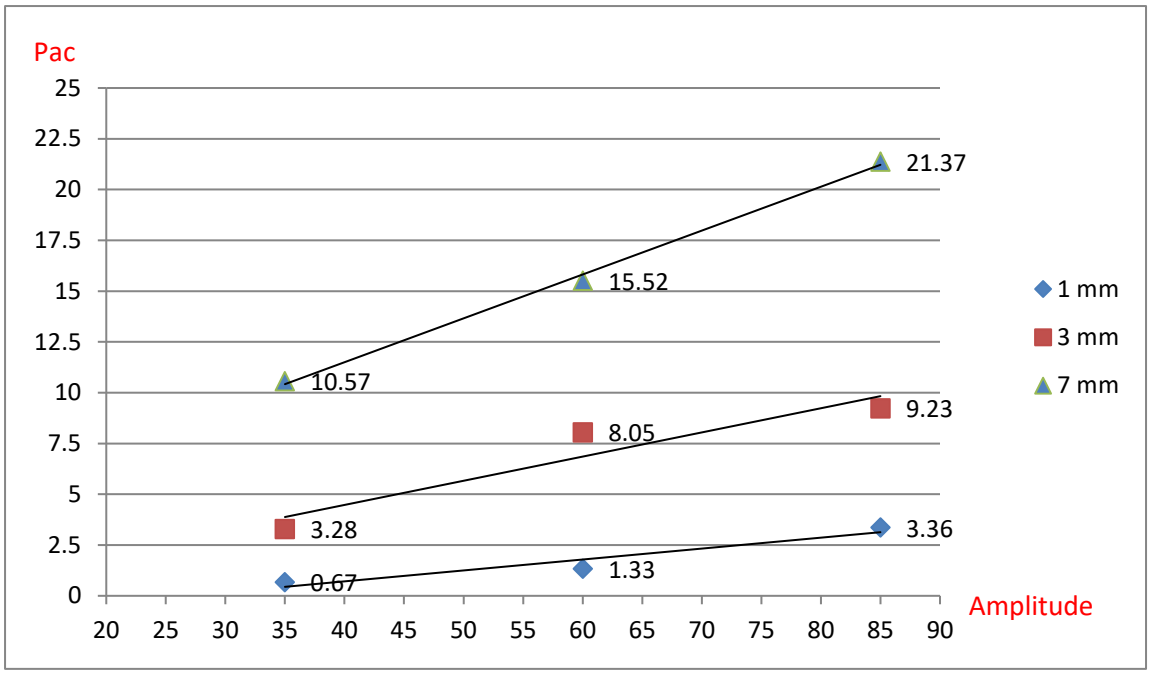


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

In this procedure for dispersion of IRMM-384 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to match these values before proceeding with the dispersion procedure.

6.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 6.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

6.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

To produce 10 mL of dispersant weigh an empty 22 mL glass vial and add approximately 20 mg of Sodium hexametaphosphate (SHMP) using a spatula. Reweigh the vial and calculate by difference amount of SHMP before adding sufficient ultrahigh purity water to give a concentration of 2mgmL^{-1}: Vortex (2'). This solution will hereafter be referred to as solution A.

Weigh approximately 300 mg of IRMM-384 into a 22 mL glass vial and add sufficient solution A to give a concentration of 50mgmL^{-1}. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (10'). This will hereafter be referred to as solution B.

Sonication using probe sonicator: Take the 22 mL glass vial containing 6 mL of solution B and mount the probe sonicator head inside the vial as shown in Figure 2.

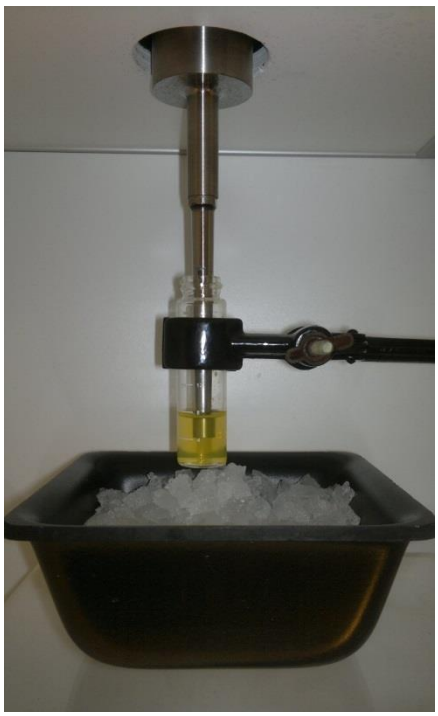


Figure 2: Photograph illustrating the mounting of probe sonicator in sample

The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. The sample should then be sonicated at a amplitude of 75 % and 50 % cycle time for 15 minutes (Hielscher UPS200S). At this amplitude setting the peak power output is 18 W which corresponds to measured mean power of 7.9 W when using a 50 % cycle time. For use with other types of sonicator correct settings should be determined from calibration curves determined by the method described in chapter 13. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low amplitude (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Sonication using vial sonicator: Place 2 mL of solution C in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 3).The vial should be sonicated for 10 min at 75 % amplitude with the cycle time being set at 50 %. Cooling cannot be applied in the case of vial sonicator and the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

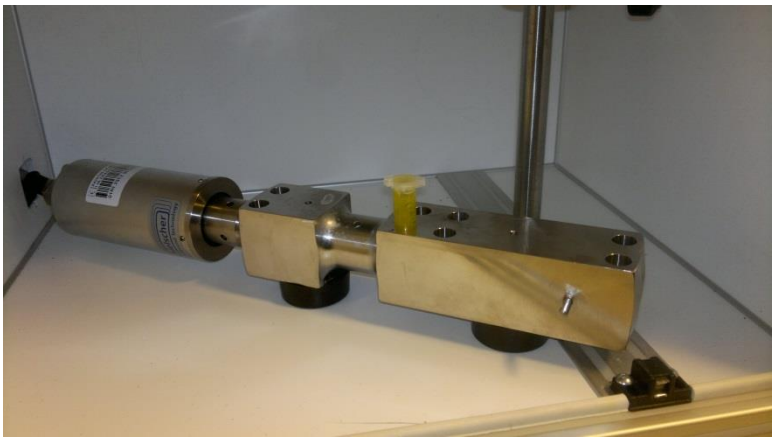


Figure 3: Positioning of sample in vial-sonicator

6.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 6.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 6.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

6.4.7 Recovery of dispersions after aging beyond verified period of stability.

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 6.4.5. In the case of materials which have been allowed to age for longer than 30 minutes it is possible that some degree of re-agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-384, material aging of up to 6 days may be

fully reversed if the sample vial is vortexed for 2 minutes and then treated for 10 minutes in a laboratory scale bath sonicator at room temperature.

6.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

6.5 Validation status

This method has not yet been subjected to validation

6.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

6.7 Information on expected particle size distribution

A typical mass based particle size distribution of the (vial sonicator) re-dispersed CaCO3 is shown in Figure 4, while Table 1 lists typical values of mean particles size obtained using the probe and vial sonicator methods.

Table 1: Mean particle size values of dispersed $CaCO_3$ as determined by CLS

	Sonication Method			
Mean Particle Size in nm of IRMM-384 by CLS (weight-size distribution)	USP 7 mm 15'	VS 10' (*)		
	348	392	359	361

(*) results of three different analyses

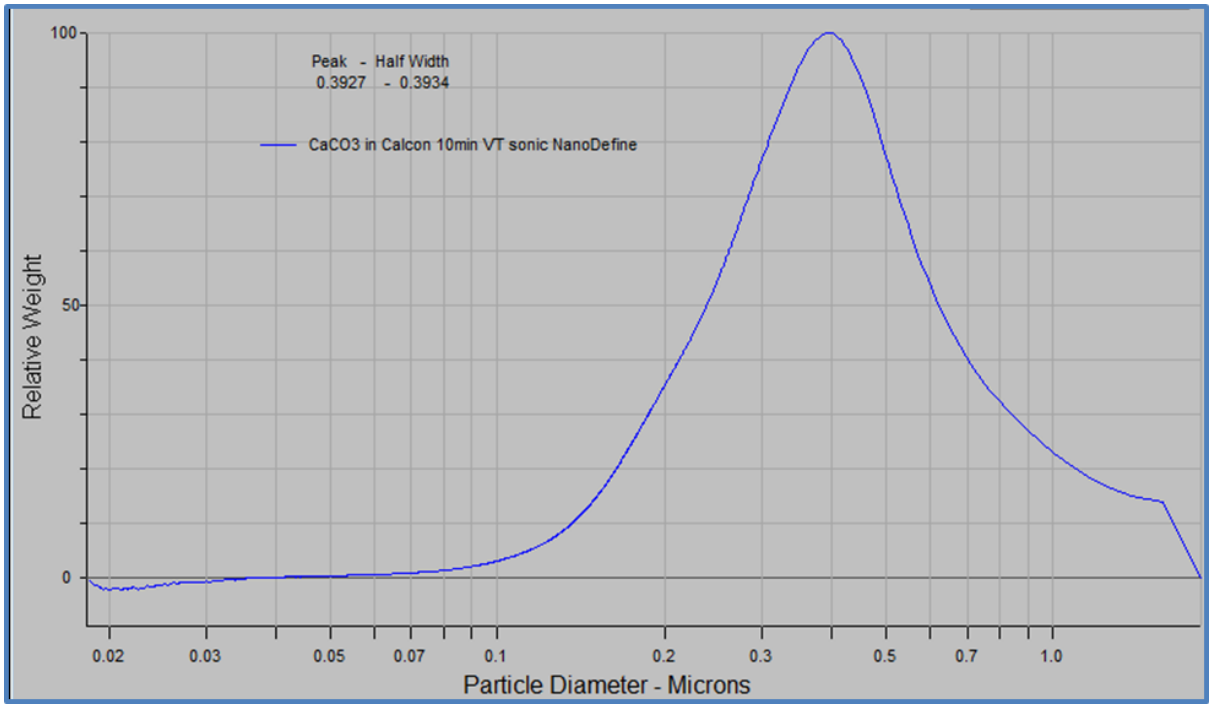


Figure 4: Typical particle size distribution of dispersed $CaCO_3$ determined by CLS

7 SOP for production of an aqueous based dispersion of IRMM-385 (Kaolin)

7.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a stable water-based dispersion of IRMM-385 starting from dry powder form.

7.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based dispersion of Kaolin (NanoDefine designation IRMM-385). The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution does not significantly change over a time period of at least 30 minutes from completion of the dispersion procedure.

7.3 Abbreviations

EM	Electron Microscopy
NM	Nanomaterial
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

7.4 Description

The following SOP describes a method for the preparation of small volumes (10 mL) of an aqueous dispersion of IRMM-385, Kaolin, designated at a concentration of 2.56 $mgmL^{-1}$. The procedure foresees starting from a dry powder sample of IRMM-385 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used, and using this data, adjust the power settings of the sonicator to produce an output value which is specified in the procedure.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine), and again after a rest period of 10, 20 and 30 minutes (aged). The particles stay in

dispersion and precipitation is not observed. Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation.

The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

7.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 20 kHz and equipped with a probe with a tip diameter of approximately 13 mm (e.g. Vibracell 75041 ultrasonifier, Fisher Bioblock Scientific, Aalst, Belgium)
- Adjustable volume pipettes of 100 µL with disposable tip
- Pipettes of 10 mL
- Box for ice-cooling of samples during sonication
- Vortex mixer

7.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

7.4.3 Material Supplies

- 20 mL glass vial (e.g. 10560503-X500, Wheaton Science Products, Millville, New Jersey, distributed by Fisher Scientific) with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs
- Parafilm M
- Flask rings (e.g. Heathrow scientific lead rings)
- Vial holder

7.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm^{-1}, 0.2 µm in-line filtration)
- Kaolin distributed by IRMM with project ID no. IRMM-385
- Ice-water mixture for cooling the sample during sonication

7.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

For laboratories equipped with a TEM additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

7.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium). This sonicator is fitted with a probe head with a diameter of 13 mm diameter. The power characteristics of this sonicator probe have been experimentally determined. The resulting calibration curves can be seen in Figure 1. The output energy in function of the sonication time and the output power in function of the selected amplitude are shown in Figure 1A and Figure 1B, respectively.

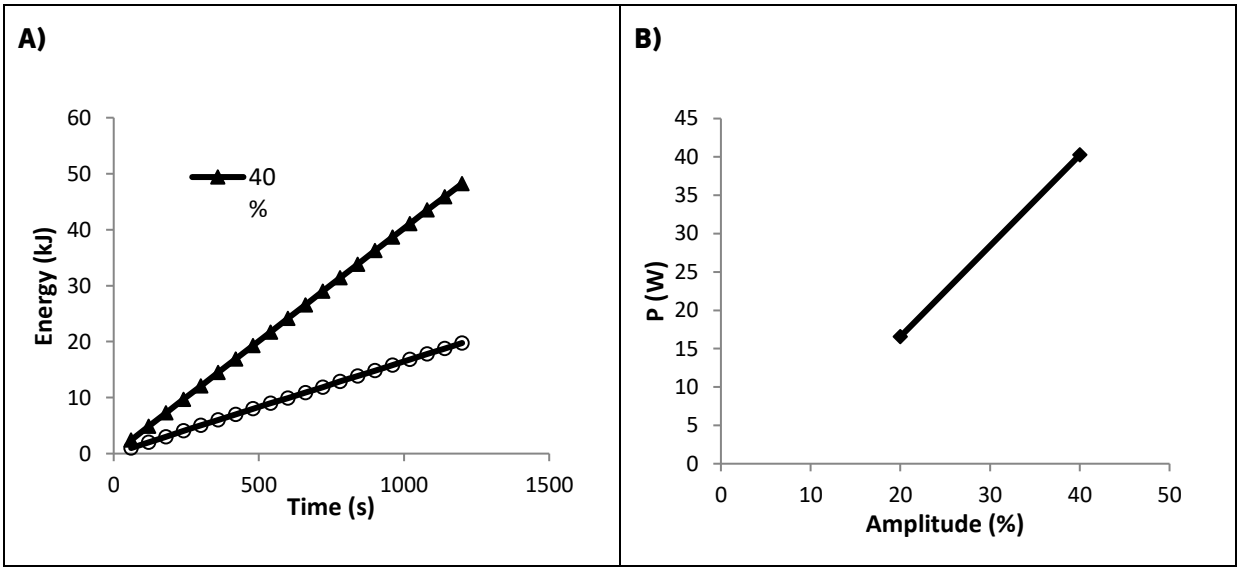


Figure 1: Calibration curves for the probe sonicator Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium) fitted with a probe head with a diameter of 13 mm, showing (A) the output energy in function of the sonication time and (B) the output power in function of the selected amplitude

In this procedure for the dispersion of IRMM-385, the 13 mm probe head (CV33) is positioned in the bottom half of the dispersion and the sonicator is operated at a set amplitude value of 40 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 40 W. Sonication is stopped after 10 minutes, when an added specific energy of 25 ± 2 kJ is read out from the sonicator apparatus.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine the correct amplitude setting required to produce an output of 40 W. The amplitude setting of the sonicator should be adjusted to this value before proceeding with the dispersion procedure.

7.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 7.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

7.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh approximately 25.6mg of IRMM-385 into a 20 mL glass vial and add 10 mL of pure water to give a concentration of 2.56 $mgmL^{-1}$. It is recommended that an Ionizer is used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Wrap parafilm M around the 20 mL glass vial containing the dispersion to avoid movement during sonication, and place the vial in the vial holder (Figure 2A). Place the vial holder in the box for ice-cooling using the flask rings (Figure 2B). Add a mixture of crushed ice and water in the box to cool the dispersion during sonication. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. Mount the probe sonicator head inside the vial (Figure 2C). The probe head should be immersed in the dispersion to a depth of at least 1 cm. The sample should then be sonicated at a constant power of 40 W for 10 minutes. The correct power setting should be determined from the calibration curve. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (20 %) for 5 minutes in high purity ethanol and then 5 minutes in water before being dried with a flow of clean compressed nitrogen or air.

As can be seen in Figure 2A the 20 mL glass vial containing the dispersion is placed in the vial holder. Parafilm M is wrapped around the vial to avoid sliding during sonication. The vial and vial holder are placed in the box for ice-cooling using the flask rings (Figure 2B). A mixture of crushed ice and water is added in the box to cool the dispersion during sonication. The vial is fully immersed in the cooling water (Figure 2C). The probe sonicator head is immersed in the dispersion to a depth of at least 1 cm.

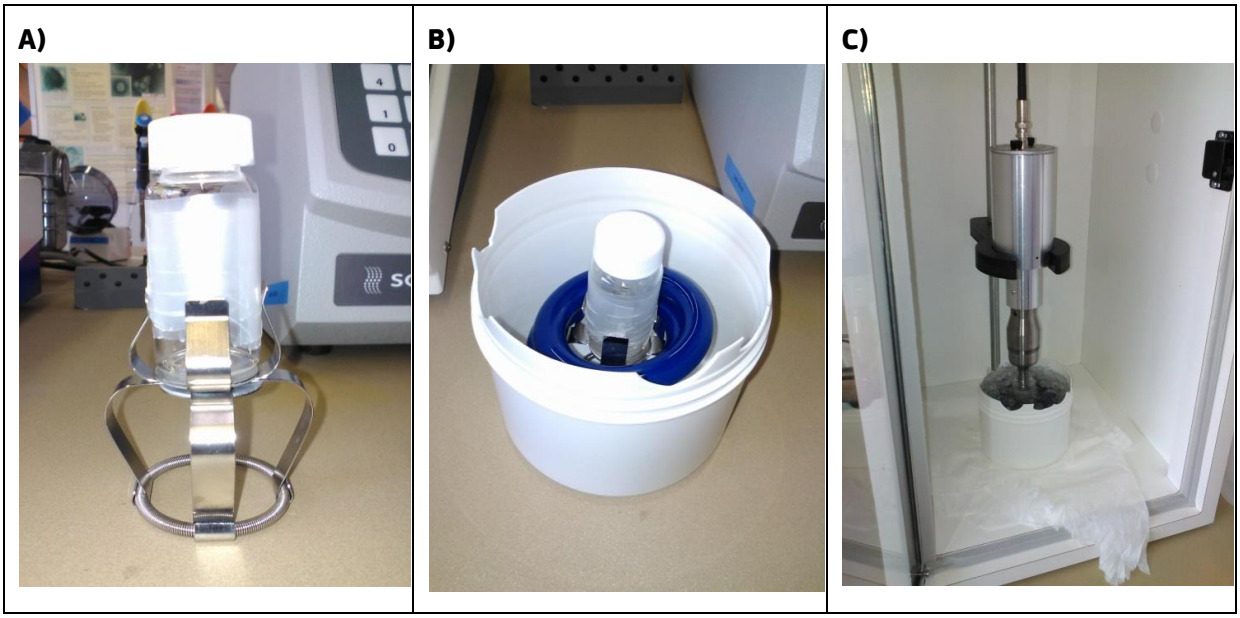


Figure 2: Setup for sonication using the probe sonicator

7.4.6 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that in section 7.7.

To become suitable for TEM analysis, the dispersion has to be diluted 10 times after sonication to obtain a concentration of 0.256 $mgmL^{-1}$. TEM specimens can be prepared following the grid-on-drop method[1]. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. The grid is then placed on 15 µL of dispersion during 10 minutes, and is rinsed 2 times afterwards with distilled water.

TEM evaluation of the dispersion quality shows the smallest dispersible particles (referred to as single constituent particles) and some small agglomerates thereof (consisting of 2-10 particles) (Figure 3 and Figure 4). If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 7.7 of this SOP, the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

7.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps has been verified visually, and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 7.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur.

7.5 Validation status

This method has not yet been subjected to validation

7.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

7.7 Information on expected particle size distribution

The qualitative TEM analysis describes the physico-chemical characteristics of the particles, such as the aggregation/agglomeration state, and the size and shape of the free single particulates, aggregates and agglomerates. Table 1 summarizes the qualitative TEM analysis of IRMM-385.

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.[1-3] shows representative TEM images of IRMM-385. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Table 1: Summary of the qualitative TEM analysis of IRMM-385 Kaolin

Examined property	Description
Distribution of particles on the grid?	evenly distributed
Concentration of particles on the grid?	OK
Aggregation/agglomeration state?	single constituent particles, aggregates and agglomerates
Sub-fraction	no sub-fraction of smaller particles/contaminants is present in the sample
Manually measured size of the constituent particles?	the size ranges from 25 nm to 750 nm
Manually measured size of the aggregates/agglomerates?	the size ranges from 100 nm to 7.5 µm
2D shape of the PP?	irregular polygonal
2D shape of the aggregates/agglomerates?	fractal-like or more complex
Surface structure of the constituent particles?	rough
Surface structure of the aggregates/agglomerates?	rough
Diffraction contrast?	diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles
Efficiency of the dispersion protocol?	allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 4A). A log-normal curve is fitted iteratively the scatter plot (Figure 4B). It should be noted that TEM is probably not able to measure the smallest dimension (platelet thickness) of all particles due to preferential orientation of platelet-like particles on the grid.

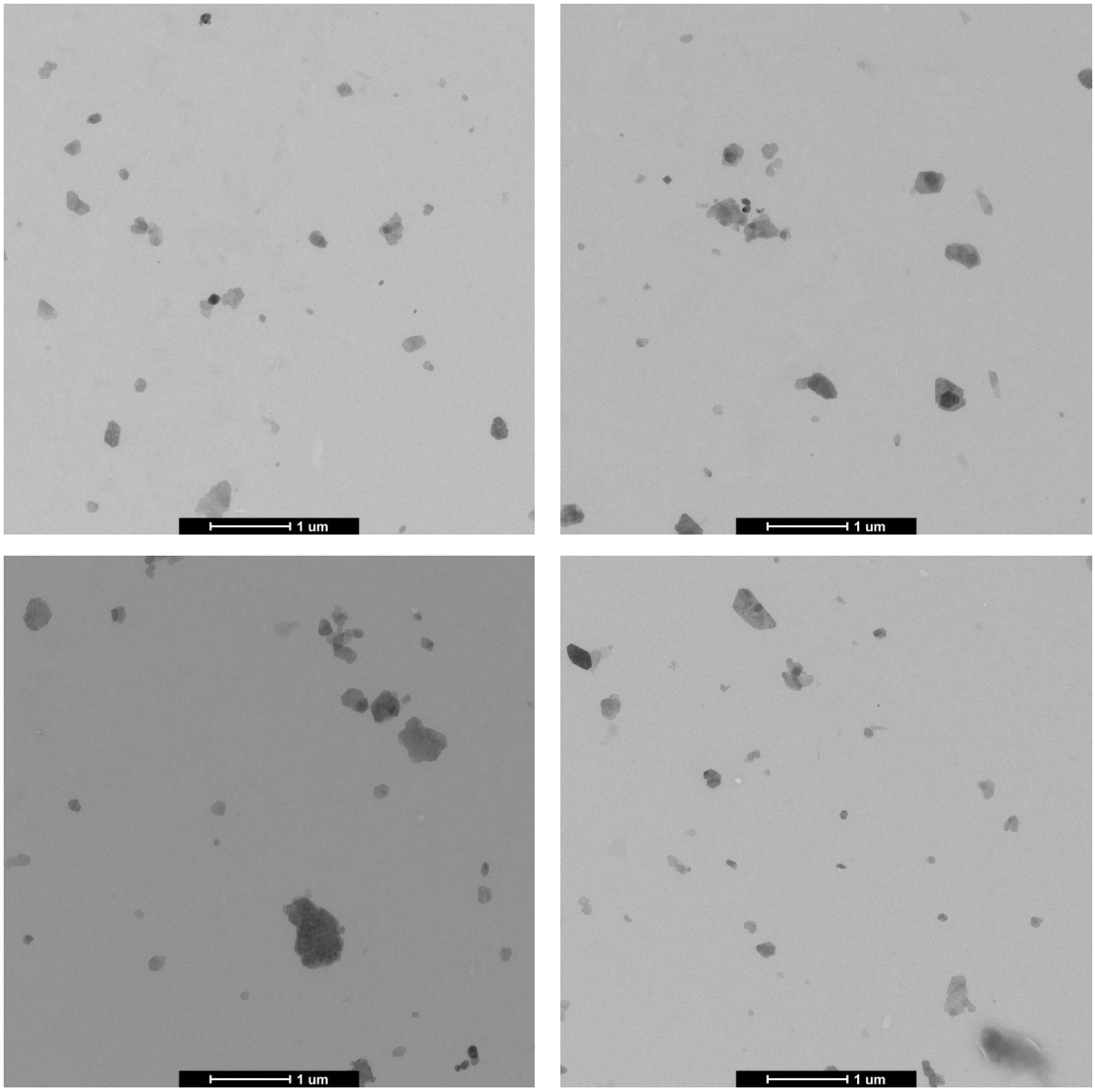
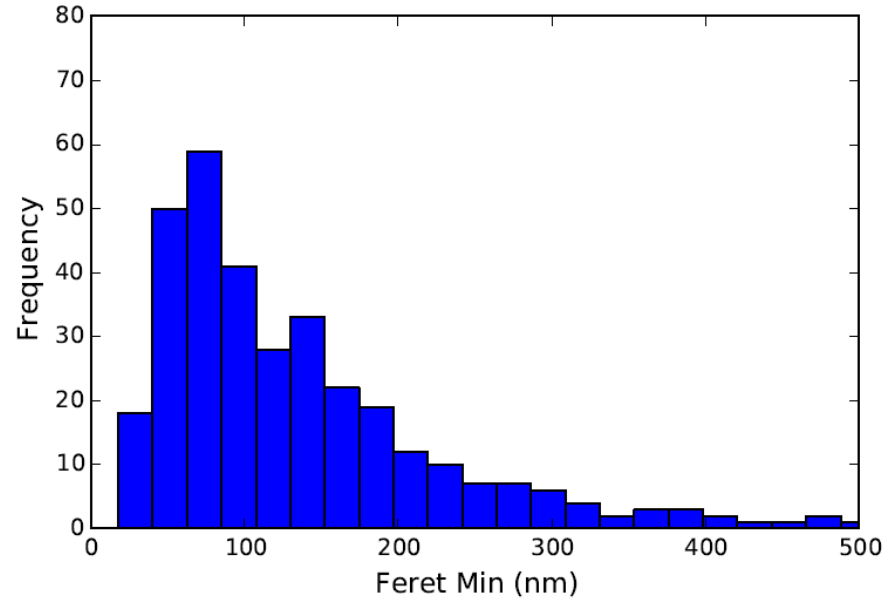
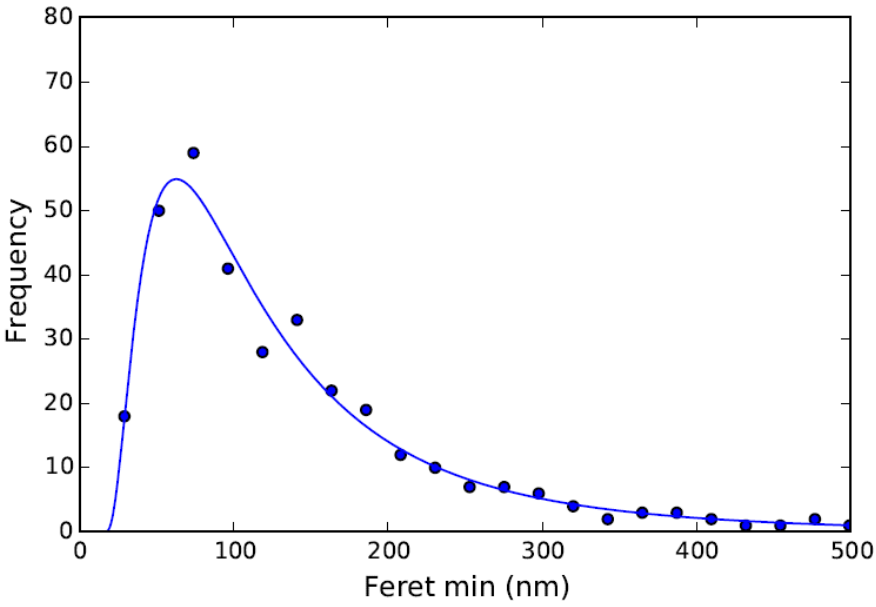


Figure 3: Representative TEM images of the dispersed particles of IRMM-385 Kaolin



A



B

Figure 4: Size distribution (Feret min) of the single particulates of IRMM-385 Kaolin obtained by quantitative TEM analysis represented by a histogram in the upper panel and in the lower panel by a scatter plot with a fitted log-normal function

7.8 References

[1] E. Verleysen, E. Van Doren, N. Waegeneers, P.-J. De Temmerman, M. Abi Daoud Francisco, J. Mast, TEM and SP-ICP-MS Analysis of the Release of Silver Nanoparticles from Decoration of Pastry. Journal of agricultural and food chemistry, 63(13), 3570-3578 (2015).

[2] E. Verleysen, P.-J. De Temmerman, E. Van Doren, M. Abi Daoud Francisco, J. Mast, Quantitative characterization of aggregated and agglomerated titanium dioxide nanomaterials by transmission electron microscopy. Powder Technology 258, 180-188 (2014).

[3] P.-J. De Temmerman, E. Van Doren, E. Verleysen, Y. Van der Stede, M. Francisco, J. Mast, Quantitative characterization of agglomerates and aggregates of pyrogenic and precipitated amorphous silica nanomaterials by transmission electron microscopy, Journal of Nanobiotechnology, 10 (2012).

8 SOP for production of an aqueous based dispersion IRMM-386 (Opaque Pigment Yellow 83)

8.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-386 starting from dry powder form.

8.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-386 opaque Pigment Yellow 83. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

8.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
MALS	Multi-angle Light Scattering
MeOH	Methanol
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

8.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 mgmL^{-1} of IRMM-386) of IRMM-386, Pigment Yellow 83 (Opaque grade). The procedure foresees starting from a dry powder sample of IRMM-386 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the commercial surfactant NEKAL BX. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and amplitude values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

8.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 3mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

8.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

8.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

8.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- Pigment Yellow 83 opaque grade distributed by IRMM with project ID no. IRMM-386
- High purity methanol (analytical grade)
- Ice-water mixture for cooling the sample during sonication.
- Surfactant: 30 wt% aqueous solution of NEKAL-BX (Sodium Butyl naphthalene sulphonate (CAS No. 25638-17-9)

8.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

8.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was

Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

In this procedure for dispersion of IRMM-386 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude and cycle time settings are required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

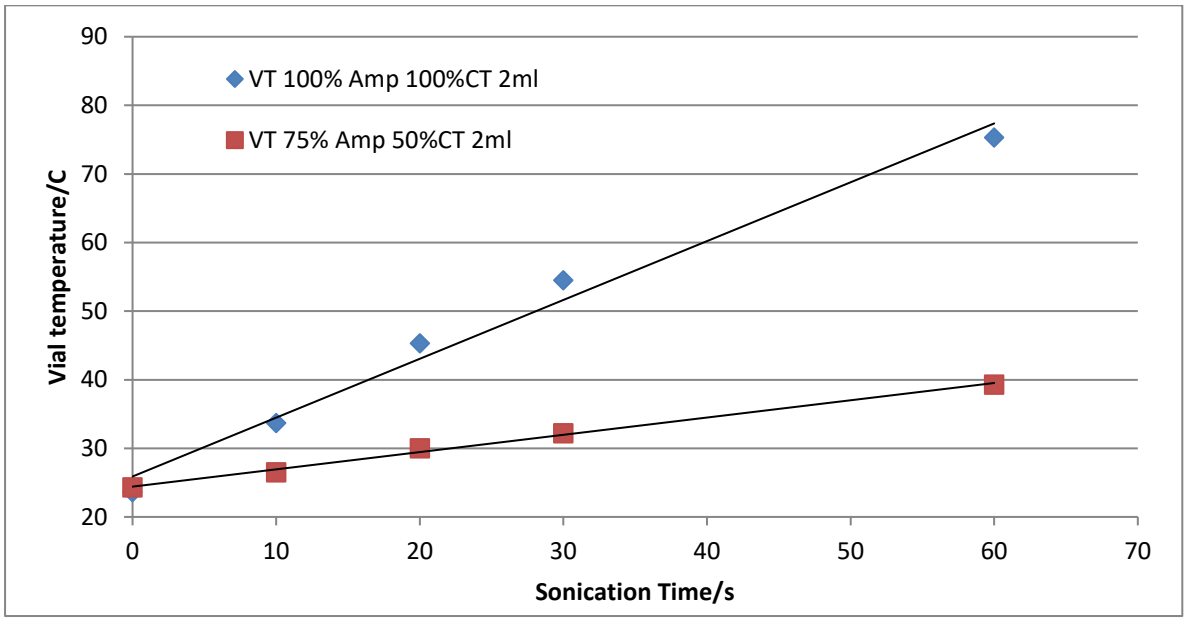


Figure 1: Temperature increase of 2 mL water in vial sonicator at (a) 100 % Amplitude and 100 % cycle-time and (b) 75 % amplitude and 50 % cycle time. Specific power absorbed is (a) 3.8 WmL^{-1} and (b) 1.1 WmL^{-1} respectively

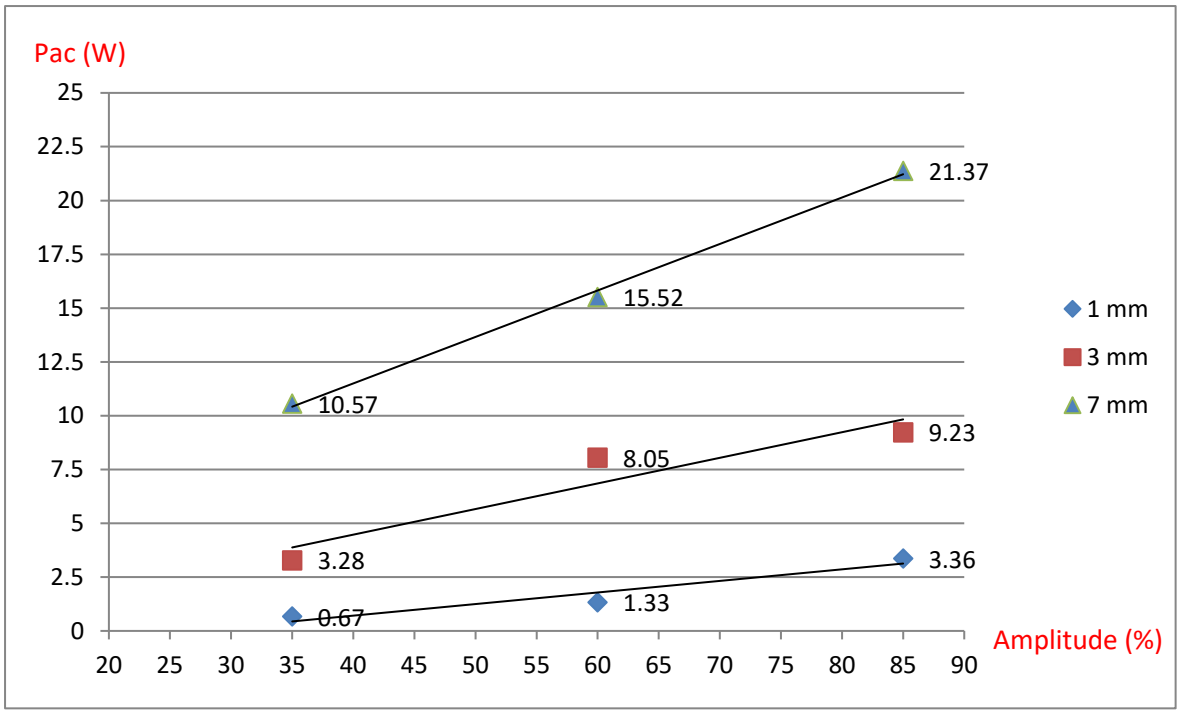


Figure 2: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time Acoustic power absorbed when using different probe diameters and amplitude settings

8.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 7). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

8.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 15 µL of Nekal BX solution (30 wt%) using a pipette. Reweigh the vial and calculate by difference the amount of NEKAL BX before adding sufficient pure methanol to give a concentration of 0.5 $mgmL^{-1}$: This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-386 into a 22 mL glass vial and add sufficient pure methanol to give a concentration of 1 $mgmL^{-1}$. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing

(2') and then sonicating in USB (2'): Add solution A to solution B in a ratio of 10 $\mu L mL^{-1}$. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): This will hereafter be referred to as solution B.

Prepare a heated water bath under a chemical safety hood and heat to 40-50 °C. Suspend the lower half of vial in the water bath until the MeOH evaporates leaving a layer of surfactant coated particles on the bottom of the vial. Add sufficient MilliQ water to get 10 $mgmL^{-1}$ solids in water and seal the vial with a suitable lid. Re-disperse the solids into the water by immersing the bottom half of the vial in a USB and sonicating for 2 minutes or until the solids appear uniformly distributed in the water. This will hereafter be referred to as solution C.

Take an empty 22 mL vial and add 5.94 mL of pure water followed by 60 µL of solution C to give a final concentration of 0.1 $mgmL^{-1}$ IRMM-386 in water. This will hereafter be referred to as solution D.

Sonication using probe sonicator: Take the 22 mL glass vial containing solution D and mount the probe sonicator head inside the vial as shown in Figure 3. The probe head should be immersed in the solution to a depth of approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is fully immersed in the cooling water. In this procedure the sonicator used was a Hielscher UPS200S and this was operated in pulsed mode with an amplitude of 75 % and a cycle time of 50 % which corresponds to a peak power of 18 W, mean adsorbed power of 7.8 W (50 % cycle time) and 1.3 WmL^{-1} when normalised to the specified volume of 6 mL. A sonication time of 20 minutes was determined to be the optimum treatment time for this material under the described conditions.

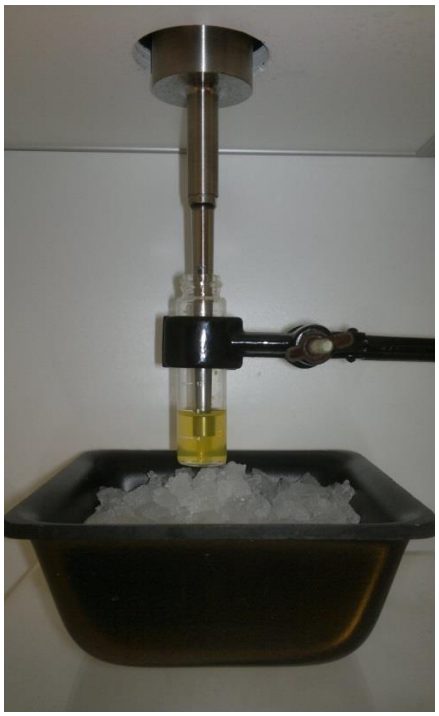


Figure 3: Photograph showing recommended positioning of probe sonicator in sample

When attempting to use a probe sonicator which is different from the model used in the development of this method users must firstly determine the power output characteristic of their

own instrument using the method described in chapter 13. From this data, instrument settings should be determined which can approximate the power output values detailed above.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

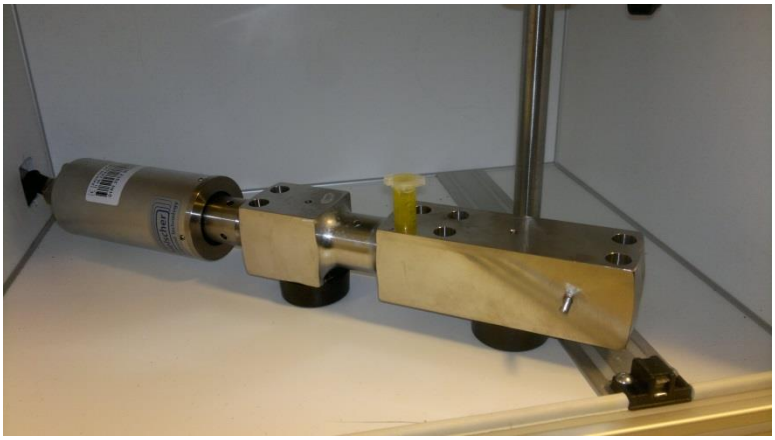


Figure 4: Positioning of sample in vial-sonicator

Sonication using vial sonicator: Place 2 mL of solution D in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 4).The vial should sonicated for 15 min at 75 % amplitude with the cycle time being set at 50 %. As cooling cannot be applied in the case of vial sonicator the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

8.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 8.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in chapter 13 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

8.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in the previous

section 8.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-386, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and the treated for 10 minutes in a laboratory scale bath sonicator at room temperature. (See Figure 5).

8.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

8.5 Validation status

This method has not yet been subjected to validation

8.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

8.7 Information on expected particle size distribution

Figure 5 shows a typical mass based particle size distribution of the (vial sonicator) as-dispersed IRMM-386 Pigment Yellow 83 (opaque grade) together with particle size distribution of the same material after 24 hours ageing and re-dispersion by 10 minutes USB. Table 1 lists typical values of mean particle size obtained using the probe and vial sonicator methods.

Table 1: Mean particle size of dispersed IRMM-386 by CLS (weight-size distribution)

	Sonication Method	
Mean Particle Size in nm of IRMM-386 by CLS (weight-size distribution)	USP 7 mm 15'	VS 15'
	326	240

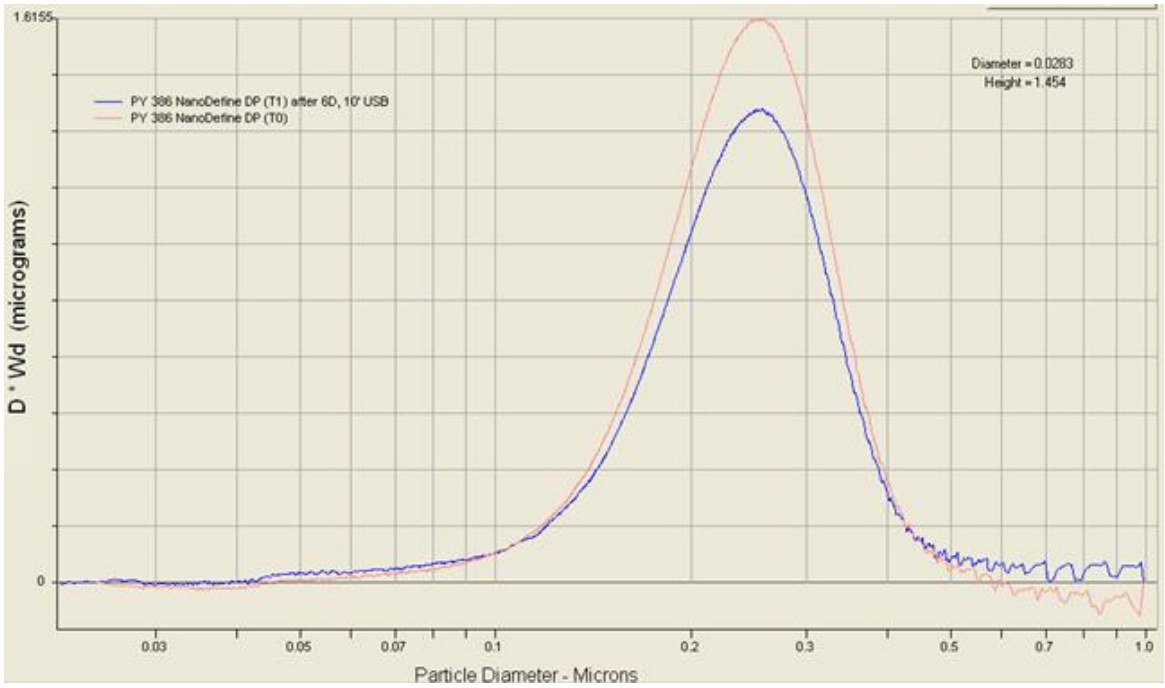


Figure 5: CLS determined particle size distribution analysis of IRMM-386 Pigment Yellow 83 (opaque grade) as-dispersed (T0) and after 24 h ageing followed by 10 min bath sonication

9 SOP for production of an aqueous based dispersion of IRMM-387 ($BaSO_4$ (ultrafine grade))

9.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-387 starting from dry powder form.

9.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-387, ultrafine grade $BaSO_4$. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS, does not significantly change (according to DLS measurements) over a time period of at least 60 minutes from completion of the dispersion procedure.

9.3 Abbreviations

DLS	Dynamic Light Scattering
MALS	Multi-angle Light Scattering
NM	Nanomaterial
PDI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

9.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 $mgmL^{-1}$) of IRMM-387, $BaSO_4$ (ultrafine grade). The procedure foresees starting from a dry powder sample of IRMM-387 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing the commercial stabilising agent sodium hexametaphosphate (SHMP). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and is suited to the production of a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particle size values while longer treatment times will either degrade the quality (re-formation of larger aggregates/agglomerates) or will not provide a significant further reduction in the mean size. Similarly, the amount of SHMP used for dispersion has a considerable influence on the quality of the dispersion and obtained particle size distribution. This procedure has been developed to produce the lowest mean particle size distribution for dispersion in 2 $mgmL^{-1}$ SHMP. The use of lower concentrations of SHMP will lead to IRMM-387 dispersions with larger mean particle size values.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 60 minutes (aged) with the results showing no major variation in the means size distribution.

9.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

9.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

9.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

9.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- $BaSO_4$ (ultrafine grade) distributed by IRMM with project ID no. IRMM-387
- Ice-water mixture for cooling the sample during sonication
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity ≥ 96 %, e.g. 305553 Aldrich)

9.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

9.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W.

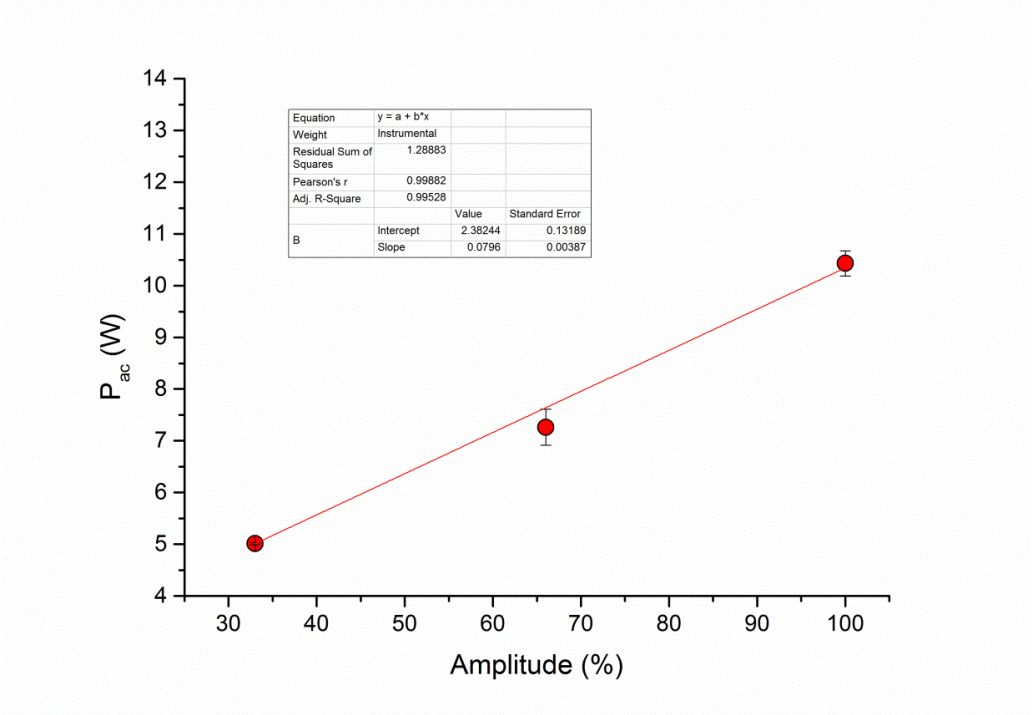


Figure 1: Calculated delivered output power P_{ac} of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to Pac = 7.6 W (in this example: amplitude of 66 %)

The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 µm). The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curve can be seen in Figure 1. In this procedure for the dispersion of IRMM-387 the sonicator was operated at a set amplitude value of 66 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 7.6 W. Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-

power curve must be done in order to determine what is correct amplitude setting required to produce an output of 7.6 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

9.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 9.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

9.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare stabilising agent solution (2 $mgmL^{-1}$ SHMP) by dissolving the appropriate amount of SHMP powder into MilliQ water. Shake vigorously to ensure that all powder is solubilized. Subsequently, filter the prepared SHMP solution using a 0.2 µm filter to ensure that no large particulates are present.

Weigh approximately 15.6 mg of IRMM-387 into a 20 mL glass vial. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of SHMP solution to give an IRMM-387 concentration of 2.6 $mgmL^{-1}$ (6 mL for exactly 15.6 mg of IRMM-387, adjust volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Take the 20 mL glass vial containing the 2.6 $mgmL^{-1}$ IRMM-387 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant power 7.6 W for 5 minutes. The correct power setting should be determine from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

9.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 9.8.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 9.8 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

9.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of IRMM-387–$BaSO_4$ dispersions prepared by this method have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 h following completion of the primary dispersion procedure outlined in section 9.4.5. At this time no further information is available of the re-dispersability of the solution after ageing for longer time periods.

9.5 Reporting of results

The main objective of the procedure does not foresee any specific metrological step. Consequently it has not been deemed necessary to detail any step relating to the reporting of results.

9.6 Validation status

This method has not yet been subjected to validation

9.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

9.8 Information on expected particle size distribution

The DLS results obtained for IRMM-387 dispersions prepared according to procedure described in section 9.4 are summarized in Table 1, Figure 3, Figure 4, Table 2 and Figure 5. The data shown includes the whole set of results obtained at different sonication times with fixed amplitude settings (22.5 kHz probe sonicator, 66 % max. amplitude, 6.4 mm probe) and is reported herein so that the end-user of this SOP can evaluate his own results. The increase in sonication time leads to a progressive decrease in mean particle size (Zave and Peak1 mean values, Table 1, Figure 3, Table 2 and Figure 5). As described in the procedure in section 9.4.5, the optimized time of sonication was found to be 5 min. This sonication time was the minimum time required in order to obtain a stable IRMM-387 dispersion (no sedimentation). The use of treatment times longer than 5 min may result in even lower mean particle size values, but is not advised since the PDI was observed to increase with the extent of treatment (suggesting that the suspension becomes more polydisperse).

Table: 1: Summary of the DLS results obtained for IRMM-387 – $BaSO_4$ (ultrafine grade) suspensions in MilliQ water and 2 $mgmL^{-1}$ hexametaphosphate (N=5) prepared at different probe sonication times

Sonication Time	0 min	1 min	2 min	3 min	**5 min**	8 min	16 min
Intensity-weighted mean diameter: by DLS (Z_{ave}, cumulants method)	127.8 ± 0.2	127.5 ± 0.7	125.9 ± 0.9	125.0 ± 1.0	**124.3 ± 1.2**	122.7 ± 0.6	118.8 ± 1.0
Polydispersity index by DLS (PDI, cumulants method)	0.135 ± 0.006	0.133 ± 0.002	0.126 ± 0.012	0.132 ± 0.008	**0.136 ± 0.006**	0.151 ± 0.006	0.161 ± 0.003

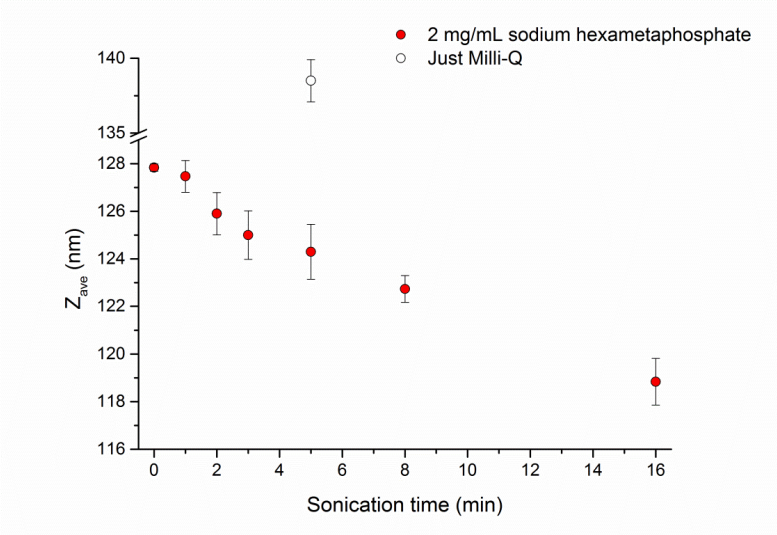


Figure 3: Z_{ave} values obtained by DLS for IRMM-387 – $BaSO_4$ (ultrafine grade) suspensions in MilliQ water (N=2) and 2mgmL^{-1} hexametaphosphate (N=3) prepared at different probe sonication times

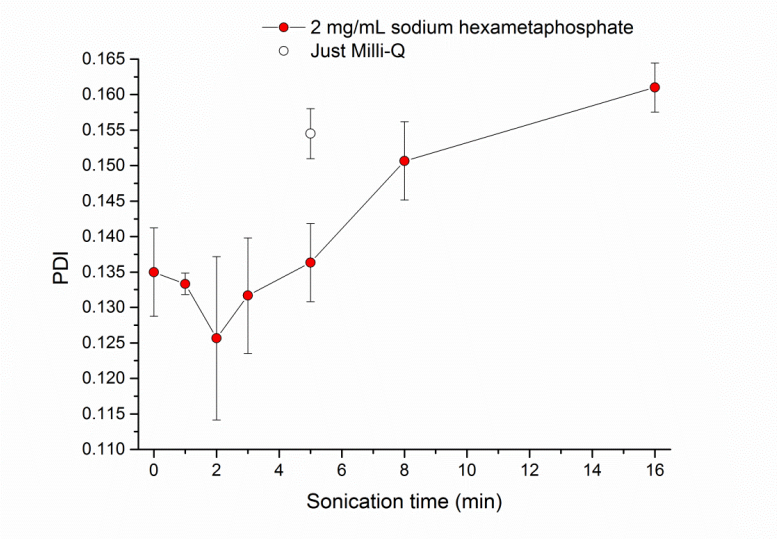


Figure 4: PDI values obtained by DLS for IRMM-387 – $BaSO_4$ (ultrafine grade) suspensions in MilliQ water (N=2) and 2mgmL^{-1} hexametaphosphate (N=3) prepared at different probe sonication times

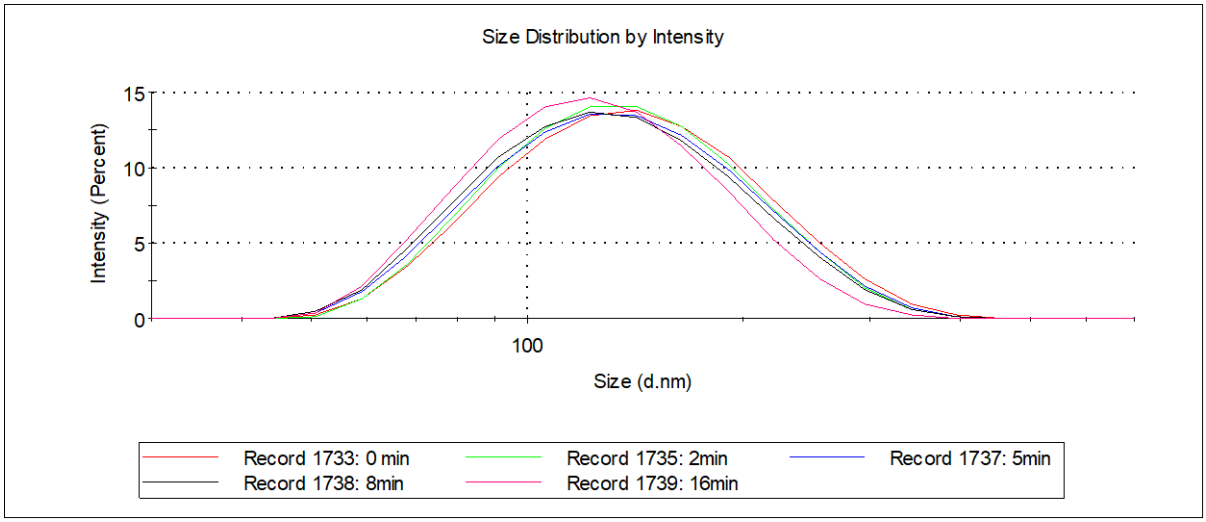


Figure 5: Intensity-weighted size distribution obtained by DLS for IRMM-387 – $BaSO_4$ (ultrafine grade) suspensions in 2 $mgmL^{-1}$ hexametaphosphate (N=3) prepared at different probe sonication times

Table: 2 Mean diameter corresponding to the major peak of the intensity-weighted size distribution obtained by the NNLS method (DLS) for dispersed IRMM-387– $BaSO_4$ (ultrafine grade)

Sonication Time	0 min	1 min	2 min	3 min	**5 min**	8 min	16 min
Peak mean	148.6 ± 1.7	146.8 ± 2.0	144.3 ± 2.6	143.5 ± 2.1	**143.5 ± 2.4**	140.3 ± 0.7	131.8 ± 2.5

10 SOP for production of an aqueous based dispersion of IRMM-388 (Coated TiO_2)

10.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-388 starting from dry powder form.

10.2 Scope

The scope of this SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-388, Titanium Dioxide TiO_2. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

10.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No.25638-17-9)
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

10.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 $mgmL^{-1}$ of IRMM-388) of IRMM-388, Titanium Dioxide (TiO_2). The procedure foresees starting from a dry powder sample

of IRMM-388 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a the commercial surfactant Sodium hexametaphosphate (Calgon). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjusting the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

10.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

10.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

10.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM and any chemicals in powder form
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

10.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$, 0.2 µm in-line filtration)
- Titanium Dioxide (TiO2) distributed by IRMM with project ID no. IRMM-388
- Ice-water mixture for cooling the sample during sonication
- Surfactant: aqueous solution of Sodium hexametaphosphate (Calgon) (CAS No. 10124-56-8)

10.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

10.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 $WmL^{-1}$for 2 ml sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

In this procedure for dispersion of IRMM-384 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the

instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

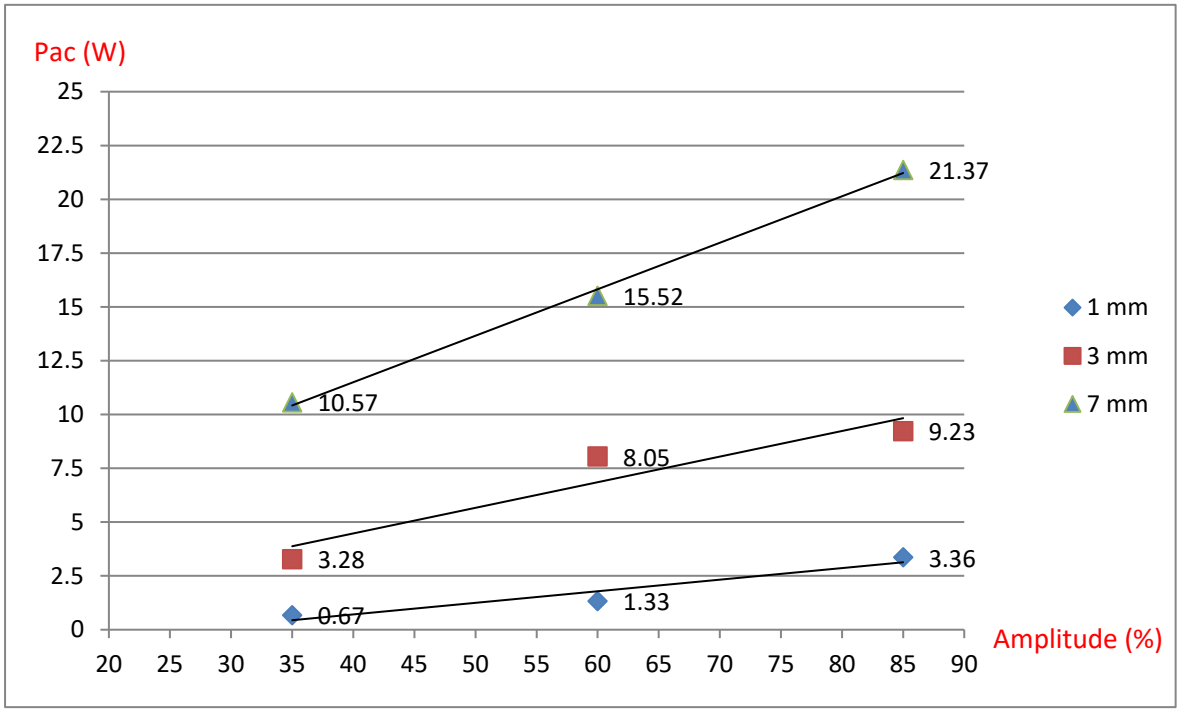


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

10.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 10.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

10.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 20 mg of Sodium Hexametaphosphate (SHMP) using a spatula. Reweigh the vial and calculate by difference amount of SHMP before adding sufficient ultrahigh purity water to give a concentration of 2 $mgmL^{-1}$ and vortex for 2 minutes. This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-388 into a 22 mL glass vial and add sufficient solution A to give a concentration of 1 $mgmL^{-1}$. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (10'). This will hereafter be referred to as solution B.

Take an empty 22 mL vial and add 5.94 mL of pure water followed by 60 µL of solution B to give a final concentration of 0.1 $mgmL^{-1}$ IRMM-388 in water. This will hereafter be referred to as solution C (6 mL Volume).

Sonication using probe sonicator: Take the 22 mL glass vial containing solution C and mount the probe sonicator head inside the vial as shown in Figure 2.

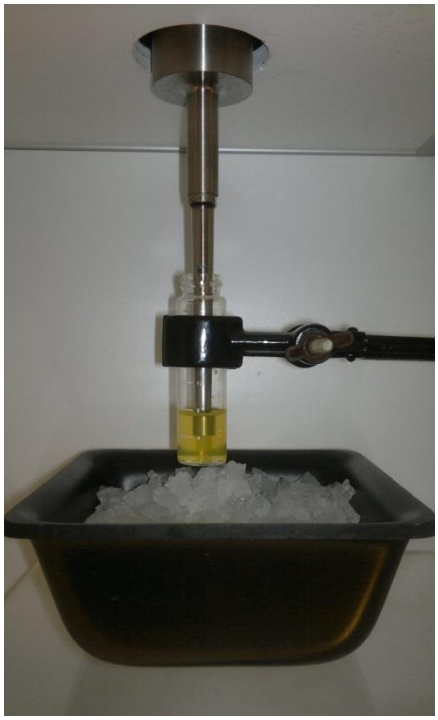


Figure 2: Photograph illustrating the mounting of probe sonicator in sample

The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. If using Hielscher UPS200S with 7 mm probe the sample should then be sonicated at an amplitude of 75 % and 50 % cycle for 20 minutes.

The correct sonication settings should be determined from the calibration curves determined by the method described in chapter 13. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low amplitude (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

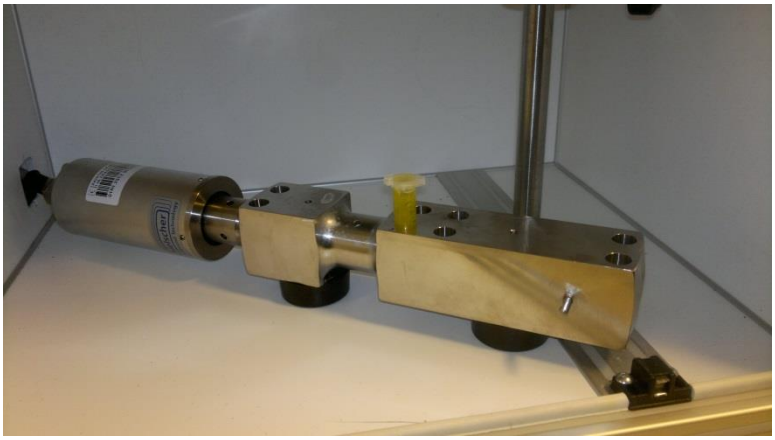


Figure 3: Positioning of sample in vial-sonicator

Alternative to step 5: Sonication using vial sonicator: Place 2 mL of solution C in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 3).The vial should be sonicated for 15 min at 75 % amplitude with the cycle time set at 50 %. Cooling cannot be applied in the case of vial sonicator and the use of 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

10.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that noted in section 10.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 10.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

10.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 10.4.5. In

the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-388, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and then treated for 10 minutes in a laboratory scale bath sonicator at room temperature.

10.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

10.5 Validation status

This method has not yet been subjected to validation

10.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

10.7 Information on expected particle size distribution

Table: 1: DLS size measurement of IRMM-388 (TiO_2) dispersed in SHMP solution

	Sonication Method		
Mean Particle Size in nm of IRMM-388 by CLS (weight-size distribution)	USP 7 mm 20' (*)		VS 15'
	316	319	365

(*) results of two different analyses

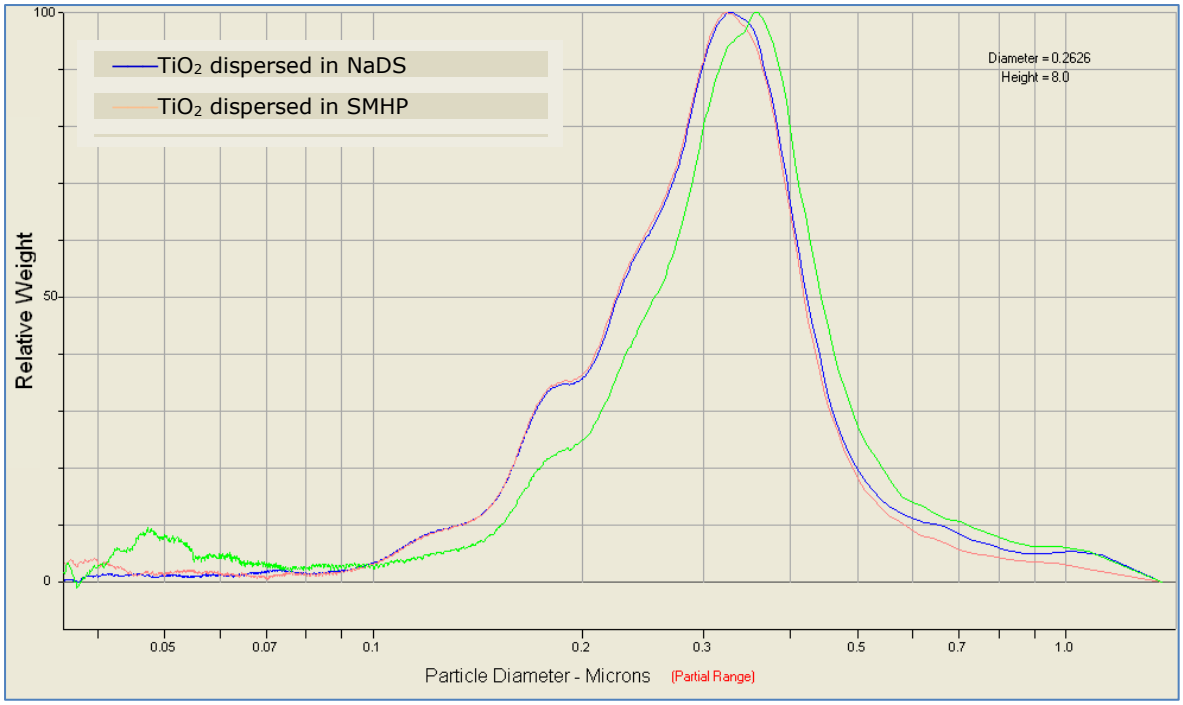


Figure 4: CLS measurement of IRMM-388 (TiO_2) dispersed in solutions pure water, SMHP and NaDS

11 SOP for production of an aqueous based dispersion of IRMM-389 (basic methacrylate copolymer)

11.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a stable water-based dispersion of IRMM-389 starting from dry powder form.

11.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-389, basic methacrylate copolymer. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are for use with suitable particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by LD or AC-CLS, does not significantly change (according to LD or AC-CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

11.3 Abbreviations

AC	Analytical Centrifugation
CLS	Centrifugal Liquid Sedimentation
LD	Laser diffraction
NaDS	Sodium Dodecyl Sulphate
NM	Nanomaterial
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator

11.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (20 mL) of a surfactant stabilised aqueous suspension (10 $mgmL^{-1}$ of IRMM-389) of IRMM-389 basic methacrylate copolymer.

The procedure foresees starting from a dry powder sample of IRMM-389 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) to disperse the solid NM into high purity water containing a low concentration of the surfactant NaDS and the supporting wetting agent stearic acid. The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator with variable power output to supply the mechanical energy necessary.

The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value

which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document. When this procedure is conducted using a probe sonicator the batch volume which is produced is 20 mL while the alternative method using a vial sonicator permits the production of 2 mL batches.

The particle size distributions of the method has been evaluated by laser diffraction and found to be comparable. The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (< 35 min). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 5 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 1 day.

11.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.01 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Stirring device

11.4.2 Recommended optional equipment

- Particle size measurement instrument (e.g. LD or CLS)

11.4.3 Material Supplies

- 500 mL glass bulk for the NaDS solution
- 50 mL glass beaker. The beaker should have an inner diameter of ca. 4 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves and other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

11.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm^{-1}, 0.2 µm in-line filtration)
- Basic methacrylate copolymer distributed by IRMM with project ID no. IRMM-389
- Stearic acid powder (CAS No. 57-11-4)
- sodium dodecyl sulphate powder (CAS No. 151-21-3)
- Ice-water mixture for cooling the sample during sonication

11.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with LD or AC-CLS instruments additional is information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

11.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Hielscher UPS200S with nominal maximum power of 200 W. This sonicator could be fitted with probe heads with diameters of 1 mm, 3 mm or 7 mm diameter. The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1.

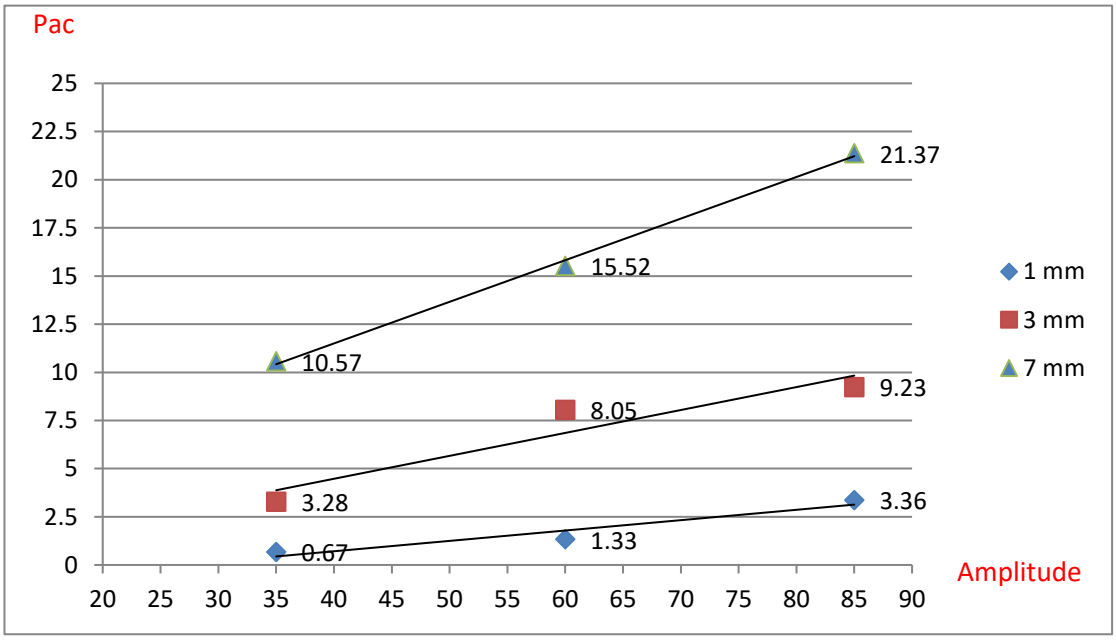


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

In this procedure for dispersion of IRMM-389 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator the operator must determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 18 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

11.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 11.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

11.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare a stabilising agent solution (1 wt%) by dissolving the appropriate amount of sodium dodecyl sulphate powder (NaDS) into ultrapure water (18.3 $M\Omega cm^{-1}$), e.g. 5 g NaDS to 495 g ultrapure water. Shake the glass bulb to ensure that all powder is solubilized. Subsequently filter the solution using a 0.2 µm filter to ensure that no particles are present.

Weigh sufficient substance to produce a final suspension with a mass concentration of about 10 $mgmL^{-1}$ IRMM-389 (e.g. 200 mg of IRMM-389 powder for finally 20 mL suspension).

Weigh sufficient stearic acid (e.g. Sigma Aldrich 75366) to produce a final suspension with concentration of about 0.15 $mgmL^{-1}$ (e.g. 30 mg of stearic acid powder for finally 20 mL suspension)

Add corresponding volume of the NaDS solution (e.g. 2 mL of NaDS solution for finally 20 mL suspension) to produce in the following step a suspension with a NaDS concentration of 0.1 wt%.

Add sufficient ultrapure water (18.3 $M\Omega cm^{-1}$) to reach the mass concentration 10 $mgmL^{-1}$ and a NaDS concentration of 0.1 wt%. (e.g. 17.7 mL ultrapure water for 200 mg IRMM-389). Take care on the floating particles and do not blow them away when an Eppendorf pipette is used.

Homogenize the suspension by brief stirring for at least 20 min. Take care that all floating particles were brought into suspension. Start with low rotational speed and increase the speed slowly that no floating particles were blown away.

Treat sample with high power ultrasonic (e.g. probe sonication). The Influence of sonication is rather low. Reproducible results were generated with sonication using a Hielscher UP200S (200 W source) equipped with a 7 mm probe operating 70 % amplitude (constant current input: 334mA) for 2 min. The probe tip shall be hold 1-2 cm below the liquid surface. Ice cooling during the sonication is required. The ice bath should be positioned such that the lower half of the beaker is immersed in a mixture of crushed ice and water. The beaker should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water.

The resulting solution should now be suitable for testing. If necessary the suspension should be diluted with ultrapure water for individual measurement.

The probe sonicator head used should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air. Avoid any contact between ethanol and the suspension as IRMM-389 will dissolve in ethanol.

11.4.6 Optional verification of dispersion quality

Where the operator has access to DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 11.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (LD/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 11.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

11.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepare in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in the previous section 11.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-389, material aging of up to 1 day may be fully reversed if the sample is treated with ultrasonic power for 5 minutes in a laboratory scale bath sonicator at room temperature.

11.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

11.5 Validation status

This method has not yet been subjected to validation

11.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) and related operating instructions of sodium dodecyl sulfonate and basic methacrylate copolymer to be aware of known hazards relevant in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

11.7 Information on expected particle size distribution

Figure 2 below shows an image of particles of IRMM-389 basic methacrylate copolymer taken by SEM using the drop-on-grid method. Clearly visible are larger particles with sizes of about 20 µm and smaller ones with diameters of approximately 3 µm.

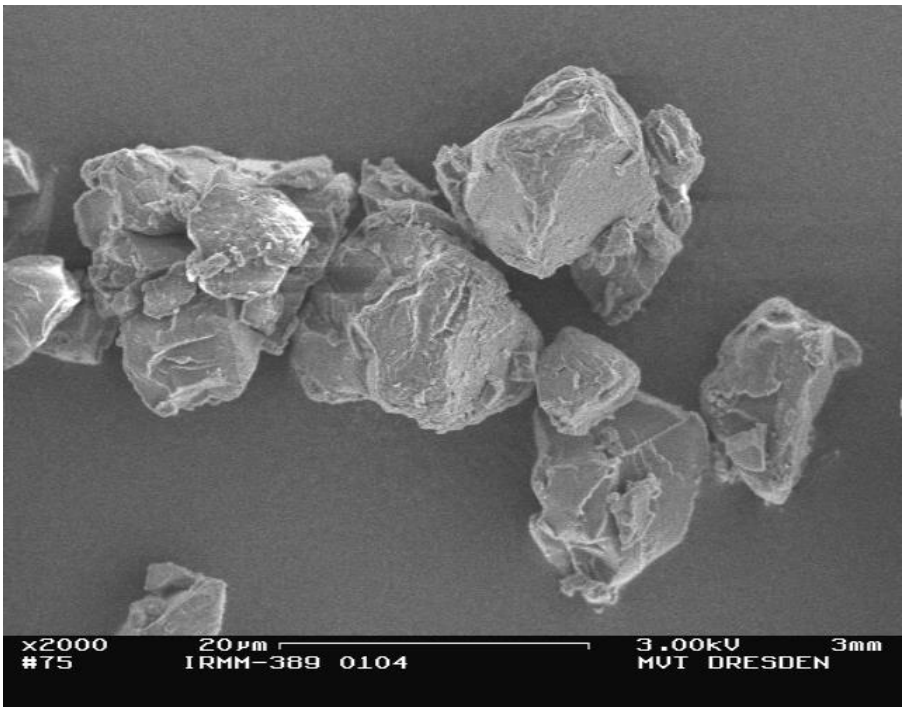


Figure 2: SEM image of IRMM-389

In table 1, values of measured particles sizes (modal value, median value) by laser diffraction are given to enable evaluation of results obtained when using this SOP. The suspension was achieved as described in section 11.4. The treating time for ultrasonic power input was varied but no significant shift was detected.

Table: 1 Expected particle size values after various ultrasonic power input times

Ultrasonic Probe Sonication	$x_{mod,3}$	$x_{10,3}$	$x_{50,3}$	$x_{90,3}$
	µm	µm	µm	µm
5 min USP	1.426	1.797	8.948	19.800
5 min USP	1.417	1.815	9.135	19.955
5 min USP	1.393	1.897	9.646	21.311
10 min USP	1.421	1.695	8.449	19.290
10 min USP	1.406	1.776	9.096	20.778
15 min USP	1.361	1.953	9.913	19.205
15 min USP	1.364	1.938	9.656	17.917
25 min USP	1.383	1.893	9.406	17.456
25 min USP	1.398	1.884	9.307	17.569
25 min USP	1.399	1.838	9.072	17.194

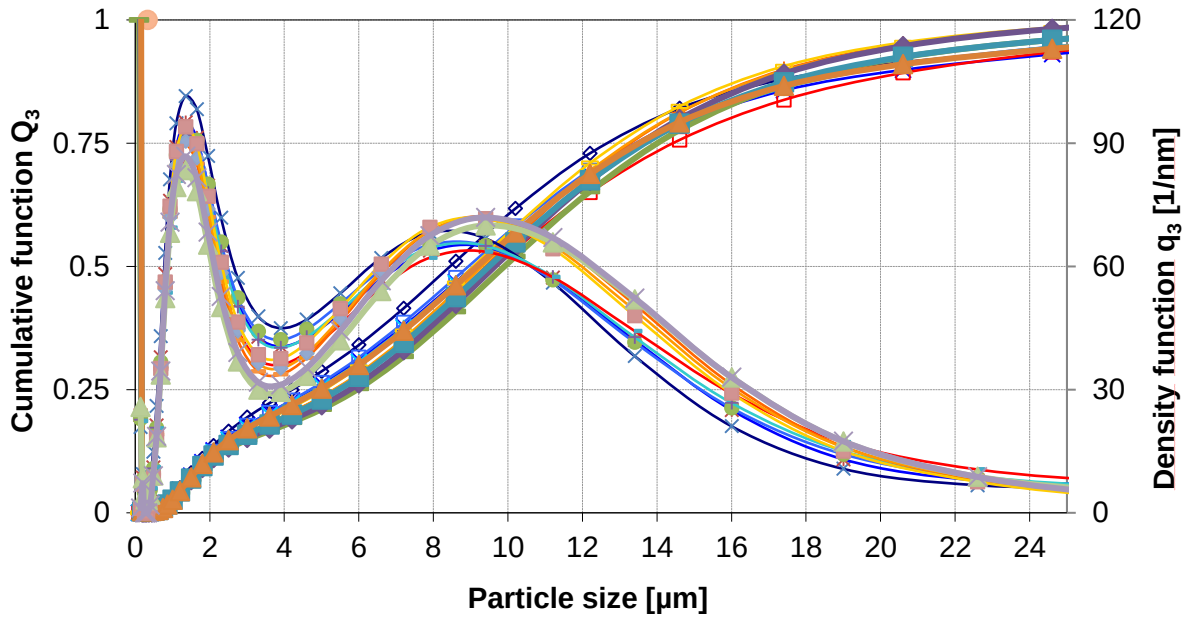


Figure 3: LD-analysis of aqueous dispersion of IRMM-389 with NaDS and stearic acid. No significant shift of peaks is visible in the mass weighted PSD by varying the ultrasonic power input

12 SOP for production of an aqueous based dispersion of BAM-11 Zeolite

12.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a water-based dispersion of BAM-11 (Zeolite powder) starting from dry powder form.

12.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based suspension of BAM-11, Zeolite powder. The method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution.

The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their relatively large size, the particles sediment rapidly. This instability is not compatible with several of the particle size measurement methods adopted in the NanoDefine project. The protocol remains useful for methods such as EM, where the sample is transferred to a solid carrier by sedimentation.

12.3 Abbreviations

AFM	Atomic Force Microscopy
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

12.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 $mgmL^{-1}$) of BAM-11, zeolite powder. The procedure foresees starting from a dry powder sample of BAM-11 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and has been developed to produce a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<60 minutes). The use of a shorter time may produce measurably larger mean particle size values. The described sonication procedure leads to the release of small particles from the zeolite material (as compared to the mean particle size value). It is unclear whether these particles can be considered as constituent particles or pieces of material broken off of the original particles. The release of these small particles increases with the extent of sonication time and power.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine). The physico-chemical properties of this material do not allow obtaining a stable dispersion for direct measurement in dispersion (e.g. by techniques such as DLS). Due to their relatively large size, the particles sediment rapidly and during measurement. The protocol remains useful for methods such as EM where specimens can be prepared by transferring a fraction of the sample to a solid carrier by sedimentation. The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

12.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W.
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication.
- Stirring device

12.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. TEM)

12.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

12.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm^{-1}, 0.2 µm in-line filtration)
- Zeolite powder distributed by BAM with project ID no. BAM-11
- Ice-water mixture for cooling the sample during sonication

12.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with LD or AC-CLS instruments additional is information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

12.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W.

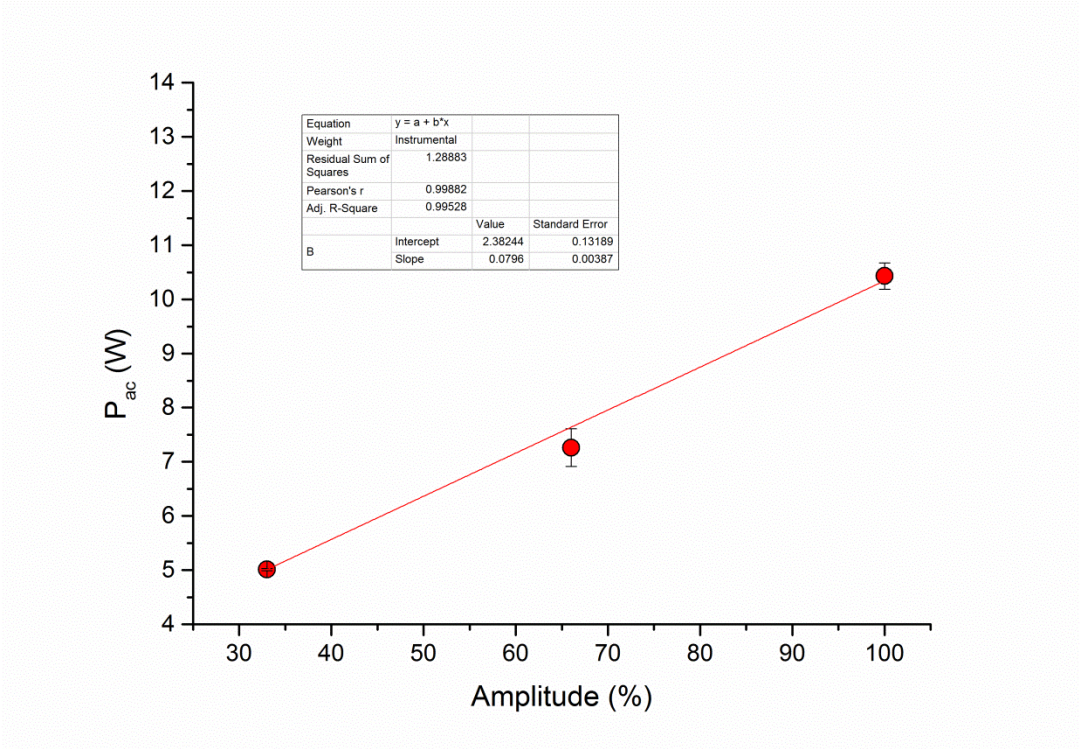


Figure 1: Calculated delivered output power Pac of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to Pac = 10.3 W (in this example: amplitude of 100 %)

The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 µm.). The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1. In this procedure for the dispersion of the BAM11 materials the sonicator was operated at a set amplitude value of 100 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 10.3 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator the operator must determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 10.3 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

12.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 12.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

12.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh approximately 15.6 mg of BAM-11 into a 20 ml glass vial. It is recommended that an ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of ultrapure water to give a BAM-11 concentration of 2.6 $mgmL^{-1}$ (6 mL for exactly 15.6 mg of BAM-11, adjust volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

12.4.5.4 Sonication using probe sonicator

Take the 20 mL glass vial containing the 2.6 $mgmL^{-1}$ BAM-11 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant amplitude setting corresponding to Pac of 10.3 W for 25 minutes. The correct power setting should be determine from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing. If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

12.4.6 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that shown in section 12.7.

TEM specimens can be prepared following the drop-on-grid method[1]. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. Just after sonication, a droplet of 10 µL BAM-11 dispersion (2.6 $mgmL^{-1}$) is then placed on the grid and left for 10 minutes. Subsequently, the excess fluid is removed using a strip of filter paper.

The described laboratory scale method produces a water-based dispersion of BAM-11 starting from this material in a dry powder form. The presented protocol allows dispersing the material down to a combination of single constituent particles, and smaller and larger aggregates/agglomerates. The constituent particle size ranges from 20 nm to 3 µm (see section 12.7 of this SOP). The constituent particles are heterogeneous in shape. The morphology of the constituent particles of the NM can be irregular polygonal, rectangular or circular. Some of the apparent differences in constituent particle shape might be the result of projection of similar particles with different orientations. The surface of the constituent particles is generally rough. For the agglomerates, the size ranges from 100 nm to several µm, measured manually on the TEM images. In most cases, the agglomerates tend to have a complex 2D structure. Diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles. During the development of this procedure it was observed that the sonication procedure resulted in the release of small particles from the zeolite material (as compared to the mean particle size value). It is unclear whether these particles have to be considered as constituent particles or pieces of material broken off of the original particle. The release of these small particles increases with the extent of sonication time and power.

If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 12.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

12.4.7 Recovery of dispersions after aging beyond verified period of stability

The physico-chemical properties of zeolite powder (BAM-11) do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

12.4.8 Reporting of results

Reporting of results should be done in a way all measurements and analysis of results can repeated.

12.5 Validation status

This method has not yet been subjected to validation

12.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) and related operating instructions of sodium dodecyl sulfonate and basic methacrylate copolymer to be aware of known hazards relevant in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

12.7 Information on expected particle size distribution

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.[2-4]. Figure 3 shows representative TEM images of BAM-11. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 3, left panel). A log-normal curve is fitted iteratively to the scatter plot (Figure 3, right panel), and gives estimates for the mode, height, width and asymmetry of the distribution (Table 2). The errors on these parameters are determined as described by Wojdyr[5] and Wolberg[6]. The median of the Feret min distribution is 82.41nm and the mode Feret min distributions is 46.24 ± 0.26nm. A sub-fraction of smaller particles was visible when imaging the sample (size 10-50nm). It is unclear whether the smaller particles have to be considered as constituent particles or pieces of material broken off of the original particles.

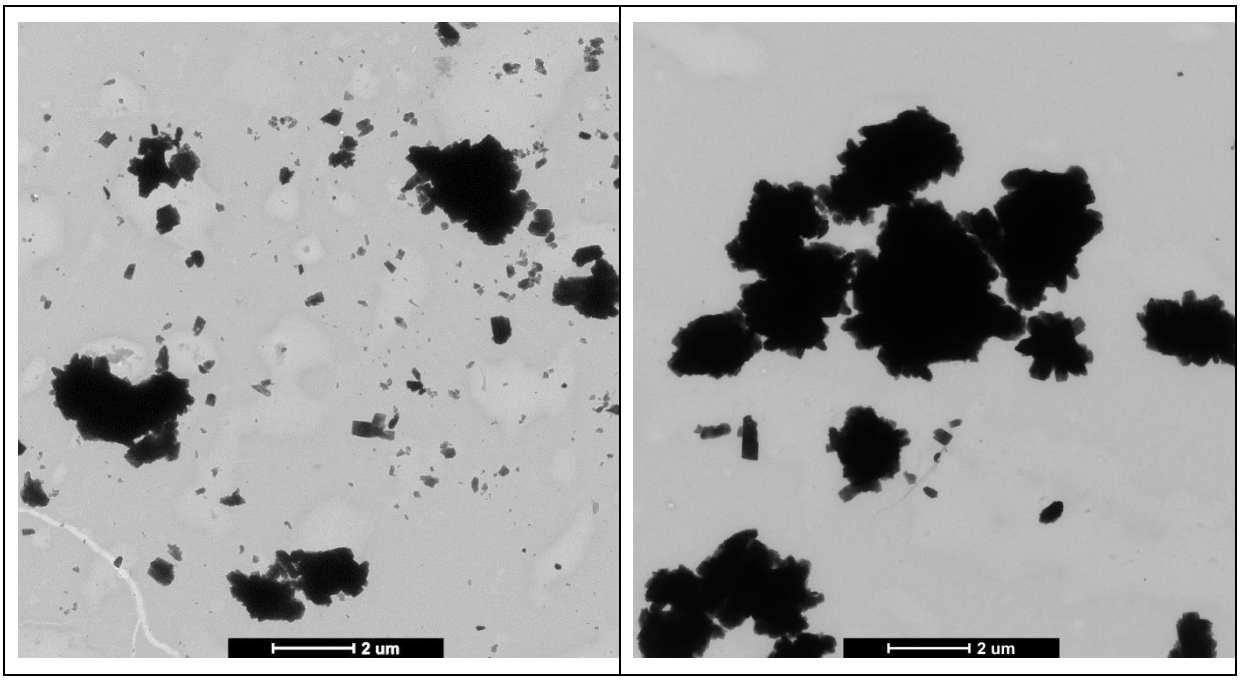


Figure 3: Representative TEM images of the BAM-11 Zeolite powder particles dispersed using the presented SOP

Table 1: Statistics on the Feret min distributions: best fit parameters (height, mode, width and asymmetry) of the log normal functions fitted to the distributions and median values of the datasets

Parameter	**Median Value**
Number of particles	2254
Height	924.65 ± 37.20
Height normalized	0.410 ± 0.016
Mode	46.24 ± 0.26 nm
Width	39.62 ± 3.74 nm
Asymmetry	1.47 ± 0.06
Median	82.41 nm

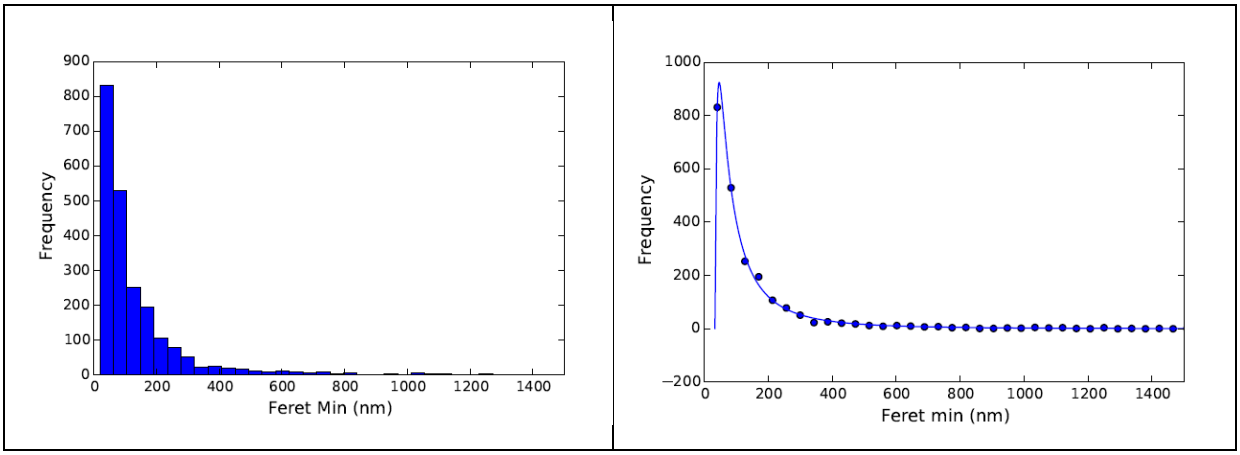


Figure 4: Left panel: representative size distribution (Feret min) of BAM-11 zeolite powder obtained by quantitative TEM. Right panel: scatter plot of the Feret min distribution of BAM-11, zeolite, fitted log normal function

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13 Generic SOP for calorimetric calibration of an ultrasonic probe sonicator

13.1 Aim

The aim of this SOP is to describe a calorimetric based experimental method to determination the level of ultrasonic energy which a generic probe sonicator system can transfer into a liquid.

13.2 Scope

This scope of the SOP is to provide a general method for determination of delivered power of probe sonicators for use in harmonising the description and application of nanoparticles inter-laboratory comparison of nanoparticle dispersion. The results obtainable from this SOP may be used to improve the inter-laboratory transferability of Standard Operating Procedures for the dispersion of dry nanomaterials into liquids by allowing better harmonisation of the sonication conditions. The following protocol and recommendations have been based on the National Institute of Standards and Technology (NIST) publication by Taurozzi et al.[1].

13.3 Abbreviations

No abbreviations required

13.4 Description

When it is required, starting from a dry powder, to produce liquid dispersions of nanoparticles with a minimum amount of residual agglomerated material it is often necessary to use high intensity sonication as the main means of supplying mechanical energy to break-up agglomerates. The efficiency, effectiveness and speed which such agglomerates can be broken up into smaller particle assemblies depends on many instrumental and experimental factors including source frequency, probe size and shape, volume of liquid treated, temperature and treatment time. For the purposes of describing and harmonising suitable reproducible methods for dispersing specific materials it is desirable to be able to define the ultrasonic energy applied on the basis of some experimentally measureable values which can be defined independently of the instrument used. Using this procedure it is possible to obtain estimates of the effective acoustic power output from generic laboratory probe sonicators when operating at a variety of instrument settings. Once the power output characteristics of an instrument are known then it becomes possible to adjust these values to match those of a dispersion procedure developed using another type of probe sonicator but whose power output characteristic have been measured in the same manner.

13.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Laboratory scale balance with maximum weight boundary greater than 700 g and a weighing accuracy of ±0.1 g or better
- A variable power sonicator operating at 22.5 kHz (please report deviating frequencies) equipped with the probe tip which that will be used in implementing the nanoparticle dispersion protocols. For the purposes of dispersing nanomaterials it is recommended that the sonicator should have nominal power output in the range 50-500 W and be fitted with a 6-7 mm diameter probe

- Water; thermally equilibrated to fume-hood air temperature (Nanopure-filtered water or MilliQ-filtered water or similar; resistivity 18.2 $M\Omega cm^{-1}$)
- 600 mL tall form borosilicate glass beaker with approximate dimensions of 150 mm in height and 80 mm in diameter
- Thermal insulation foam or similar to wrap beaker to reduce heat loss(optional)
- Digital thermometer with metal or glass sheathed thermocouple probe capable of a measurement accuracy better than ±0.1 °C
- Digital timer capable of measurement accuracy better than ±1 s

13.4.2 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for undertaking the measurements necessary to characterise the power output of the sonicator probe.

13.4.2.1 Calibration of delivered acoustic power from calorimetry

1. Measure the weight of the empty 600 mL tall form beaker and then add 500 mL de-ionized water
2. Measure the total weight of the filled beaker and then calculate the amount of water by difference
3. Immerse the sonicator probe ca. 2.5 cm below the liquid surface
4. Immerse a temperature probe connected to a temperature meter in the liquid and position it approximately 1 cm away from the sonicator probe as shown in Figure 1
5. Use a hook/clamp to fix the beaker, so it cannot move during the measurement
6. Let the liquid temperature stabilise at room temperature and note the equilibrium temperature
7. Select a sonicator output setting (e.g. 'amplitude in µm' or '% of amplitude'; usually set by a dial in the sonicator power module), operating in continuous mode and record the water temperature increase for the initial 5 minutes with minimum resolution of 30 s
8. Using the recorded temperature values, create a temperature vs. time curve (see Figure 2) and obtain the best linear fit for the curve using last squares regression
9. With the obtained slope $\left(\frac{\Delta T}{\Delta t}\right)$, the delivered acoustic power P_{ac} (W) can be calculated from the following equation:
10. $P_{ac} = \frac{\Delta T}{\Delta t} M C_P$
    where T and t are temperature (K) and time (s), respectively, C_P is the specific heat of the liquid ($4.18 Jg^{-1}K^{-1}$ for water) and M is the mass of liquid (g)
11. Repeat steps 7-9 with new sonicator output settings after exchanging the water in the beaker for at least three power settings (2 repetitions for each setting). Plot the calculated delivered acoustic power values P_{ac} over the chosen output setting values (Figure 3)

Figure 1: Photograph of probe sonicator and thermocouple positioning for calorimetric calibration (thermal insulation removed for clarity)

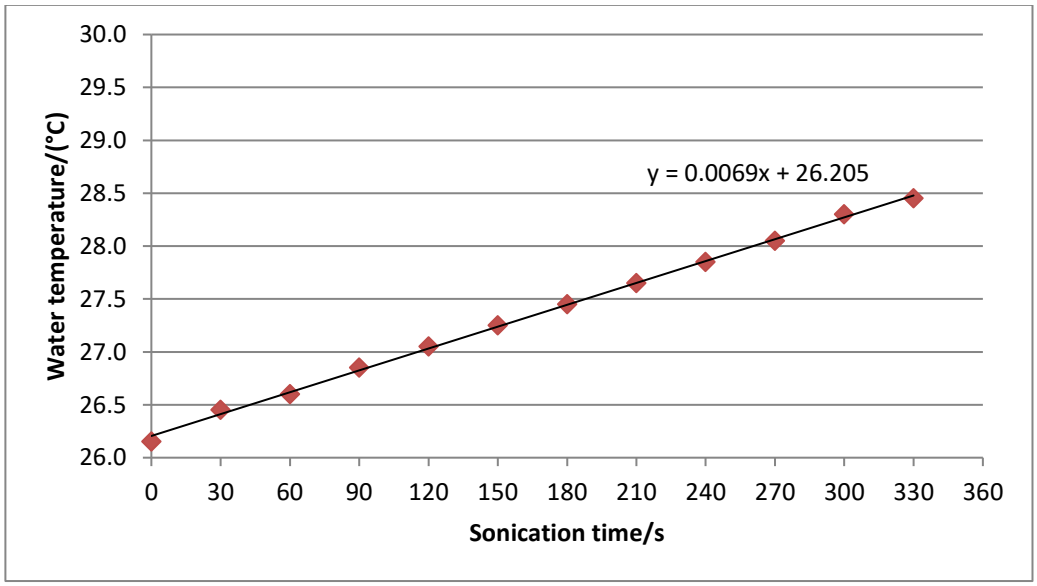


Figure 2: Temperature vs. time curve for 500 mL H_2O sonicated using a Hielscher UPS200S ultrasonic disruptor fitted with 7 mm probe operating at 60 % amplitude

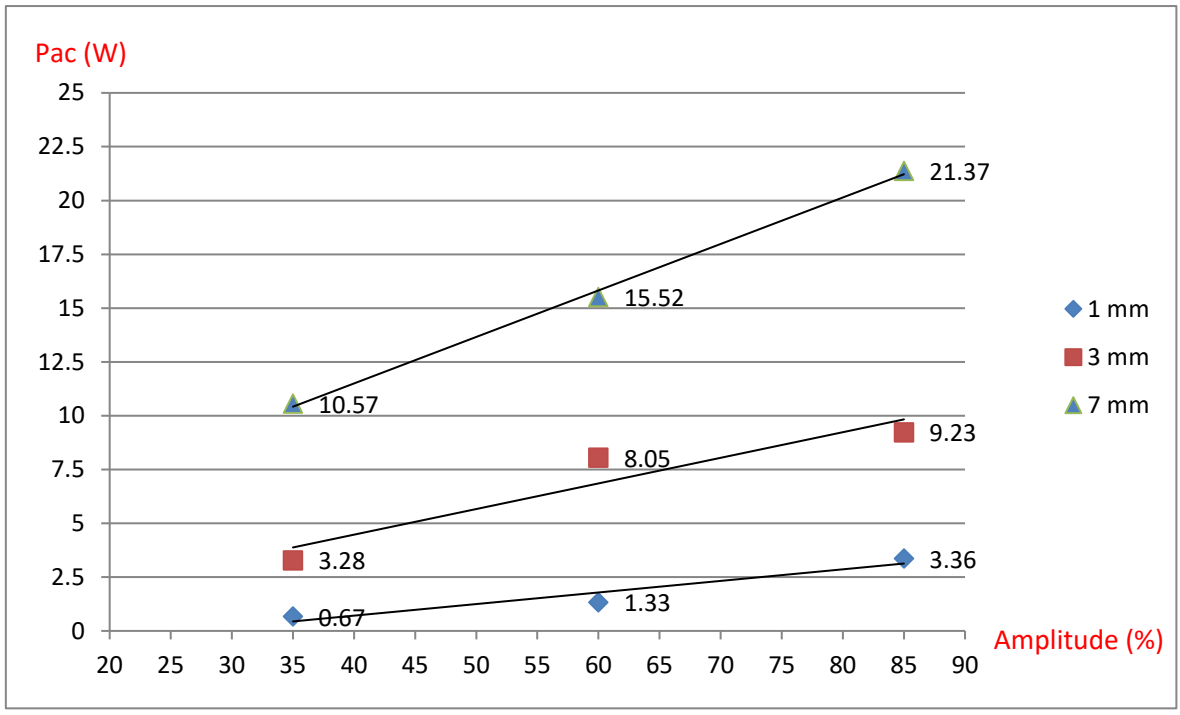


Figure 3: Example of calorimetrically determined sonication power

13.4.3 Reporting of results

No additional recommendations are currently available regarding the reporting of results.

13.5 Validation Status

This method has not yet been subjected to validation within the NanoDefine project

13.6 HSE Issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

13.7 References

[1] Taurozzi J, Hackley V, Wiesner M. Preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. *National Institute of Standards and Technology*. 2012. Available at: http://nvlpubs.nist.gov/nistpubs/specialpublications/nist.sp.1200-2.pdf

14 Properties of recommended materials

Recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within NanoDefine.

Code material	**ρ, gcm^{-3}**	**m_{405nm}**	**m_{470nm}**	**m_{530nm}**	**m_{633nm}**	**m_{670nm}**	**m_{865nm}**
ID-19 (PSL mono)	1.05(x)	1.624	1.608	1.598	1.587	1.584	1.576
ID-20 (PSL 3-mod)	1.05(x)	1.624	1.608	1.598	1.587	1.584	1.576
ID17 (ERM-FD304, SiO_2)	2.305	1.469	1.463	1.459	1.455	1.454	1.45
ID-18 (SiO_2 3-mod)	2.305	1.469	1.463	1.459	1.455	1.454	1.45
ID-16 (BAM-nano-Au)	19.3	1.46-1.96i	1.28-1.88i	0.569-2.26i	0.155-3.36i	0.140-3.74i	0.175-5.48i
ID-21 (BAM-nano-Ag)	10.5	0.170-2.03i	0.142-2.64i	0.140-3.15i	0.140-3.98i	0.140-4.27i	0.140-5.75i
IRMM-384 ($CaCO_3$)	2.657	1.551	1.541	1.534	1.525	1.522	1.507
IRMM-387 ($BaSO_4$ UF)	(4.4)						
RMM-381 ($BaSO_4$ fine)	(4.4)						
IRMM-388 (coated TiO_2)	3.99	2.955/2.737	2.735/2.567	2.639/2.493	2.554/2.429	2.536/2.415	2.480/2.373
IRMM-385 (kaolin)	2.61						
IRMM-383 (nano steel)	7.8*	1.85-3.07i*	2.29-3.27i*	2.58-3.31i*	2.85-3.39i*	2.91-3.44i*	3.11-3.82i*
IRMM-382 (MWCNT)	2.05						
IRMM-380 (Y83 nano)	1.484	1.47-0.457i	1.92-0.42i	1.93-0.07i	1.75-0.029i	1.73-0.023i	1.72-0.03i
IRMM-386 (Y83 opaque)	1.5	1.47-0.457i	1.92-0.42i	1.93-0.07i	1.75-0.029i	1.73-0.023i	1.72-0.03i
IRMM-389 (BMA)	1.13(x)	1.391	1.387	1.384	1.381	1.381	1.378
BAM-11 (zeolite)	2.07						
BAM 12a-1 (fumed SiO_2)	2.2	1.469	1.463	1.459	1.455	1.454	1.45
water (H_2O)	0.997	1.343	1.338	1.335	1.332	1.331	1.328

Generic SOPs for methods

15 Generic SOP for DLS method for sample preparation and measurement of substances in suspension

15.1 Aim

This document describes the Standard Operating Procedure (SOP) to be used to determine the particle size and particle size distribution of diluted suspensions of nanomaterials. The principle is based on the estimation of the kinetics of the relaxation process by application of the principles of light scattering and Stokes-Einstein law.

15.2 Scope

This SOP describes the use of a dynamic light scattering (DLS) method to perform particle size measurements. The procedure is applicable for the determination of particles suspended in stable aqueous suspensions. Depending on the optical properties and effective density of the dispersed materials, particle sizes in a range from 1 nm to 5 µm in diameter can be measured at mass concentrations of $1 mgL^{-1}$ to $1 gL^{-1}$. The SOP includes information on the sample preparation, method parameters, data evaluation and reporting of the number-based hydrodynamic diameter after conversion from intensity-based particle size distribution.

This SOP is intended for the determination for the NanoDefine materials given in Table 1. The operating conditions were chosen with respect to the mean particle sizes in the sub-micrometre range.

It can also be applied to comparable types of powders (e.g. inorganic insoluble salts), considering that adaptions for the sample preparation and measurement might be needed. Depending on the material properties (e.g. particle size, effective density, hydrophilic or hydrophobic behaviour), adaption have to be done regarding sample preparation, filtration, delay and measurement time.

15.3 Abbreviations

DLS	Dynamic light scattering
IRMM	Institute for reference materials and measurement [c]
NP	Nanoparticle(s)
RI	Refractive index
PDI	Polydispersity index
PTFE	Polytetrafluoroethylene
SOP	Standard Operating Procedure
SHMP	Sodium hexametaphosphate, Calgon, (CAS No. 10124-56-8)
TSPP	Tetra sodium pyrophosphate
USB	Ultrasonic bath

15.4 Description

DLS measurement is based on the principle that smaller particles are in faster Brownian motion than smaller ones. Dynamic light scattering allows the measurements of this translational diffusion. The hydrodynamic diameter can by determined by application of the Stokes-Einstein

[c] Currently: Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, European Commission

equation and assuming that the sample being investigated consists of a set of non-interacting spherically shaped particles. This method is only applicable to dilute suspensions without particle-particle interaction[1]. The DLS technique probes this particle motion in liquids by optical principles. A laser beam illuminates the particles. The light scattered from the particles has a time-dependent phase imparted to it from the time-depended position. Measured over time the random particle motion forms a distribution of optical phase shifts or spectral frequency shifts. These shifts are determined by comparison with all scattered light (self-beating mode). The optical signals received from the particles are intensity weighted. ISO 22412[2] gives more information on this technique and more setups of DLS. Two types of data analysis algorithms have been established: Cumulants method (e.g. CONTIN algorithm) and distribution method (e.g. NNLS algorithm). The cumulants method is independent from the refractive index[3] chosen and leads to a harmonic mean diameter of the intensity-based particle size distribution. The distribution method lead to intensity weighted size distribution and this distribution can be converted into the number-based size distribution applying Mie's theory. DLS is a calibration free system which does not require calibration. An already suspended reference solution shall be used to carry out the qualification of the instrument. This SOP gives information about how to apply DLS to the NanoDefine materials, but it can also be applied to chemically and optically comparable types of samples. The instructions for sample preparation are described briefly in Chapters 3 and 9.

Table 1: NanoDefine materials in the non-nano range analysed by DLS

Sample	Description	Sample identifier
S1	$BaSO_4$ fine grade	IRMM-381
S2	$CaCO_3$	IRMM-384
S3	Kaolin	IRMM-385
S4	Coated TiO_2	IRMM-388

15.4.1 Materials and methods

This section provides information on the required chemicals, samples and analytical instrumentation needed for both sample preparation and DLS measurement (characterisation and quantification).

NOTE: The refractive index of the dispersed particles must be known for calculation of the number-weighted particle sizes and their distributions. For the NanoDefine substances (Table 1) the information on the refractive indices are given in Table 6

15.4.1.1 Chemicals

Chemicals required for sample preparation are detailed in Table 2 and substances for DLS measurement are listed in Table 3.

Table 2: Chemicals for sample preparation

Chemical	Description	Manufacturer/Provider
Deionised water	deionised water, filtered at 0.2 µm, final resistivity 18.2 MΩcm^{-1} at 25 °C	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm) Millipore, Billerica, MA, USA or equivalent
SHMP	sodium hexametaphosphate, analytical grade or better (CAS 10124-58-8)	e.g. Sigma Aldrich
TSPP	tetrasodium pyrophosphate, analytical grade or better (CAS 7722-88-5)	e.g. Sigma Aldrich

Table 3: Substances for DLS measurement

Chemical	Description	Manufacturer/Provider
Reference solution	e.g. polystyrene 46 nm suspension	e.g. JRC, Fisher Scientific

15.4.1.2 Instrumentation

Table 4 gives an overview on Instrumentation and accessories required for sample preparation. Table 5 names the instrumentation and software packages required for measurements by DLS.

Table 4: Instrumentation and accessories required for sample preparation

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance (readability at least 0.1 mg)	e.g. XPE205, Mettler Toledo, Vienna, Austria or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication device with a probe (e.g. with probe 7 mm and able to operate at 70 % amplitude) or a sonication device with similar energy input	e.g. Hielscher, Germany or equivalent
Calibrated pipettes	3 pipettes (20-200 µL, 0.1 - 1.0 mL, 1.0 – 5.0 mL), equipped with suitable plastic tips	e.g. Eppendorf, Hamburg, Germany or equivalent
Stirrer	Stirrer or magnetic stir bars and stirrer device	e.g. Mettler Toledo or equivalent
Filtration membranes and filtration equipment	Hydrophilic PTFE filtration membranes, nominal pore size 1.0 µm and equipment for filtration, e.g. holder, vacuum pump	e.g. Merck
Ice bath	Mixture of crushed ice and water	-

Table 5: Instrumentation, consumables and software packages for DLS measurements

Instrument	**Description**	**Manufacturer/Provider**
DLS instrument	Instrument for dynamic light scattering	e.g. Malvern, Sympatec, Anton Paar
Cuvettes	Transparent cuvettes/cells for samples, recommended by the DLS manufacturer	e.g. Sarstedt
Software	**Description**	**Manufacturer/Provider**
Instrument software	e.g. DTS, Windox, Kaliope	e.g. Malvern, Sympatec, Anton Paar
Spreadsheet calculation programme	e.g. Excel	e.g. Microsoft

15.4.1.3 Reagents for sample preparation and measurements

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found in Chapters 3, 6, 7 and 10 of this report.

Note: When choosing another liquid than aqueous solution, the user shall take care the material of the cuvette is inert to the liquid. Especially organic and acidic solvents should not be used if the user cannot ensure that the material of the cuvette is chemically resistant against the given solvent.

The suspension for qualification of the instrument is prepared according to the manufacturer's SOP of the reference material. In other cases, the suspension for qualification is prepared by giving one drop of a pre-dispersed suspension with a referenced particle's size (e.g. polystyrene 46 nm by Thermo Scientific) into a clean cuvette and adding with 2 mL 10 mM NaCl solution. After gently shaking the suspension for one minute, the suspension shall be treated in an ultrasonic bath for 30 seconds.

15.4.2 Preparation procedure

15.4.2.1 Suspension preparation

The preparation of suspension of the materials given in Table 1 can be found in Chapters 3, 6, 7 and 10 of this report. Suspensions suitable for DLS measurement must be stable at least in the period between sample preparation and the end of the measurement against sedimentation and formation of agglomerates. If there are doubts on the homogeneity, e.g. are visible supernatant or sediment, the measurement would be carried out on a not representative sample.

Using the HPPS by Malvern, the (calculated) derived count rate of the pure dispersion medium shall never exceed 100 kcps, whereas a value below 50 kcps is preferred. The critical value is depending on the laser intensity. In general, the signal to noise ratio should be at least 10.

NOTE: The user must ensure that the beakers and cuvettes are free of contamination or other particles that may contribute or interfere with the measurements.

15.4.2.2 Filtration of suspensions

Regarding the non-nano materials given in Table 1, filtration is recommended for IRMM-385 only. A volume of 20 mL with a sample content of 100 ppm kaolin should be prepared. The suspension should be filtrated through a hydrophilic PTFE membrane with a nominal pore size of 1.0 µm. The user shall not touch the membrane with hands.

NOTE: Filtration of suspensions leads to the deposition of a high quantity of particles much smaller than the nominal pore size of the filtration membranes. Only suspension with expected particle sizes close to 100 nm or below this value shall be filtrated. In other cases the impact of filtration is very high regarding the number-based median diameter and filtration could lead to false positive classification according to the EC NM definition of a nanomaterial.

15.4.2.3 Dilution of sample

The sample should have a concentration that leads to a detection signal with a derived count rate of at least 1000 kcps. Due to concentration effects that are expressed by multiple scattering and particle-particle-interactions, the maximum concentration must be determined empirically. The measured particle size shall be constant for different concentration levels (e.g. 100 ppm, 1000 ppm). If required, dilution of the sample must be performed with a particle-free diluent of the same refractive index, ionic strength, surfactant concentration, pH etc. as the original dispersion medium. A diluent of different physicochemical properties may change the surface chemistry of the particles.

NOTE: The working range (concentration) is varying on the instrument used, especially the light source and detector chosen, and the detection angle (90°, backscattering)

15.4.3 Performing measurements in DLS

In this chapter, the details of the generic measurement process are described. The user always has to follow the manual and manufacturer's guidelines.

NOTE: DLS instruments use a laser source, which can cause eye damage! Never look into the laser beam! DLS instruments have to be maintained according to the manufacturer's instructions.

15.4.3.1 Set-up

According to the manufacturer's guidelines the instrument has to be connected to the PC and communication between the instrument and the computer has to be ensured. The optical devices inside the instrument have to be clean and free of dust. The user shall not touch the surfaces of the cuvettes and inside of the instrument required for measurements. Typically, a period of 30min is required to stabilise the laser intensity.

15.4.3.2 Daily Performance Check

In general, DLS instruments are calibration-free. Most manufacturers advise the user to check the performance with a reference material annually. The user should take care that the optical components inside the measurement cell are clean and free of dust. If there is dust on the optical components a brush can be used to carefully clean the surfaces.

15.4.3.3 Typical measurement conditions

For selection of instrument settings, it is required to consider the scattering behaviour of the suspension that leads to visible turbidity of the suspension. For the NanoDefine materials given in Table 1 the recommended measurement conditions are given below, see Table 6.

The cuvette position recommended is for the HPPS instrument only. Using another instrument, the user shall check if the measurement volume is close to wall and reduce optical paths of incident and scattered light to a minimum if required. In other cases, the central position of measurement zone is preferred.

NOTE: If stability cannot be ensured (e.g. with polymers) or special material effects (e.g. hydrogel swelling) or any other reason (e.g. experiments in toxicology studies prefer a temperature of 37 °C), which require a certain temperature range; the user should reflect on temperature, known material properties at the temperature.

Table 6: Recommended measurement conditions

Operating temperature:	25 °C
Cuvette type:	acrylic cuvette 10 x10 x 45 mm
Equilibration time:	5 min
Cuvette position:	-1 mm
Intensity of illuminating light	automatic
Measurement duration:	60 s
Delay between measurements:	0 s
Setting of inversion algorithm/ mode for size analysis	general purpose

15.4.3.4 Delay time before measurement

Regarding the non-nano materials given in Table 1, a delay time between sample preparation and measurement is recommended for IRMM-384 and IRMM-385 only. A sample volume of 1.5 mL has to be taken from the recently prepared suspension and given into a cuvette. This cuvette shall be stored in the laboratory at a place protected from vibration and shocks and without direct sunlight. After a period of 24 h the cuvette can be inserted into the DLS instrument. The user shall take care not to homogenise the suspension.

15.4.3.5 Measurement description

The time between sample preparation and inserting the cuvettes has to be as short as possible to avoid the disintegration of the homogeneous particle concentration inside the samples before the measurement has started. The volume needed for measurement shall be looked up in the instrument manual. Typically a volume of 1.5 mL is used. During the preparation steps of filling the cuvette and inserting into the instrument, it is important that the user does not touch the surfaces foreseen for light transmission.

A typical sequence for preparation and measurement is as follows:

- Starting the instrument and stabilisation of the laser
- Equilibrating the temperature of the instrument
- Measurement of the deionised water used for preparation
- Measurement of a reference suspension in the expected size range, e.g. polystyrene 46 nm
- Measurement of blank samples with dispersing agents
- Measurement of the sample

Evaluation of results

The instrument software will guide the user and calculate most of the results. This section provides briefly information on the fundamental equations and the required parameters to calculate the results. DLS intrinsically determines intensity-weighted size distributions. Two data analysis algorithms have been established: Cumulants method and distribution method, e.g. CONTIN or NNLS algorithm:

The cumulants algorithm assumes a second-degree polynomium and leads to a material independent mean diameter and the polydispersity index (PDI). Both values need to be reported for every measurement.

The distribution method applies a multi-exponential fitting to the measured correlation function. This leads to a particle size distribution from which a median particle size can be determined (intensity-based hydrodynamic median diameter). Software typically also computes number- and volume-weighted size distribution. For this purpose, an optical model for scattering intensity is included in the inversion algorithm (Mie theory for electromagnetic scattering at spheres.

If the software provides the opportunity to calculate the number-based particle size distribution from the raw data, the user should follow this wizard. The standard deviation shall be calculated from the cumulants diameter and additionally from the number-based median diameter results.

Determination of intensity-based particle size

Please note that DLS does not determine the particle size in a direct way, but the translational diffusion coefficient due to Brownian motion that correlates to the particle diameter.

The investigated suspension is illuminated by a monochromatic and coherent light source with the wavelength λ_0. The light is scattered by the particles and detected at an angle with respect to the incident radiation. The observed scattered intensity I(t) will fluctuate with time correlated to the Brownian motion of the dispersed particles. Analysis of these intensity fluctuations as a function of time provides information on the motion of the particles. In correlation analysis, this analysis is carried out with a correlator which constructs the time autocorrelation function $G^{(2)}(\tau)$ of the scattered intensity

$$G^{(2)}(\tau) = \langle I_S(t)\, I_S(t+\tau)\rangle$$

Here $I_S(t)$ is the scattered intensity of beam at time t and $I_S(t+\tau)$ is the scattered intensity of beam at time $t+\tau$. For polydisperse samples, the correlation function of the scattered intensity is related to the normalised field autocorrelation function $g^{(1)}(\tau)$, where A is a factor reflecting the baseline of scattering light or a time- independent constant proportional to the square of the time averaged scattered intensity and B is an instrumental factor.

$$G^{(2)}(\tau) = A[1 + B\,|\,g^{(1)}(\tau)\,|^2]$$

The field autocorrelation function $g^{(1)}(\tau)$ is related to the normalized distribution function of decay rates $C(\Gamma)$:

$$g^{(1)}(\tau) = \int_0^\infty C(\Gamma) \exp(-\Gamma\tau)\, d\Gamma \quad \text{with} \quad \int_0^\infty C(\Gamma)\, d\Gamma = 1$$

The decay rates Γ relate to the translational diffusion coefficients of spherical particles in Brownian motion:

$$\Gamma = Dq^2$$

Here, D is the translational diffusion coefficient of the set of illuminated particles and q is the modulus of the scattering vector, given by this equation:

$$q = 4\pi n \sin(\theta/2)/\lambda_0$$

In this equation, n is the refractive index of the dispersion medium and λ_0 is the wavelength of the laser in a vacuum. The particle diameter x is calculated by rearranging the Stokes-Einstein equation to give the following equation, assuming that the sample being investigated consists of a set of non-interacting spherically shaped particles. Here k_B is the Boltzmann constant; T is absolute temperature; η is the dynamic viscosity of the dispersing medium.

$$x = \frac{k_B T}{3\pi\eta D}$$

If any other temperature is chosen the values for liquid density ρ_F and viscosity n have to be adapted for Stokes' equation. The literature recommends a dynamic viscosity n of 0.890 mPa s at 25 °C for water.

The result is the median diameter of the intensity based particle size distribution ($x_{50,int}$).

15.4.3.6 Conversion into number-based particle size distribution

The instrument software will guide the user for calculation of the number-based particle size distribution following Mie light scattering theory. The software also will allow the user to calculate the volume-based size distribution too. The recommended values for the refractive indices (RI) of the NanoDefine materials and water are given in the table below. The refractive indices are given this way:

$$\text{RI} = \text{real part} \pm \text{imaginary part i}$$

Some templates may also call the imaginary part of the refractive index 'absorption'.

(NOTE: The adaption of Mie's solution is associated with assumptions, e.g. spherical particles)

Table 6: Recommended RI values for NanoDefine non-nano materials for a wavelength of 633 nm

Sample	Description	RI
S1	$BaSO_4$ fine grade	1.64
S2	$CaCO_3$	1.66
S3	Kaolin	1.56
S4	Coated TiO_2	2.77
H_2O	Water	1.332

If the refractive index of the sample is unknown then approximate values may be found in many databases, e.g. the open source database at www.refractiveindex.info.

NOTE: If the investigated particulate material consists of core-shell-particles the user shall ask the material's supplier on the optical properties of the sample or use an open source data base.

After the step of data evaluation the data should be exported to a commonly used file format for spreadsheet calculation such as EXCEL®. The resulting diameter is the hydrodynamic median diameter of the number-based particle size distribution $x_{50,0}$.

15.4.4 Reporting of results

In order to allow full interpretation and reproduction of the measurement results, the analysis report shall include at least the following:

- The average particle size by cumulants method and the PDI
- Information on sample preparation, especially the concentration of sample, the suspending liquid and volume, and the dispersing agent and its concentration, the method of dispersion including the dispersion time, the amount of energy added and net power density
- Information on measurement instrument and applied settings, especially the instrument type, the temperature und the cuvette position
- Information on parameters for data analysis, especially the refractive index used of the sample and water
- Information on data fitting and correction

and information required by the guidelines of Good Laboratory Practice (date of analysis, laboratory, operator's name, identification of the sample, page numbering, name and signature of person authorising the analysis report, ...).

For validation purposes, the number-based median diameter shall be used and additionally the average particle size by cumulants method and the corresponding PDI need to be reported

15.5 Validation status

This method is not validated yet.

15.6 HSE issues

DLS instruments have to be maintained according to the manufacturer's instructions. Never use a DLS instruments if you are not sure that the laser is shielded properly.

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations. Each chemical/particulate material should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

Waste disposal: According to the safety regulation of the nanomaterials, the suspending liquid and the particulates and other waste from cleaning or preparations must be collected and eliminated in a manner respects all necessary norms relating to safety and environmental protection.

15.7 References

[1] Babick F. Suspensions of Colloidal Particles and Aggregates. Springer. 2016

[2] ISO 22412:2017 Particle size analysis - Dynamic light scattering (DLS)

[3] http://refractiveindex.info/

16 SOP for Cuvette-AC method for sample preparation and measurement of $BaSO_4$ and comparable types of powders in suspension

16.1 Aim

This document describes the Standard Operating Procedure (SOP) to be used to determine the particle size and particle size distribution of diluted suspensions of nanomaterials based on the principles of centrifugal sedimentation and Stokes' law.

16.2 Scope

This SOP describes the use of a cuvette-based analytical centrifuge or a cuvette photocentrifuge (ISO 13318-2[1]) with turbidity detection to perform particle size analysis measurements. The procedure is applicable for the determination of particles suspended in stable aqueous and a lot of non-aqueous suspensions. Depending on the optical properties[2, 3] and effective density of the dispersed materials, particle sizes in a range from about 5 nm to 10 µm in diameter can be measured at mass concentrations of 0.1 gkg^{-1} to 10 gkg^{-1}. The SOP includes information on the sample preparation, method parameters, data evaluation and reporting of the number based Stokes‘ median diameter after conversion from extinction based particle size distribution

This SOP is primarily intended for the determination for the NanoDefine materials $BaSO_4$ (Table 1) for measurements of instruments of the LUMiSizer® range. The operating conditions were chosen with respect to particles sizes distributions in the sizes range 30 nm – 1 µm.

It can also be applied to comparable types of powders and cuvette-type centrifugation instruments, considering that adaptions for the sample preparation and measurement might be needed. Depending on the material’s properties (e.g. particle size, effective density, hydrophilic or hydrophobic behaviour) adaptions have to done regarding sample preparation, rotational speed, operating time and detection frequency.

16.3 Abbreviations

AC	Analytical centrifugation
CLS	Centrifugal liquid sedimentation
IRMM	Institute for reference materials and measurement
NP	Nanoparticle(s)
RI	refractive index
RRI	relative refractive index
SOP	Standard Operating Procedure

16.4 Description

Centrifugal liquid sedimentation is based on the simple principle that larger particles sediment faster than smaller ones if they have the same effective density[4]. Measurement of the sedimentation rate allows, through the application of a modified Stokes’ equation (ISO 13318-1[5]), the determination of a spherical-equivalent Stokes diameter. The described method is only applicable to dispersion fluids at low Reynolds numbers and particles that sediment in an unhindered fashion.

The cuvette-AC technique, also referred to as the homogeneous incremental technique, measures the sedimentation rate of particles. Under influence of a centrifugal field, particles which are initially uniformly dispersed throughout the test sample, will segregate depending on their size and density. By means of a CCD line sensor, the intensity of the transmitted light is detected across the entire length of the sample cell as function of time and position. In a homogenous sample the initial transmission is at its minimum at each position, what corresponds to the homogeneous concentration of particles. During measurement, particles settle through the liquid, and the intensity of the transmitted light gradually increases. The progress of sedimentation is stored in the time- and space-resolved transmission profiles. In order to determine the particle size, the transmission values are converted into extinction values by using the maximum transmission value as obtained for the last profile (i.e. which should correspond to the particle-free medium/supernatant).

The cumulative light extinction-weighted particle size distribution is obtained through equations (10) to (13) of ISO 13318-1. Apart from the specified centrifuge type, instruments differ with regard to the measurement of particle concentration. There are also integrating detectors, which measure the total amount of particles above/ below a certain position. Cuvette-AC is a calibration-free system which does not require calibration for sedimentation rate determination. An already suspended reference substance shall be used to carry out the qualification of the instrument.

This SOP gives information about how to apply cuvette-AC to the NanoDefine materials, but it can also be applied to chemically comparable types of samples. The instructions for sample preparation described are in Chapters 3 and 9 of this report.

Table 1: NanoDefine representative test materials for analysis by cuvette-AC

Sample	Description	Sample type
S1	$BaSO_4$ fine grade	IRMM-381
S2	$BaSO_4$ ultrafine grade	IRMM-387

16.4.1 Materials and methods

This section provides information on the required chemicals, samples and analytical instrumentation needed for both sample preparation and cuvette-AC measurement (characterisation and quantification).

NOTE: The effective density and the refractive index of the dispersed particles must be known for calculation of the number-weighted particle sizes and their distributions. For the NanoDefine substances (Table 1) the information on density is given in Table 8. The refractive indices for 5 wavelengths are given in Table 9.

16.4.1.1 Chemicals

Chemicals required for sample preparation are detailed in Table 2 and chemicals required for measurement are detailed in Table 3.

Table 2: Chemicals for sample preparation

Chemical	Description	Manufacturer/Provider
Deionised water	deionised water, filtered at 0.2 µm, final resistivity 18.2 MΩcm^{-1} at 25 °C	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm) Millipore, Billerica, MA, USA or equivalent
SHMP	sodium hexametaphosphate, analytical grade or better (CAS 10124-58-8)	e.g. Sigma Aldrich

Table 3: Chemicals for Cuvette-AC measurement (see also Table 2)

Chemical	Description	Manufacturer/Provider
Reference solution	e.g. polystyrene 50 nm suspension	e.g. JRC, Fisher Scientific

16.4.1.2 Instrumentation

Instrumentation and accessories required for sample preparation is detailed in Table 4, instrumentation and software packages required for measurements by cuvette-AC are detailed in Table 5.

Table 4: Instrumentation and accessories required for sample preparation

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance (readability at least 0.1 mg)	e.g. XPE205, Mettler Toledo, Vienna, Austria or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication device with a probe (e.g. UDS probe 7 mm and able to operate at 70 % amplitude) or a sonication device with similar energy input	e.g. Hielscher, Germany or equivalent
Calibrated pipettes	3 pipettes (20-200 µL, 0.1-1.0 mL, 1.0 – 5.0 mL), equipped with suitable plastic tips	e.g. Eppendorf, Hamburg, Germany or equivalent
pH electrode	pH electrode range pH 0-14	e.g. Mettler Toledo or equivalent
Stirrer	Stirrer or magnetic stir bars and stirrer device	e.g. Mettler Toledo or equivalent

Table 5: Instrumentation, consumables and software packages for cuvette-AC measurements

Instrument	**Description**	**Manufacturer/Provider**
Cuvette-AC	Instrument for integral sedimentation technique	e.g. Beckman, LUM
Cuvettes	Transparent cuvettes/cells for samples, recommended by the cuvette-AC manufacturer	e.g. Beckman, LUM
Software	**Description**	**Manufacturer/Provider**
Instrument software	e.g. SEPVIEW	e.g. Beckman, LUM
Spreadsheet calculation programme	e.g. Excel	e.g. Microsoft

16.4.1.3 Reagents for sample preparation and measurements

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found in Chapters 3 and 9.

Note: When choosing another liquid than aqueous solution, the user shall take care the material of the cuvette is inert to the liquid. Especially organic and acidic solvents should not be used if the user cannot ensure that the material of the cuvette is chemically resistant against the given solvent.

16.4.2 Procedure

16.4.2.1 Preparation

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found briefly in Chapters 3 and 9. Suspensions suitable for sedimentation measurement must be stable at least in the period between sample preparation and the end of the measurement against formation of agglomerates. The time between sample preparation and inserting the cuvettes has to be as short as possible to avoid the disintegration of the homogeneous particle concentration inside the samples before the measurement has started.

16.4.2.2 Dilution of sample

The sample should have an optical transmission in the range 30 - 60 % with regard to the light source wavelength. Due to concentration effects, the concentration level shall not exceed 0.25 % (v/v). If required, dilution of the sample must be performed with a particle-free diluent of the same refractive index, ionic strength, surfactant concentration, pH etc. as the original dispersing medium. A diluent of different physicochemical properties may change the surface chemistry of the particles.

NOTE: The user must ensure that the beakers and cuvettes are free of contamination or other particles that may contribute or interfere with the measurements.

16.4.3 Performing measurements in Cuvette-AC

In this chapter, the details of the measurement process are described. The user always has to follow the manual and manufacturer's guidelines.

NOTE: Centrifuges have to be maintained according to the manufacturer's instructions. Never use a centrifuge if you are not sure that the centrifuge and the rotor are maintained properly.

16.4.3.1 Set-up

A runnable cuvette centrifuge consists of the cuvettes, an appropriate rotor and the instrument. According to the manufacturer's guidelines the rotor has to be connected to the instrument and communication between the instrument and the computer has to be ensured. The optical devices inside the instrument have to be clean and free of dust. The user shall not touch the surfaces of the cuvettes and inside of the instrument required for measurements.

16.4.3.2 Daily Performance Check

In general, these instruments are calibration-free. Most manufacturers advice the user to check the performance with a reference material annually.

The user should take care that the optical components inside the instrument (laser or LEDs, detectors) are clean and free of dust. If there is dust on the optical components a brush can be used to carefully clean the surfaces. The rotor should rotate easily without any obstacles.

16.4.3.3 Typical running conditions and tune settings for specific samples

Typical tune settings for all samples are reported below:

Operating temperature:	25 °C
Cuvette type:	thin (2 mm for LUMiSizer® cuvettes)
Light factor:	1

NOTE: If there is the possibility for negative stability effects (e.g. with polymers) or special material effects (e.g. hydrogel swelling) or any other reason (e.g. experiments in toxicology studies prefer a temperature of 37 °C), which require a certain temperature range; the user should reflect on temperature, known material properties at the temperature and should ensure stability of the temperature during measurement.

For selection of instrument settings it is required to have estimations on the upper and lower limit of the size distribution. The lower limit may correspond to the size of the constituent particles. The upper limit depends on the state of aggregation and agglomeration. The rotational frequency shall be chosen that it is possible to detect the largest assumed particles. The operating time shall be chosen that the smallest assumed particles are settled down. For NanoDefine the recommended running and measurement conditions are given in Table 6.

Table 6: Recommended running and detection conditions for $BaSO_4$ NanoDefine samples (IRMM-381 and IRMM-387) relevant for LUMiSizer® instruments

Sample	Description	Detection intervals	Rotational frequency
S1	$BaSO_4$ fine grade	250 x 5 s, 250 x 10 s, 250 x 20 s, 250 x 45 s	2000 rpm
S2	$BaSO_4$ ultrafine grade	250 x 5 s, 250 x 10 s, 250 x 20 s, 250 x 45 s	3000 rpm

If there is the opportunity of choosing the wavelength, it is recommended to use a wavelength which gives the substance the highest optical contrast to the liquid, which means a high relative refractive index. For many cases this means to measure at rather low values of the wavelength. In case of using the LUMiSizer, a wavelength of 470 nm is recommended.

16.4.3.4 Measurement description

Several prepared samples can be measured at the same time. Subsamples need to be measured at least in three replicates. Recommended are at least six replicates.

The volume needed for measurement shall be looked up in the instrument manual. During the steps of filling and closing the cuvette and inserting into the rotor, it is important that the user does not touch the surfaces foreseen for light transmission. The rotor has to be loaded symmetrically.

A typical sequence for preparation and measurement is as follows:

Equilibrating the temperature of the instrument

Normalisation

Inserting the sample

Measurement of the sample

Removing the sample

Evaluation of results: The instrument software will guide the user and calculate most of the results. This section provides information on the fundamental equations briefly and the required parameters to calculate the results. If the software provides the opportunity to calculate the number based particle size distribution directly from the raw data, the user should follow this wizard. The way of data evaluation is instrument specific. The user shall follow the instrument specific manual provided by the manufacturer. The standard deviation shall be calculated from the repeated extinction based and additionally from the number based median diameter results.

16.4.3.5 Determination of extinction based particle size distribution

For analysis of transmission the Lambert-Beer law shall be applied. For sedimentation, Stokes' law has to applied to calculate the particle size x from the sedimentation coefficient.

$$x = \sqrt{\frac{18 \cdot \eta_F \cdot S}{\rho_P - \rho_F}}$$

The specific sedimentation coefficient, *S*, (Svedberg and Rinde 1924) is calculated from the sedimentation velocity, *v*, and the machine specific parameters of rotational frequency ω and distance r from the centre of rotation to the detection position. This parameter is given in the instrument's documentation.

$$S = \frac{v}{r \cdot \omega^2}$$

The recommended values for the NanoDefine materials and water are given in the table below. If any other temperature is chosen the values for liquid density ρ_F and viscosity n have to be adapted for Stokes' equation.

Table 7: Recommended properties of water at 25 °C

Property	**value**
Dynamic viscosity η	0.890 mPa s
Density ρ_F	0.997 gcm^{-3}

Table 8: Recommended values for density for $BaSO_4$ (IRMM-381 and IRMM-387) samples at 25 °C

Sample	**Description**	**Solid density ρ_P [gcm^{-3}]**
S1 / S2	$BaSO_4$	4.4

NOTE: If it is a porous particle the density ρ_P chosen has to be averaged with the density of the medium ρ_F depending on the porosity. If it is a core-shell-particle the density ρ_P chosen has to be averaged between to two materials depending on the mass ration of the materials.

NOTE: If the concentration of the sample in the suspension was chosen above 0.25 % (v/v), a correction by applying a hindrance function is needed, e.g. reported by Richardson and Zaki 1954.

The result is the median Stokes diameter of the extinction based particle size distribution ($x_{50,ext}$).

16.4.3.6 Conversion into number based particle size distribution

The instrument software will guide the user for calculation of the mass based particle size distribution following Mie light scattering theory. The software also will allow the user to calculate the number based size distribution too. The recommended values for the refractive indices (RI) of the NanoDefine materials and water are given in the table below for commonly used wavelengths. The refractive indices are given this way:

$$RI = real\ part - imaginary\ part\ i$$

Some templates may also call the imaginary part of the refractive index 'absorption'.

NOTE: The adaption of Mie's solution is associated with assumptions, e.g. spherical and optical isotropic particles.

Table 9: Recommended RI values for $BaSO_4$ (IRMM-381 and IRMM-387) at selected wavelength

Sample	Description	RI at 405 nm	RI at 470 nm	RI at 530 nm	RI at 633 nm	RI at 670 nm	RI at 865 nm
S1 / S2	$BaSO_4$	1.697	1.668	1.652	1.634	1.63	1.617

If the refractive index of the sample is unknown then approximate values may be found in many databases, e.g. the open source database at www.refractiveindex.info.

NOTE: If it is a core-shell-particle the user shall ask the material's supplier on the optical properties of the sample or use an open source data base.

After the step of evaluation the data should be exported to a commonly used file format for spreadsheet calculation such as EXCEL®.

The result is the Stokes' median diameter of the number-based particle size distribution $x_{50,0}$.

Reporting of results

In order to allow full interpretation and reproduction of the measurement results, the analysis report shall include at least the following:

- Information on sample preparation, especially the suspending liquid and volume, and the dispersing agent and its concentration, the method of dispersion including the dispersion time and amount of energy added and net power density
- Information on measurement instrument and applied settings, especially the instrument type, the cuvette dimensions or cuvette identification and the centrifugal speed.
- Information on parameters for data analysis, especially the powder solid density and the refractive index used of the sample and water, temperature and viscosity
- Information on data fitting and correction

and information required by the guidelines of Good Laboratory Practice (date of analysis, laboratory, operator's name, identification of the sample, page numbering, name and signature of person authorising the analysis report, ...).

For validation purposes, the number based median diameter shall be used and additionally the extinction based median diameter reported

16.5 Validation status

The validation parameters of the method were determined successfully regarding the Stokes' diameters $x_{50,0}$ and $x_{50,3}$ for both grades of $BaSO_4$ particles IRMM-381 and IRMM-387. The working range regarding sample content is from 0.6 gkg^{-1} – 2.6 gkg^{-1} for IRMM-381 and 0.6 gkg^{-1} – 10 gkg^{-1} for IRMM-387. The upper limits were set with regard to the initial transmission and possible multiple scattering, whereas the lower limits were set due too large uncertainties (RSD) and too large deviations to the expected number-based median diameter. The lower limit is 0.1 gkg^{-1} by choosing 10 mm cuvettes, but the user should also note that this could lead to other sedimentation flow conditions and use of thicker cuvettes is not recommended in general.

The intermediate precision was determined to 9.7 % for IRMM-381 and 9.4 % for IRMM-387: It should be noticed that the results of the f-tests led to a rejection of the null hypotheses for the

number-based diameter $x_{50,0}$ for IRMM-381 and for the volume-based diameter $x_{50,3}$ for IRMM-387. In the case of the nanomaterial IRMM-387, the average number-based median could be determined certainly and the number-based median results of the 15 measurements are randomly distributed. The determination of the related volume-weighted median diameters does not fulfil the null hypothesis the reason for this could be the presence of rare particles in the sub-micrometre size range caused by erosion of the ultrasonic device and the following sample taking. The user might take samples not the same way every day and therefore decreases the chance of catching the same amount of rare large particles. The user should keep this in mind and handle the results with care if the volume based median diameter is required for any reason. In the case of the non-nanomaterial IRMM-387 this effect changes. The volume-based median diameters of the 15 measurements are randomly distributed, whereas the null hypotheses for the number-based median determination was rejected. Reasons for this are probably related to the sample preparation and the data treatment. The user should take care that the volume specific energy input by de-agglomeration with ultrasonic devices needs to be constant even if different devices of one type are used. In the case of broadly distributed particle size distributions, e.g. IRMM-381, the user should take care to keep a very reproducible procedure on determining the amount of the smallest particles as this fraction is dominating the position of the number-based median.

Tests on trueness, linearity and selectivity could not be carried out for several reasons.

16.6 HSE issues

Centrifuges have to be maintained according to the manufacturer's instructions. Never use a centrifuge if you are not sure that the centrifuge and the rotor are maintained properly.

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations. Each chemical/particulate material should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

16.7 References

[1] ISO 13318-2:2007 Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method

[2] M. J. Weber, Handbook of optical materials. CRC Press, Boca Raton, 2003. (based on: J.H. Weaver, E. Colavita, D.W. Lynch, and R. Rosei, Phys. Rev. Sect. B, 19:3850, 1979) [3] http://refractiveindex.info/

[4] Lerche, D., Sobisch, T., 2007. Consolidation of concentrated dispersions of nano- and microparticles determined by analytical centrifugation. Powder Technology 174:46-49

[5] ISO 13318-1:2001 Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines

17 SOP for analysis of Fe_2O_3 in Polyethylene Matrix with Electron Microscopy methods

17.1 Aim

The aim of this SOP is to provide and determine the sample preparation protocols and quantitative methods for fully automatic particle size distribution (PSD) analysis for Fe_2O_3 nanoparticles embedded in high density polyethylene (PE) matrix.

17.2 Scope

This SOP describes the use of an ultramicrotome (Leica EM UC6) for sample preparation, a transmission electron microscope (TEM) operated in scanning mode (STEM, Hitachi HD-2700) for imaging and NanoDefine ParticleSizer for analysis. The Fe_2O_3 in PE matrix was manufactured by industrial partners and received as small cylinder shaped rods. The dimensions of the rods were ~ 2 mm x 5 mm. The mass ratio of the hematite nanoparticles was 5 % (g/g). The NPs were ~ 40 nm in diameter and were agglomerated into complex 3D structures. The scope of the sample preparation can be extended to any nanocomposite soft material that can be cut by an ultramicrotome; and the analysis guidelines are valid for any complex nanoparticle agglomerates.

17.3 Abbreviations

DF	Dark field
DMSO	Dimethyl sulfoxide
HAADF	High angle annular dark field
PE	Polyethylene
PSD	Particle size distribution
SOP	Standard operating procedure
STEM	Scanning TEM
TEM	Transmission electron microscope

17.4 Description

17.4.1 Materials and methods

Ultramicrotome: Leica EM UC6

Hotplate: Gerhardt Hotplate

TEM: Hitachi HD-2700 200kV

Software:

Digital Micrograph™ script for magnification calibration:

Find Cross grating distance

Online: http://portal.tugraz.at/portal/page/portal/felmi/DM-Script/DM-Script-Database

ImageJ plug-in for particle size distribution analysis:

Nanodefine ParticleSizer

Solvents for sample preparation:

MilliQ H_2O: Millipore Advantage A10

DMSO: Analysis Emsure® Acs from EMD Millipore

17.4.2 Performing measurements

17.4.2.1 Sample preparation

The Fe_2O_3 in PE rods were prepared using cryo ultramicrotomy (Leica EM UC6). The rods were first embedded in epon block and trimmed to get a smooth cutting surface. The diamond knife temperature was set to -30 °C and sample temperature -130 °C. Low cutting speed was applied 0.4 – 0.8 mmmin^{-1}. The sections were left floating in a mixture of H_2O/DMSO at 75 °C and then transferred to TEM grids. This procedure results in crushed sections with a wavy structure. The TEM grids were then placed on top of a clean pin mount SEM holder and heated (Gerhardt Hotplate) for 1 h at 120 °C to straighten the sections.

17.4.2.2 Measurement description

The most prominent TEM calibration related to PSD analysis is the magnification calibration. This was done using a standard cross grating sample and a custom written automatic software (Find cross grating distance) for the pixel size calculation. The eucentric height has to be accurately determined such that no large defocus deviation occurs. The microscope should be additionally well aligned (user alignments) for optimized imaging conditions. Dark field (DF) or high angle annular dark field (HAADF) mode is recommended. The magnification should be chosen such that the smallest estimated particles are at least 10 pixels across (here at least 40 kX). The number of images should be chosen such that the PSD contains at least 1000 particles.

17.4.2.3 Evaluation of results

Due to the complex 3D structure of the agglomerates it is recommended to use single particle mode of the NanoDefine ParticleSizer and irregular watershed with high convexity threshold (> 0.9). All images should be visually checked and possible agglomerates should be removed.

17.5 Validation status

This method is not yet validated.

17.6 HSE issues

DMSO: Flammable liquid and vapour. Keep away from hot surface, sparks and other ignition sources. Take precautionary measures against static discharge. Wear protective latex gloves, protective clothing, eyes and face protection.

TEM: The user should be trained and guided to proper and safe usage of a TEM.

Ultramicrotome: The user should be trained and guided to proper and safe usage of an ultramicrotome.

18 SOP for extraction of TiO_2 from Sunscreen for analysis with Electron Microscopy

18.1 Aim & Scope

The aim of this SOP is to describe a mineral charge extraction protocol using solvents applied to cosmetic matrix.

Although allowing the detection of nanoparticles, direct observation of the finished product by EM does not readily give access to particle size distribution. To circumvent this limitation, extraction of particles from the organic matrix, prior to the determination of particle size distribution, by means of EM or other techniques, has been investigated. Three samples were delivered to the NanoDefine consortium: *i* ID 13a is the actual representative sample, containing 4 % NanoTiO_2, with an aluminium salt based surface treatment, as a UV filter, plus: micro-Titanium and Iron oxides for colouring purpose; *ii* ID 13b, is a simplified formula, containing 4 % NanoTiO_2, with an aluminium salt based surface treatment, as a UV filter (same particles as for ID 13a); iii A blank formula, without mineral particles (noted ID blank) has been provided. ID 13b and ID blank were delivered to the Consortium for the purpose of helping for the extraction of Nano-particles from ID 13a. The organic components are identical for the three samples.

18.2 Abbreviations

Rpm	rounds per minute
QSF	quantity sufficient for
g	gravitational constant
FFP3	Filtering Facepiece Particles 3
Dry ice	frozen CO_2
Vortex mixer	allow to mix liquid +solid with vibration
Centrifugal apparatus	allow to separate solid and liquid
Magnetic stirrer	allow to mix liquid and solid with mechanic movement
PTFE	Polytetrafluoroethylene (chemically inert)
SOP	Standard Operating Procedure

18.3 Description

18.3.1 Materials and methods

Centrifugal apparatus: SIGMA supplier/ SIGMA centrifuge 3 K 30/ controlled temperature system (set temperature to 23 °C) / max 28200 rpm / equipped with 12155 rotor 4x 85 mL - max radius 9 cm- min radius 2.1 cm-angle 30°-max speed 20000 rpm- max gravitational 40248 g / AUREAU VERITAS controlled every years

Vial for centrifugal: SIGMA supplier /Polycarbonate tube 85 mL standard screw cap diameter 38x104 mm

Vortex mixer apparatus: Heidolph supplier/ REAX2000 / 2400 x 1/min

Magnetic stirrer apparatus: 2mag magnetic^{e}motion supplier / MIX15

Rod for magnetic stirrer: PTFE coated ovoid shape in order to fit to the bottom centrifugal polycarbonate tube

Ultrasound bath apparatus: Bransonic supplier / US Bransonic 3510E-DTH, 100 W 42 kHz ± 6 %

Solvents used:

- Absolute Ethanol - VWR Chemicals supplier/ AnalaR NORMAPUR –ref 20821.296

-Water for HPLC - CARLO ERBA supplier / filtered through 0.1 µm membrane

Freeze dryer apparatus: Thermo Supplier / Lyolab A

Freezing mix: dry ice + acetone (normal quality)

Weighing apparatus: METTLER supplier / AT261 deltaRANGE scale / 205 g to 0.1mg or 62 g to 0.01mg

18.3.2 Sample preparation

18.3.2.1 Sample preparation

Particle extraction protocol:

In a centrifuge tube (60 mL /polycarbonate)

- Add magnetic stirrer (ovoid shape)
- Weigh 5 g of finished product
- Add absolute ethanol (QSF: 50 g)

Cycle 1

- Vortex mixer: 30 seconds
- Magnetic stirrer: 15 min (700 rpm)
- Ultrasonic bath: 15 min
- Centrifugation; 20 000 g (14026 rpm): 15 min
- Remove slowly the liquid phase
- Add absolute ethanol to solid phase residue (QSF: 50 g)

Cycle 2 = Cycle 1

Cycle 3 = Cycle 1

Cycle 4

- Vortex mixer: 30 seconds
- Magnetic stirrer: 15 min (700 rpm)
- Ultrasonic bath: 15 min
- Centrifugation; 20 000 g: 15 min
- Remove slowly the liquid phase
- Add 30 mL of water
- Magnetic stirrer: 5 min (700 rpm)

- Pour into a freeze drying flask (rinsing out with 10 mL of water)
- Freeze the dispersion with a bath full of a mix of dry ice and acetone
- Freeze drying during 12 h.

18.3.2.2 Measurement description

The insoluble fractions have been weighed for the three products, and corresponding percentage determined in mass:

ID 13a: the insoluble fraction corresponds to 11.1 wt% of the initial formula

ID 13b: the insoluble fraction corresponds to 7.7 wt% of the initial formula

ID blank (blank formula): the insoluble fraction corresponds to 3.3 wt% of the initial formula

18.4 HSE issues

Solvent absolute Ethanol: Highly flammable liquid and vapour:

- keep away from hot surface, sparks and other ignition sources - take precautionary measures against static discharge

- wear protective gloves/ protective clothing/ eyes protection/ face protection.

Extractions residue: as residues may contain nanoparticles

- Wear protective gloves/ protective clothing/ eyes protection/ face protection/ mask protective FFP3.

- Place weighing apparatus in a protective area such as an Erlab laboratory hood / Captair flex XLS 392 with 2 filter HEPA UP17

Ultrasound apparatus: use EAR protection

Freezing mix (dry ice + acetone):

-Dry ice: due to the low temperature (-78 °C) wear temperature protective gloves/ protective clothing/ eyes protection/ face protection.

-Acetone: Highly flammable liquid and vapour- keep away from hot surface, sparks and other ignition sources: take precautionary measures against static discharge and wear protective gloves/ protective clothing/ eyes protection/ face protection.

19 Size characterisation of suspended particles by AUC-RI with speed ramp option

19.1 Aim

The aim of this SOP is to determine the number and mass based median particle size, the particle size distribution (PSD) and the mass concentration (C) of suspended micro- and/or nanoparticles, based on the principles of Analytical (Ultra) Centrifugation with Rayleigh interference Refractive Index detection (AUC-RI).

19.2 Scope

This SOP describes the use of the AUC-RI to perform particle size and particle concentration analysis measurements by measuring the refractive index radial profiles during sedimentation by means of Analytical (Ultra) Centrifugation).

Specifically for polydisperse samples or samples of unknown size range, this SOP includes also the optional operation of the AUC-RI in rotor speed ramp mode, or g-sweep.

The SOP was validated for SiO_2 and $BaSO_4$. In case of $BaSO_4$, both nano and non-nano forms (median in number metrics either below or above 100 nm) were analysed. The method is applicable to all particles that do disperse but not dissolve in the suspension medium, and which have a density contrast >0.05 gcm^{-3} and refractive index increment >0.01 cm^3g^{-1} in the specific suspension medium.

The technique can also be used to measure the molecular mass of proteins or dissolved macromolecules. However, note that these types of measurements are outside the scope of the current SOP.

The data acquisition part of this SOP specifies parameters for the AUC-RI model 'XLI' from Beckman-Coulter (Palo Alto, USA), currently the only commercial provider of AUC-RI instruments. The basic principles of analytical centrifugation are described by ISO 13318, which covers methods for determining the particle size distributions of particulate materials, by centrifuges other than the AUC-RI, so that the ISO 13318 only covers the size range 0.1 µm to 5 µm, whereas the higher centrifugal acceleration of the Beckman XLI extends the range to smaller sizes down to 1 nm.

19.3 Definitions

As far as possible, terminology follows ISO 13318-1:2001(E)) 'Determination of particle size distribution by centrifugal liquid sedimentation methods — Part 1: General principles and guidelines' and terminology developed in NanoDefine..

N = Centrifuge speed or rotational frequency, in units of '1/min'

ρ = skeleton density of the material, in units of 'gcm^{-3}' (= true particle density in ISO 13318)

ρ_1 = liquid density of the suspension medium, in units of 'gcm^{-3}'

η = viscosity of the suspension medium, in units of 'Pa*s'

s = sedimentation coefficient, in units of 'Svedberg', where 1 Svedberg = 10^{-13} sec

λ= wavelength of the RI detector, in nm

l = optical path length in the AUC cell, in m

C = cumulative (mass) concentration, in units of 'mgmL^{-1}'

C_{RI} = total (mass) concentration represented by PSD, in units of 'mgmL^{-1}'

$x_{50,3}$ = Median diameter in volume metrics, in units of 'nm'

$x_{50,0}$ = Median diameter in number metrics, in units of 'nm'

Q_3 = cumulative size distribution in volume metrics

Q_0 = cumulative size distribution in number metrics

PSD = Particle Size Distribution

DLS = Dynamic light scattering

CLS = Centrifugal liquid sedimentation

SHMP = Sodium hexametaphosphate

19.4 Description

19.4.1 Materials and methods

- Beckman XLI or other AUC with RI detector.
- 4-hole (An-60-Ti) or 8-hole (An-50-Ti) analytical rotor
- Double-sector cells, preferably with sapphire windows of 0° oriented optical axis.
- Standard laboratory equipment for sample preparation, filling and cleaning of cells.
- The suspension medium (e.g. water or sodium hexametaphosphate solution) should be of high purity and must be free of particles (e.g., passed through a membrane filter with appropriate cut-off)
- For $BaSO_4$ in water: Stabilising agent: sodium hexametaphosphate (SHMP)
- Sonication equipment able to deliver at least 30 W sonication power (as measured by calorimetry, Taurozzi et al.[1]), for instance:
    - Preferably tip sonicator, e.g. Hielscher UPS200S operated at pulsed mode with an amplitude of 75 % and a cycle time of 50 %, and thus produces a mean absorbed power of 7.8 W, or 1.3 WmL^{-1}.
    - or vial tweeter, e.g. Hielscher 250 W Ultrasonic Processor UIS250v head fitted with VialTweetersonotrode vial sonicator with an amplitude setting of 75 % and cycle time of 50 %.(Calorimetrically determined power input is 1.0-1.1 Wml^{-1} mean energy absorbed) or equivalent.
    - or equivalent equipment.
- Software requirements: Sedfit, freeware, available at
- http://www.analyticalultracentrifugation.com/tutorials.htm
- Computer (hardware) requirements to use Sedfit: PC with at least 500 MHz, 256 MB RAM, Pentium or Xeon processor (Cyrix 3 processors may not work). Multi-core processors are supported and can significantly speed up many computations.
- Windows NT, 95, 98, XP, 2000, or Vista, at least 2 MB of disk space.

19.4.2 Performing measurements

19.4.2.1 Operation of the equipment

- As described by AUC-RI operating instructions
- Use an empty double-sector cell, set N to the speed that is appropriate for the specific material (see below: measurement description), open the details menu, select laser setup, adjust laser delay and duration for optimum visibility of fringes. Stop the rotor.

  - The recommended criterion for an 'appropriate' speed is a duration of sedimentation within approximately 30 minutes to 2 h. This ensures that the $x_{50,3}$ size is well within the limits of size range detection.
  - If the size of the sample is unknown, the speed ramp option can save a lot of time for identification of the appropriate speed. Alternatively, the appropriate speed can be determined iteratively by repeated fixed speed measurements.
- Use 25 °C as measurement temperature.
- To avoid artefacts by sample sedimentation inside the AUC cells due to time-consuming temperature equilibration before data acquisition, ensure that the rotor and cells are at ±0.1 °C the same temperature as the AUC before filling the cells.

- Only for the *optional* AUC-RI-ramp operation[d], to eliminate the need to select a speed that is appropriate for the specific sample, the speed ramp requires setup parameters. Specifically for the Beckman XLI, this SOP includes predefined setup files:
  - Copy the RampNanoDefine.EQU and RampNanoDefine.SCN files into the XLI software directory.
  - The RI laser delay has to be adjusted once for the local machine. This step is essential for the extended ramp that starts from N=1100 rpm, but is recommended also for the standard ramp from N=3,000 rpm.
  - Select File, select open, select the RampNanoDefine.SCN file.
  - Use an empty double-sector cell, set speed to N=3000 rpm, open the details menu, select laser setup, adjust laser delay and duration for optimum visibility of fringes. Check whether the selected laser delay and duration provide good visibility of fringes also at the highest rotor speed.
  - Select file, select save. The XLI operating software will recall this setting also after restart. Stop the rotor.

19.4.2.2 Sample preparation

The suspension medium (e.g. water) should be of high purity and must be free of particles (e.g., passed through a membrane filter with appropriate cut-off).

- Specifically for colloidal silica the suspension should be analysed without dilution.
- Specifically for $BaSO_4$, the materials under study are supplied as dry powders which require re-dispersion in aqueous media using an ultrasonic probe prior to use with the AUC. All information necessary to produce suitable samples of IRMM-381 and IRMM-387 for AUC analysis is documented Chapters 3, 9 and 13. Chapter 13 contains details of the procedure which must be followed to determine the correct power settings for a tip (probe) sonicator, while Chapters 3 and 9 of this report details the exact dispersion procedure, including sonication, to be followed for the materials IRMM-381 and IRMM-387. The liquid dispersions (6 ml batches of 2.6 $mgmL^{-1}$ suspensions) are prepared by mixing defined quantities of dry $BaSO_4$ powders with water containing 2.0 $mgmL^{-1}$ of Sodium hexametaphosphate (SMPH), homogenised by vortexing, de-agglomerated using high intensity probe sonication. The resulting dispersion should be diluted with further SMPH solution to produce a final analyte concentration of 1 $mgmL^{-1}$. The SMPH in the solution is present as an aid to de-agglomeration during sonication and later as a

[d] Two files to represent the recommended speed ramp that is applicable to all commercial Beckman XLI instruments (from 3k rpm to 50k rpm, or adapted for specific materials with known size ranges):

- Speedsteps.equ (for data acquisition by import in XLI - EQU interface)
- Speedsteps.txt (for evaluation of data in Sedfit_ramp)

An extended speed ramp (from 1.1k rpm to 55k rpm) that extends the upper detection limit above 1 µm sizes requires an adaptation of the EPROM of the XLI, and is available upon request from BASF Material Physics.

stabiliser. The sonication steps were done using values of ultrasonic power and treatment times which were specific to each material. Table 1 shows the variations in sample preparation parameters for the two $BaSO_4$ materials.

- The suspensions are stable for at least 1 h.

Table 1: Variations in sample preparation parameters for the two $BaSO_4$ materials

Sample	**SHMP conc.**	**Tip (probe) sonicator**				**Vial treater****		
		Temp.	**Typical final volume**	**Power output***	**Time**	**Temp.**	**Typical final volume**	**Time****
Non-nano $BaSO_4$ IRMM-381	2 $mgmL^{-1}$	Ice bath	6 mL	10.3 W	20 min	Not controlled	1.7 mL	Time should be optimised for the individual equipment**
Nano $BaSO_4$ IRMM-387	2 $mgmL^{-1}$	Ice bath	6 mL	7.6 W	5 min		1.7 mL	

* The sample preparation SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given Chapter 13 of this document.

** The operator has to have access to a DLS or CLS instrumentation. The dispersion has to be evaluated by DLS or CLS at various treatment times and the results compared with that shown in section 2.7. If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

If applied to other materials with specific sample preparation protocols, these must fulfil the following requirements:

- The suspension medium can be water, optionally with dispersing agents, but can also be organic solvents.
- The resulting samples must be at least 0.5 mL volume of particle suspension in a medium of known density ρ_1 and known viscosity η. The particles should be suspended at a concentration in the range of 0.1 $mgmL^{-1}$ to 10 $mgmL^{-1}$. The validation showed that the widest size range can be covered at a concentration of 1 $mgmL^{-1}$.
-
- → fill 380 µL ±10 µL of sample into the sample sector of the double-sector cell.
- → fill 380 µL ±10 µL of suspension medium without particles into the reference sector of the double-sector cell
  - Matching volumes will match the meniscus so that solvent compression is identical on both sides, thus reducing baseline tilt that would lead to artificial signal around s_{min}.
  - For the conventional operation at fixed speed, it is possible to fill 400 µL ±10 µL into the reference sector of the double-sector cell. The solvent compression is not an issue at fixed speed and the mismatch of the menisci is advantageous in the data fitting to locate the position of the meniscus visually.
  - *Optionally*, the medium in the reference cell is not pure water or pure solvent, but contains the same concentration of dispersing agents as the sample. The option can help to exclude any ambiguity of the assignment of components potentially observed

at low sedimentation coefficients, because the AUC-RI does detect surfactant micelles as separate component.

→ seal cells and insert the cells into the rotor, insert rotor into AUC instrument, activate evacuation and temperature equilibration.

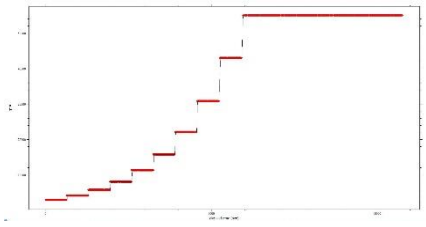
- avoid times longer than 30 minutes until data acquisition due to potential sedimentation of large particles onto the lower cell window, potentially leading to artefacts.

19.4.2.3 Measurement description

- AUC-RI is an absolute technique, requiring no signal response calibration by means of a particle size standard. As specified by the manufacturer, the magnification factor of the optical detection system must be calibrated by means of the counterbalance cell.
- Perform three measurements from three sample preparations, i.e. one analysis per prepared suspension.
- For the conventional operation at fixed speed, the appropriate speed needs to be verified by comparing the Q_3(fixed speed) to a Q_3(speed ramp): The $x_{50,3}$ values should match within 20 %.
    - Specifically for colloidal silica, the indicative speed is 15,000 rpm
    - Specifically for $BaSO_4$, the indicative speed is between 1,000 rpm (detection range 20 nm to 2 µm) and 6,000 rpm (detection range 2 nm to 200 nm).
    - The recommended criterion for an 'appropriate' speed is a duration of sedimentation within approximately 30 minutes to 2 h. This ensures that the $x_{50,3}$ size is well within the limits of size range detection. A complete fixed speed measurement generates between 50 and 500 scans, typically around 150.
- For the *optional* speed ramp operation: The present ramp is intended for the measurement of 2 samples in the same run, using either a 4-hole or 8-hole rotor.
    - The detection range is at least 5 nm to 1 µm for $BaSO_4$, or wider, depending on menicus matching (19.4.2.2), g(s) truncation (19.4.3.3) and extended ramp (19.4.2.1).
    - In principle, up to 8 samples can be measured simultaneously in a single run, if the ramp is adapted accordingly with fewer RI scans per speed step.

→ Select *File*, select *open*, select the RampNanoDefine.SCN file. The software will now load the predefined ramp from the RampNanoDefine.EQU file. Verify by selecting *Method*

For *XL settings* → *Speed*, enter the final speed of the ramp (50,000 rpm).



→ *Start Method Scan*. Data acquisition is finished after 3 h. The long running time at the final speed ensures that particles down to 1 Svedberg (around 1 nm diameter) are detected. The power-law shape of the ramp is optimal for homogeneous information content across the entire size range.[e] The specific ramp was developed for NanoDefine applications.

Data is stored in #.IP*n* files, with # the running number of scans, and *n* indicating the cell number. Each file represents the radially resolved interference fringe shift between

[e] Ma J, Zhao H, Sandmaier J, Liddle JA, & Schuck P (2016) Variable field analytical ultracentrifugation: Gravitational sweep sedimentation velocity. Biophysical journal 110(1):103-112.

sample cell and reference cell. The RampNanoDefine.EQU file is designed to acquire a scan about every 15 seconds (depending on the specific machine), resulting in a number of 500 scans per cell per measurement.

19.4.3 Evaluation of results

19.4.3.1 Conversion from interference fringe shifts to sedimentation coefficient distribution

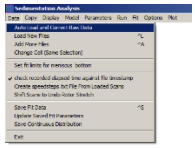
For generic introduction to AUC evaluation by the Sedfit software, refer to http://www.analyticalultracentrifugation.com/tutorials.htm

Specifically for evaluation of fixed speed measurements, use Sedfit Version 14 or later (download at http://www.analyticalultracentrifugation.com/download.htm)

- Choose -> *data* -> *Load new scans; Load* all scans 1 to 500
- Investigate raw data critically and load the appropriate number of scans (1 to n) again based on the remaining signal at the centre of the cell (0.05 fringes at mid-cell is considered as limit below which noise dominates). High number of measurements containing no information on sedimentation but contributing to noise decrease the quality of fit and increase analysis time.

Specifically for evaluation of speed ramp measurements, the methodology is described in literature[e].

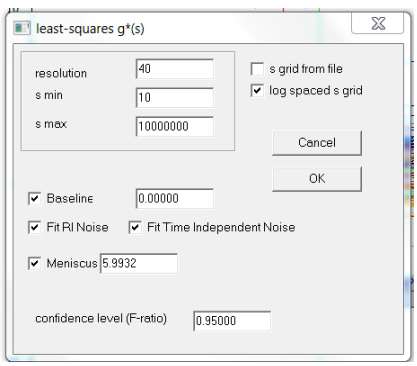
- Use version 'sedfit-lsgofs.exe'. The algorithm is described in the publication, but the specific version was developed for polydisperse distributions in NanoDefine cooperation.
    - Request a copy from Peter Schuck, NIH Bethesda, USA.
    - Unlike conventional XLI operation at fixed speed, the meniscus will shift between scans. This is not an indication of leaking cells, but is due to rotor stretching and solvent compression, and is corrected by the software.



- Choose -> *data* -> *Auto Load and Correct Raw Data*
- *Load* all scans 1 to 500; choose rotor (4-hole or 8-hole); close by ESC the ramp graphics. Investigate raw data critically and re-load the appropriate number of scans (1 to n) again based on the remaining signal at the centre of the cell (0.05 fringes at mid-cell is considered as limit below which noise dominates). High number of measurements containing no information on sedimentation but contributing to noise decrease the quality of fit and increase analysis time.

For both conventional fixed speed and the optional speed ramp, proceed to fitting:

- Choose *model* ls-g*(s) ('least squares fitting of sedimentation coefficient distribution')
- Set *parameters*
    - For fixed speed: start with range s_{min}=1 to s_{max}=10^7 Svedberg, resolution = 40. Reduce s_{max} if SedFit alerts that the upper detection limit is exceeded.
    - For speed ramp: s_{min}=1 Svedberg; s_{max} =



10^7 Svedberg, resolution = 40

- Select log spaced s grid

- Set apparent meniscus and bottom, set meniscus fitting boundaries.
  - Set the fitting range to use the entire length of the cell, excluding approx. 0.3 mm from the apparent meniscus, and approx. 1 mm from the apparent bottom.

→ Perform '*fit*'. It will result in a low-resolution ls-g*(s), only serves to find correct meniscus.

- Do not change the position of meniscus, but increase the *resolution* to 250

→ Perform '*run*'. It will result in a high-resolution ls-g*(s) distribution.

- Do NOT close the program, but proceed to quality control checks.

19.4.3.2 Quality control checks (still within the Sedfit evaluation software)

- Select *Display*, select *Subtract all systematic noise*, critically check residuals.
  - If residuals of the early scans show jumps of interference fringes within the scan, the turbidity of the sample was too high for a useful interference pattern.
    - '*delete scan*', knowing that early scans represent larger particles.
    - Alternatively, repeat measurement at lower particle concentration.
  - If residuals diverge at the bottom, outside the fitting range, do not worry.
  - If residuals diverge at the meniscus, e.g. by an apparent baseline drift, this influences the distribution at lower s-values. Check meniscus settings. Check meniscus matching between sample and reference (only a single meniscus visible).
  - If residuals show a wavy pattern, this might be due to turbulence by insufficient temperature equilibration.
  - If residuals are 'jigsaw pattern exceeding 20 % of signal', the s_{min}/s_{max} range can be adapted for optimal resolution of the PSD (although the actual median is not influenced significantly):
    - *RUN* repeatedly, reducing s_{max} from 10^7 to 10^6, 10^5, 10^4 Svedberg until the ls-g*(s) distribution extends around $s_{max}/10$.
- Optionally, save a screenshot to document the residuals.
- Select *Data*, *Save continuous distribution*, default file name is 'newdat.dat', can be changed.

The resulting file is the distribution of sedimentation coefficients g*(s) in volume metrics, which is the intrinsic metric of the RI detector.

19.4.3.3 Conversion from sedimentation coefficient distribution to number metric distribution

- Load g(s) distribution from the 'newdat.dat' file into a suitable software, e.g. excel.
- Optionally, truncate the lowest range of s values as appropriate. This serves to remove artifacts from baseline drift, but of course it reduces the detectable size range.
  - Appropriate truncation ranges can be identified by measuring pure suspension medium with zero particle concentration.

  - Often, truncation of the lowest s-decade can be appropriate, e.g. if s_{min}=1 Svedberg, delete g(1 Svedberg) to g(10 Svedberg).

- Convert each sedimentation coefficient s_i to particle diameter D_i. Typically (ISO 13318), this conversion assumes spherical shape with homogeneous density and applies the Stokes equation:

$$D_i = \sqrt{\frac{18\eta s_i}{\varrho - \varrho_1}}$$

  - For other shapes, specific relationships between s and D can be calculated based on frictional drag and centrifugal forces, but are beyond the scope of the present SOP. (see Wohlleben, J Nanopart Res. 2012, 14:1300)

- To generate a size distribution, integrate the g_i values to C, knowing the refractive index increment dn/dc. Please note that if dn/dc is unknown, this has no influence on the median diameters!

$$C_i = C_{i-1} +\cdot\, g_i \frac{B}{dn/dc}$$

  - Specifically for a wavelength of the RI detector of ⍰=675 nm and an optical path length of l=12 mm through the AUC sample cell (XLI standard parameters), the integration parameter B=0.05625. It is calibration-free and scales linearly with wavelength and inversely with optical path length.

  - The cumulative size distribution in volume metrics is given by the D_i and C_i columns. Due to the measurement principle, D_i are the end-point, not mid-point intervals.

  - The C_{RI}=C_{imax} value is the actual concentration of particles represented by the PSD, in units of mgmL^{-1}.

  - Normalize and read at Q_3 = 50 % the median diameter in volume metrics, $x_{3,50}$. Analogously, read $x_{3,10}$, $x_{3,90}$.

$$Q_{3,i} = \frac{C_i}{C_{RI}}$$

    - As optional fitting-free cross-check, convert the raw data fringe shift Δj to absolute concentration.

$$C_{RI,fitfree} = \frac{\lambda \cdot \Delta j}{\frac{dn}{dc} \cdot l}$$

- By differentiation of Q_3, obtain q_3.

$$q_{3,i} = \frac{Q_{3,i} - Q_{3,i-1}}{log(D_i/D_{i-1})}$$

- Convert to Q_0, assuming a diameter – mass relation (typically spheres).

$$Q_{0,i} = Q_{0,i-1} + \frac{g_i}{\frac{\pi}{6} 10^{-21} \rho D_i^3}$$

- Normalize and read at $Q_0^{norm} = 50\%$ the median diameter in number metrics, $x_{50,0}$.

- $Q_{0,i}^{norm} = \frac{Q_{0,i}}{Q_{0,max}}$
  - By differentiation of Q_0, obtain q_0.

$$q_{0,i} = \frac{Q_{0,i} - Q_{0,i-1}}{log\left(D_{0,i}/D_{0,i-1}\right)}$$

19.4.4 Quality control checks based on concentration

- Does $Q_{3,max}$ match the concentration of particles in sample preparation?
  - If $Q_{3,max}$ is more than 10 % higher than the concentration of particles in sample preparation, either the dn/dc is incorrect (which has no consequences on the PSD or on the classification by the EC nanodefinition), or there is considerable adsorption of dispersing agent onto the particles (which might distort the PSD)
  - If $Q_{3,max}$ is more than 10 % lower than the content of particles in sample preparation, the PSD might not be representative for the material, because significant parts of the material have dissolved or have diameters larger than the upper detection limit.

19.4.5 Reporting of results

- PSD reporting:
  - plot Q_0^{norm} (unitless, normalized to 1) and q_0 in one graph over a log Diameter axis in nm.
  - Plot C (in $mgmL^{-1}$) and q_3 in one graph over a log Diameter axis in nm.
- Report $x_{50,0}$ in nm.
- Report $x_{50,3}$ in nm and C_{RI} in $mgmL^{-1}$

19.5 Validation status

Validation of the AUC-RI method showed that the method is able to properly identify as nano/non-nano materials the tested nano $BaSO_4$ and SiO_2 and non-nano $BaSO_4$ samples. Trueness of the method was not investigated because of the lack of appropriate reference material. Thus, relative measurement uncertainty was calculated as a combination of intra-day and day-to-day variation related uncertainties. The resulting standard measurement uncertainty values determined for $x_{50,0}$ and $x_{50,3}$ fall in the expected range (below the 20 %target uncertainty) and were below 12 % for all three test materials.

The total observed concentration C_{RI} (synonymously designated as Q3,max) was significantly reduced by imperfections of sample preparations from powders. In contrast, for ID-18 as ideally pre-dispersed sample we observed C_{RI} = 96 % of the specified concentration, with a relative measurement uncertainty of 5.9 %. This is an acceptable performance.

The method is robust for temperature changes in the +/- 1 °C range.

The working (particle size) range of the fixed speed experiments was appropriate for the characterisation of the samples. The extended ramp method improved the detection of both smaller and larger particles. The standard ramp program failed to detect larger particles in case of IRMM-381 showing that rotation speed is a very sensitive parameter of the AUC-RI method. The

option of the SOP (first ramp measurement to identify suitable speed, then decisive measurement at (this) fixed speed) is thus seen as most robust implementation.

19.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards and exposure limits relevant to all chemical substance used in the procedure described here.

Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can produce damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

AUC Cells may be deteriorated in aggressive suspension medium, leading to leakage. The producer Beckman provides a compatibility table to verify that the cells are suitable for the solvent and pH.

The Beckman XLI is an *ultra*centrifuge (AUC), and as such some countries (e.g. Germany) require yearly inspection in disassembled state by qualified personnel (typically the Beckman service engineer). Rotors are recommended to be used only for a period of ten years by the manufacturers.

19.7 References

[1] Taurozzi, J. S., Hackley, V., Wiesner. M. Preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. National Institute of Standards and Technology. 2012. Available at: http://nvlpubs.nist.gov/nistpubs/specialpublications/nist.sp.1200-2.pdf

20 Particle size distribution measurement of $BaSO_4$ using Line-Start Disc Centrifuge with Optical Detection

20.1 Aim

The aim of this SOP is to determine the number and mass based median particle size and the particle size distribution (PSD) of suspended micro- and/or nanoparticles, based on the principles of line-start Centrifugal Liquid Sedimentation (CLS) with optical detection.

20.2 Scope

This SOP details methods for determining the particle size distribution of particulate materials by means of centrifugal sedimentation in liquid using optical detection and quantification. Specifically, this SOP refers to the use of a line start disc centrifuge (CPS UHR24000 disc centrifuge) with optical detection by a light beam from a 405 nm laser diode light source. The methods are applicable to liquid dispersible powders in which all particles have the same density and comparable shapes and do not undergo chemical or physical change in the suspension liquid. It is necessary that the particles have a density higher than that of the liquid used in the density gradient. This SOP is primarily intended for the determination of the particle size distributions of the fine and ultrafine $BaSO_4$ materials IRMM-381 and IRMM-387. Depending in the chosen operating speed measurement size range for $BaSO_4$ particulates should be range from 2 µm to 70 nm (8000 rpm) and from 800 nm to 30 nm (16000 rpm).

20.3 Terms, definitions and symbols

As far as possible, terminology follows ISO standards listed in Table 4 below.

Table 4: ISO standards relative to centrifugal liquid sedimentation methods

Reference	Title
ISO 13318-1:2001	Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines
ISO 13318-2:2007	Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method

Abbreviations

AC	Analytical centrifuge
AUC	Analytical Ultra Centrifuge
CLS	Centrifugal liquid sedimentation
Disc-AC	Line-start Disc-centrifuge
DLS	Dynamic Light Scattering
EtOH	Ethanol
NM	Engineered Nanomaterial
NP	Nanoparticle(s)
PQ	Performance qualification

PQ	Performance Qualification Test
PS	Probe Sonicator
PSD	Particle Size Distribution
PVC	Polyvinylchloride
RI	Refractive index
SHMP	Sodium HexaMetaPhosphate
SOP	Standard Operating Procedure
VS	Vial-sonicator also referred to as Vial-tweeter
USB	Ultrasonic Bath
USP	Ultrasonic Probe

20.4 Description

The general measurement principles of determining the particle size distribution by line-start centrifugal liquid sedimentation (CLS) are described in ISO 13318 which is applicable to powders that can be dispersed in liquids, colloidal suspension of solid particles and some emulsions. In particular the use of disc centrifuge with optical detection is detailed in ISO 13318-2. The method is applicable to powders in which all particles have the same effective density and comparable shapes and do not undergo chemical or physical change in the suspension liquid. It is usually necessary that the particles have a density higher than that of the liquid of the density gradient.

Depending on the density of the particles, and at a rotational speed of 18000 rpm to 20000 rpm, the working range of the disc centrifuges and cuv2ette centrifuges as considered in ISO 13318-2 covers the size range of approximately 0.1 µm to 5 µm. At higher rotational speeds (e.g. 24000 rpm) the lower limit of the working range can be 20 nm or lower.

In the disc-sedimentation version of CLS, the instrument is based on a hollow transparent rotating disc containing a liquid of increasing density into which a small volume of dispersed particles is injected and then undergo forced sedimentation through centrifugal force. By the use of suitable detectors it is possible to measure the sedimentation rate and to determine the particle size distribution. In the Disc-AC method, which is more correctly known as 'Line-start CLS', particles are injected into the centre of the rotating disc. Once the particles enter the density gradient they sediment radially outwards at a speed which is a function of their density and Stokes diameter. At a certain point in time the particles pass through a narrow beam of light which shines through a region near the outside edge of the rotating disc. As the particles pass through the light beam, the amount of light transmitted to the detector decreases due to absorption and scattering (extinction) by the particles. From the time of sample injection the variation in light extinction is continuously recorded as a function of sedimentation time. During a measurement sequence the method parameters such as sedimentation distance, refractive index, density and viscosity of the density gradient do not always remain constant. Since the true values of these method parameters cannot be easily assessed manufacturers of the major Disc-AC instruments recommend performing a calibration measurement prior to each sample measurement. Such calibration must be done with monodisperse particles of which their size and effective density are accurately known. The light extinction-weighted particle size distribution can be converted by the operating software into a mass-weighted particle size distribution. This conversion, which is based on the application of Mie light scattering theory, requires that the complex value of the particle refractive index is accurately

known. Finally, a number-weighted distribution can be calculated from the mass-based distribution using the particle density and geometric and shape factors as input parameters.

The following procedure is primarily designed for the determination of particles size distributions of IRMM-381 fine and IRMM-387 ultrafine grade $BaSO_4$ materials of the NanoDefine project. The selection of the instrument parameters have been chosen to meet the following criteria.

- The rotational speed should be chosen such to ensure that the sedimentation time for the smallest particles expected in the sample does not exceed 30 minutes. At longer measurement times baseline drift can become significant and in that case a subtraction of the baseline may be needed to allow reliable determination of the PSD.
- Throughput times for the full measuring cycle should be approximately 60 minutes. The full measurement cycle includes the calibration step, the sample measurement step and an additional rest period of 20-30 minutes to allow the sedimentation of any potential residual fine particulates, of which their sizes are below the detection limit, and to re-stabilise the density gradient.
- The rotational speed should be chosen such to ensure that the sedimentation time for the largest particles in the sample is not less than 0.5 s.

On the basis of these criteria and the expected sample size ranges of the two materials, rotational speed values of 8000 rpm and 16000 rpm have been considered suitable for the IRMM-381 and IRMM-387 materials respectively as detailed in Table 5.

Table 5: Estimated particle sizes at selected sedimentation times and rotational speeds*

Speed	Particle size at t=0.5 s	Particle size at t=30 min	Particle size at t=60min
8000 rpm	2000 nm	30 nm	20 nm
16000 rpm	800 nm	15 nm	10 nm

* Assuming operation with sedimentation gradient as described in section 20.5.4.5

20.5 Materials and Methods

20.5.1 Instruments and Equipment

- Disc centrifuge with optical detection and line-start capability. e.g. CPS UHR24000, DC20000 or DC18000 disc centrifuges, or equivalent.
- Laboratory scale analytical balance with maximum load greater than 100 g and a readability of ±0.1 mg or better.
- Variable volume pipettes 1-20 µl, 20-200 µl, 100-500 µl, 100-5000 µl,
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a recommended tip diameter of approximately 6-7 mm. The sonicator should have a nominal declared acoustic output at least 100 W. A protocol describing the recommended procedure for measuring the effective acoustic energy output characteristics of a probe sonicator is detailed in Chapter 13 of this document.
- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- High purity (resistivity 18.2 $M\Omega cm^{-1}$).filtered water (0.1 µm or 0.2 µm filter) water; thermally equilibrated to fume-hood air temperature.
- Digital thermometer with metal sheathed thermocouple probe capable of a measurement accuracy better than ±0.1 °C.

- Digital timer capable of measurement accuracy better than ±1 s.

20.5.2 Chemicals and consumables

- 2 ml plastic microcentrifuge tubes with sealing lid for use with vial-sonicator
- Disposable graduated plastic syringes (1 ml) with flat ended needle
- Disposable plastic spatula for weighing of NM.
- Disposable anti-static plastic weighing boats or similar for weighing of NM and any chemicals in powder form.
- Disposable powder-free nitrile gloves.
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs.
- High purity filtered water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega cm^{-1}$ at 25 °C, 0.2 µm in-line filtration).
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity ≥ 96 %, e.g. 305553 Aldrich).
- Sucrose
- Dodecane
- Aqueous particle size calibration standard suitable for use with Disc-AC

20.5.3 Sample dispersion

The $BaSO_4$ materials under study are supplied as dry powders which require re-dispersion in aqueous media prior to use with the Disc-AC. All information necessary to produce suitable samples of IRMM-381 and IRMM-387 for Disc-AC analysis is documented in Chapters 3 and 9.

The liquid dispersions (6 ml batches) are prepared by mixing defined quantities of dry $BaSO_4$ powders with water containing 2.0 $mgml^{-1}$ of Sodium HexaMetaPhosphate (SMPH), homogenised by vortexing, de-agglomerated using high intensity probe sonication. The resulting dispersion should be diluted with further SMPH solution to produce a final analyte concentration of 1 $mgml^{-1}$. The SMPH in the solution is present as an aid to de-agglomeration during sonication and later as a stabiliser. The sonication steps were done using values of ultrasonic power and treatment times which were specific to each material.

Chapter 13 contains details of the procedure which must be followed to determine the correct power settings for a probe sonicator while Chapters 3 and 9 of this report details the exact dispersion procedure to be followed for the materials IRMM-381 and IRMM-387. The optimum sonication times quoted in Table 6 were determined experimentally by comparing Disk-AC measured particle size distributions of samples treated for times ranging from 1 to 60 minutes at the quoted power output values. The sonication times were chosen to correspond with the minimum time beyond which no further significant decrease in mean particle size could be detected by Disc-AC. As the efficiency of sonication can vary significantly with instrument type, probe geometry and sample volume it is strongly recommended that Disk-AC be used to verify that the particles size distribution (maximum in the weight distribution) achieved using the quoted sonication times is within 10 % of those noted in Table 6. In the case that the particle size obtained is significantly higher than the quoted values, other samples should be prepared using longer and shorter sonication times and analysed to determine the sonication time which produces the minimum mean particle size in the shortest time.

Table 6: Summary of dispersion parameters for each material

Material	Material concentration (sonication)	Material concentration (AC analysis)	Surfactant	Batch volume	Sonication power used*	Sonication time (approx.)	Disc-AC determined size**
IRMM-381	(2.6 $mgmL^{-1}$)	(1 $mgmLl^{-1}$)	SMPH (2 $mgmL^{-1}$)	6 mL	10.3 W (1.8 WmL^{-1})	20 min	480 nm
IRMM-387	(2.6 $mgmL^{-1}$)	(1 $mgmL^{-1}$)	SMPH (2 $mgmL^{-1}$)	6 mL	7.6 W (1.26 WmL^{-1})	5 min	70 nm

* Chapter 13

** Particle diameter size corresponding to the peak maximum in the weight based particle size distribution.

20.5.4 Instrument Operation

General operation of the instrument is detailed in manufacturer's instruction manual.

20.5.4.1 Preparation

For maximum stability of the optical system it is recommended that the instrument is powered-up at least 1 hour before attempting to make any measurement. After having injected the density gradient solutions, an equilibration period of at least 30 minutes must be applied. Furthermore, as the interior of the instrument, including the disc and its density gradient, heats up due to the friction between the rotating disc and the air. the instrument should be allowed to operate for sufficient time as to allow a stable temperature to be reached in the enclosure of the disc. This temperature should be verified and any temperature sensitive parameters used by the instrument software adapted to the observed temperature.

20.5.4.2 Choice of the rotational speed

The rotational speed shall be chosen so that it is possible to detect the smallest expected particles, and respect maximum and minimum times as noted above in section 20.4. This may be done using either the instrument software or from first principle using Stokes' law to estimate the sedimentation time. For measurements undertaken using disc-centrifuges by CPS instruments (e.g. CPS UHR24000, DC20000 or DC18000 disc centrifuges) rotational speeds of 8000 rpm and 16000 rpm are recommended for IRMM-381 and IRMM-387 respectively. For alternative instruments the operator should choose speeds appropriate to the particle size ranges noted in Table 7.

20.5.4.3 Instrument input variables and limitations

The correct operation of the instrument and subsequent data treatment requires the preparation of measurement procedures containing a series of parameters relevant to the sample materials and the measurement condition. For the two materials, IRMM-381 and IRMM-387, the input variables used for the two materials and their respective procedures are listed in Table 7 together with the expected approximate upper and lower size limits.

Table 7: Instrument procedure parameters and range limits

Parameter	**IRMM-381 $BaSO_4$**	**IRMM-387 $BaSO_4$**
Preferred rotational speed	8000 rpm	16000 rpm
Particle density [g/cm^3]	4.4	4.4
Particle refractive index	1.697	1.697
Particle absorption value	0	0
Non-sphericity factor	1	1
Sedimentation gradient	Aqueous sucrose gradient (a) 8-24 wt% or (b) 0-8 wt%	Aqueous sucrose gradient a) 8-24 wt% or (b) 0-8 wt%
Mean density of gradient	(a) 1.045 gmL^{-1} or (b) 1.007 gmL^{-1}	1.045 gmL^{-1} or 1.007 gmL^{-1}
Refractive index at optical detector	(a) 1.357 or (b) 1.344	(a) 1.357 or (b) 1.344
Viscosity of gradient	(a) 1.2 cps or (b) 1.0 cps	(a) 1.2 cps or (b) 1.0 cps
Estimated measurement range limits		
Upper size limit (t = 0.5 s)	2 µm	0.8 µm
Lower measurement limit (t = 30 min)	30 nm	15 nm
Minimum diameter (t = 60 min)	20 nm	10 nm

20.5.4.4 Calibration

A particle size standard is used to perform a calibration run before each unknown sample is analysed. This calibration step is an integral part of the material specific measurement procedure which must have been pre-defined in the instrument software before starting any measurement. When the measurement procedure is initiated the instrument software obliges the operator, as a first step, to perform a calibration run using a suitable particle size standard. The data obtained from the calibration run is then automatically used by the instrument software to elaborate the particle size distribution of the unknown sample which is measured in the second step of the pre-defined material specific procedure.

When using aqueous liquid gradients, a PVC standard of known particle size and particle density can be used for calibration. The particles of the calibration standard should be spherical and have a narrow monomodal size distribution. The instrument procedure requires that that the following parameters (typically from the calibration standard certificate) be included in the material specific measurement procedure.

a) Peak diameter

b) Half height peak width

c) Particle density

20.5.4.5 Preparation of the aqueous sucrose gradient (8-24 % or 0-8 %)

The disc-AC operates with fluids that can be used to form a density gradient inside the disc. These fluids can be dilute solutions of sucrose in high purity water (resistivity 18.2 $M\Omega cm^{-1}$ at 25 °C, 0.2 µm in-line filtration) possibly with a very low concentration of surfactant (SHMP). A density gradient is built-up in the rotating disc by sequentially injecting a series of sucrose solutions with different, decreasing sugar concentrations as described below. Finally, a small volume of a water immiscible, low density, oil (e.g. dodecane) is injected into the disc to produce a thin film on top of the aqueous layer which acts as barrier to evaporation of the water. The density gradient created in this fashion serves to stabilise sedimentation of particles improving accuracy and reproducibility of the results. To prepare a gradient the following procedure can be used.

- prepare two solutions of sucrose with the maximum and minimum concentration to be used in the gradient (typically 8 wt% to 24 wt% or 0 wt% to 8wt%).
- prepare a rack of 9 empty centrifuge vials of volume 2 mL
- add suitable volumes of the two sucrose solutions to the vials so as to produce in each a volume of (typically) 1.6 mL and concentration which are evenly distributed between the maximum and minimum e.g. 8 wt%, 10 wt%, 12 wt%, ...20 wt%, 22 wt%, 24 wt%.
- insert the disc closure cap correctly and close the security door of the disc centrifuge
- inject the 1.6 mL of the highest density solution into the disc
- Set the desired rotational speed and start the instrument. Wait till the instrument reaches constant speed. (If the disc is accelerating while injecting the sucrose solutions, the gradient will be disrupted.)
- Inject 1.6 mL of each different sucrose solution in order of decreasing concentration. i.e. 22 wt%, 20 wt% ... 8 wt% etc.
- inject 0.5 mL of dodecane in the disc as a cap fluid to reduce water evaporation
- leave the gradient to stabilise for 30 minutes before injecting samples

20.5.4.6 Performance Qualification (PQ) Test

It is recommended that each time a new sucrose gradient is prepared at least one measurement of a known material be undertaken to check that the sedimentation gradient has been correctly formed and is stable. Comparison of the size distribution with previous performance evaluation measurements should be made to verify the correct operation of the system. The choice of test materials for use in the PQ is at the discretion of the test laboratory provided it is compatible with the test material ($BaSO_4$) and stabiliser (SMPH) being used in the trial.

20.5.4.7 Operation of CPS Disc centrifuge

- Select a predefined operating procedure for the material and gradient to be used.
- Select 'Operate Analyser', and follow the on-screen instructions as follows
- Introduce sample ID, then click on Start
- Wait till instrument completes measurement of background and requests the injection of the calibration standard
- Inject an appropriate, known, volume of the of the standard (100-200 µL) with a 1 mL syringe in the disc and simultaneously press the space bar to start the data acquisition for the calibration standard.

- After completion of the calibration run, inject an appropriate, known, volume (100-200 µL) of sample with a 1 mL syringe in the disc and simultaneously press the space bar to start the data acquisition for the sample. The measurement must be left to run until either the procedure is completed or the measurement is terminated manually by the operator
- Once the measurement is finished, click on Next Sample before starting a new measurement.
- Before starting a new sample it is important to ensure that sufficient time passes that all the particles from the previous sample have passed through the field of the optical detector.
- If no new measurement has to be carried out, stop the disc by clicking on STOP on the main menu and wait until the safety interlock opens confirming the disc has stopped rotating and the disc may be accessed for cleaning.
- The gradient fluid in the disc may then be carefully removed by suction through a thin plastic tube using either a syringe or a suitable liquid pump.
- After removal of the disc closure cap, the liquid chamber should then be thoroughly rinsed using clean water (with trace or surfactant) while rotating the disc by hand. The rinsed water should be removed by suction and a piece of soft non-abrasive paper tissue (optical wipe) should then be inserted into the chamber until contacting the outside edge and slowly rotated to remove any residual liquid in the chamber. The above step of rinsing and wiping should be repeated at least once with pure water and finally done using ethanol or ethanol/water mix before final drying with a soft tissue.
- The exterior front and back faces of the disk should be carefully wiped using a moistened soft tissue or cloth to ensure that the surfaces is clean of dust or other residues such as sucrose particularly in the region where the detector light beam passes.

20.5.4.8 Reporting

The data shall be presented in graphical and tabular form. The report should contain at least the following data

- identification of the sample
- the date of test,
- identification of the operator and the testing institute and a unique report identification
- Information on sample preparation, especially the suspending liquid, its temperature, density, viscosity and volume, and the dispersing agent and its concentration, the method of dispersion including, where used, the sonication time and specifications of the sonication device.
- Information on measurement instrument and operational settings, especially the gradient used and the rotational speed.
- Information on the calibration and PQ materials used
- Information on parameters for data analysis, especially the effective density and the complex refractive index
- Information on any instrument defined software operations for data smoothing or compensation of baseline shift.
- Measurement data files: Where the instrument software permits, the participant laboratories are requested to make available data files (in electronic format) containing the measured particle size distributions according to one of the following 2 options

  Data Export Option 1: Export the data of each sample analysis data files as ASCII text file containing the following fields (Sedimentation Time, Optical Signal, Diameter, Weight, Number). If any field is not available this should be stated in the report.

Date Export Option 2: For users of CPS disc-centrifuge instruments a copy of the complete sub-directory of the measurement procedures used should be made available in the original electronic format or alternatively as a single zipped file.

20.5.4.9 Evaluation of results

The instrument software will calculate the majority of the results. In cases where the instrument software is unable to provide the data in an appropriate tabular or graphical form, ASCII data files will be exported and elaborated in suitable excel files. The calculation of each cumulative mass and number base size distribution should be done over the specific size ranges for the different materials under examination, see Table 8.

Table 8: Size ranges to be used in calculating cumulative size distribution

Limit	IRMM-381	IRMM-387
Minimum size	70 nm	30 nm
Maximum size	2000 nm	400 nm

PSD reporting

1. Plot $Q_3(x)$ (norm) (unitless, normalized to 1) and q_3 in one graph over a log diameter axis in nm.
2. Plot $Q_0(x)$ (norm) (unitless, normalized to 1) and q_0 in one graph over a log diameter axis in nm.
3. Plot instrument optical signal vs particle size
4. Report $X_{50,0,}$ in nm. Report $X_{50,3}$ in nm.
5. Report $X_{90,0}$ in nm. Report $X_{90,3}$ in nm.
6. Report $X_{10,0}$ in nm. Report $X_{10,3}$ in nm.
7. Report particle size at peak maximum in the weight, number and optical density distributions.
8. Report X_{max}, X_{min}

Measurement data files

Where the instrument software permits, the participant laboratories are requested to provide data files containing the measured particle size distributions according to one of the following 2 options:

Data Export Option 1: Export the data of each sample analysis data files as ASCII text file containing the following fields (Sedimentation Time, Optical Signal, Diameter, Weight, Number). If any of these fields is not available this should be stated in the report.

Date Export Option 2: For users of CPS disc-centrifuge instruments the complete sub-directory of each measurement procedure should be made available in the original format or alternatively as a single zipped file.

20.6 Validation Status

This method has been subjected to an intra-laboratory validation within the NanoDefine project.

20.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards and exposure limits relevant to all chemical substance used in the procedure described here.

Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can produce damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

Residues and waste materials must be disposed of according to local environmental and safety regulations.

21 Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software

21.1 Aim

This SOP describes an off-line method for automated image analysis of particulate materials imaged by electron microscopy, which has been developed in the NanoDefine project, referred to as the 'NanoDefine ParticleSizer'.

21.2 Scope

The off-line method for automated image analysis allows determining automatically the distributions of the characteristic size and shape properties (section 21.7) of constituent particles in aggregates and agglomerates, or present as single particles, from EM images. This application is specifically designed in the scope of implementing the EC-definition of a nanomaterial. The median value of the number-based distribution of the minimal external dimension of the constituent particles is assessed.

21.3 Application domain

This SOP describes an off-line method for automated image analysis of particulate materials, and requires that the material has been representatively brought on an EM grid e.g. by following the SOP 'SOP/NANoREG/D2.10/TEMSpePrep' entitled 'Preparation of EM-grids containing a representative sample of a dispersed NM'[f]. In addition, the SOP requires that representative images of the material have been recorded by an electron microscopy based imaging technique such as TEM, SEM or STEM, e.g. following the SOP 'SOP/NANoREG/D2.10/TEMIma' entitled 'Transmission electron microscopic imaging of nanomaterials'[g]. The ParticleSizer can also be applied on representative EM micrographs obtained in an alternative way.

The SOP can be used to characterise particulate materials (single particles and aggregated/agglomerated particles) and measure the size and shape properties of the electron microscopic projections of the constituent particle of the material.

In the context of implementing the EC definition of a nanomaterial[3], it produces a number based distribution of the minimal external dimension of the constituent particles, assessed as the minimal Feret diameter or short axis length of fitted ellipse. The median value of these parameters allows classifying a material as a nanomaterial according to the EC NM definition. The other measured size and shape parameters (Section 21.7) allow a detailed characterisation of the materials required for e.g. risk analysis, batch and process control.

The SOP is tested on a series of certified reference materials (CRMs), such as ERM-FD304[h] and ERM-FD100[i] and representative test materials (RTMs), such as NM-100, NM-103

[f] http://www.nanoreg.eu/images/D2.10_Protocol_for_size-distribution_analysis_of_primary_NM_particles_in_air_powders_and_liquids_-approvedpublic_for_website_final09.11.pdf

[g] https://www.rivm.nl/sites/default/files/2018-11/NANoREG%20D2.10%20SOP%2002%20Transmission%20electron%20microscopic%20imaging%20of%20nanomaterials.pdf

[h] https://crm.jrc.ec.europa.eu/p/40456/40487/By-analyte-group/Particle-pore-size/ERM-FD304-COLLOIDAL-SILICA-40-nm-nominal/ERM-FD304

[i] https://crm.jrc.ec.europa.eu/p/40456/40487/By-analyte-group/Particle-pore-size/ERM-FD100-COLLOIDAL-SILICA-20-nm-nominal/ERM-FD100

and NM-212[j], and other materials such as Au Nanoparticles and Nanorods.

21.4 Principle of the method

The SOP finds suitable configurations of the ParticleSizer for different types of materials (Table 1).

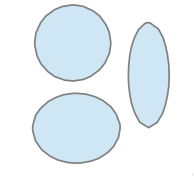
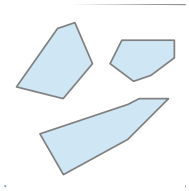
In a general procedure, the SOP helps to find suitable configurations of the ParticleSizer for different types of materials based on the amount of overlap between constituent particles (type of overlap) and the shape of the constituent particles (type of particle) (Table 1). Once a suitable configuration is selected, the images are automatically analysed.

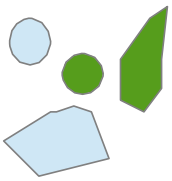
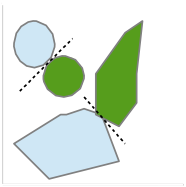
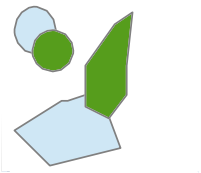
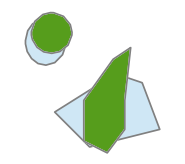
During an automatic analysis, the software automatically detects and analyses particles on an image. The grey value is the criterion for the recognition of a particle. Therefore, in order to have successful particle detection, the particles must clearly stand out from the background. All detected particles can be automatically measured. A wide array of measured parameters can be chosen (Section 21.7).

An overview of the processing pipeline implemented in the ParticleSizer is presented in Section 21.9.

[j] https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository

Table 1: ParticleSizer classification of materials based on A) the shape of the constituent particles (Type of particle) and B) amount of overlap between constituent particles (Type of overlap)

A) Type of particle	Name	Description
	Ellipsoidal	The outline of the particles can roughly be approximated by an ellipse in 2D images.
	Irregular	The outline of the particles is irregular and cannot be approximated by an ellipse in 2D images.

B) Type of overlap	Name	Description
	None	No overlap of the particles.
	Touching	These particles touch and do not overlap.
	Slightly overlapping	The particles show a low degree overlap.
	High or complete overlapping	These particles have a high degree of overlap.

21.5 Definitions, abbreviations and norms

CRM	Certified Reference Material
EM	Electron microscopy
NP	Nanoparticle
NRBS	noise reduced and background subtracted
OTB	Object-to-Background
RTM	Representative test material
SOP	Standard Operating Procedure
TEM	Transmission electron microscopy

21.6 System requirements and installation

The ParticleSizer couples image analysis by Fiji[8] with data analysis by R Development Core Team[7]. Online manuals can be found on the following websites: http://fiji.sc/ for Fiji and https://www.r-project.org/ for R.

To install the ParticleSizer software, the following procedure has to be followed:

1. Download the latest fiji http://fiji.sc/#download
2. Activate the Biomedgroup update site[k]
3. Add a new update site[l] with the name 'psizer' and url http://sites.imagej.net/Ndef-psizer/. By adding the psizer update site, the ParticleSizer software is updated automatically when Fiji is up- dated.
4. Do the update. Now the particle sizer should be installed.
5. (Optional): It is recommended to install R to get better plots. When this point is skipped, a stripped-down plot will be shown.
    - 5.1 Download the latest R: https://cran.uni-muenster.de/
    - 5.2 Download the Rserver package: https://rforge.net/bin/windows/contrib/3.0/Rserve_1.8-0.zip
    - 5.3 Download the MASS package:

      https://cran.r-project.org/bin/windows/contrib/3.2/MASS_7.3-45.zip
    - 5.4 Start R and select the packages downloaded in 5.2 and 5.3 via '*Start Packages -> Install packages from local zip files*'

21.7 Procedure to analyse sample images

21.7.1 Start Fiji

To start Fiji, simply open your Fiji folder and click on Image-J.exe

To open the ParticleSizer, the following procedure has to be followed:

1. Open Fiji

[k] A manual how to follow a update site could be find here: http://fiji.sc/How_to_follow_a_3rd_party_update_site

[l] A manual how to add a new update site could be find here: http://fiji.sc/How_to_follow_a_3rd_party_update_site#Add_update_sites

2. Go to plugins
3. Select nanodefine
4. Select particlesizer

21.7.2 Determine a basic suitable configuration

The software allows the user to set up a configuration suitable for a specific 'type of particle' and 'type of overlap' combination. The default configuration can be observed by activating the Settings Manager: Plugins → NanoDefine → SettingsManager and is illustrated by Figure 1. A default configuration is readily provided and can be applied on simple models with 'none' or 'touching' particles (Figure 2).

The default configuration can be split into 5 categories: mode selection, segmentation, Ellipse shape constraints, shape constraints and miscellaneous (Misc). For each option a default value is defined. The user has the option to optimize the settings for the images that will be analysed. A detailed description of each of these settings is given in Annex (Section 8).

The default values are as follows, see Figure 1A and 1B.

In a typical image analysis, the default settings in the Settings Manager have to be optimized for a certain 'type of particle' and 'type of overlap' combination.

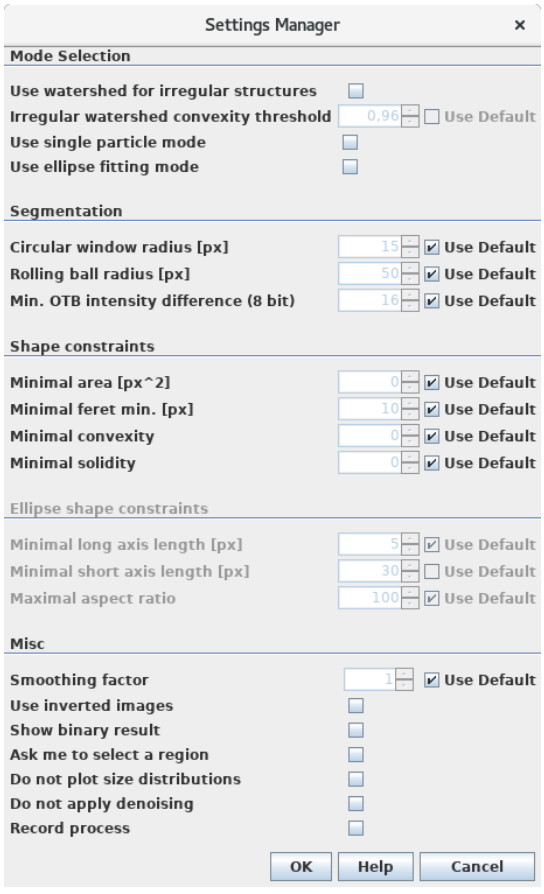


Figure 1A: Settings Manager of the ParticleSizer with default configuration

Mode selection:
Use watershed for irregular structures: off
Irregular watershed convexity threshold: 0.7
Use single particle mode: off
Use ellipse fitting mode: off

Segmentation:
Circular window radius: 1.5 % of the image width
Rolling ball radius: 15 % of the image width
Min. OTB intensity difference: 16

Shape constraints:
Minimal area: 0 px
Minimal Feret min: 10 px
Minimal convexity: 0
Minimal solidity: 0

Ellipse shape constraints:
Minimal long axis length: 5 px
Minimal short axis Length: 5 px
Maximal Aspect ratio: 100

Misc:
Smoothing factor: 1
Show binary result: off
Ask me to select a region: off

Figure 1B: Default settings of the ParticleSizer

Figure 2 shows the general procedure to determine a suitable configuration. The first step is to determine the type of overlap based on visual inspection of the particles. A distinction is made between 'none', 'touching', 'slightly overlapping' or 'high or complete overlapping' particles. The second step is to determine the type of particle based on visual inspection of the particles. A distinction is made between 'ellipsoidal' or 'irregular' particles. Besides the image quality the combination of overlapping type and particle type determines the configuration.

Depending on the selected 'type of particle' and 'type of overlap' combination, a suitable algorithm is selected for image analysis. These can be 'Default', 'Irregular Watershed', 'Ellipse fitting' or 'Single particle mode'.

For 'Irregular watershed', 'Ellipse fitting', 'Single particle mode', the boxes 'Use watershed for irregular structures', 'Use ellipse fitting mode' or 'Use single particle mode' have to be checked, respectively. The method can be readily applied on 'none', 'touching' or 'slightly overlapping' particles. For difficult materials, different modes can be combined. For particles with a high degree of overlap, single particle mode can be used on the condition that the single particles have the same physical properties as the constituent particles in aggregates/agglomerates. If this is not the case, the large aggregates/agglomerates have to be separated by optimizing the sample preparation.

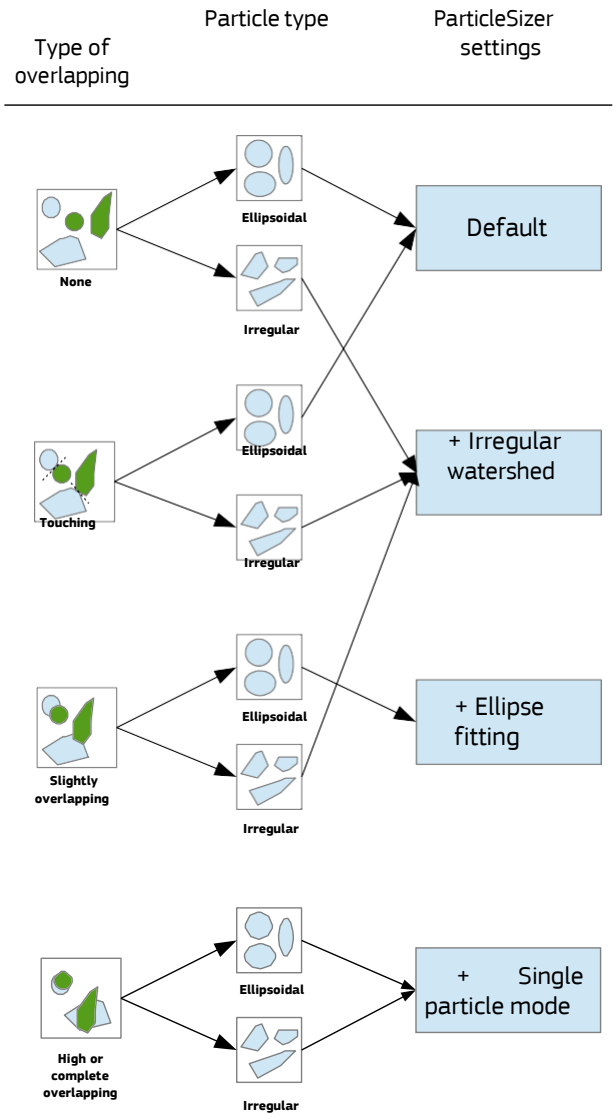


Figure 2: Procedure to determine suitable mode

The following general rules should be ensured: If the image contains a scale bar, this could interfere the analysis process. Therefore the largest possible region which does not contain the scale bar has to be selected. To do this, please check the option 'Ask me to select a region'.

The software expects bright particles on a dark background. If this is not the case, please check the option 'Use inverted images'.

21.7.3 Image analysis

To apply the ParticleSizer to your sample data, select 'Plugins → NanoDefine → ParticleSizer'.

A single image can be opened by 'File → Open'. If a whole image series (stack) should be analysed it can be opened by '*File → Import → Image Sequence*'. The option '*Convert to 8-Bit Grayscale*' should be checked. If the whole sequence does not fit in the memory, check 'Use virtual stack' in the sequence options.

21.7.4 Optional: Optimize the analysis

Certain artefacts might occur during the image analysis, which can have an influence on the results. The following strategies give advice how to proceed in those cases.

21.7.4.1 Some low contrast particles are not detected

Open the configure dialog by 'Plugins → NanoDefine → Settings Manager', decrease the 'Min. OTB intensity difference' by 2, and apply the ParticleSizer again. Repeat this until all particles are detected. By lowering the OTB difference it could happen that some small parts of the background are detected as particles. Experience shows that these objects are rather small and could be filtered out by increasing 'Minimal area' or 'Minimal Feret min'.

21.7.4.2 Background is detected as particles

IF the artefacts are as large as the smallest particles, then increase the 'Min. OTB intensity difference' by 2, press OK and apply the ParticleSizer again. Repeat this until you get reasonable results.

IF the artefacts are smaller than the smallest particles, then increase the 'Minimal Feret min' diameter until they are removed. The minimal Feret diameter can be estimated by measuring the smallest dimension of the largest artefact with the line tool.  If the images are scaled, than press 'ALT' while using the line tool to get it in pixels.

21.7.4.3 Use 'record process' to analyse the segmentation processing

If you check the option 'record process' every single interim image (segmentation step) is recorded. In the top left of the corner is written which step was recorded. This option can be used to determine which settings have to be optimized. Please note that the 'record process' option only works for a single image, not for a complete stack.

21.8 Output of results

There are several geometrical features which are calculated and saved into a results table by the Particle - Sizer software. The table could be exported by the user. The geometrical features are defined as follows:

- **Area (A):** The area enclosed by the outer contour of the particle.

- **Area convex hull (C):** The area enclose by the convex hull of the outer contour of the particle.
- **Perimeter (P):** The perimeter of the outer contour of the particle.
- **Perimeter convex hull (H):** The perimeter of the convex hull of the particle.
- **Maximum Feret diameter:** The maximum distance between the two parallel tangents touching the particle outline in all directions.
- **Minimum Feret diameter:** the minimum distance between the two parallel tangents touching the particle outline in all directions.
- **Long side minimum bounding rectangle (L):** The larger side of the minimum bounding rectangle.
- **Short side minimum bounding rectangle (S):** The smaller side of the minimum bounding rectangle.
- **Aspect ratio:** Defined as L/S
- **Area / Perimeter ratio:** Defined as A/P
- **Circularity:** Defined as P^2/A
- **Elongation:** Defined as $1-S/L$
- **Convexity:** Defined as H/P
- **Solidity:** Defined as A/C
- **Number of holes:** The number of holes inside a particle.
- **Thinness ratio:** Inversely proportional to the circularity and normed. It is defined as $4\pi A/P^2$
- **Contour temperature:** It has a strong relationship to the fractal dimension, defined as

  $(\log(\frac{2P}{P_H}))^2$
- **Fractal dimension:** Estimated fractal dimension by the box count algorithm. The default box-sizes are '2,3,4,6,8,12,16,32,64'.
- **Maximum inscribed circle diameter:** Computes the largest inner circle of a particle.

The default plot is the minimum Feret diameter (Figure 3).

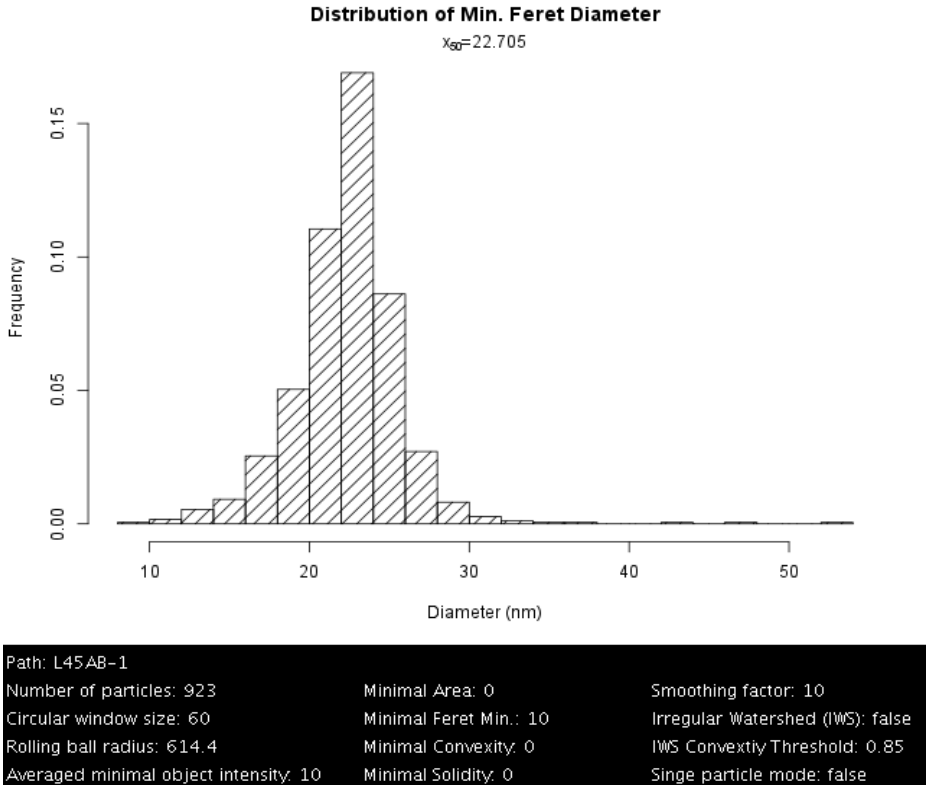


Figure 3: Size distribution of the minimal Feret diameter for a polystyrene sample

However, if the ellipse fitting mode is used, the short axis length is reported which is equivalent to the minimum Feret diameter. Directly below the plot, all used settings are documented for easily reproduction. Below the title the median value is outputted. By right clicking on the histogram and selecting 'Modify Plot', the X-axis and Y-axis labels and the number of bins can be altered. In addition, a function selected in the Fit Distribution field can be fitted through the histogram. Other geometric features could be plotted by selecting in Results table 'Results → Distribution'. Finally the plot could be exported by 'File → Save as'. Furthermore the segmented particles are visualized by an overlay (red lines) on top on the input image (Figure 4)

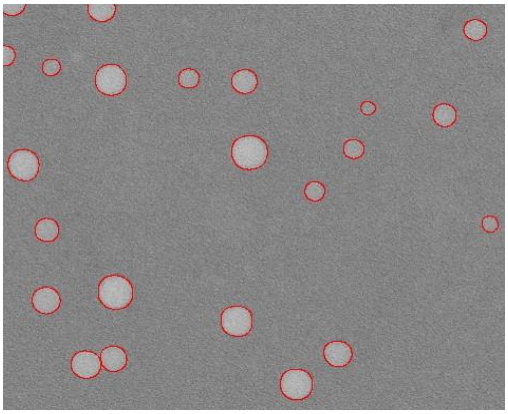


Figure 4: Segmented particles shown as overlay (red circles)

21.9 Description of adjustable settings in the Settings Manager

Mode selection:

- Use watershed for irregular structures: If selected, the mode for irregular structures is used. See section 0 for details.
- Irregular watershed convexity threshold: A threshold, which determines the amount of splitting up agglomerates. If the convexity of a particle is larger than this threshold, the splitting is stopped for this object. If the convexity is smaller than this threshold, the Software tries to split the particle again.
- Use single particle mode: If selected, the single particle mode is used. See section 21.10.5 for details.
- Use ellipse fitting mode: If selected, the ellipse fitting mode is used. See section 21.10.4 for details.

Segmentation:

- Circular window radius: This is a parameter of the local thresholding technique. The ParticleSizer does not use a global threshold to binarize the image. Instead it uses a local threshold which is estimated for a specific circular region with the configured radius.
- Rolling ball radius: The background is removed by rolling a ball with this radius over the surface (intensity interpreted as height) of the image. It should be at least as large as the largest object in image which does not belong to the background.
- Min. OTB intensity difference: Objects which have an object-to-background (OTB) intensity difference in the noise-reduced and background subtracted image (see section 9.2) lower than this threshold are considered as artefacts and are removed.

Shape constraints:

- Minimal area: Minimum area in pixels. Particles smaller than this threshold are removed.
- Minimal Feret min: Minimal Feret diameter in pixels. Particles smaller than this threshold are removed.
- Minimal convexity: The convexity is defined as the ratio of the perimeter of the convex hull of the particle and the perimeter of the particle. It lies between 0 and 1. The convexity increases with larger values. Particles smaller than this threshold are removed.
- Minimal solidity: Defined as the ratio of the particle area and the area of the convex hull of the particle. It lies between 0 and 1. The solidity increases with larger values. Particles smaller than this threshold are removed.

Ellipse shape constraints:

- Minimal long axis length: The length in pixels of the major direction of the fitted ellipse. Ellipses smaller than this threshold are removed.
- Minimal short axis length: The length in pixels of the minor direction of the fitted ellipse. Ellipses smaller than this threshold are removed.
- Maximal aspect ratio: Ratio of the length of major and minor axis. Ellipses with an aspect ratio larger than this value are removed.

Misc:

- Smoothing factor: It sometimes occurs that the estimated standard deviation of the noise is lower than the true value. The smoothing factor is a multiplicative factor for the estimated standard deviation.
- Use inverted images: The ParticleSizer expects bright objects on a darker background. If images show the opposite, then this option should be checked.
- Show binary result: If selected the ParticleSizer shows the binary result.
- Ask me to select a region: If selected, the software allows you to select a specific region to analyse.

21.10 The ParticleSizer pipeline

Sections 21.10.1 and 21.10.2 give an overview of the processing pipeline implemented in the ParticleSizer. The methods 'watershed for irregular structures', 'single particle mode' and 'ellipse fitting' in the category segmentation are most important for this SOP and will be described more detailed in the sections below.

21.10.1 Segmentation pipeline

The flow scheme of the segmentation pipeline is given in Figure 5. The standard deviation σ of the noise of recorded EM images is estimated using Immerkaer's method[5] and used to adapt an efficient, parallelized noise filter called 'non local means' [1, 2]. To identify particles on EM images, the background is removed using the rolling ball algorithm (parameterization de- pends on the image size) implemented in ImageJ. If the noise standard deviation is higher than the threshold T_1 a small median filter is applied to homogenize the particles. The result is the noise reduced and back-ground subtracted (NRBS) image. The NRBS image is then binarized by a local adaptive threshold technique[6] and saved as 'Pre-Watershed image'. If single particle mode (SPM) is selected, all particles with a convexity smaller than T_2 are removed. When the SPM is deactivated the agglomerates are split into constituent particles by a user selected technique which provides an initial identification of particles (segmentation). In post-processing steps possible artefacts introduced by the segmentation procedure are removed before geometrical features and size distributions are extracted. The post-processing steps are described in the next section.

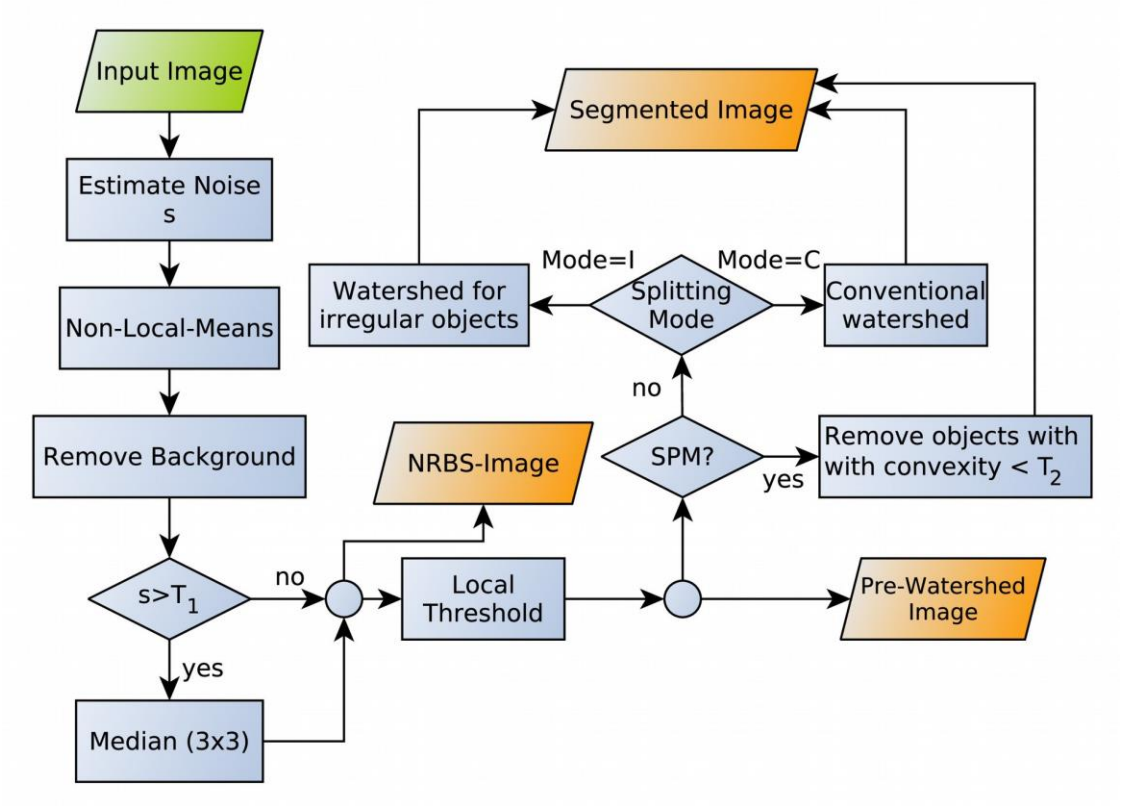


Figure 5 Pre-processing pipeline

21.10.2 Post-Processing pipeline

The flow scheme of the post-processing pipeline is given in Figure 6.

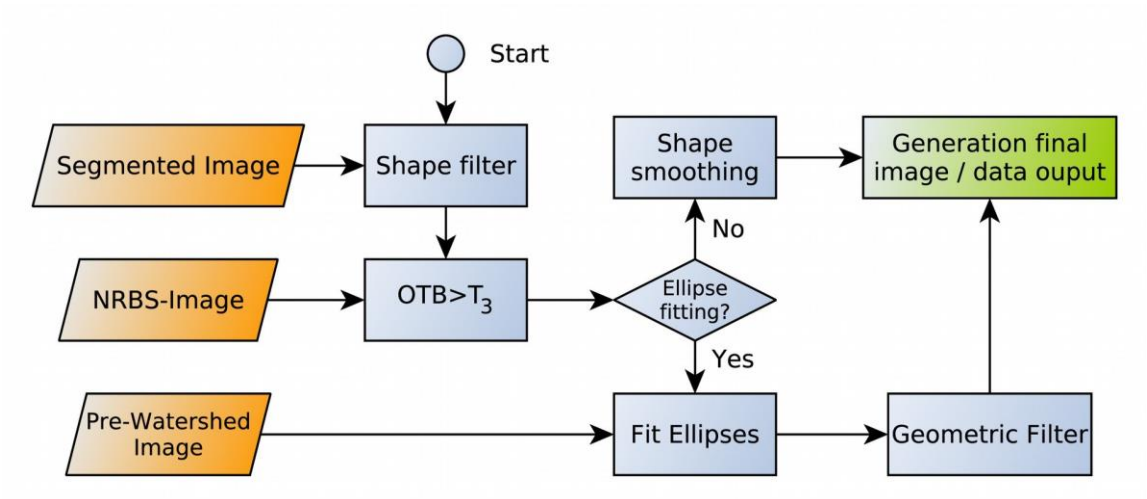


Figure 6: Post-processing pipeline

Starting from the segmented image resulting from the segmentation pipeline, all objects which are outside of user-defined limits of the geometrical features are removed. Default values are provided but the software also allows for an individual adaption of the limits to account for non-standard images. In a second step objects which have an object-to-background (OTB) intensity

difference in the NRBS Images lower than T_3 (as configured in the settings manager) are considered as artefacts and are removed. When ellipse fitting is activated the ellipses are fitted to the particle boundaries to expose potential overlapping. However, the segmentation process often results in objects showing rough boundaries, which is corrected by applying a shape smoothing algorithm using Fourier descriptors when ellipse fitting is deactivated. Geometrical features extracted from the remaining objects are listed in a results table, and a particle size distribution based on the minimal Feret diameter (including also X50 values) is displayed in a graphical format. The complete results can also be exported for more detailed processing, if required.

21.10.3 Irregular watershed

The irregular watershed technique combines a conventional watershed splitting with a morphological erosion. The procedure starts with the binary image I. The image L with watershed lines is calculated the following way:

$$L = I \cap W(I)$$

where W() is a conventional watershed splitting based on the euclidean distance map and $\cap$ the logical AND operation.

In a next step connected components of image I are eroded. The erosion of a connected component is stopped when a convexity larger than a user defined threshold (0.7 is used by default) is reached or when the component is fully eroded. This results in image E which contains those connected components which fulfil the convexity condition. In the final step watershed lines in L which are crossing objects in E are rejected. This method successfully splits overlapping irregular objects but prevents over-segmentation.

21.10.4 Ellipse fitting

The combined approach couples a conventional watershed splitting based on the euclidean distance map with the direct ellipse fitting method. Figure 7 illustrates the principle of the method: The objects in the input image (Figure 7a) are split by the watershed technique (Figure 7b).

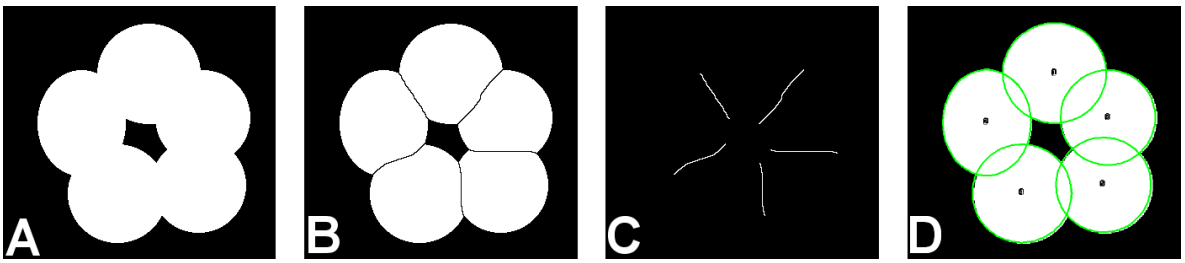


Figure 7: Ellipse fitting method (A) Input image of 5 overlapping circles, (B) splitted objects by conventional watershed technique, (C) the extracted watershed lines and (D) the ellipses fitted to the contours of the objects in image B rejecting all contour points which have a watershed line (image C) in the direct neighbourhood

By combining Figure 7a and Figure 7b by a logical XOR operation the watershed lines are extracted (Figure 7c). Contour lines of the objects in image B are then extracted and all

contour points which have a watershed line in the direct neighbourhood are rejected. Finally an ellipse is fitted[4] to the remaining contour data of the objects (Figure 7d).

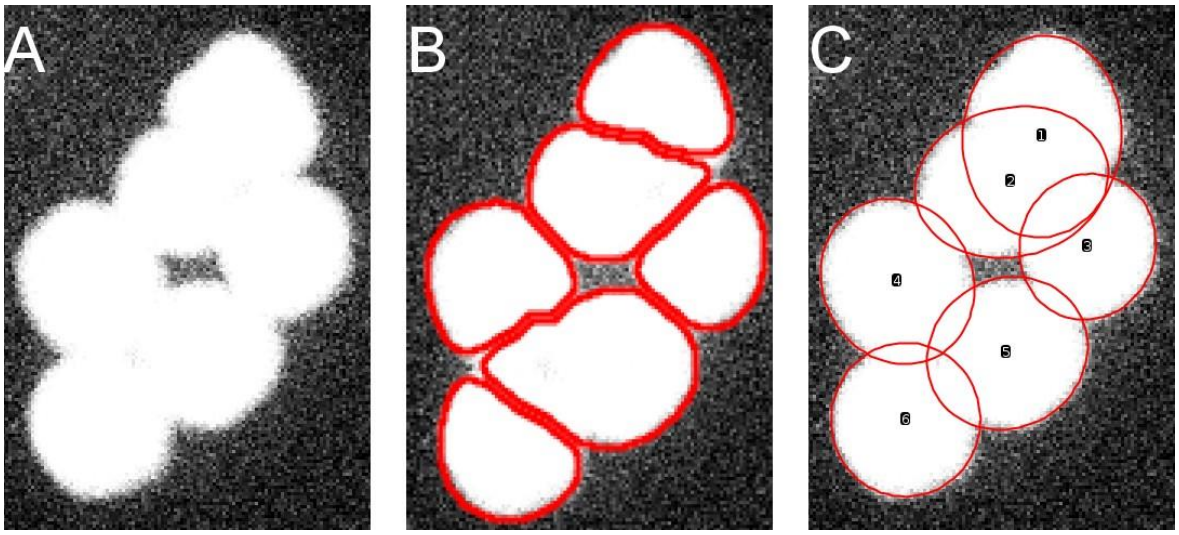


Figure 8: Au nanoparticle agglomerate analysed by the ParticleSize software in (B) default mode and (C) ellipse fitting mode

Figure 8 shows a comparison of the conventional watershed method with the ellipse fitting approach using an Au nanoparticle agglomerate. The results demonstrate that the ellipse fitting provides a better estimate of the effective size of ellipsoidal overlapping particles compared to the conventional watershed approach.

21.10.5 Single particle mode

In cases, where agglomerates cannot be well dispersed, the proper segmentation of the agglomerates may not be possible. For such cases, 'single particle mode' (SPM) is implemented in the ParticleSizer software. In the SPM, agglomerates are rejected and only constituent particles - defined as particles with a high convexity

- are included in the analyses. In the context of the ParticleSizer software the term 'high convexity' is defined as follows:

A particle has a high convexity when the ratio of the perimeter of the convex hull and the perimeter of the outer contour is larger than 0.7.

21.11 References

[1] Buades, A., Coll, B. & Morel, J.-M., 2005. A Non-Local Algorithm for Image Denoising. In *2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR'05)*. IEEE, pp. 60–65.

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[4] Fitzgibbon, A., Pilu, M. & Fisher, R.B., 1999. Direct least square fitting of ellipses. *IEEE Transactions on Pat- tern Analysis and Machine Intelligence*, 21(5), pp.476–480.

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Generic SOPs for nanomaterials in products

22 SOP for analysis of TiO_2 particles from sunscreen by AF4-MALS-ICP-MS

22.1 Aim

The purpose of this SOP is to provide the protocols for the sample preparation of sunscreen material containing TiO_2 particles, and instructions for the quantitative determination of characteristic parameters of particle size distributions of TiO_2 particles using AF4-MALS-ICP-MS.

22.2 Scope

This protocol describes the sample preparation procedure for TiO_2 particles present in sunscreen and particle analysis by means of AF4-MALS-ICP-MS. The method allows determining $r_{h,mode}$ (MALS), $r_{h,mode}$ (ICP-MS), and mass-based PSD (d_{10}, d_{50}, d_{90}).

The described procedure is applicable to sunscreen samples studied in the NanoDefine project, as well as TiO_2 particles present in sunscreen samples containing other UV blocker (e.g. ZnO). All steps required for the analysis are summarized in Figure 1.

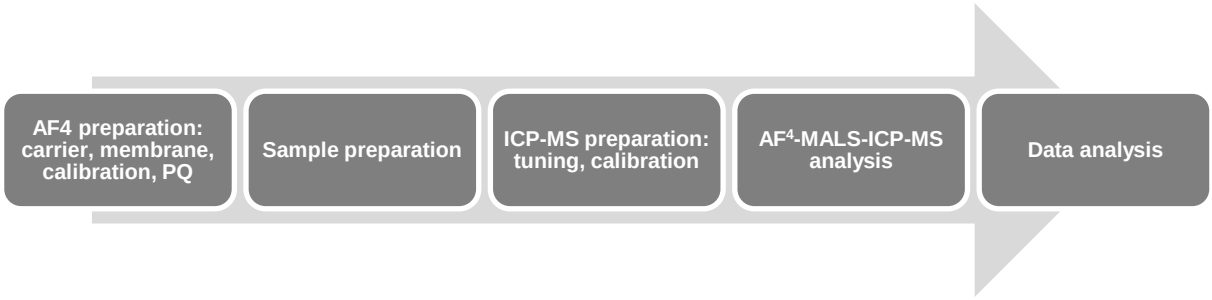

Figure 1: Overview of the different steps included in the measurement procedure for extraction of TiO2 particles from sunscreen and AF4-MALS-ICP-MS analysis.

Definitions

AF4	Asymmetric flow field-flow fractionation
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
MALS	Multi-angle light scattering
NaDS	Sodium dodecyl sulphate
NP	Nanoparticles
PQ	Performance qualification

22.3 Description

22.3.1 Materials and methods

22.3.1.1 Essential equipment

- Analytical balance (0.1 mg precision)
- Laboratory scale bath sonicator
- Shaker

- Vortex
- pH meter
- ICP-OES system
- AF4 system coupled to MALS detector
- ICP-MS system

22.3.1.2 Recommended optional equipment

- Vacuum pump for filtration of aqueous solutions.

22.3.1.3 Material supplies

- Glass/plastic vial (or similar, for ~20 mL sample volume) with screw top or other appropriate stopper
- Volumetric flask (e.g. 100 mL, 250 mL)
- Disposable plastic spatula for weighing
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Anodisc 0.02 µm nominal pore size membrane filters or similar for vacuum filtration of aqueous solutions (see section 22.3.2)
- Disposable (powder-free) nitrile gloves
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including engineered nanomaterials

22.3.1.4 Chemicals

- Ultrapure water (demineralised water of 18.2 MΩcm^{-1} resistivity; purified by reverse osmosis and sanitization)
- Sunscreen samples
- Sodium dodecyl sulphate (NaDS) powder (purity ≥ 98.5 %)
- FL-70™ (in the absence of FL-70™ NovaChem 100 might be used)
- Dishwashing detergent (Denk mit Ultra by DM, in further text cleaning agent)
- NaOH solution (0.01 M, 0.1 M and 1 M) - prepared from analytical grade NaOH and ultrapure water
- NaCl (0.03 M) prepared from analytical grade NaCl and ultrapure water

22.3.2 Additional chemicals/solutions to be prepared

0.2 % (m/v) NaDS solution

Prepare 0.2 % (m/v) NaDS solution by dissolving the appropriate amount of NaDS powder into ultrapure water (e.g. add 0.2 g of NaDS in volumetric flask and fill it by ultrapure water). Shake vigorously to ensure that all powder is solubilized. Adjust pH to 8.5-9 by using previously prepared NaOH solution and filter through a 0.02 µm nominal pore size membrane.

0.1 % (m/m) NaDS solution

Dilute 0.2 % (m/m) NaDS solution (pH=8.5-9) by a factor of 2 and filter through a 0.02 µm nominal pore size membrane.

0.5 % (v/v) FL70™ (or NovaChem 100)

Prepare 0.5 % (v/v) FL70™ by adding 0.5 mL of FL70™ solution in 99.5 mL of water. Filter the solution through the 0.02 µm nominal pore size membrane.

1 % (v/v) cleaning agent

Prepare 1 % (v/v) cleaning agent by adding 1 mL of dishwashing cleaning liquid (Denk mit Ultra by DM) in 99 mL of water.

22.3.3 Performing measurements

22.3.3.1 Sample preparation

a. Weigh an empty glass/plastic vial, press tare and add approximately 10 mg of sunscreen using a spatula;

b. Reweigh the vial and calculate by difference the effective amount of sunscreen added. Then add 1 % (v/v) cleaning agent to give a concentration of 1 $mgmL^{-1}$;

c. Shake the sample for 10 min horizontally or until the sample has a homogeneous appearance;

d. Sonicate the sample for 15 min in a laboratory scale bath sonicator;

e. Slightly shake it before transferring 2 mL of the sonicated sample to an empty glass/plastic vial and add 2 mL of 0.2 % NaDS solution (pH = 8.5-9) (note: record the weight the mass of sample and surfactant added);

f. Sonicate the sample for 5 min in a laboratory scale bath sonicator;

g. Leave over night;

h. Slightly shake the sample and sonicate for 15 min in a laboratory scale bath sonicator;

i. Slightly shake the sample and dilute the sample further by taking 1 mL of sample and 4 mL of 0.1 % NaDS solution (note: record the weight of the mass of sample and surfactant added).

j. Sonicate for 2 min in a laboratory scale bath sonicator.

22.3.3.2 Recovery of dispersions after aging beyond verified period of stability

Where the operator has access to DLS instrumentation it is strongly recommended that the dispersion state is evaluated by DLS before AF4-MALS-ICP-MS analysis.

The temporal stability of the dispersions prepared in the previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 hour after completion of the final sample preparation step. Therefore, before each measurement homogenisation in a sonication bath for 2 minutes must be done.

The dispersion state of a sample aged for 1 day can be reversed to its original state by 10 min of bath sonication before measurements.

Additional, aging of 2 weeks is allowed for the sample after sample preparation step f. The aging can be fully reversed if glass vial with the sample is slightly manually shaken, treated for 15 min in a laboratory scale bath sonicator at room temperature. Final dilution step (step h-j) should be done before AF4-MALS-ICP-MS analysis.

22.3.3.3 Measurement description

The AF4 separation channel must have a length of 275 mm, and must be equipped with a 350 µm spacer and a 10 kDa regenerated cellulose membrane. For particle size characterisation (samples including quality control checks) the AF4-system has to be coupled online with light scattering

detection (e.g. MALS, DLS). Measurement conditions of AF4 analysis are presented in Table 9. A flow condition in the channel has to be set up as following: the detector flow rate to 1.0 $mLmin^{-1}$; the cross flow rate and the focus flow rate of 0.60 $mLmin^{-1}$; the injection flow of 0.1-0.2 $mLmin^{-1}$. As carrier liquid a mixture of 0.025 % (v/v) FL-70 has to be used.

The AF4 size-retention time calibration is performed using polystyrene (PS) latex beads standards (at least 4 calibrants from working range of 20 nm to 270 nm are selected for calibration purposes). Please note that the carrier liquid for the PS standards should be a mixture of 0.025 % (v/v) FL70™ (or NovaChem 100) and 0.003 M NaCl. Standards must be run under optimized run conditions every time when membrane is changed. After running the PSS, the data should be immediately analysed in order to construct the size-retention time calibration curve. The slope and the correlation coefficient (r value calculated using regression analysis) must be equal or higher than 0.990. For performance criteria monodisperse PS material (with a diameter in the range of 50 nm to 200 nm) should be run on weekly basis. If the obtained peak modes deviate strongly (e.g. deviation > 50 %) operator should change the membrane or reconsider troubleshooting (extensive flushing of the channel in focusing mode, changing tubing, adjusting focusing valve, etc.).

The r_h (mean of 3 independent replicate results for every sample) is determined from the size-retention time calibration curve. The retention times (e.g. 90° MALS signal), for every standard correspond to the mode of the largest peak present in the fractogram.

AF4-MALS-ICP-MS measurement conditions are summarized in Table 9. For Ti mass quantification during AF4 analysis an ICP-MS is coupled online to the outflow of the MALS detector. A splitter and a peristaltic pump provide the volumetric flow rate of 0.30 mL/min required by the ICP-MS system. The flow can be monitored by a flow meter. Ti mass quantification is done using the time resolved analysis mode. For calibration of the ICP-MS system solutions of 0 μgL^{-1}; 1.25 μgL^{-1}; 2.5 μgL^{-1}; 5 μgL^{-1}; 12.5 μgL^{-1} and 50 μgL^{-1} Ti are prepared using a dilution media of 0.025 % FL-70 and a Ti stock solution of 1000 mgL^{-1}. The sample uptake speed is adjusted to a flow rate of 0.3 mL/min. CSV-data files can be exported from ICP-MS software (Ti signal) into an Excel spread sheet. The correlation coefficient must be > 0.990.

Finally, all collected data sets (AF4 calibration, MALS signals, MALS fittings and ICP-MS data) can be copied into the spread sheet. The output parameters are:

- hydrodynamic size distribution based on AF4 calibration (mode),
- mass based particle size distribution (mode, d_{10}, d_{50}, d_{90}) and
- retention time.

Table 9: Measurement conditions for AF4-ICP-MS analysis

AF4 parameter#	**unit**	**value**
Tip to tip channel length	[mm]	275
Spacer thickness	[µm]	350
Focus flow rate	[mL min^{-1}]	0.60
Injection flow	[mL min^{-1}]	0.1-0.2
Focus time	[min]	2

Injection + focus time	[min]	10
Focus time	[min]	2
Elution time	[min]	50
Detector flow rate	[mL min^{-1}]	1.0
Cross flow rate	[mL min^{-1}]	0.60
Membrane		regenerated cellulose, 10 kDa
Carrier liquid #		0.025 % (v/v) FL-70™
Injection volume	[µL]	50-100 µL of sample suspension
ICP-MS parameters	**unit**	**value**
RF power	[W]	1600
Sample depth	[mm]	10
Gas flow rates		
Carrier	[L min^{-1}]	1.06
Dilution	[L min^{-1}]	0.40
Collision gas He	[mL min^{-1}]	4.5
Sample uptake rate	[mL min^{-1}]	0.3 (established by split flow)
Isotopes monitored		^{47}Ti
Dwell time	[ms]	250-2000

Size-retention time calibrations of the AF^4 channel are performed under identical run conditions, with the only exception being for a carrier composition 0.025 % FL-70 and 0.003 M NaCl.

22.3.4 Evaluation of results

Data analysis is performed by evaluating Ti bulk mass recovery, and particle size distribution.

Ti bulk mass recovery ($rec_{Ti,bulk}$; eq. 1) is defined as the ratio between Ti mass concentration after the sample preparation procedure ($c_{Ti,sample,ICP-OES}$) and initial Ti mass concentration ($c_{Ti,initial}$). The initial concentrations are calculated from the Ti mass concentration in the stock solutions and dilution caused by sample preparation. Ti mass concentration after preparation procedure is determined using ICP-MS or ICP-OES analysis directly after sample preparation, final dilution of 100x in 0.025 % FL-70 and addition of 200 µL of 5 M HNO_3 in 10 mL of sample.

$$rec_{Ti,bulk} = c_{Ti,sample,ICP-OES} / c_{Ti,initial} \quad \text{(eq. 1)}$$

Particle size distribution obtained by AF4 separation and MALS analysis is derived from the measured fractograms. The spreadsheet is used for converting the retention times to hydrodynamic sizes using the calibration function determined in fractionations of the 50 nm – 200 nm diameter PS latex bead standards, after subtracting the time of the void peak. The ICP-MS is calibrated on daily basis for Ti (as representative element in sunscreen). In addition Fe and Al are also chosen as representative elements for the complex sunscreen samples (BAM-13a) which contains iron oxides in addition to TiO_2. Calibration functions for converting the ICP-MS signals to concentrations is set up by plotting the averaged intensities of standard solutions against the standard concentrations, after subtracting the background signal. The signal intensities in the

fractograms are then converted into concentration values. A size distribution is obtained based on particle mass.

22.3.5 Reporting of results

Reporting of the final results is presented below:

$rec_{Ti,bulk}$ [%]

$r_{h,mode}$ (MALS) [nm]

$r_{h,mode}$ (ICP-MS) [nm]

PSD (d_{10}, d_{50}, d_{90}) [nm]

22.4 Validation status

This method has been subjected to in-house validation.

22.5 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in this SOP.

Personnel should utilize all necessary precautions to avoid exposure to chemical and nanomaterials.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

23 Sample preparation and spICP-MS analysis of TiO_2 particles in sunscreen products

23.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of TiO_2 nanoparticles in sunscreen products using single particle ICP-MS (spICP-MS).

23.2 Scope

The procedure is applicable for the determination of TiO_2 nanoparticles in sunscreen products. The procedure may also be applicable for other nanoparticles consisting of metal or metal oxides (e.g. Ag, Au, Al_2O_3, SiO_2, etc.) in aqueous suspensions or consumer products with a composition comparable with sunscreen products (e.g. cosmetic creams, toothpaste, etc.). In those cases additional quality control samples shall be incorporated. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter (ESD)) in the range of 10 to 1000 nm and mass concentrations in the range of 1 to 1000 ng L^{-1} in the final extract/suspension can be determined. The mass concentration range can be extended by further dilution of the prepared extracts or suspensions.

23.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.

23.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

A sub-sample of the sunscreen product is collected and diluted in a diluting agent, which stabilises the nanoparticles in suspension. This first suspension is then diluted further in one or more steps before instrumental analysis.

spICP-MS is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised, and produce a plume of element ions which travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD (equivalent spherical diameter), is calculated from the particle's mass. The number of peaks that are recorded during the

time scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine Manual, Part 2: Evaluation of methods.

23.4.1 Materials and methods

23.4.1.1 Materials, chemicals and reagents

Chemicals required for sample preparation and measurement are detailed in Table 1.

Table 1: Chemicals for sample preparation and measurement

Chemical	**Description**	**Manufacturer/Provider**
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm)	e.g. Millipore, Billerica, MA, USA or equivalent
Triton X-100	Nonionic surfactant, laboratory grade	e.g. Sigma Aldrich, St Louis, MO, USA or equivalent
MelPers® 2450	Deflocculant for inorganic pigments (solid content 49-51 %)	BASF, Ludwigshafen, Germany
HNO_3	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Ionic Standard Solutions	Titanium ionic standards in 3 % nitric acid (1 g L^{-1})	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Laboratory instruments and material are listed in table 2.

Table 2: Laboratory instruments and materials

Instrument	**Description**	**Manufacturer/Provider**
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 µL, 10-100 µL, 100-1.000 µL)	e.g. Eppendorf, Hamburg, Germany

Analytical instruments and software are listed in table 3.

Table 3: Analytical instruments and software

Instrument	Description	Manufacturer/Provider
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of nickel cones (nanoparticle suspensions) and platinum cones (products)	e.g. ICAP-Q, Thermo Fisher Scientific, Waltham, MA, USA or equivalent
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
Qtegra npQuant	Qtegra software with integrated nano-application tool for nanoparticle measurements and data evaluation	Thermo Fisher Scientific, Waltham, MA, USA
RIKILT SPC spreadsheet[1]	Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

The following reagents are needed:

Diluting agent. The diluting agent for sunscreen samples is prepared by weighing 5 g of MelPers® 2450, and 5 g of Triton-X 100 in a clean glass bottle and add 1 L of ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 μgL^{-1}). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 μgL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL^{-1}). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standards of ionic titanium solutions (100 μgL^{-1}). Assuming the ionic standard solution has a concentration of 1 gL^{-1}, pipet 50 µL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mg L^{-1}. Repeat this step, pipetting 1 mL of the 1 mgL^{-1} standard to 5 mL ultrapure water in a 10 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 100 μgL^{-1}. Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic titanium solutions (0.2 – 10 μgL^{-1}). Prepare the calibration curve ionic standards according to table 1.Pipet the volumes of the stock standard of 100 μgL^{-1} to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix shall be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted to 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
5 mL	10 μgL^{-1}	2 weeks
2.5 mL	5 μgL^{-1}	2 weeks
1 mL	2 μgL^{-1}	2 weeks
0.5 mL	1 μgL^{-1}	2 weeks
0.25 mL	0.5 μgL^{-1}	1 week
0.1 mL	0.2 μgL^{-1}	1 week

23.4.2 Performing measurements

23.4.2.1 Preparation of products

The sample preparation of sunscreen samples consists of a matrix dilution. The following steps will be performed:

1. Weigh approximately 50 mg of the product in a 50 mL polyethylene tube and add 50 mL of the diluting agent.
2. Vortex the tube until the sunscreen is completely detached from the tube walls.

Sonicate the tube in a water bath for 10 minutes at room temperature.

3. First dilution. Vortex and shake the suspension before collecting a 50 µL subsample. Dilute the subsample of 50 µL in a 50 ml PE tube with approximately 25 mL of ultrapure water. Fill to the 50 mL mark with ultrapure water.
4. Sonicate the first dilution in a water bath for 10 minutes at room temperature
5. Second dilution. Vortex and shake the diluted suspension before a subsample is collected. Collect a subsample of 25 µL and dilute in a 50 ml PE tube with approximately 25 mL of ultrapure water. Fill to the 50 mL mark with ultrapure water.

(NOTE: This procedure was prepared for sunscreen products containing TiO_2 nanomaterial in a concentration of approximately 50 g Ti/kg product. For other nanomaterials or other matrices (e.g. facial creams, toothpaste etc.) the method may need adjustments.)

23.4.2.2 ICP-MS set-up and calibration

ICP-MS performance check

The instrument has a performance check and an autotune function which are designed to replace the manual checks and tuning procedures and the short term stability test. If the criteria of the performance check are not met, a tuning, autotune or manual tune, is performed to optimize the instrument.

A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention shall be paid to the cleanliness of the sample introduction system of the ICP-

MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels is subsequent analysis leading to erroneous results. Analyse an ultrapure water sample and a blank matrix sample to determine the background signals. In neither of the two the number of observed particles shall exceed a number of 10.

Settings of the ICP-MS system

- Forward power : 1550 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Gas flows : plasma, 13 $Lmin^{-1}$
  nebulizer, 1.1 $Lmin^{-1}$

- Rinsing liquid flow rate : 1 $mLmin^{-1}$

- Sample flow rate : 0.35 $mLmin^{-1}$
- Data acquisition : time resolved analysis (TRA) mode (npQuant)
- Dwell time : 3 ms
- Total acquisition time : 60 s
- Isotope monitoring : Au (m/z 197), Ti (m/z 48)

(NOTE: For elements with potentially polyatomic interferences the application of another measurement mode (e.g. KED) or reaction cell (CCT) can improve results)

In general the number of peaks in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan shall not exceed 2000. If this number is exceeded, the aqueous sample extract shall be diluted and re-analysed. For the instrumental settings used in this procedure a particle number concentration in the range of $2x10^6$ to $2x10^8$ particles L^{-1} results in useful measurement data.

23.4.2.3 Measurement description

Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected of particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL^{-1} standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL^{-1})

Determination of the response of the analyte

A mass calibration shall be performed using working standards in table 1 (6 concentrations in the range of 1 to 50 μgL^{-1} and a blank) under the same measurement conditions as for spICP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient shall be >0.99.

(NOTE: When possible the composition of the size calibrant matrix shall be matched to that of the prepared samples)

Analysis of sample dilutions

Sunscreen and other facial creams containing TiO_2 NP also contain few large aggregates or agglomerates of these particles. The presence of such an aggregate or agglomerate may strongly influence the result of individual measurements. Therefore, each sample dilution shall be analysed in triplicate. Results will be calculated as the average of the three determinations.

Sample list

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

1 Blank
2 Ionic standard 1
3 Ionic standard 2
4 Ionic standard 3
5 Ionic standard 4
6 Ionic standard 5
7 Ionic standard 6
8 Nanoparticle standard (NIST AuNP 60 nm)
9 Blank
10 Sample 1, rep 1
11 Sample 1, rep 2
12 Sample 1, rep 3
13 Sample 2, rep 1

: :

17 Sample 10, rep 2
18 Sample 10, rep 3
19 Blank
20 Ion standard 4
21 Nanoparticle standard (NIST AuNP 60 nm)
22 Blank
23 Sample 11, rep 1
24 ….

: :

40 Blank
41 Ionic standard 1
42 Ionic standard 2
43 Ionic standard 3
44 Ionic standard 4
45 Ionic standard 5
46 Ionic standard 6

(NOTE: When high particle concentrations are expected a ultrapure water sample can be placed after each sample to minimize and check on possible carry-over of analytes)

23.4.3 Evaluation of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing[1]. This spreadsheet and a SOP how to use it are freely available from the RIKILT website[2]. The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration is calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

23.4.4 Reporting of results

The final results of the calculations within the spreadsheet are expressed as follows:

- Particle mass concentration (ngL^{-1})
- Particle number concentration ($particleL^{-1}$)
- Particle size (nm) as ESD
- Ionic concentration (ngL^{-1})

In addition a graph of the particle's size distribution is presented.

23.5 Validation status

This method is validated.

23.6 HSE issues

Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses. Each chemical/particle shall be treated as a potential health hazard and exposure to these chemicals/particles shall be minimized.

23.7 References

[1] Peters, R., Herrera-Rivera, Z., Undas, A., van der Lee, M., Marvin, H., Bouwmeester, H. and Weigel, S. *Single particle ICP-MS combined with a data evaluation tool as a routine technique for the analysis of nanoparticles in complex matrices. J Anal. At. Spectrom.*, 2015, 30:1274-1285

[2] Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research. https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm

23.8 Performance characteristics

Table 1 gives the performance characteristics of the method for the detection and characterisation of TiO_2 NP in sunscreen product BAM-13A.

Table 1: Performance characteristics of the method for the detection and characterisation of TiO_2 NP in sunscreen

Linearity	0.5 to 50 μgL^{-1} based on ionic concentrations
Working range	size: from LOD_S up to 500 nm concentration: from LOD_C up when proper dilution is applied
LOD	LOD_S: 20 nm TiO_2 LOD_C: 12 $mgkg^{-1}$ product
LOQ	LOQ_S: 23 nm TiO_2 LOQ_C: 30 $mgkg^{-1}$ product
Repeatability	size: 2.4 % number concentration: 8.0 % mass concentration: 19.9 %
Intermediate precision	size: 4.0 % number concentration: 16.8 % mass concentration: 21.3 %
Trueness at 1.0VL and 0.5 VL*	84 % and 82 % for mass concentration
Ruggedness	determination of particle size not rugged for proper mixing (vortex) and setting of dwell time
Specificity/selectivity	yes/yes
Stability	the intermediate dilution is stable for at least 7 days
Measurement uncertainty, ux (Ux)	size: 5 % (11 %) number concentration: 20 % (41 %) mass concentration: 34 % (67 %)

24 Sample preparation and spICP-MS analysis of TiO_2 nanoparticles in suspensions

24.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of TiO_2 nanoparticles in suspensions using single particle ICP-MS.

24.2 Scope

The procedure is applicable for the determination of TiO_2 nanoparticles in suspensions. The procedure may also be applicable for other nanoparticles consisting of metal or metal oxides (e.g. Ag, Au, Al_2O_3, SiO_2, etc.) in aqueous suspensions. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter (ESD)) in the range of 10 to 1000 nm and mass concentrations in the range of 1 to 1000 ngL^{-1} in the final suspension can be determined. The mass concentration range can be extended by further dilution of the suspensions.

24.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.

24.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

In case of suspensions dilution before instrumental analysis will often be required.

Single particle ICP-MS (spICP-MS) is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised, and produce a plume of element ions which travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD (Equivalent spherical diameter), is calculated from the particle's mass. The number of peaks that are recorded during the time scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine NanoDefine Manual, Part 2: Evaluation of methods.

24.4.1 Materials and methods

24.4.1.1 Materials, chemicals and reagents

Chemicals required for sample preparation and measurement are detailed in Table 1. Table 2 lists laboratory instruments and materials and Table 3 the analytical instruments and software.

Table 1: Chemicals for sample preparation and measurement

Chemical	Description	Manufacturer/Provider
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm)	e.g. Millipore, Billerica, MA, USA or equivalent
SHMP	Sodium hexametaphosphate (2 gL^{-1})	e.g. Fisher Scientific, Pittsburgh, PA, USA or equivalent
HNO_3	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Ionic Standard Solutions	Titanium ionic standards in 3 % nitric acid (1 gL^{-1})	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Table 2: Laboratory instruments and materials

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or aquivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 µL, 10-100 µL, 100-1.000 µL)	e.g. Eppendorf, Hamburg, Germany

Table 3: Analytical instruments and software

Instrument	Description	Manufacturer/Provider
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of nickel cones (nanoparticle suspensions) and platinum cones (products)	e.g. ICAP-Q, Thermo Fisher Scientific, Waltham, MA, USA or equivalent
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
Qtegra npQuant	Qtegra software with integrated nano-application tool for nanoparticle measurements and data evaluation	Thermo Fisher Scientific, Waltham, MA, USA
RIKILT SPC spreadsheet[1]	Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

Reagents

Diluting agent. The diluting agent for the preparation of suspensions is prepared by weighing 2 g of sodium hexametaphosphate in a clean glass bottle and add 1 L of ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 μgL^{-1}). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 μgL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL^{-1}). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standards of ionic titanium solutions (100 μgL^{-1}). Assuming the ionic standard solution has a concentration of 1 gL^{-1}, pipet 50 µL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mgL^{-1}. Repeat this step, pipetting 1 mL of the 1 mgL^{-1} standard to 5 mL ultrapure water in a 10 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 100 μgL^{-1}. Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic titanium solutions (0.5 – 50 µg L^{-1}). Prepare the calibration curve ionic standards according to table 1.Pipet the volumes of the stock standard of 100 µg L^{-1} to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix should be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted to 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
25 mL	50 μgL^{-1}	2 weeks
5 mL	10 μgL^{-1}	2 weeks
2.5 mL	5 μgL^{-1}	2 weeks
1 mL	2 μgL^{-1}	2 weeks
0.5 mL	1 μgL^{-1}	2 weeks
0.25 mL	0.5 μgL^{-1}	1 week

24.4.2 Performing measurements

24.4.2.1 Sample preparation

For the preparation of suspensions solid materials need to be suspended and the resulting suspensions need to be diluted prior to instrumental analysis.

24.4.2.2 Preparation of nanoparticle suspensions

The dispersion of nanomaterial powders is performed according to the following steps:

1. Prepare the initial stock by weighing 5 ± 2 mg of the nano-powder in a 50 mL glass vial. Add 5 mL of sodium hexametaphosphate (2 gL^{-1}) to produce a final concentration of ~1 $mgmL^{-1}$.
2. Homogenize the dispersion by brief vortexing (30 seconds).
3. Sonicate the dispersion in ultrasonic bath for 10 minutes.
4. Dilute with sodium hexametaphosphate (2 gL^{-1}) to a total volume of 50 mL and a final concentration of ~100 μgmL^{-1}
5. Sonicate the second stock (~100 μgmL^{-1}) for 15 minutes with an sonication probe (power: 4W) in an ice bath

   Note: Sonication is a critical step in the preparation of the suspension. In case of doubt extend the sonication time (15 min) to 30 min.
6. For spICP-MS analysis dilute the sample 100,000 times in ultrapure water to a final concentration of ~1,000 ngL^{-1}.

(NOTE: This procedure was written for the preparation of a suspension a TiO_2 nanomaterial. For other nanomaterials the method may need adjustments)

24.4.2.3 ICP-MS set-up and calibration

ICP-MS performance check

The instrument has a performance check and an autotune function which are designed to replace the manual checks and tuning procedures and the short term stability test. If the criteria of the

performance check are not met, a tuning, autotune or manual tune, is performed to optimize the instrument.

A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention should be paid to the cleanliness of the sample introduction system of the ICP-MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels is subsequent analysis leading to erroneous results. Analyse an ultrapure water sample and a blank matrix sample to determine the background signals. In neither of the two the number of observed particles shall exceed a number of 10.

Settings of the ICP-MS system

- Forward power : 1550 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Gas flows : plasma, 13 $Lmin^{-1}$
  nebulizer, 1.1 $Lmin^{-1}$
- Rinsing liquid flow rate : 1 $mLmin^{-1}$
- Sample flow rate : 0.35 $mLmin^{-1}$
- Data acquisition : time resolved analysis (TRA) mode (npQuant)
- Dwell time : 3 ms
- Total acquisition time : 60 s
- Isotope monitoring : Au (m/z 197), Ti (m/z 48)

(NOTE: For elements with potentially polyatomic interferences the application of another measurement mode (e.g. KED) or reaction cell (CCT) can improve results)

(NOTE: Use platinum cones when analysing samples containing fluorine)

In general the number of peaks in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan should not exceed 2000. If this number is exceeded, the aqueous sample extract should be diluted and re-analysed. For the instrumental settings used in this procedure a particle number concentration in the range of $2x10^6$ to $2x10^8$ particles L^{-1} results in useful measurement data.

24.4.2.4 Measurement description

Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected of particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL^{-1} standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL^{-1})

Determination of the response of the analyte

A mass calibration should be performed using working standards in table 1 (6 concentrations in the range of 1 to 50 μgL^{-1} and a blank) under the same measurement conditions as for spICP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient should be >0.99.

(NOTE: When possible the composition of the size calibrant matrix should be matched to that of the prepared samples)

Sample list

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

1. Blank
2. Ionic standard 1
3. Ionic standard 2
4. Ionic standard 3
5. Ionic standard 4
6. Ionic standard 5
7. Ionic standard 6
8. Nanoparticle standard (NIST AuNP 60 nm)
9. Blank
10. Sample 1
11. Sample 2

: :

24. Sample 9
25. Sample 10
26. Blank
27. Ion standard 4
28. Nanoparticle standard (NIST AuNP 60 nm)
29. Blank
30. Sample 11

: :

44. Blank
45. Ionic standard 1
46. Ionic standard 2
47. Ionic standard 3
48. Ionic standard 4
49. Ionic standard 5
50. Ionic standard 6

(NOTE: When high particle concentrations are expected a ultrapure water sample can be placed after each sample to minimize and check on possible carry over of analytes)

(NOTE: If the material that is suspended has a wide polydispersity, or if large aggregates or agglomerates can be present, it is advised to analyse the final suspensions in triplicate to minimize the effect of these large particle structures)

24.4.3 Evaluation of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing[1]. This spreadsheet and a SOP how to use it are freely available from the RIKILT website[2]. The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration is calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

24.4.4 Reporting of results

The final results of the calculations within the spreadsheet are expressed as followed:

- Particle mass concentration (ngL^{-1})
- Particle number concentration (particle L^{-1})
- Particle size (nm) as ESD
- Ionic concentration (ngL^{-1})

In addition a graph of the particle's size distribution is presented.

24.5 Validation status

This method is validated.

24.6 HSE issues

Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses. Each chemical/particle should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

24.7 References

[1] Peters, R., Herrera-Rivera, Z., Undas, A., van der Lee, M., Marvin, H., Bouwmeester, H. and Weigel, S. *Single particle ICP-MS combined with a data evaluation tool as a routine technique for the analysis of nanoparticles in complex matrices. J Anal. At. Spectrom.*, 2015, 30:1274-1285

[2] Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research. https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm

24.8 Performance characteristics

The Table 1 below gives the performance characteristics of the method for the detection and characterisation of TiO_2 NP in suspensions.

Table 1: Performance characteristics of the method for the detection and characterisation of TiO_2 NP in suspension

Linearity	0.5 to 10 µg/L based on ionic concentrations and based on other results extended to 0.5 to 50 µg/L
Working range	size: from LOD_S up to 500 nm concentration: from LOD_C up when proper dilution is applied
LOD	LOD_S: 25 nm TiO_2 LOD_C: 250 pg/L in suspension
LOQ	LOQ_S: 30 nm TiO_2 LOQ_C: 750 pg/L in suspension
Repeatability	size: 3.2 % number concentration: 23 % mass concentration: 20 %
Intermediate precision	size: 7.9 % number concentration: 24 % mass concentration: 21 %
Trueness	83 %
Robustness	robust sonication time and setting of dwell time
Specificity/selectivity	yes/yes
Stability	the suspension is stable for at least 2 days
Measurement uncertainty u_x (U_x)	size: 9 % (18 %) number concentration: 40 % (81 %)* mass concentration: 33 % (66 %)*

* Represents the sum of sample preparation and measurement, as performed in the NanoDefine project

25 Sample preparation and spICP-MS analysis of Al_2O_3 nanoparticles in toothpaste

25.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of Al_2O_3 particles in toothpaste products using single particle ICP-MS (spICP-MS).

25.2 Scope

The procedure is applicable for the determination of Al_2O_3 particles in toothpaste. The procedure may also be applicable for other particles consisting of metal or metal oxides (e.g. Ag, Au, TiO_2, SiO_2, etc.) in consumer products with a composition comparable with toothpaste. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter, ESD) in the range of 50 to 500 nm and mass concentrations in the range of 1 to 1000 ng L^{-1} in the final extract/suspension can be determined. The mass concentration range can be extended by further dilution of the prepared extracts or suspensions.

25.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.
ESD	Equivalent spherical diameter (for particle size)

25.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

In case of toothpaste a sub-sample is collected and dispersed in a diluting agent, which stabilises the nanoparticles in suspension. This first suspension is then diluted further in one or more steps before instrumental analysis.

Single particle ICP-MS (spICP-MS) is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised. Produced the plume of element ions travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD, is calculated from the particle's mass. The number of peaks that are recorded during the time

scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine Manual, Part 2 : Evaluation of methods.

25.4.1 Materials and methods

25.4.1.1 Chemicals, equipment and instruments

The chemicals required for sample preparation and measurement are detailed in Table 1. Table 2 lists laboratory instruments and materials and Table 3 the analytical instruments and software.

Table 1: Chemicals for sample preparation and measurement

Chemical	**Description**	**Manufacturer/Provider**
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm)	e.g. Millipore, Billerica, MA, USA or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Al_2O_3 particles standard	e.g. powdered Al_2O_3 particles, ESD 135 nm	e.g. US Research Nanomaterials, Houston, TX, USA
Dispersion agent	Sodium hexametaphosphate (2 gL^{-1})	e.g. Fisher Scientific, Pittsburgh, PA, USA or equivalent
HNO_3	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Ionic Standard Solutions	Aluminium ionic standards in 3 % nitric acid (1 gL^{-1})	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Table 2: Laboratory equipment

Instrument	**Description**	**Manufacturer/Provider**
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Magnetic plate	Magnetic plate used with magnetic stirrers to disperse the toothpaste	e.g. AG Germany or equivalent
Magnetic stirrers	Egg-shaped PTFE-coated magnetic stirrers used to disperse the toothpaste (L 15 mm, W 6 mm)	e.g. VWR Collection, Randor, PA, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 µL, 10-100 µL, 100-1.000 µL)	e.g. Eppendorf, Hamburg, Germany

PE Tubes	PE centrifuge tubes used in sample preparation (50 ml) and measurement (12 ml)	e.g. VWR Collection, Randor, PA, USA or equivalent

Table 3: Analytical instruments and software

Instrument	**Description**	**Manufacturer/Provider**
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of platinum cones	e.g. Perkin Elmer Nexion 350D or equivalent, Waltham, MA, USA.
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
RIKILT SPC spreadsheet[1]	Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

25.4.2 Methods

25.4.2.1 Stock and working solutions

Rinsing solvent (3 %). The ICP-MS system is rinsed after each measured sample therefore it is wise to prepared larger volume of the rinsing solvent. Fill the 8 L container with ultrapure water up to 2/3. Add ca. 370 ml of 65 % nitric acid. Fill with ultrapure water to 8 mL and homogenize. The rinsing solvent can be stored at room temperature for at least 1 week.

Dispersion agent (2 gL^{-1}). The dispersion agent for the preparation of toothpaste sub-sample is prepared by weighing 2 g of sodium hexametaphosphate in a clean glass bottle and fill to 1 L with ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved, and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 µg L^{-1}). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 μgL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL^{-1}). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL^{-1}. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standard of Al_2O_3 particles (2 gL^{-1}). If necessary the recovery of the Al_2O_3 can be approximated by spiking the diluted toothpaste dispersions with Al_2O_3 particles standard dispersed beforehand according to the modified NanoGenotox protocol[2]. Briefly, weigh 20 mg of the powdered particle standard in 12 ml glass vial. Pre-wet the powder with 30 µL ethanol and vortex briefly. Add dispersion agent in two steps. First step, add 570 µL of dispersion agent followed by brief vortexing and second step add 4.4 mL of dispersion agent followed again by brief vortexing.

Sonicate the dispersion for 15 min using probe sonicator (4 Watt). Rinse the probe with 5 mL dispersion agent. This standard is stable for x. Vortex prior usage.

Stock standards of ionic aluminium solutions (1000 μgL^{-1}). Assuming the ionic standard solution has a concentration of 1 gL^{-1}, pipet 50 µL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mg L^{-1}. Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic aluminium solutions (0.5 – 50 μgL^{-1}). Prepare the ionic working standards according to Table 1. Pipet the volumes of the stock standard of 1000 μgL^{-1} to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix should be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted in 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
2.5mL	50 μgL^{-1}	2 weeks
1 mL	20μgL^{-1}	2 weeks
0.5 mL	10 μgL^{-1}	2 weeks
0.25 mL	5 μgL^{-1}	2 weeks
0. 05 mL	1 μgL^{-1}	2 weeks
0.025 mL	0.5 μgL^{-1}	1 week

25.4.2.2 Sample preparation

1. Weigh approximately 0.5 g of the product in a 50 mL PE centrifuge tube and fill to 50 mL with dispersion agent. (dispersed sub-sample of toothpaste)
2. Place in the sub-sample of toothpaste magnetic stirrer and place the PE centrifuge tube on magnetic plate. Disperse the toothpaste for 15 min.
3. First dilution. Vortex 10 sec the suspension. Fill a 50 ml PE centrifuge tube with 25 ml ultrapure water and pipette 250 µL of dispersed sub-sample of toothpaste into the ultrapure water. Fill to the 50 mL mark with ultrapure water.
4. Second dilution. Vortex 15 sec and shake the diluted suspension. Fill a 50 ml PE centrifuge tube with 25 ml ultrapure water and pipette 100 µL of diluted dispersed sub-sample of toothpaste into the ultrapure water. Fill to the 50 mL mark with ultrapure water.

(NOTE: This procedure was prepared for toothpaste products containing Al_2O_3 in a concentration around 10 gkg^{-1}. For other nanomaterials or other matrices (e.g. facial creams, toothpaste etc.) the method may need adjustments)

25.4.2.3 Measurements

Settings and tuning of the ICP-MS (performance check)

Settings of the ICP-MS system

- Forward power : 1600 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Sample, skimmer cone : platinum
- Gas flows : plasma, 13 $Lmin^{-1}$
  nebulizer, 1.1 $Lmin^{-1}$
- Rinsing liquid flow rate : 1 $mLmin^{-1}$
- Sample uptake rate : 0.35 $mLmin^{-1}$
- Data acquisition : time resolved analysis (TRA) mode
- Dwell time : 3 ms
- Acquisition time : 60 s
- Isotopes monitored : Au (m/z 197), Al (m/z 27)
- DRC parameters : 0.6 $mlmin^{-1}$ cell gas (ammonia), RPq 0.5

The ICP-MS instrument should be tuned and its performance check should be performed according to the manufacturer guidelines. A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention should be paid to the cleanliness of the sample introduction system of the ICP-MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels in subsequent analysis leading to erroneous results. Analyse an ultrapure water sample after each sample and a blank matrix sample to determine the background signals should be implemented in the analysis sequence. In neither of the two the number of observed particles shall exceed a number of 10.

In general the number of peaks of the analysed diluted dispersed toothpaste sample in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan should not exceed 2000. If this number is exceeded, the aqueous sample extract should be diluted and re-analysed.

(NOTE: In case of toothpastes containing fluoride, the fluoride can seriously damage the cones of the ICP-MS. This is certainly the case when non-platinum cones are used and therefore the use of platinum cones is advised)

Measurement description

a. Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL^{-1} standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL^{-1})

b. Determination of the response of the analyte

A mass calibration should be performed using working standards of ionic aluminium prepared according to Table 1 (6 concentrations in the range of 0.5 to 50 μgL^{-1} and a blank sample) under the same measurement conditions as for spICP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient should be >0.99.

c. Samples of interest

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

1. Blank
2. Ionic standard 1
3. Ionic standard 2
4. Ionic standard 3
5. Ionic standard 4
6. Ionic standard 5
7. Ionic standard 6
8. Nanoparticle standard (NIST AuNP 60 nm)
9. Blank
10. Sample 1
11. Sample 2

: :

25. Sample 9
26. Sample 10
27. Blank
28. Ion standard 4
29. Nanoparticle standard (NIST AuNP 60 nm)
30. Blank
31. Sample 11

- ....

: :

44. Blank
45. Ionic standard 1
46. Ionic standard 2
47. Ionic standard 3
48. Ionic standard 4
49. Ionic standard 5
50. Ionic standard 6

25.4.3 Data processing and reporting of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing[1]. This spreadsheet and a SOP how to use it

are freely available from the RIKILT website[2]. The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration are calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

The final results of the calculations within the spreadsheet are expressed as followed:

- Particle mass concentration (ngL^{-1})
- Particle number concentration (particle L^{-1})
- Particle size (nm) as ESD
- Ionic concentration (ngL^{-1})

In addition a graph of the particle's size distribution is presented.

25.5 Validation status

This method was validated in-house.

25.6 HSE issues

Protective clothing (lab coat, safety glasses, and gloves.) is required. Each chemical/particle should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized. If possible standards, chemicals and reagents should be prepared in a fume hood.

25.7 References

[1] Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research. https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm

[2] NanoGenotox, Standard operating procedures for characterisation of the selected manufactured nanomaterials and dispersions thereof June, 2011

25.8 Performance characteristics

Table 1 below gives the performance characteristics of the method for the detection and characterisation of Al_2O_3 particles in toothpaste products using single particle ICP-MS (spICP-MS).

Table 1: Performance characteristics of the method for the detection and characterisation of Al_2O_3 nanoparticles in toothpaste

Linearity	0.5 to 50 µg/L based on ionic concentrations
Working range	size: from LOD_S up to 500 nm concentration: from LOD_C up when proper dilution is applied
LOD	LOD_S: 48 nm TiO_2 LOD_C: 61 mg/kg product
LOQ	LOQ_S: 70 nm TiO_2 LOQ_C: 180 mg/kg product

Repeatability	size: 5.3 % number concentration: 5.8 % mass concentration: 2.3 %
Intermediate precision	size: 15 % number concentration: 24 % mass concentration: 29 %
Trueness at 1.0VL and 2.0 VL*	81 % and 64 % for mass concentration
Ruggedness	the determination of particle mass concentration is not rugged for proper mixing (stirring)
Specificity/selectivity	yes/yes
Stability	the intermediate dilution is stable for at least 4 days
Measurement uncertainty, u_x (U_x)	size: 17 % (34 %) number concentration: 25 % (50 %) mass concentration: 33 % (66 %)

*VL was 10 g Al_2O_3 NP/kg product

26 General Conclusions

This report presents 23 Standard Operating Procedures, SOPs, developed in NanoDefine.

NanoDefine developed 11 detailed material specific protocols designed to produce liquid dispersions of the NanoDefine priority substances such that the resulting dispersions are stable and contain only or mainly constituent particles. The priority substances are: IRMM-380 (Pigment yellow 83 (transparent grade)), IRMM-381 ($BaSO_4$ (fine grade)), IRMM-382 (MWCNT), IRMM-383 (Nano steel), IRMM-384 ($CaCO_3$ (fine grade)), IRMM-385 (Kaolin), IRMM-386 (Pigment yellow 83 (opaque grade)), IRMM-387 ($BaSO_4$ (ultrafine grade)), IRMM-388 (Coated TiO_2 (pigment grade)), IRMM-389 (Basic methacrylate copolymer particles (BMC)) and BAM-11 (Zeolite powder).

An additional 13 SOPs have been developed:

i) a generic SOP for calorimetric calibration of the sonicators,

ii) the DLS method, which was developed for four of materials (IRMM-381 ($BaSO_4$ (fine grade)), IRMM-384 ($CaCO_3$ (fine grade)), IRMM-385 (Kaolin), IRMM-388 (Coated TiO_2 (pigment grade)). It can also be applied to comparable types of materials, considering that adaptions might be needed,

iii) the Cuvette-AC method which was developed for two materials (IRMM-381 ($BaSO_4$ (fine grade)), IRMM-387 ($BaSO_4$ (ultrafine grade)), and which can also be applied to comparable types of materials, considering that adaptions might be needed,

iv) a method for the analysis of Fe_2O_3 in polyethylene matrix with electron microscopy,

v) a method for the analysis of TiO_2 in sunscreen with electron microscopy,

vi) SOPs for size characterisation of suspended particles by AUC-RI with speed ramp option,

vii) particle size distribution measurement of $BaSO_4$ using Line-Start Disc Centrifuge with Optical Detection,

viii) measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software,

ix) analysis of TiO_2 particles from sunscreen by AF4-MALS-ICP-MS,

x) sample preparation and spICP-MS analysis of TiO_2 nanoparticles in sunscreen products,

xi) sample preparation and spICP-MS analysis of TiO_2 nanoparticles in suspensions, and

xii) sample preparation and spICP-MS analysis of Al_2O_3 nanoparticles in toothpaste.

26.1 Dispersion protocols

The dispersion procedure is a pivotal step in the process of making measurements of the particle size distribution. It is thus necessary that dispersion procedures are reproducible fit to the purpose of analysis/characterisation and lead to no/insignificant contamination. Of course it is also important that dispersion procedures are effective and efficient. The final dispersion obtained by these dispersion procedures should have a particle size distribution, which is as close as possible to the true distribution of constituent particles.

The issue of dispersion is particularly important in the evaluation of nanoparticle size as many nanomaterials are normally found in the form of dried powders which need to be brought into

stable liquid dispersions before they can be measured by many of the most common particle size measuring methods such as dynamic light scattering, angular light scattering, centrifugal liquid sedimentation and analytical centrifugation. To achieve comparable results, the procedures for bringing the materials into dispersion should as far as possible be harmonised and standardized. A first step towards this is to develop SOPs and NanoDefine developed 11 SOPS for dispersion protocols, which take into account material specificities and are based on aqueous dispersion. When developing these protocols it was found that agglomerated materials cannot be adequately dispersed by the use of low energy mixing (stirring/shaking) or by the use of ultrasonic bath (USB). Instead it was necessary to apply the high energy methods probe sonication (USP) or vial sonication (VS). Thus, the 11 SOPs all include a probe sonication dispersion protocol, as the probe-sonicator was the most common apparatus among the laboratories participating, and furthermore selected materials ((IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 ($CaCO_3$), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO_2)) have an associated protocol for vial-sonication.

The cup-horn sonicator was not available to the participating laboratories and is thus not included in the analysis.

Sonication is effective for dispersing the test material, but it introduces a significant variable in the process as a wide variety of different sonication instruments exists with different nominal power and probe size. To reduce that variability a SOP 'Generic SOP for calorimetric calibration of an ultrasonic probe sonication' was developed to allow ensuring a better harmonisation of the power output when using significantly different sonicator types or probe types/sizes for the dispersion compared to those used in the development of in the optimised protocols.

For the materials ((IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 ($CaCO_3$), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO_2)) for which procedures using both probe and vial sonication were developed it was noted that the treatment times required (to achieve comparable levels of dispersion) were generally similar provided that the volume specific ultrasonic energy input were comparable. This indicates that it is not sufficient to consider only the power output of a sonicator, and that also the volume of sample which is being treated needs to be considered. For example to achieve a similar level of dispersion in a similar time (15-20 min) the vial sonicator supplied a much lower power of 2.2 W compared to the 7.8 W value output by the probe sonicator. However, when considering that in the first example the sample volume was 2 mL while in the second case it was 6 mL it can be seen that the actual specific power expressed as WmL^{-1} are actually quite similar (1.1 WmL^{-1} compared to 1.3 WmL^{-1}). This observation underlines the importance of respecting the technical details of dispersion protocols and in particular care should be taken to ensure that the sonication power used is appropriate to the volume and concentrations of the sample being treated. If it is necessary to deviate significantly from the volumes and concentrations specified in the protocols it would be critical that sonication times and energies be adequately adapted and optimised by the use of appropriate methods. It is also relevant to note that while insufficient sonication must be avoided it is equally important to avoid excessive treatment times or energies as this can lead to a loss in the number of the small and intermediate particulates/aggregates presumably by irreversible fusion of the particulates. This phenomenon has not been detailed in this report but it has been specifically observed to occur with TiO_2, $BaSO_4$ and $CaCO_3$ treated for periods of greater than 2 hours rather than the normal times of 20-30 minutes.

26.2 Possible contamination by probe sonicator

Despite being the most commonly available probes, sonication probes should be used cautiously, as probe material may be released due to wear of the probe, starting after only a few hours of use of the probe. Furthermore, this wear may not be easy to detect, as the residues (fine grey-white powder) may not be easily detected by visual inspection of the final dispersions.

If a laboratory regularly produces nanoparticle dispersions for metrological applications, such as those of NanoDefine, then it is advisable that, when appropriate equipment is available and sample volumes and temperature sensitivity permit, non-contact methods of sonication should be adopted to avoid any risk of this problem. Non-contact systems are e.g. vial or cup sonicators.

In cases where direct contact probe sonication is to be used then it may be preferable to use a probe sonicator which has an exchangeable tip so that this may be easily inspected and replaced whenever necessary. In this way regular substitution may be undertaken with a lower cost than in the case of substituting mono-block probes.

26.3 Material- and technique- dependent observations

For two of the priority materials which are platelets (IRMM-383 (nano steel) and IRMM-385 (Kaolin)), systematic optimisation of the dispersion protocols using DLS or CLS was not feasible and of limited relevance as these materials are likely to be limited to EM or BET analysis, which both require limited optimisation for colloidal stability. Consequently, the protocol development for these materials has mainly concentrated on achieving sufficient de-agglomeration in simple aqueous media to make them suitable for the preparation of TEM samples without the need for long term stability.

Also for IRMM-382 (MWCNT) the argument about analysis being limited to TEM applies, but as these materials are strongly hydrophobic and composed of tangled bundles which cannot easily be separated it was necessary to undertake a more detailed optimisation of the protocols as the use of a surfactant is critical. In this case, the commonly used 'universal' surfactant BSA was explored and found to useable but the dispersions tended to re-agglomerate and consequently two other alternatives were examined. The first, Triton-X100, was probably the most effective in stabilising higher concentrations of the MWCNT in aqueous solution but a relatively high level of surfactant in solution was required and foaming during probe sonication may be produced. The second material, tannic acid, was able to stabilise only lower concentrations of the MWCNT but the relative concentration of additive need was also lower and the solutions did not produce problems of foaming.

For mineral type products (TiO_2, $CaCO_3$ and $BaSO_4$) a single, commonly used dispersant, Sodium Hexametaphosphate (SHMP) was found to be generally applicable. More complex procedures using more exotic surfactants or combinations of surfactant were necessary only in the case of the organic materials (basic methacrylate co-polymer (IRMM-389) and the diarylide Pigment Yellow 83 (IRMM-380 and IRMM-386)).

Finally, BAM-11 (zeolite) was tested with a number of stabilising agents but no advantage was found over the use of pure water. Overall, this material could be dispersed in solution but the resulting particulates had sizes from the nanorange to the micrometre range.

26.4 Sample preparation for NM in products

A SOP for analysis of Fe_2O_3 in Polyethylene Matrix with Electron Microscopy methods was developed (Chapter 17). It illustrates protocols for preparation of products for microscopy methods and covers sample preparation and fully automatic particle size distribution analysis of Fe_2O_3 nanoparticles in high density polyethylene. The scope of the sample preparation can be extended to any nanocomposite soft material that can be cut by an ultramicrotome; and the analysis guidelines are valid for any complex nanoparticle agglomerates.

Three SOPs for analysing the presence of TiO_2 in sunscreen were developed, one by electron microscopy (Chapter18), one by AF4-MALS-ICP-MS (Chapter 22), and one by spICP-MS (Chapter 23). Also for TiO_2 a SOP for sample preparation and spICP-MS analysis of TiO_2 nanoparticles in suspensions (Chapter 24) was developed.

One SOP for sample preparation and spICP-MS analysis of Al_2O_3 nanoparticles in toothpaste (Chapter 25) was developed.

Two SOPs for characterisation were developed: a SOP for measuring particle size distribution measurement of $BaSO_4$ using Line-Start Disc Centrifuge with Optical Detection (Chapter 20), and a SOP for size characterisation of suspended particles by AUC-RI with speed ramp option (Chapter 19).

Finally a SOP for measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software (Chapter 21) has been presented.

SOPs for products were found to be highly targeted towards each product, and it is evaluated that a SOP would be needed for each combination of <product, NM, analytical technique>.

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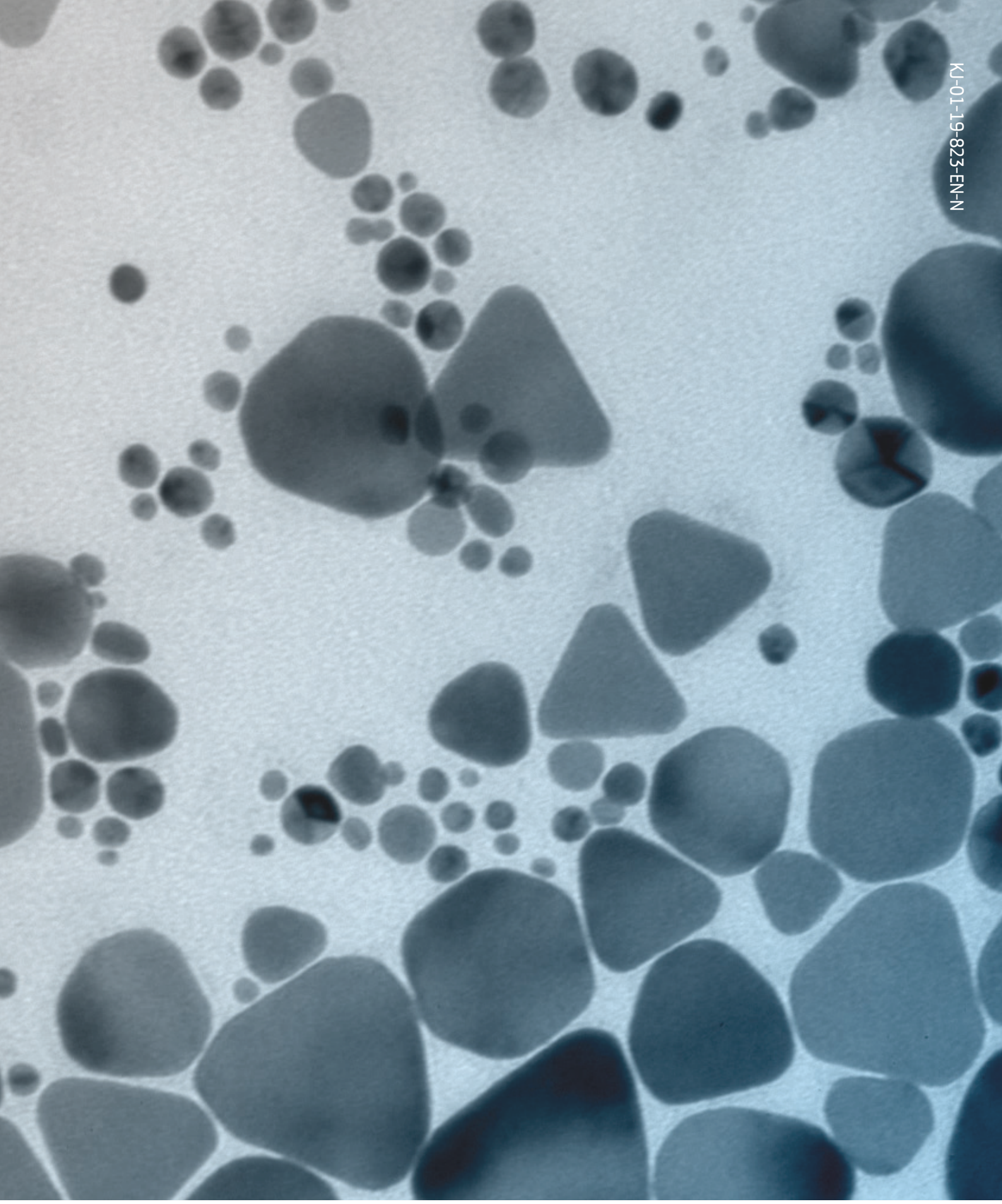

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