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Development of a specific OECD Test Guideline on Particle Size and Particle Size Distribution of Nano-materials

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Development of a specific OECD Test Guideline on Particle Size and Particle Size Distribution of Nanomaterials

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Kurzbeschreibung

Im Rahmen des Forschungsprojekts wurde eine neue OECD-Prüfrichtlinie (TG) für die Bestimmung von Partikelgrößen und Partikelgrößenverteilungen von Nanomaterialien entwickelt, da die existierende OECD TG 110 zur Bestimmung von Partikelgrößen in Bezug auf den anwendbaren Größenbereich und die gegebenen Methoden veraltet ist bzw. den Nanometerbereich < 200 nm nicht abdeckt. Mit ihrem Anwendungsbereich von 1 bis 1000 nm deckt die neue Prüfrichtlinie (TG PSD) die gesamte Nanoskala ab. Die TG PSD ist für partikel- und faserförmige Nanomaterialien anwendbar. Durch die, in der TG PSD vorgeschriebene, paarweise Messung von Faserdurchmesser und -länge ermöglicht diese TG zum ersten Mal Fasern hinsichtlich ihrer größenabhängigen Gefahrstoffeigenschaften zu unterscheiden. Die Messanweisungen aller enthaltenen Methoden wurden im Rahmen von zwei getrennten Ringversuchen validiert, da bei der Anwendung der Methoden eine Unterscheidung zwischen Partikeln und Fasern gemacht werden muss.

Neben Angaben zum Inhalt und Struktur der TG PSD, befasst sich der vorliegende Abschlussbericht mit den wesentlichen Schritten, Überlegungen und organisatorischen Aspekten bei der Entwicklung der Prüfrichtlinie. Darüber hinaus werden Einblicke in die Auswahl, Vorbereitung und Prävalidierung der im Ringversuch verwendeten Testmaterialien gegeben. Schließlich werden die wichtigsten Ergebnisse aus den Ringversuchen und ihre Auswirkungen auf die TG PSD vorgestellt.

Abstract

In this research project, a new OECD Test Guideline (TG) for the determination of “Particle Size and Particle Size Distributions of Nanomaterials” was developed as the existing OECD TG 110 is considered to be outdated in terms of applicable size range (not covering sizes <200 nm) and methods. By its scope with an applicable size range from 1 to 1000 nm the new Test Guideline (TG PSD) covers the whole nanoscale. The TG PSD is applicable for particulate and fibrous nanomaterials. The prescribed, pairwise measurement of fibre diameter and length in the TG PSD allows for the first time to differentiate fibres with regard to their size-dependent hazard properties. Measurement instructions for each included method were validated within two separated interlaboratory comparisons, as a distinction between near spherical particles and fibres when applying the methods has to be made.

Besides information on content and structure of the TG PSD, this final report outlines essential steps, considerations and organisational aspects during the development of the TG. Insights into the selection, preparation and prevalidation of test materials used in the interlaboratory comparison are given. Finally, main results of the interlaboratory comparisons and their impacts on the TG PSD are presented.

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List of Abbreviations

AEG	Advisory Expert Group
AFM	Atomic Force Microscopy
Ag	Silver
Au	Gold
AUC	Analytical Ultra Centrifuge
CLS	Centrifugal Liquid Sedimentation
DFSTEM	Dark-field Scanning Transmission Electron Microscopy
DLS	Dynamic Light Scattering
DMAS	Differential Mobility Analysis System
FFF	Field Flow Fractionation
ILC	Interlaboratory Comparison
JEG	Joint Expert Group
MWCNT	Multi-walled Carbon Nanotubes
OECD	Organisation for Economic Co-operation and Development
PSL	Polystyrene
PTA	Particle Tracking Analysis
SAXS	Small-Angle X-Ray Scattering
SEM	Scanning Electron Microscopy
SiC	Silicon Carbide
SiO_2	Silica
SOP	Standard Operation Procedure
sp AMS	Single Particle Aerosol-Mass Spectrometry
sp ICP-MS	Single Particle Inductively Coupled-Mass Spectrometry
SPSF	Standard Project Submission Form
TEM	Transmission Electron Microscopy
TG	OECD Test Guideline
TG PSD	TG on Particle Size and Particle Size Distribution of Nanomaterials
TiO_2	Titanium Dioxide
TRPS	Tuneable Resistive Pulse Sensing
UAS	Ultrasonic Attenuation Spectroscopy
WNT	Working Group of the National Coordinators for the Test Guidelines Programme
WPMN	Working Party on Manufactured Nanomaterials
XRD	X-Ray Diffraction
ZnO	Zinc Oxide

Zusammenfassung

Die "Working Party on Manufactured Nanomaterials" (WPMN) der OECD begleitet seit mehreren Jahren die internationale Diskussion zu Sicherheitsaspekten von hergestellten Nanomaterialien. Sie hat durch die Erarbeitung von nanospezifischen Prüfrichtlinien (TG), Leitfäden und einer Vielzahl weiterer Berichte einen aktiven Beitrag zum sicheren Umgang mit Nanomaterialien geleistet.

Die TG zur Bestimmung der Partikelgrößenverteilung und der Faserlängen- und Durchmesserverteilung (OECD TG 110) wurde von der OECD-WPMN als eine der TG identifiziert, die nanospezifisch aktualisiert werden muss. Die bevorzugte Alternative zur Aktualisierung war eine neue TG für die Bestimmung der Partikelgröße und Partikelgrößenverteilung von Nanomaterialien (TG PSD). Das UBA beauftragte die BAuA (Projektkoordinator) und die BAM mit der Erarbeitung einer entsprechenden Prüfrichtlinie. Mit Abschluss des Projektes wurde der Entwurf der neuen TG der Joint WPMN/WNT Expert Group (JEG) bei der OECD zur Stellungnahme vorgelegt. Dennoch werden die Projektpartner BAM - zuständig für partikelförmige Materialien - und - BAuA - zuständig für faserförmige Materialien - die Kommentierungsphase bis zur endgültigen Verabschiedung der TG durch die OECD weiterhin aktiv unterstützen. Dies wird voraussichtlich innerhalb eines Jahres nach Beendigung dieses Projektes geschehen.

In Vorbereitung der neuen OECD TG PSD wurden verschiedene Messmethoden, die für die Messung von Nanomaterialien geeignet sind, für die Aufnahme in die TG in Betracht gezogen. Aus diesen Methoden wurden diejenigen ausgewählt, die hinreichend verbreitet sind und zuverlässig und reproduzierbar für die Analyse von hergestellten Nanomaterialien eingesetzt werden können. Bei der Auswahl der Methoden wurde zwischen Methoden für partikuläre und faserige Nanomaterialien unterschieden. Diese Trennung wurde auch im Entwurf der TG PSD beibehalten und orientiert sich an der Struktur der TG 110. Für partikuläre Nanomaterialien wurden die Rasterkraftmikroskopie (AFM), die Zentrifugale Flüssigkeitssedimentation (CLS), die Dynamische Lichtstreuung (DLS), das Differential Mobility Analysis System (DMAS), das (Nano-)Partikel Tracking System (PTA/NTA), die Röntgenkleinwinkelstreuung (SAXS), die Rasterelektronenmikroskopie (SEM) und die Transmissionselektronenmikroskopie (TEM) in den Entwurf der TG PSD aufgenommen und die Vergleichbarkeit und Reproduzierbarkeit der Prüfvorschriften in einem Ringversuch (ILC, interlaboratory comparison) validiert. Eine weitere Methode, die Einzelpartikel-Massenspektrometrie mit induktiv gekoppeltem Plasma (sp-ICP-MS), wurde zur Aufnahme in den Anhang C der Prüfrichtlinie vorgeschlagen, da sie für eine Reihe von Nanomaterialien relevant ist, jedoch im Rahmen des ILC aufgrund der gerätespezifischen Limitierungen fehlender Referenzstandards nicht auf Vergleichbarkeit und Reproduzierbarkeit validiert werden konnte. Für diese Methode liegen allerdings mehrere Validierungen außerhalb dieses Projektes für spezifische Nanomaterialien vor.

Für faserförmige Nanomaterialien wurden die Rasterelektronenmikroskopie (SEM) und die Transmissionselektronenmikroskopie (TEM) in den Entwurf der TG PSD aufgenommen. Beide Methoden können gleichzeitig die Faserlänge und den Durchmesser bestimmen. Messanweisungen für beide Methoden wurden in einem ILC validiert.

Neben der Auswahl der Methoden war es auch notwendig, den Größenbereich zu definieren, für den die TG PSD anwendbar sein soll. Dieser geht über (gesetzlich) definierte Obergrenzen für Nanomaterialien hinaus, beispielsweise die der REACH-VO oder Biozid-VO. Nur so kann durch eine oder mehrere Messungen festgestellt werden, ob sich die untersuchte Substanz innerhalb oder außerhalb der gesetzlichen Grenzen befindet. Der Größenbereich wurde für Partikel- und Faserdurchmesser auf 1-1000 nm und die Obergrenze für die Faserlänge auf 20 µm festgelegt. Eine Faser ist definiert als ein Objekt mit einem Verhältnis von Länge zu Durchmesser (Aspektverhältnis) ≥ 3. Damit ergibt sich eine Überschneidung mit TG 110, die eine untere Messgrenze von 200 nm beschreibt. Der Messbereich der einzelnen im Entwurf der TG PSD beschriebenen Methoden wird in den meisten Fällen nicht den gesamten spezifizierten Größenbereich abdecken. Daher kann es notwendig sein, mehr als eine Messmethode oder einen Messaufbau zu verwenden, um den gesamten Größenbereich abzudecken. Die anwendbaren Methoden beruhen auf unterschiedlichen Messprinzipien und können sich in ihren Ergebnissen unterscheiden. Daher ist es in der Regel notwendig, mehr als eine Methode zu verwenden. Eine

dieser Methoden sollte eine bildgebende Methode sein, insbesondere REM oder TEM. Eine weitere Messmethode sollte eine gute Statistik für die Anzahlgrößenverteilung gewährleisten.

Für die Validierung des Entwurfs der TG PSD wurden zwei ILCs durchgeführt, eine für partikuläre und eine für faserige Materialien. Die internationalen Laboratorien nahmen freiwillig am ILC mit eigenen Mittel teil. Die an den Vergleichsmessungen beteiligten Laboratorien verwendeten eine oder mehrere der im Entwurf der TG PSD vorgeschlagenen Methoden. In einem Validierungsplan erhielten die Teilnehmenden genaue Anleitungen zur Anwendung der Methoden und zur Vorbereitung der Testmaterialien. Die jeweiligen Einzelergebnisse der Teilnehmenden wurden von BAM und BAuA gesammelt, bewertet und verglichen. Bei signifikanten Ergebnisabweichungen wurden die jeweiligen Teilnehmenden kontaktiert, um mögliche Ursachen zu diskutieren. Die Ergebnisse und wichtigsten Schlussfolgerungen der Ringversuche sind in diesem Abschlussbericht in gekürzter Form dargestellt. Die vollständigen Ergebnisse und alle Schlussfolgerungen sind im Validierungsbericht zusammengefasst. Nach Verabschiedung der TG durch das zuständige OECD-Gremium wird der Validierungsbericht zusammen mit der TG veröffentlicht.

Die angenommene TG wird in Abschnitt 1 der OECD-Richtlinien für die Prüfung von Chemikalien (https://doi.org/10.1787/20745753) veröffentlicht.

Summary

The OECD "Working Party on Manufactured Nanomaterials" (WPMN) has been accompanying the international discussions on safety aspects of manufactured nanomaterials for several years. It has made an active contribution to the safe handling of nanomaterials through the development of nano-specific Test Guidelines (TG), Guidance Documents and a large number of other reports.

The TG for determining particle size distribution and fibre length and diameter distribution (OECD TG 110) has been identified by the OECD-WPMN as one of the TGs that needs to be updated nanospecifically. The preferred alternative for updating was a new TG for the determination of particle size and particle size distribution of nanomaterials (TG PSD). The UBA commissioned the BAuA (project coordinator) and the BAM to develop a corresponding test guideline. With the end of the project duration the draft new TG was submitted to the Joint WPMN/WNT Expert Group (JEG) at the OECD for comments. Nevertheless, the project partners BAM - responsible for particulate materials - and - BAuA - responsible for fibrous materials will continue to actively support the commentary phase until the final adoption of the TG by the OECD. This is expected to happen within one and a half year after this project has come to its end.

In preparation of the new OECD TG PSD, various measurement methods suitable for measuring particle size and size distribution of nanomaterials were considered for being included in the TG. From those methods the ones that are sufficiently widespread and can be used in a reliable und reproducible way for the analysis of manufactured nanomaterials were selected. In the selection of methods, a distinction was made between methods for particulate and fibrous nanomaterials. This separation was also retained in the draft TG PSD and is based on the structure of TG 110. For particulate nanomaterials, atomic force microscopy (AFM), centrifugal liquid sedimentation (CLS), dynamic light scattering (DLS), differential mobility analysis system (DMAS), (nano)particle tracking system (PTA/NTA), small angle X-ray scattering (SAXS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were included in the draft TG PSD and the comparability and reproducibility of the measurement instructions was validated in an interlaboratory comparison (ILC). A further method, single particle mass spectrometry with inductively coupled plasma (sp-ICP-MS), was proposed to be included in Annex C of the testing guideline, as it is relevant for a number of nanomaterials. For this method several validations outside of this project are available for specific nanomaterials, but reproducibility and comparability could not be validated within the ILC. However, for fibrous nanomaterials, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were included in the draft TG PSD. Both methods can simultaneously determine fibre length and diameter. Measurement instructions for both methods were validated in an ILC.

Besides selecting the methods, it was also necessary to define the size range for which the TG PSD will be applicable. This range surpasses the upper limit of the size ranges currently used within (the regulatory) definition of nanomaterials. This is the only way to determine, by means of one or more measurements, whether the substance under investigation is within or outside such size ranges of currently applied definitions. The size range was set at 1-1000 nm for particle and fibre diameters and the upper limit for fibre length at 20 µm. A fibre is defined as an object with a length to diameter ratio (aspect ratio) ≥ 3. This results in an overlap with TG 110, which describes a lower measurement limit of 200 nm. The measuring range of the individual methods described in the draft TG PSD will in most cases not cover the entire specified size range. Therefore, it may be necessary to use more than one measurement method or measurement setup to cover the full size range. The applicable methods are based on different measurement principles and may differ in their results. It is therefore usually necessary to use more than one method. One of these methods should be an imaging method, especially SEM or TEM. Another measuring method should ensure good statistics for the number size distribution.

For the validation of the draft TG PSD, two ILCs were carried out, one for particulate and one for fibrous materials. Participation in the ILC was voluntary. The laboratories participated in the comparison measurements with one or more methods. In a validation plan the participants were given precise instructions on how to apply the methods and how to prepare the test materials. The respective individual results of the participants were collected, evaluated and compared by BAM and BAuA. In case of significant deviations in results, the respective participants were contacted to discuss possible causes. The results and most important conclusions of the interlaboratory comparisons are presented in abridged form in this final report. The complete results and all conclusions are summarised in the validation report. After adoption of the TG by the competent OECD body, the validation report will be published together with the TG.

The adopted TG will be published in Section 1 of the OECD Guidelines for the Testing of Chemicals (https://doi.org/10.1787/20745753).

1 Introduction

The OECD "Working Party on Manufactured Nanomaterials" (WPMN) has been accompanying the international discussions on safety aspects of manufactured nanomaterials for several years. It has made an active contribution to the safe handling of nanomaterials through the development of nano-specific Test Guidelines (TG), Guidance Documents and a large number of other reports. The TG for determining particle size distribution and fibre length and diameter distribution (OECD TG 110) from 1981 was identified by the OECD WPMN as one of the TGs that needs to be updated. Germany, represented by BMU also identified the need and agreed to take the lead in the development of a new TG on particle size distribution determination with the focus on nanomaterials. The German Environmental Agency (UBA) coordinated the activity and started the project leading to this report and the corresponding draft TG. The project itself, coordinated by BAuA, was pursued with the project partners BAM - responsible for particulate materials - and - BAuA - responsible for fibrous materials. The process of developing the draft TG along with the main decisions, steps and results are summarised in the following chapters.

These project partners agreed to further support the commentary phase of the TG development until the final adoption of the TG by the OECD. This is expected to happen within one and a half year after this project has come to its end.

2 Development of the Test Guideline

This chapter summarises the formal steps necessary for the development of a TG and presents general considerations which were necessary in the preparatory work and during the drafting of the TG PSD. The structure of the submitted draft TG PSD is based on other recently published OECD TGs and on the structure of TG 110, which also makes a distinction between methods for particulate and fibrous materials. In addition, there are two main reasons in terms of content for such a separation. a) A large number of the measuring methods considered are based on particle-shaped or spherical test objects in their measuring principle. The interpretation of the measurement data is not possible for fibrous measurement objects. b) Fibrous materials show size-dependent hazard properties, the so-called fibre toxicity. The ratio of fibre diameter and length is particularly important and both quantities should be measured in pairs according to the present draft of the TG. Deviations from this are only possible in exceptional cases.

2.1 Standard Project Submission Form (SPSF)

At the beginning of the project, the project partners prepared a draft "Standard Project Submission Form" (SPSF). This is the application to include the project in the Work plan for the TGs Programme at OECD. The draft was discussed with an expert group accompanying this project and submitted to the OECD "Working Group of National Co-ordinators of the TGs programme" (WNT) by UBA on 15 November 2017. The response from the OECD WNT was consistently positive, with comments from various delegations. The responses to the comments and the revised draft were submitted by 28 February 2018. Further comments on the SPSF were made by the delegations from Canada and BIAC at the 18th meeting of the OECD-WPMN and were responded following to the OECD WPMN. The SPSF was included in the Work plan for the Test Guidelines Programme by the OECD WNT on 26 April 2018.

An SPSF must already indicate what type of document is to be prepared, a TG or a Guidance Document to supplement a TG. In order to develop a TG, it is mandatory to carry out an ILC. In this ILC, the reproducibility and comparability of the measurement principle contained in the draft TGs is validated. Only if the measuring principle is successfully validated with regard to reproducibility and comparability, to be presented in a validation report, it can be implemented as a TG. More details on the procedure of

the ILC within this project can be found in Chapter 2.7, the results are presented in Chapter 3.

2.2 Establishing an international expert group and course of TG development

As part of the development of the TG PSD, an international expert group was set up to provide support for the development of the TG and to give advice on different topics. The expert group was set up based on expressions of interest from various experts at the 17th OECD-WPMN meeting in May 2017. During the development of the TG PSD, meetings and telephone conferences were organised with the expert group at regular intervals. Furthermore, prepared documents were sent to the expert group for comments (e.g. SPSF or the draft TG PSD). The meetings or telephone conferences with the expert group are listed below with the respective topics discussed.

In 2017, two telephone conferences were held to discuss the draft SPSF, the possible choice of reference materials and methods.

At the 18th meeting of the OECD-WPMN in February 2018, first results based on literature research and preliminary tests for the development of the TG PSD were presented and then discussed in depth in the plenary session. The discussion yielded good suggestions for further work on the TG PSD.

The first draft of the TG and the validation plan for the ILC were sent to the expert group and the participants in the ILC at the end of January 2019. This first draft was commented by the members of the expert group. The validation plan was commented by the participants of the ILC.

The international expert group met on 19.2.2019, following the 19th meeting of the OECD-WPMN in Paris. At this meeting, open questions of the TG were discussed. The most important points were:

- Use of the term nanoscale
- Definition of the measurement objects in the particle section
- Integration of international standards in the TG PSD
- Introduction of a length limit for fibres (to 20 µm)

The received comments and the results of the expert group meeting were incorporated into an improved draft TG PSD. Most changes were particular improvements relevant for the test performance in the then upcoming interlaboratory comparison.

In autumn 2019 the expert group was expanded to become the "Joint Expert Group (JEG) on physical chemical characterisation" of the OECD-WPMN and OECD-WNT. On 18 December 2019, back-to-back to an OECD-WPMN workshop in Paris, a meeting of the JEG took place. There, the draft TG PSD was presented along with first results from the ILC.

In August 2020, a revised draft of the TG PSD and a first draft of the validation report were sent to JEG for comments.

In November and December 2020, a series of four virtual meetings with the JEG was held to discuss the comments received between August 2020 and October 2020. From those comments and the discussions, a final draft TG was prepared to be submitted to the WNT.

2.3 Definitions and size range

According to DIN 1319-1 (DIN 1995), the measurand is the physical quantity to which a measurement applies. DIN 1319-1 uses the term measurand in a general sense but additional also as a specific term. For the OECD TG PSD, the particle diameter and fibre diameter / fibre length were selected as the main measurands.

In addition to the measurand, also a primary measurand exists. The primary measurand corresponds to the direct result resolved from the measurement principle, without mathematical/physical conversion (e.g. volume, mass, surface, cross-section). This primary measurand determines the weighting of a size distribution and is given as an index with the measurand.

The final measured value depends not only on the measurand but also on the measurement goal. The measurement goal thus decides whether, for example, the mean diameter or the size distribution is to be determined.

At the first meeting of the international expert group, the size range to be covered by the TG PSD was discussed. It was agreed that the lower limit for fibre and particle diameters should be 1 nm and the upper limit should exceed 100 nm. It was also agreed that there should be an overlap with OECD TG 110 (OECD 1981). The OECD TG 110 defines as lower limits: 350 nm particle diameter, 200 nm fibre diameter, 5 µm fibre length.

The common definitions of nanomaterials usually set an upper limit for the diameter of 100 nm, in exceptions 300 nm or 500 nm. Therefore, 1000 nm was chosen as a reasonable upper measurement limit for the TG PSD. This makes it possible to map the entire size distribution, even above the defined nanoscale range. The levelling of the size distributions as well as agglomerates or aggregates are thus largely within the validity range of the TG.

An upper limit for the fibre length was originally not planned. From the discussion between the project participants and the expert group, an upper limit for the fibre length of 20 µm was set, which relates to technical limits of image resolution, image size and aspect ratio of the images of electron microscopy.

The proposed measurement limits of 1-1000 nm for particle and fibre diameter and 20 µm for fibre length were discussed and confirmed by the expert group at the second meeting. These limits were defined in the SPSF.

Particles can have arbitrary shape and show different dimensions. Following the OECD TG 110, it was decided to limit the TG to fibres and nearly spherical particles. The proposed definition for fibres was an aspect ratio ≥3, following the definition of “nanoparticle” in ISO 80004-2, and for a nearly spherical particle an aspect ratio of ≤ 2 (ISO 2015 B). The international expert group has decided that an aspect ratio of up to < 3 can also be considered as nearly spherical. The aspect ratio of ≥ 3 thus also defines the lower fibre length as 3 nm.

Another important point for defining the size range is the structure of nanomaterials. According to the definition of nanomaterial in ISO 80004-1(ISO 2015 A) the outer equivalent diameter is decisive for the characterisation of particle size. This includes any coating of the nanomaterials with stabilising agents or functional groups. This ISO definition was adopted in the OECD TG PSD.

For the definitions of particle, aggregate, agglomerate, primary particle, the definitions in ISO 80004-2 were proposed and accepted by the international expert group. At the meeting of the JEG of the OECD-WNT in December 2019, it was finally decided that the OECD as an international organisation should follow existing and agreed definitions used in ISO standards as far as possible. This resulted in the following texts for the TG definitions for the second commenting round of the JEG:

agglomerate [(ISO 2015 B); 3.4**]**
collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components
Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

aggregate [(ISO 2015 B); 3.5**]**
particle comprising strongly bonded or fused particles where the resulting external surface area maybe significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

nanomaterial [(ISO 2015 A); 2.4**]**
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale
Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.
Note 2 to entry: See also engineered nanomaterial, manufactured nanomaterial and incidental nanomaterial.

nanoparticle [(ISO 2015 B); 4.4]
nano-object with all external dimensions in the *nanoscale* where the lengths of the longest and the shortest axes of the nano-object do not differ significantly
Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as *nanofibre* or *nanoplate* may be preferred to the term nanoparticle.
Note 2: The terms *nanofibre* or *nanoplate* are intended to be used instead of the term nanoparticle [ISO 80004-4].

particle [(ISO 2015 B); 3.1**]**
minute piece of matter with defined physical boundaries
Note 1 to entry: A physical boundary can also be described as an interface.
Note 2 to entry: A particle can move as a unit.
Note 3 to entry: This general particle definition applies to nano-objects.

primary particle [(ISO 2015 B); 3.2**]**
original source particle of agglomerates or aggregates or mixtures of the two

Revisions to the definitions used for this TG had to be done to pick up the feedback received from members of the JEG for the Draft TG which was then submitted for the first WNT commenting round in July 2021.

2.4 Structure of the Test Guideline

At the beginning of the current draft TG PSD, the scope, significance and use of the TG PSD are presented, initial considerations and limitations of the TG PSD are given. The following section deals with aspects of sample preparation. However, due to the variety of different nanomaterials, the TG PSD does not include explicit preparation instructions. Next, all included methods are presented. First for particles and then for fibres the applicability, requirements and limitations of the methods are explained before validated instructions for carrying out and evaluating the measurement are given. Finally, the TG PSD provides a structure for the test report. The appendix contains definitions and documentation standards according to ISO standards and additional information on the evaluation of fibre samples. To better visualize the structure of the TG PSD, Figure 1 shows the Table of content of the draft TG.

Figure 1: Table of content of the draft TG PSD (July 2021), listing first the five common chapters for particles and fibres Introduction, Definitions, Scope, significance and use, Initial considerations and Limitations, and Aspects of sample preparations. This is followed by the specific parts for particles and fibres, each naming all methods available in this TG and concluding with the two again common chapters on recommended materials for the validation of the tests and the Test report. References and Appendixes A to E conclude the Table of content.

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2.5 Method Selection

2.5.1 Particles

Based on (OECD 2016), JRC Reference Report Requirements on measurements for the implementation of the European Commission definition of the term nanomaterial (JRC 2012, Linsinger et al. 2012), (CEN 2016) and (ISO 2016) the methods known for the determination of particle size and number size distribution were identified. From these in principle available methods, a selection of methods was made on the basis of the general applicability of the method for the determination of particle size and number size distribution as well as the commonness and prevalence of the method, which were examined more closely.

For the examination method-specific standards or scientific publications were used if no method-specific standard was available. Table 1 shows the selected methods and, in italics, some additional methods that can also characterise particle size but were not further considered. The methods not further considered are methods that are not widely established or still in an experimental stage (e.g. ultrasonic attenuation spectroscopy, UAS and single particle aerosol mass spectrometry, sp-AMS), cover

For the examination method-specific standards or scientific publications were used if no method-specific standard was available. Table 1 shows the selected methods and, in italics, some additional methods that can also characterise particle size but were not further considered. The methods not further considered are methods that are not widely established or still in an experimental stage (e.g. ultrasonic attenuation spectroscopy, UAS and single particle aerosol mass spectrometry, sp-AMS), cover the size range below 100 nm only slightly (e.g. controllable resistance pulse detection, TRPS), are applicable only for a small amount of material classes and determine only the mean particle size without size distribution (e.g. X-ray diffraction, XRD), or are only capable for particle size classification and not for size determination (e.g. field flow fractionation FFF). If methods such as sp-AMS are established beyond the experimental stage, they could be included in a future revision of the TG PSD.

Table 1: Overview of the methods considered in the project and their characteristics and limitations. The methods marked in italics were not included in the ILC.

Method	Size range	Measuring medium	Size distribution	Limitations
TEM	>1 nm	Vacuum	Number based	- Time consuming measurement - Expensive to purchase and operate - Weak statistics
SEM	>5 nm	Vacuum	Number based	- Time consuming measurement - Expensive to purchase and operate - Weak statistics
DMAS	1-1000 nm	Aerosol	Number based	- No distinction between particles and agglomerates - Only for airborne materials
DLS	>1 nm	Dispersion	Volume based	- Not well applicable for polydisperse materials - No distinction between particles and agglomerates
CLS	>20 nm	Dispersion	Mass based	- No distinction between particles and agglomerates
PTA	>20 nm	Dispersion	Number based	- No distinction between particles and agglomerates - Dissolution of particles due to solubility
SAXS	5 - 300 nm	Dispersion	Volume based	- Not well applicable for polydisperse materials
sp ICP-MS	10-1000 nm	Dispersion	Number based	- Only applicable for specific materials - Dissolution of particles due to solubility
AFM	>1 nm	Powder	Number based	- Time consuming measurement - Statistics - Preferred orientation, only the height of the particles is determined
FFF	*1-200 nm*	*Dispersion*	*Detector dependent*	- *Only classification of particle sizes* - *no size determination*
UAS	*>5 nm*	*Dispersion*	*Volume based*	- *No distinction between particles and agglomerates*

Method	Size range	Measuring medium	Size distribution	Limitations
sp-AMS	*40-1000 nm*	*Aerosol*	*Number based*	- *No distinction between particles and agglomerates* - *Only for airborne materials*
XRD	*1-100 nm*	*Powder*	*None*	- *Does not determine size distribution* - *Only for crystalline materials*
TRPS	*>50 nm*	*Dispersion*	*Volume based*	- *No distinction between particles and agglomerates*

The current state of knowledge was collected for the various methods for determining particle size and number size distribution, for sample preparation, and generally for the presentation and calculation of measurement results. In order to avoid different instructions, especially standards published or in preparation for publication were considered.

For each method it was determined whether standards exist and whether they are relevant for the project. Normally, standard operating procedures (SOPs) for calibration and technically correct execution of an analysis are available for all common methods. For the majority of the methods, SOPs specifically for particle size characterisation in the nanoscale also exist or are currently being developed. The SOPs for the methods SEM, TEM and DLS also refer to existing results of other ILCs. This included supplement information to SOPs, and also resulted in an overview on already performed comparisons of particle size determination using different methods. This literature research served as a first basis for the applicability and limitations of the respective methods, which were subsequently tested further.

2.5.2 Fibres

Only imaging methods can be used to determine the length and diameter of the fibres in pairs, as other methods cannot provide information about the geometric properties of the fibres. Imaging methods with a resolution in the nanometre range are SEM, TEM, as well as AFM. Table 2 shows an overview of the properties and limitations of these methods. The AFM has not been included as a method in the TG PSD because of its disadvantages in length measurement and the low image stability for large images.

Table 2: Overview of the methods considered in the project and their characteristics and limitations. The methods marked in italics were not included in the ILC.

Method	Size range	Measuring medium	Advantages	Disadvantages
TEM	>1 nm	Vacuum	- Very high resolution possible - Method with sufficient dissemination - aspect ratios >100 possible	- Long recording times - Expensive to purchase and operate
SEM	>5 nm	Vacuum	- High resolution possible	- Long recording times - Expensive to purchase

Method	Size range	Measuring medium	Advantages	Disadvantages
			- Method with sufficient dissemination - Automated image acquisition possible - aspect ratios >100 possible	
AFM	*>1 nm*	*Powder*	- *High vertical resolution possible* - *Widely used method* - *Cheap to buy* - *3D information partially available*	- *Very long recording times, low image stability with large images* - *Larger length error* - *Lateral resolution is not sufficient* - *Aspect ratios >100 difficult*

2.6 Sample Preparation

For all methods the sample preparation can have a great influence on the subsequent analysis, thus the standards and preparation guidelines for the subsequent analysis were determined as well. The sample preparation is often carried out using a dispersion. Some methods even measure the particles in dispersion. As the preparation of a stable dispersion is strongly dependent on the material, there is no generally valid standard procedure. However, recommendations are made in the TG PSD which techniques can be generally applied for certain classes of materials to obtain a stable dispersion and how to assess whether a dispersion is stable or not (ISO 2013, OECD 2017). For size characterisation, the dispersion should be stable over the entire measurement period, i.e. there should be no relevant change in the properties of the dispersion that affects the measurement result. The particles must therefore not sediment, agglomerate or dissolve. If it is not possible to disperse the complete sample, the analysis can also be carried out on the stable part of the dispersion after separating the agglomerates or aggregates. A prerequisite for this is that the particles in the separated agglomerates or aggregates have the same properties and particle size distribution as the dispersed particles and the statistics are therefore not falsified. How a sample was dispersed must always be included in the test report of results based on the TG PSD.

2.7 Performance of the Interlaboratory Comparison

Within the framework of the development of the TG PSD, two interlaboratory comparisons were carried out to validate the TG PSD. There was one ILC for the fibre-specific part and another one for the particle-specific part. Although two separate ILCs were carried out, some organisational matters of the particle and fibre ILC were carried out together.

33 institutes participated in the particle ILC. Nine methods were included in the particle ILC. Since some institutes participated with several methods, all methods were well covered with at least five

participating laboratories. 15 institutes took part in the fibre ILC. Each participating laboratory was provided with an individually defined material priority list for the examination of the fibre samples to ensure that - despite limited laboratory capacities - an adequate number of results were obtained for each test material.

Prior to the ILC, the participants received the validation plan and extracts from the draft TG PSD. In addition, a total of three telephone conferences were held with the ILC participants. The ILC was explained in detail and open questions regarding the execution of the test were clarified. The telephone conferences offered were attended very well. A total of 29 participants jointed the telephone conferences. From received comments and output from the telephone conferences on the draft TG PSD and the validation plan some technical and scientific and additions were made. Some important adjustments are listed below:

- The valid aspect ratio for particles in the draft TG PSD was adjusted from 2 to 3 in agreement with the international expert group. This closed the gap to the fibres.

- For analysis by means of analytical centrifugation, the option of measurement with gradual increase of speed during the measurement was added.

- If possible, the sample preparation for the microscopic methods is done by spin-coating and not by the drop-on method.

- For the imaging methods SEM and TEM, the evaluation of particle systems with strongly varying particle sizes is adapted. If different magnifications for image acquisition are necessary for the reliable evaluation of the different size fractions of a particle system, the same area should be analysed for each size fraction. In this way, the ratio of the respective size fractions should be reliably determined.

- For fibre analysis, instead of counting all aggregates, it is to record for each image whether it contains non-evaluable fibre aggregates. This should allow a qualitative evaluation of the fibre aggregate frequency.

On 26 March 2019, the samples for the fibre ILC were shipped; the particle samples were shipped in the period 1-5 March 2019. The official start of the ILC was after the dispatch of the updated validation plan and draft TG PSD. The scheduled running time of the particle ILC was from 02 May 2019 to 02 July 2019 and that concerning the fibre ILC from 09 April 2019 to 10 June 2019. However, due to extended dispatch times or individual delays at some laboratories, the running times of the ILC were adjusted. The submission deadline for fibre results was postponed to 10 August 2019 and for particle results to 30 September 2019.

Following the ILCs, a first draft of the validation report was prepared. The validation report lists the results, influences on the measurement and the resulting conclusions for the revision of the draft TG PSD. The report was sent to the ILCs participants at the end of May 2020. Subsequently, the results were discussed with the participants in telephone conferences for each method. Based on this discussion, the validation report was revised. Afterwards, the report was submitted together with the draft TG PSD to the OCED-JEG in August 2020.

3 Interlaboratory comparisons

In this chapter the most important aspects and results of the interlaboratory comparisons for particle and fibre sizing are presented and discussed. A more detailed discussion of the results of the interlaboratory comparisons will be published in the context of the validation report of the TG PSD (to be published at the webpages of the OECD TG programme).

3.1 Interlaboratory comparison for particle size determination

3.1.1 Material selection and preparation

Various test materials were selected for the performance of the ILC. The focus was on the coverage of the size range of 1-1000 nm, as well as on the coverage of different material properties and material classes. The aim was to test the reproducibility and comparability of the individual methods and measurement instructions by using materials with different properties. The individual methods determine the diameter using different physical principles. This leads to the fact that some nanomaterials cannot be reliably characterised with some methods. By using different nanomaterials with different properties, the limitations of the individual methods are addressed in the ILC. An overview of the test materials and their properties is shown below in Table 3.

Table 3: Overview of test materials and their properties

	Ag	SiO_2	SiO_2	ZnO	PSL	TiO_2	PSL
Particle size (nm)	17	20	50	100	90/125	200-300	80/800
Core + shell	x					x	
Aspherical				x		x	
Amorphous		x	x		x		x
Metallic	x						
Inorganic		x	x	x		x	
Organic					x		x
Soluble				x			
Functionalisation	x					x	
Crystalline	x			x		x	
Size Mixture					x		x
Hydrophobic					x		x
Low scattering		x	x				
Powder				x		x	
Dispersion	x	x	x		x		x
Wide size distribution				x		x	x

Since not every material can be analysed with every method due to the different properties of the materials, the applicability of the individual methods was considered. Table 4 gives an overview of the suitability of the methods for characterising the respective material.

Table 4: Overview of the suitability of the individual methods for the selected test materials

Method	Ag 17 nm	SiO_2 20 nm	SiO_2 50 nm	ZnO 100 nm	PSL 90/125 nm	TiO_2 200-300 nm	PSL 80/800 nm
TEM	* Core	* Var.	* Var.	* Stat.	+	* Stat.	O Stat.
SEM	+	* Var.	* Var.	* Stat.	+	* Stat.	O Stat.
DMAS	+	+	+	O Disp.	+	O Disp.	* Lim.
DLS	* SNR	+	+	* Disp.	*	* Disp.	*
CLS	+	O SNR	O SNR	O Disp.	* SNR	O Disp.	* SNR
PTA	+	- SNR	- SNR	O Disp.	+	O Disp.	- Stat.
SAXS	* Core	+	+	- Poly	*	- Poly	- Lim.
sp ICP-MS	- SNR	- SNR	- SNR	O Disp.	- SNR	O Disp.	- SNR
AFM	+	+	+	* Stat.	*	* Stat.	O Stat.

+: suitable
*: suitable with slight restrictions
o: suitable with consideration of expert knowledge (specific protocol)
-: not suitable
Restrictions:
Core: Result refers only to the core of the particle
Disp.: Difficulties in dispersion / stability of dispersion
Lim: Outside the definite measuring range of the method
Poly: Material to polydisperse
SNR: Poor signal-to-noise ratio
Stat: Statistical inaccuracies due to agglomerates and/or overlapping particles.
Var: Material changes due to measurement conditions (e.g. radiation damage during longer analysis duration, shrinkage in vacuum)

After suitable methods for the determination of particle size and distribution of the chosen test materials were selected according to the current state of knowledge, the experimental considerations for the methods were performed. The selected test materials were characterised with the respective method with specific regard to their particle size. In this process, problems arising from sample preparation as well as general errors that may occur when using the method were identified. On the basis of this, a method-specific and material-specific and reproducible sample preparation was obtained. The parameters, limits and application errors relevant for the respective method were considered in the validation plan for information of the ILC partner laboratories and in the draft TG PSD.

3.1.2 Instructions and Standard Operating Procedure (SOP)

For the ILC, SOPs were developed for each method and the respective test materials. A basic structure was developed, with taking into consideration the SOPs of DaNa 2.0, NanoSafety and NanoDefine. The standard structure of the SOPs has the following outline:

- Scope
- Definitions
- Method overview/Principle
- Requirements
- Sample preparation
- Instrument
- Instrument calibration
- Measurement performance
- Data acquisition and calculation
- Evaluation of results
- Report of results

Each SOP contains a device-specific part which is relevant and hence included in the TG PSD. Furthermore, there is a material-specific part, which is only relevant for the materials used in the ILC and is included in the validation plan. In addition to the SOPs, a template for the submission of results was developed. In this template, the data listed in the draft TG PSD for the report are requested.

3.1.3 Feedback rates

The feedback rates to the ILC are listed in Table 5.

Table 5: Feedback rates for the different methods

Methods	SEM	TEM	AFM	DLS	CLS/AUC	PTA	SAXS	DMAS	sp ICP-MS
Registered	13	12	7	22	8	8	5	6	6
Submitted	9	11	6	21	5	6	4	6	5
Feedback rate%	69	92	86	95	63	75	80	100	83

The overall feedback rate was 82%. The following Table 6 shows the number of results that were submitted for the individual materials used in the ILC.

Table 6: Feedback rates for the different materials

Material	SEM	TEM	AFM	DLS	CLS/AUC	PTA	SAXS	DMAS	sp ICP-MS	overall
Ag	5	6	-	12	4	5	4	6	-	45
SiO_2 (20 nm)	6	5	-	13	4	-	5	5	-	41
SiO_2 (50 nm)	-	6	5	15	4	-	5	5	-	38
ZnO	2	3	3	15	3	4	-	-	-	30
PSL (90/125 nm)	6	7	6	7	3	3	2	2	-	36
TiO_2	3	4	-	13	4	5	-	3	4	36
PSL (80/800 nm)	4	3	-	10	4	-	-	3	-	24

The "-" denote that no results for this material and method were reported.

3.1.4 Overview of the results

Below the results of each material with the respective results of the methods are presented. The order of the methods in the graphs (x-axis) is based on the respective determined particle diameter:

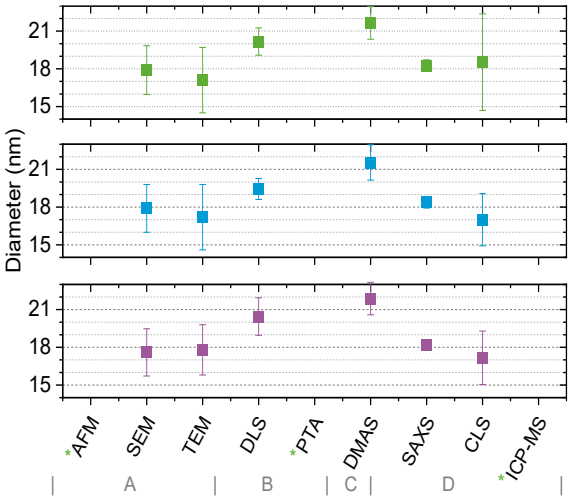
- A: Geometric equivalent diameter (AFM, SEM, TEM)
- B: Hydrodynamic equivalent diameter (DLS, PTA)
- C: Aerodynamic/mobility equivalent diameter (DMAS)
- D: Specific methods (SAXS, CLS, sp ICP-MS)

The DLS, CLS and SAXS methods determine intensity/volume-based particle size distributions. For non-spherical particles or wide size distributions this results in non-comparable values for the mean, median and modal diameter. This had to be taken into account in the ILC especially for TiO_2 and ZnO.

3.1.4.1 Silica particles (ca. 20 nm)

Figure 2 shows the comparison of the diameters retrieved from the individual methods for the silica particles (ca. 20 nm). All determined diameters are in the range of 20 ± 3 nm. The result for CLS deviates. The measurement of this material with this method is difficult, because there is only a small contrast between the particles and the dispersing solution. In general, however, a good comparability of the diameters obtained with the different methods is seen. Any deviations obtained are due to the fact that physically different diameters are determined.

Figure 2: Comparison for mean (green), median (blue) and modal (purple) diameter from the size distribution of the individual methods of silica particles (20 nm). Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable.

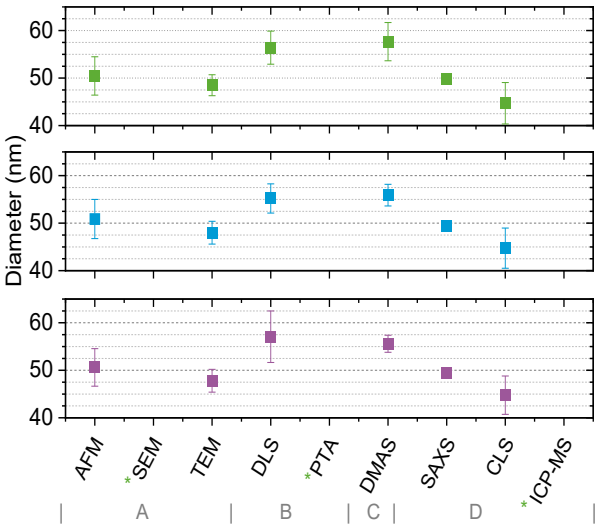


Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.2 Silica particles (ca. 50 nm)

Figure 3 shows the comparison of the diameters retrieved from the different methods for the silica particles (ca. 50 nm). All determined diameters are in the range of 50 ± 5 nm. Deviating from this is the result of CLS. The measurement of the material with this method is difficult, because there is only a small contrast between the particles and the dispersing solution. In general, however, a good comparability of the determined diameters is obtained with the different methods. Any deviations obtained are due to the fact that physically different diameters are determined with the different methods.

Figure 3: Comparison for mean (green), median (blue) and modal (purple) diameter from the size distribution of the individual methods of silica particles (50 nm). Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable.



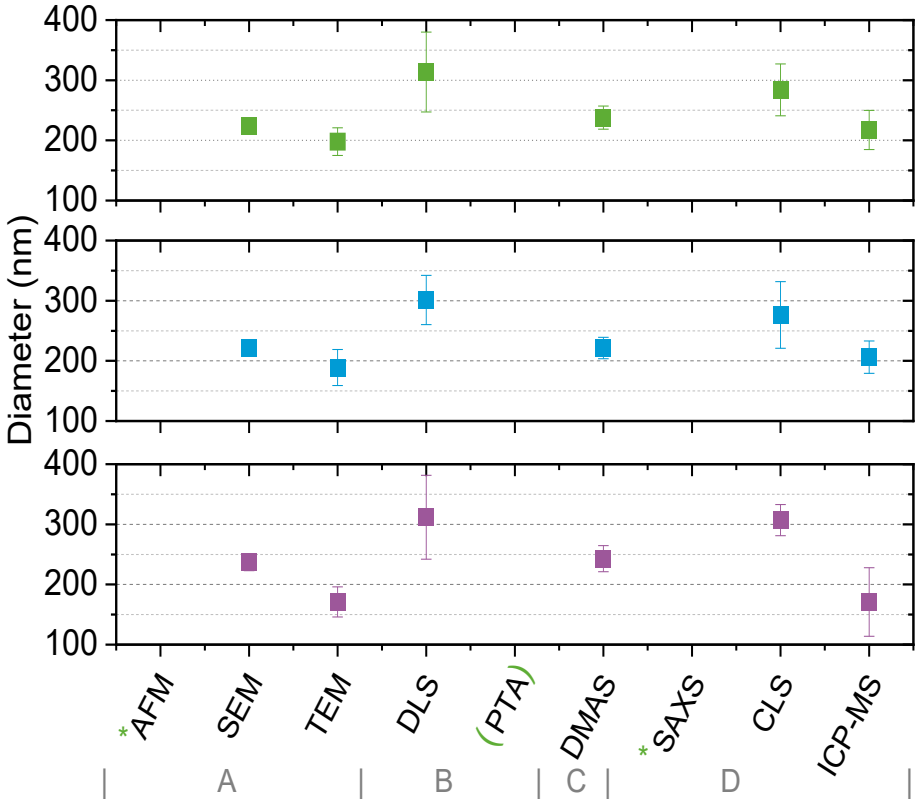
Source: Own research, Bundesanstalt für Materialforschung und -prüfung

Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.3 Titanium oxide particles (ca. 220 nm)

Figure 4 shows the comparison of the diameters retrieved from the individual methods for the titanium oxide particles. Titanium oxide is a real-life material with particles of varying shape with a wide size distribution. The material tends to agglomerate. Normally, one assumes a higher deviation in the determination of the diameter for such a material. Only for PTA it was found to have a deviation >50%. All other diameters determined are in the range of 250 ± 65 nm. Other deviations obtained are due to the fact that physically different diameters were determined.

Figure 4: Comparison for mean (green), median (blue) and modal (purple) diameter from the size distribution of the individual methods of titanium oxide particles. Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable. For methods in brackets the difference between the laboratories was more than 50% and the result is not regarded as valid.

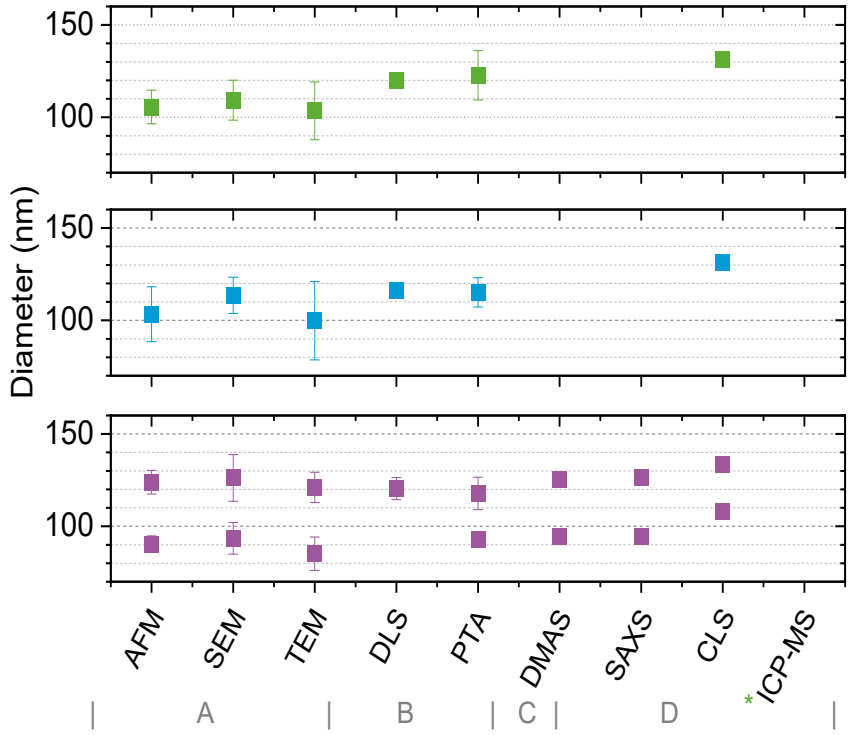


Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.4 Polystyrene particles (ca. 90 nm / 125 nm)

Figure 5 shows the comparison of the diameters retrieved from the individual methods for the polystyrene particle mixture 90/125 nm (ratio 1:1). The diameters were determined with traceable deviations. For SAXS only one result was delivered for the mean and the median diameter and thus had to be excluded. For DMAS only few results were available, which led to a high deviation of the median diameter due to the bimodal distribution. The modal diameters determined with DMAS and SAXS are in good agreement with those of other methods. The separation of the individual modes from the material could not be achieved at all with DLS and was only shown partial with PTA. The ratio of the modes could not be reliably determined with SAXS and CLS. For CLS the deviation was not calculated due to the fact that different measurement principles were applied, and the devices were not optimally equipped for a material with such low density. With the microscopic methods, well comparable diameters were determined. The obtained hydrodynamic mean and median of the diameter obtained with DLS and PTA is generally slightly higher than that of the other diameters, but well comparable within the method.

Figure 5: Comparison for mean (green), median (blue) and modal (purple) diameter from the size distribution of the individual methods of polystyrene particles (90/125 nm). Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable.

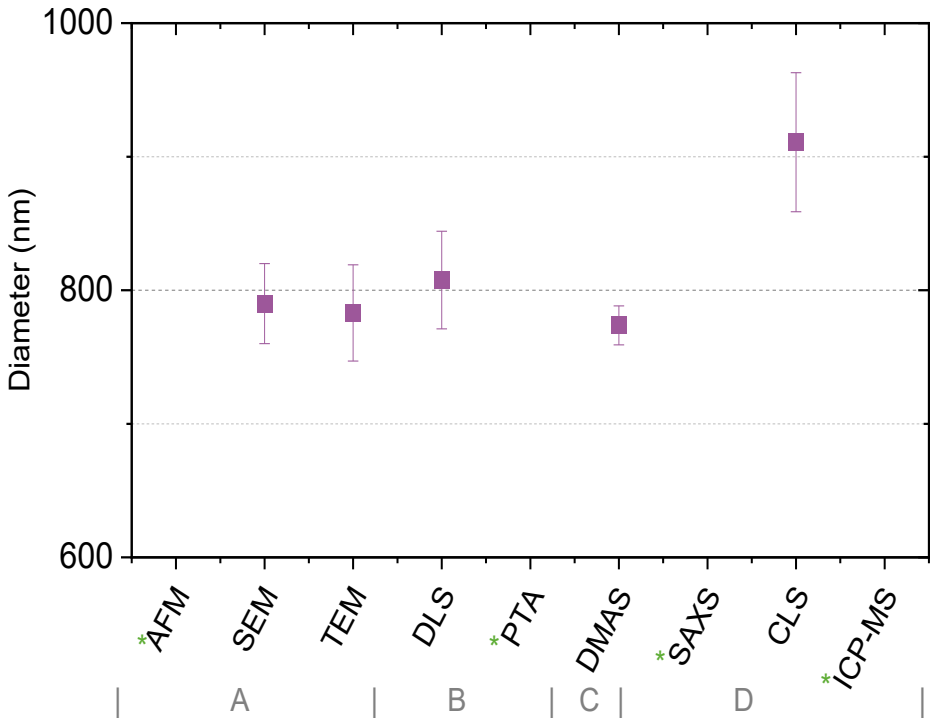


Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.5 Polystyrene particles (ca. 80 nm / ca. 800 nm)

Figure 6 shows the comparison of the diameters retrieved from the individual methods for the polystyrene particle mixture 80/800 nm (ratio 2:1). For this material, none of the methods used was able to reliably determine the bimodal particle size distribution. It is generally difficult to determine the individual particle populations and to determine the ratio correctly with such a high variance in particle size. Furthermore, particle mixtures with such a high variance in particle size are often not stable in the long term and the particles agglomerate. In some laboratories, the 80 nm particle fraction could be detected, but the ratio to the 800 nm particles could not be determined reliably. As only few data were available and these were not reliable, the total particle size distribution could not be determined for this material. However, the 800 nm particle fraction could be reliably determined and compared. The diameters for the 800 nm were determined with traceable deviations. In the following graph only the values for the 800 nm mode were used.

Figure 6: Comparison for modal (purple) diameter from the size distribution of the individual methods of polystyrene particles (800 nm). Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable.

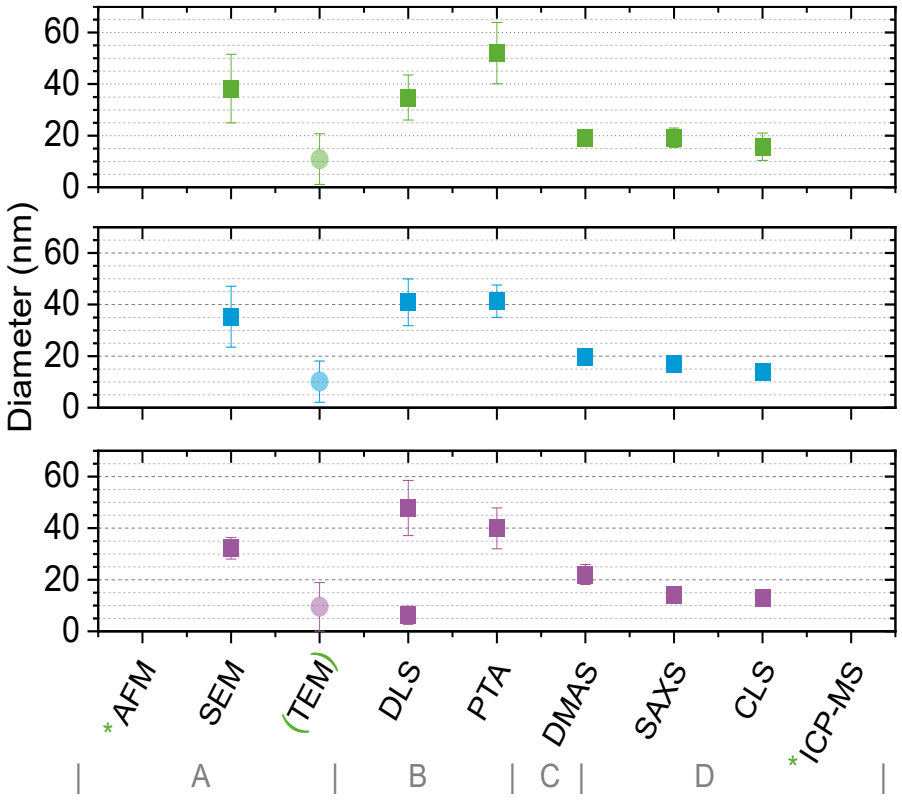


Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.6 Silver particles (ca. 18 nm)

Figure 7 shows the comparison of the diameters retrieved from the individual methods for the silver particles. This nanomaterial contains a fraction of very small particles, which leads to a bimodal character of the material and results in a higher deviation of the determined diameters between laboratories. As the particles are spherical but have a specific size distribution, this material can be considered as a transition material between calibration particles and real-life material. High deviations of the determined diameters were found for DLS and PTA in between the laboratories for the hydrodynamic diameter. The high variance can be attributed to the stabilising material contained in the material, which influences the hydrodynamic diameter. It is not clear what causes the high variance between SEM and TEM. However, TEM showed such a high variance between laboratories that the method was excluded. In some methods different modes in the size distribution could be determined, which can be attributed to the bimodal character of the material. A clear separation of the modes could only be obtained for DLS.

Figure 7: Comparison for mean (green), median (blue) and modal value (purple) of the diameter from the size distribution of the individual methods of silver particles. Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable. For methods in brackets, the difference between laboratories was more than 50%. The corresponding values are included only for information.

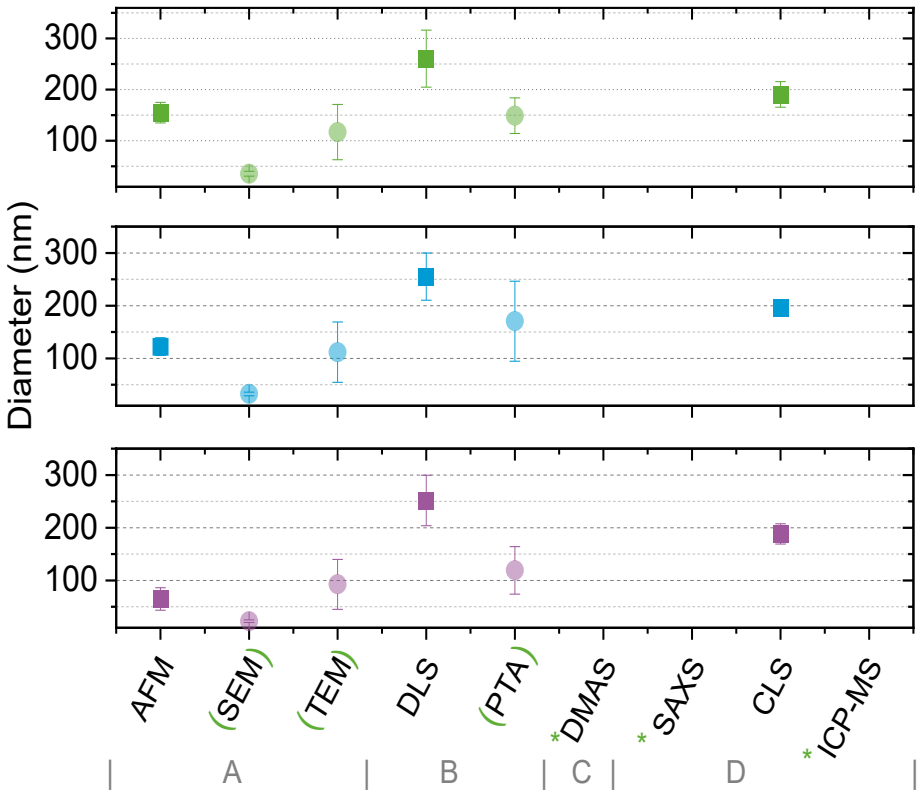


Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.4.7 Zinc oxide particles (ca. 100 nm)

Figure 8 shows the comparison of the diameters retrieved from the individual methods for the zinc oxide particles. Zinc oxide is a real-life material with particles of varying shape with a wide size distribution. The material has a high tendency to agglomerate, which leads to problems in the analysis of the material. A reliable determination of the particle size distribution for this material could only be obtained with AFM, DLS and CLS. The PTA method showed a deviation between the laboratories of more than 50%. Very few results were submitted for SEM and TEM, so that no reliable deviation could be determined here.

Figure 8: Comparison for mean (green), median (blue) and modal value (purple) of diameter from the size distribution of the individual methods of zinc oxide particles. Methods marked with a * were not part of the ILC due to incomparability of the method for this material or to keep workload manageable. For methods in brackets the difference between the laboratories was more than 50% or too few results were submitted. The corresponding values are included only for information.



Source: Own research, Bundesanstalt für Materialforschung und -prüfung

3.1.5 Impact of the interlaboratory comparison on the Test Guideline

Based on the results of the ILC the general methodology and the measurement instructions of the draft TG PSD were validated. In certain cases, some necessary refinements and clarifications to the draft TG PSD had to be made. These are listed below for the individual methods.

For AFM, it has been shown that in some cases the given pixel size is not practical to measure in a reasonable time. Nevertheless, the prescribed pixel size is not changed in the final version of the draft TG. Instead, a second way to determine particle size distribution with AFM has been added. Measurement at a higher pixel size is permitted under certain conditions. For this purpose, it must first be demonstrated by means of close-ups that there is no influence on the height determination when measuring with a higher pixel size.

For the determination of the particle size distribution by analytical centrifugation, there are three submethods which were applied in the ILC. The analytical ultracentrifuge (AUC), disc CLS and cuvette CLS. Despite slightly different approaches to the determination of size distributions, the three methods provided well comparable results. This is consistent with other ILCs, which showed good agreement of results and only minor differences between the methods (Babick et al. 2016). The deviations found were mainly related to material-specific problems in determining the size distribution with the respective sub-method. The dependence on material properties and the instrument used was already addressed in the draft TG PSD, thus, no changes based on the ILC outcomes were made to the draft TG PSD.

The determination of the particle size distribution of some laboratories by DMAS showed deviations in the reported size distributions, which are due to a different treatment of the background or correction from the contribution of the stabilising material. In the TG PSD, the measurement of the background contribution was prescribed, but instructions on how to correct these contributions were not given and were now added to the revised draft TG PSD.

When determining the particle size distribution with DLS, the evaluation algorithm used had a great influence on the particle diameters obtained. Not every algorithm can be applied to every material. The cumulant method only provides reliable particle diameters for monomodal particle systems with a narrow particle size distribution. In the ILC, some participants also used the cumulant method for bimodal and widely distributed particle systems, although this is not in accordance with the TG PSD. Therefore, paragraphs of the revised draft TG PSD have been amended to make this clear.

Within the framework of the ILC, the determination of the particle size distribution with PTA showed that the software or software version used has a great influence on the distribution obtained. Even though the revised draft TG PSD already prescribes that the software and version used must be reported, this point was further adjusted in the draft TG PSD for PTA. Furthermore, the ILC revealed the problem already noted in the revised draft TG PSD for the reliable determination of the particle size distribution of widely distributed materials. This point was further elaborated in the revised draft TG PSD.

Sp ICP-MS could only be tested with titanium dioxide in the ILC due to the material limitations of this method. For the tested material the particle size distribution could be determined with acceptable deviations between the laboratories. The size distribution obtained is also well comparable to that obtained by other methods. However, due to the small number of results for this method, the measurement instructions are not considered validated in this ILC. Other ILCs show a good applicability of the method for materials such as gold (Montoro Bustos et al. 2015), silver (Linsinger et al. 2014) and titanium oxide particles (Babick et al. 2016). In consultation with the participants in the ILC, it was decided to keep the method in the annex of the revised draft TG PSD in a modified. The JEG confirmed this procedure.

For the EM determination of the particle size distribution, some paragraphs of the draft TG PSD had to be adapted. No validation could be performed for the determination of the particle size distribution of bimodal materials with strongly varying particle size fractions. Therefore, the revised draft TG PSD was adapted and the information was marked as a non-validated recommendation. Furthermore, the specifications for automatic image evaluation were adapted. On the basis of the ILC, a complete automatic image evaluation cannot be recommended and its application in the revised draft TG PSD is clearly prohibited. Further adaptations of the draft TG, discussing the possibility of determining individual particles in agglomerates and aggregates were made after the end of this project.

In the ILC, the determination of the particle size distribution with SAXS showed a very good agreement between laboratories. Slight deviations could be attributed to different correction steps of the raw results. The instructions for correcting the raw results were adapted in the revised draft TG PSD.

3.2 Interlaboratory Comparison for Fibre Size Determination

3.2.1 Material selection and preparation

Test materials were selected for the ILC according to the following criteria:

- High number of individual fibres
- Low level of particulate impurities
- Reasonable dustiness
- Aspect ratios less than 300
- Monomodal length and diameter distribution, if possible.

Various fibrous nanomaterials were examined for their suitability as test materials: Ag, SiC, TiO_2, ZnO as well as several multi-walled carbon nanotubes (MWCNT). The MWCNTs were prepared directly from powder as aerosol on gold-coated polycarbonate pore filters. Other test materials were prepared by dispersion on silicon wafers or TEM grids.

Ag, SiC, and ZnO fulfilled the above criteria and were selected. TiO_2 was not used because with ZnO a metal oxide was already included in the ILC. To ensure that the Ag fibres had lengths below the upper limit of 20 µm, they were broken into smaller pieces by ultrasonic treatment. Some of the MWCNT nanomaterials investigated could not be brought into aerosol phase in sufficient quantity due to low dustiness. Therefore, a MWCNT nanomaterial that met all criteria was selected. In order to also be able to validate the boundary between fibres and particles by the ILC, with Au nanorods, a nanomaterial with a low aspect ratio of about 4 was included as test material. Table 7 shows the size related properties of the test materials based on manufacturer data or our own prevalidation.

Table 7: Size specifications of the test materials used based on manufacturer's specifications (*) and median values from our own pre-validation ($^+$) using SEM.

properties	Au	Ag	MWCNT	ZnO	SiC
Length [µm]	0.045*	0.8$^+$	<15* 1.0$^+$	5-50* 5.4$^+$	50-100* 7.2$^+$
Diameter [µm]	10*	20* 25$^+$	10-30* 30$^+$	50-120* 90$^+$	100-600* 140$^+$
Aspect ratio	4.5*	20$^+$	33$^+$	60$^+$	51$^+$
Width of the length distribution	1.4$^+$	1.5$^+$	2.3$^+$	2.3$^+$	3.0$^+$
Width of the diameter distribution	1.2$^+$	1.2$^+$	1.4$^+$	1.5$^+$	1.8$^+$

3.2.2 Overview of the results

13 institutes participated in the ILC for fibre size determination. They submitted a total of 55 results. The number of results is distributed among the test materials and methods as listed in Table 8:

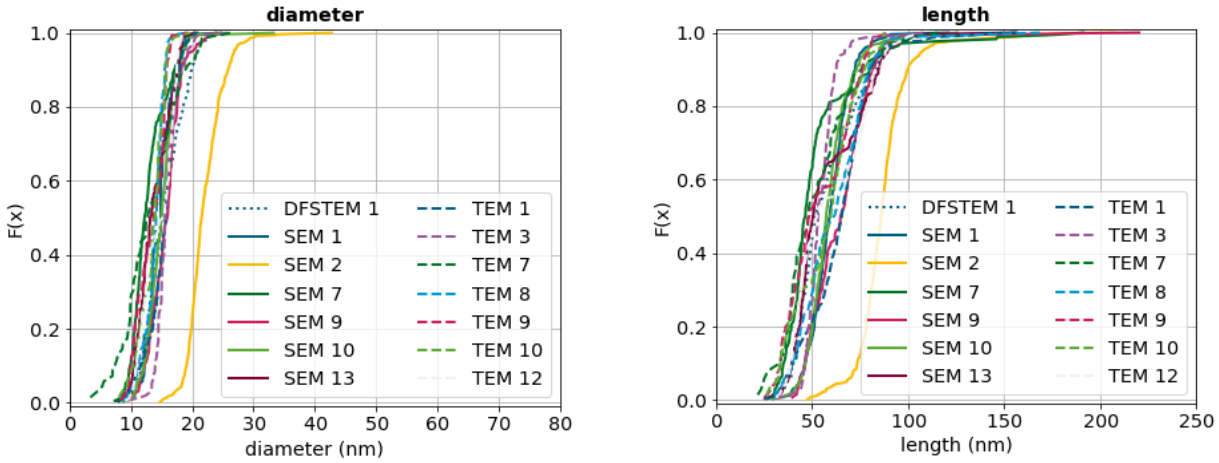
Table 8: Number of submitted results per nanomaterial and method

method	Au	Ag	MWCNT	ZnO	SiC
SEM	6	8	6	8	7
TEM	7	-	-	5	5
DFSTEM[1]	1	-	-	1	1
overall	14	8	6	14	13

For technical reasons, the nanomaterial MWCNT was only examined by means of SEM in the ILC. The pore filters used were incompatible with the TEM grids. During the ILC, the participants found that the TEM samples of Ag fibres were contaminated with sulphur. As a result, the silver fibres were sulphurised and degenerated. The TEM samples of the Ag fibres were therefore excluded from the evaluation of the results.

Using Au nanorods as an example, Figure 9 shows cumulative distribution functions for all submitted results. Plotting the cumulative distribution functions, differences between the results become apparent and outliers can be identified. In Figure 9 a clear discrepancy between the yellow curve "SEM 2" and the other results is visible. The reason for this is that short fibres were systematically not counted in the "SEM 2" measurement. This led to an overestimation of the proportion of long fibres. Since there is a positive correlation between diameter and length of a fibre, the diameter was also overestimated. Hence, the result "SEM 2" was not considered for further evaluation.

Figure 9: Cumulative distributions of all submitted results for the Au nanorods. In the left plot results for fibre diameter and in the right plot results for fibre length are shown.



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

[1] Dark-field scanning transmission electron microscopy is a TEM with a special mode of operation.

Table 9 and Table 10 provide an overview of the results of ILC for fibre length and diameter, respectively. The data given there are average values calculated from all valid results. The SEM measurements show that the results for MWCNTs have the highest variability with 42% to 45% for twice the relative standard deviation. This result was expected due to the challenging properties of the real-life material. The length determination of SiC, the test material with the longest fibres and the widest length distribution, also proved to be challenging with a relative standard deviation of 37% to 40%. The lowest variance of results was found for ZnO with a double relative standard deviation of 13% to 20%. In contrast, the TEM measurements of ZnO are much more scattered with 31% double relative standard deviation at the mean value of the diameter up to 60% at the median of the length. The measurement of SiC fibres with the TEM caused problems for the participating laboratories. Only one laboratory was able to determine the length and the diameter of enough fibres. For the other participants the maximum resolution of the instrument was not sufficient to measure the fibre lengths.

No fixed acceptable variability value can be given for the EM methods since it is influenced by several factors e.g. the operator, material properties and chosen pixel resolutions. The here given variability may be viewed as representative for analyses made following GLP.

Table 9: Overview of the results of fibre length determination.

	Au		Ag	MWCNT	Zn0		SiC	
Method	**SEM**	**TEM**	**SEM**	**SEM**	**SEM**	**TEM**	**SEM**	**TEM**
Number of valid results	5	5	7	5	5	4	4	1
Mean [nm]*	59.0 ±8.7	58.6 ±8.8	686 ±202	1390 ±620	5210 ±680	3910 ±1560	11020 ±4050	10080[2]
Relative error of the mean	0.15	0.15	0.29	0.45	0.13	0.40	0.37	-
Median [nm]*	55.8 ±14.6	56.3 ±14.1	597 ±180	1020 ±430	4080 ±600	3050 ±1840	6790 ±3030	4526[2]
Relative error of the median	0.26	0.25	0.30	0.42	0.15	0.60	0.45	-
Distribution width	1.3	1.3	1.9	2.0	2.3	2.2	2.8	4.4

*The stated absolute and relative uncertainties refer to twice the standard deviation.

[2] For SiC there is only one valid TEM measurement with pairwise length and diameter determination. Therefore, no standard deviations can be stated.

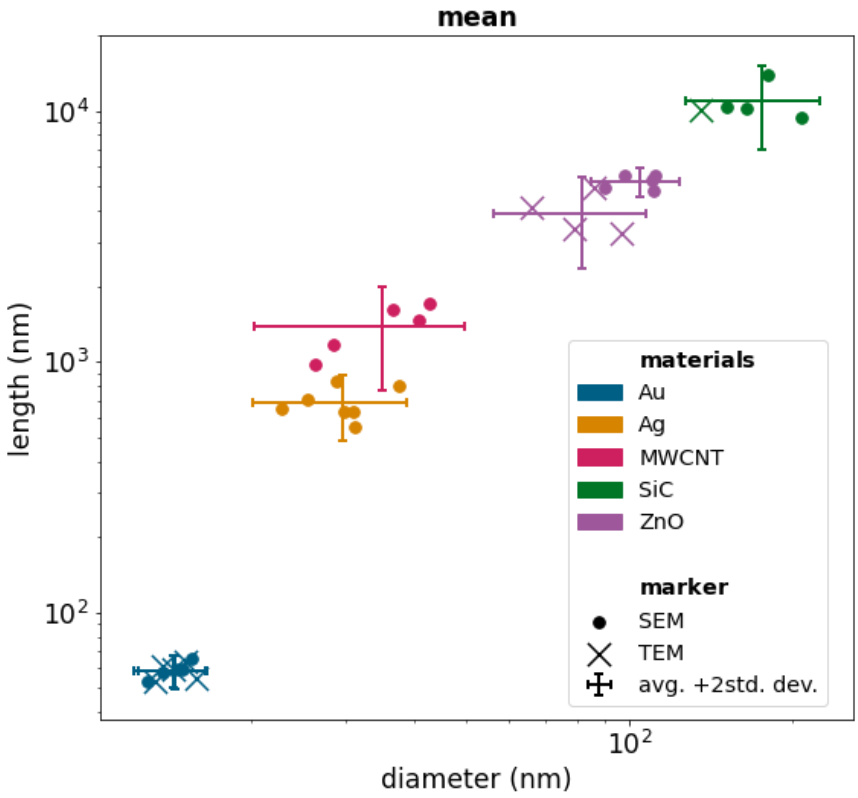
Table 10: Overview of the results of fibre diameter determination.

	Au		Ag	MWCNT	Zn0		SiC	
Method	**SEM**	**TEM**	**SEM**	**SEM**	**SEM**	**TEM**	**SEM**	**TEM**
Number of valid results	5	5	7	5	5	4	4	1
Mean [nm]*	14.4 ±2.1	14.5 ±2.1	29.5 ±9.4	35.0 ±14.7	104.2 ±19.2	81.9 ±25.7	175.6 ±48.5	135.1[2]
Relative mean error	0.15	0.14	0.32	0.42	0.18	0.31	0.28	-
Median [nm]*	14.3 ±3.0	14.9 ±1.8	29.2 ±9.1	32.8 ±14.5	93.5 ±18.5	67.7 ±32.8	148.6 ±31.8	125.1[2]
Relative median error	0.21	0.12	0.31	0.42	0.20	0.48	0.21	-
Distribution width	1.2	1.2	1.2	1.4	1.5	1.6	1.9	2.3

*The stated absolute and relative uncertainties refer to twice the standard deviation.

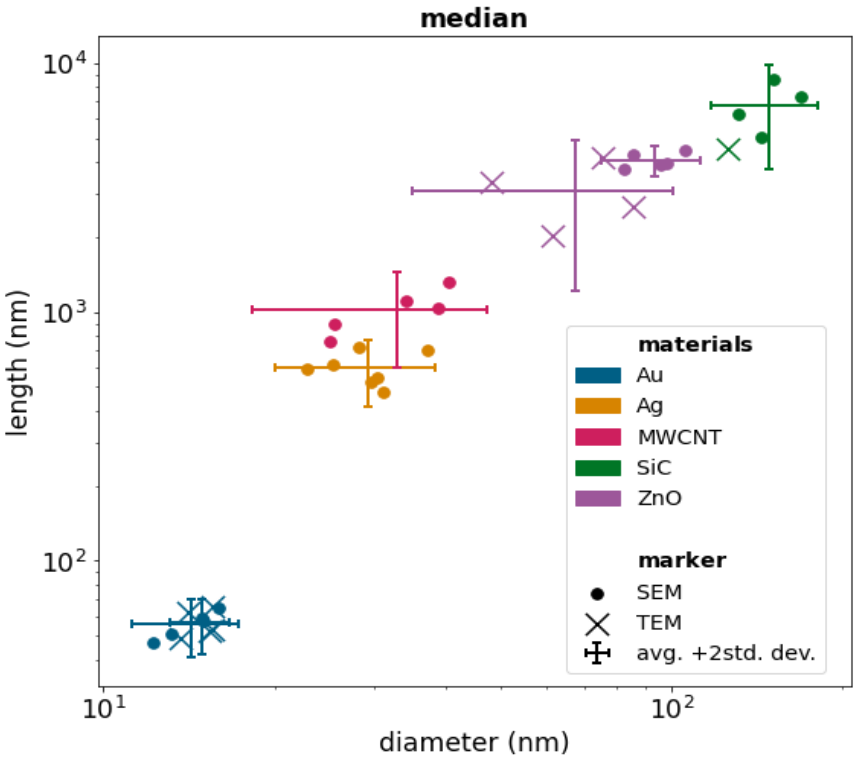
Figure 10 and Figure 11 show both the averages and the underlying individual results of means and medians in scatter plots of length vs. diameter. The axes are double logarithmic.

Figure 10: Mean values and calculated averages of length and diameter measurements of all valid results shown as length vs. diameter plot. The diagram uses a double logarithmic scale. For SiC an average value is shown only for SEM.



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

Figure 11: Medians and calculated averages of length and diameter measurements of all valid results shown as length vs. diameter plot. The diagram uses a double logarithmic scale. For SiC an average value is shown only for SEM.



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

3.2.3 Comparison of results between SEM and TEM

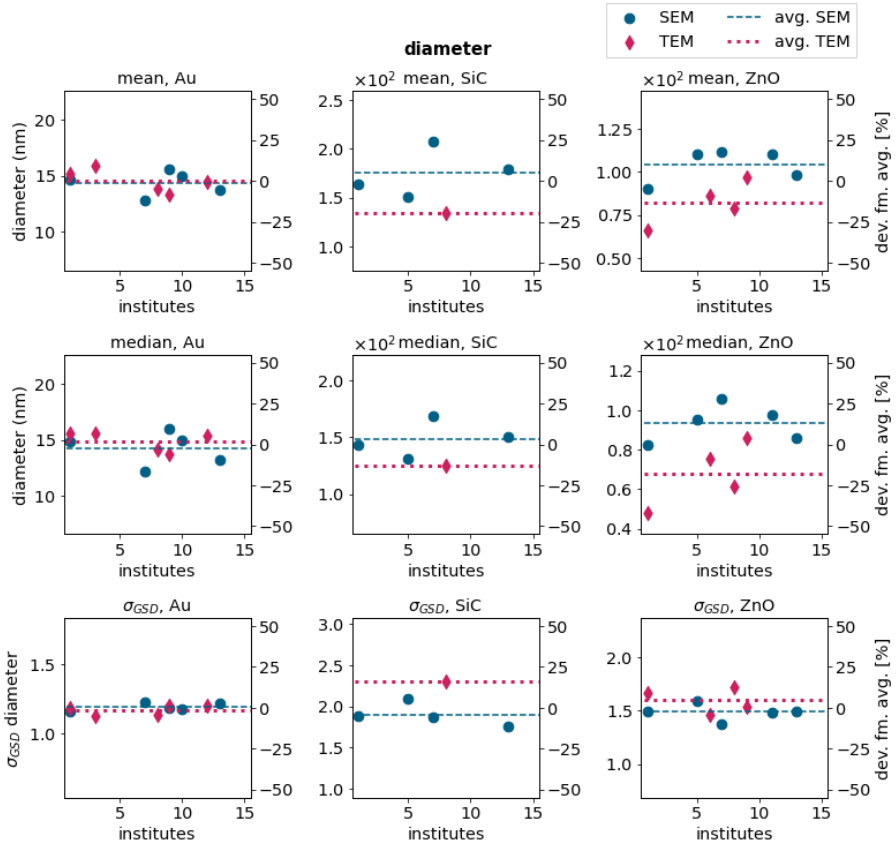
At the time of the ILC, the draft version of the TG PSD treated methods SEM and TEM together. For this reason, the extent to which the results of SEM and TEM are comparable was checked within the framework of the evaluation of the ILC. From Table 8, Table 9 and from Figure 9 to Figure 11 it can be seen that for Au nanorods the methods SEM and TEM give similar results. For SiC and ZnO, however, clear differences are visible.

The student t-test was used to determine whether there were significant differences between the methods. The test results show, with a probability of error of 5%, that there are significant differences for SiC and ZnO for the following parameters:

- SiC: mean value of the diameter distribution, geometric standard deviation of the length distribution
- ZnO: mean value and median of diameter and length distribution.

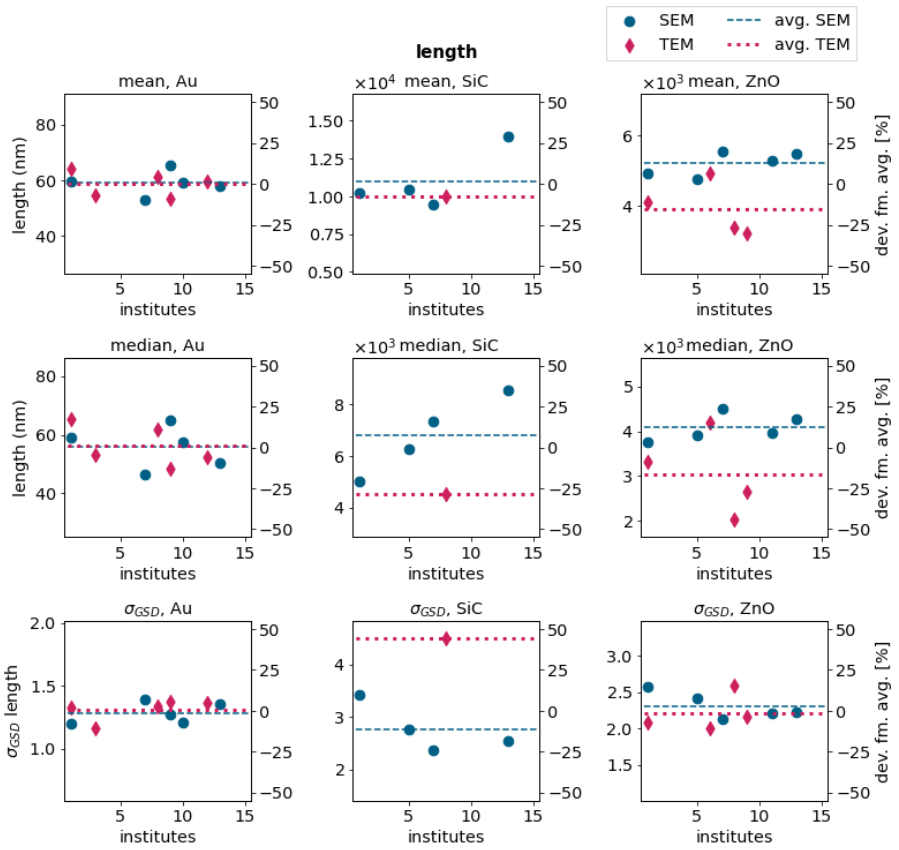
As expected from the results of the ILC, no significant differences were found for Au. Figure 12 and 13 show comparisons of different parameters of the length or diameter distributions between SEM and TEM for Au, SiC and ZnO.

Figure 12: Comparison of different parameters of diameter distribution between SEM and TEM. Absolute values are plotted on the left Y-axis and relative values on the right Y-axis



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

.Figure 13: Comparison of different parameters of the length distribution between SEM and TEM. Absolute values are plotted on the left Y-axis and relative values on the right Y-axis.



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

3.2.4 Influence of the evaluator

To estimate the influence of the evaluator on the result of the size determination, identical SEM images of the Ag fibres were sent to the participants with a request for evaluation. Seven institutes participated in this second part of the ILC. The decision to choose the Ag fibres as test material for this second part of the ILC was made because of their extremely homogeneous morphology. Therefore, the morphological characteristics of the fibres do not constitute a further uncertainty factor in this study. The results given in Table 11 and Table 12 show a noticeable reduction of the relative stand-ard deviation for length and diameter determination from 15%-16% to 6%-10%. This outcome indi-cates that the influence of the evaluator is not the main factor in the overall uncertainty. A comparison between the results of this image evaluation and the results of the ILC is also shown Figure 14.

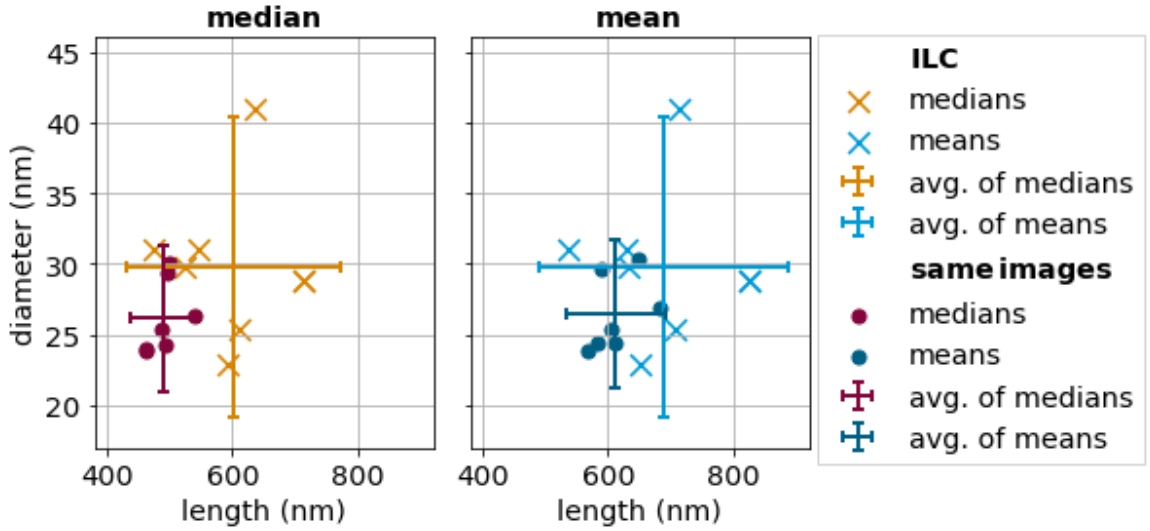
Table 11: Comparison of the results of length determination from same images and the ILC.

	same images	ILC
Mean [nm]	612	686
Relative mean error	0.07	0.15
Median [nm]	491	597
Relative median error	0.06	0.15

Table 12: Comparison of the results of diameter determination from same images and the ILC.

	same images	ILC
Mean [nm]	26.4	29.5
Relative mean error	0.10	0.16
Median [nm]	26.2	29.2
Relative median error	0.10	0.16

Figure 14: Comparison between the results of the ILC and the evaluation of same images of Ag nanowires.



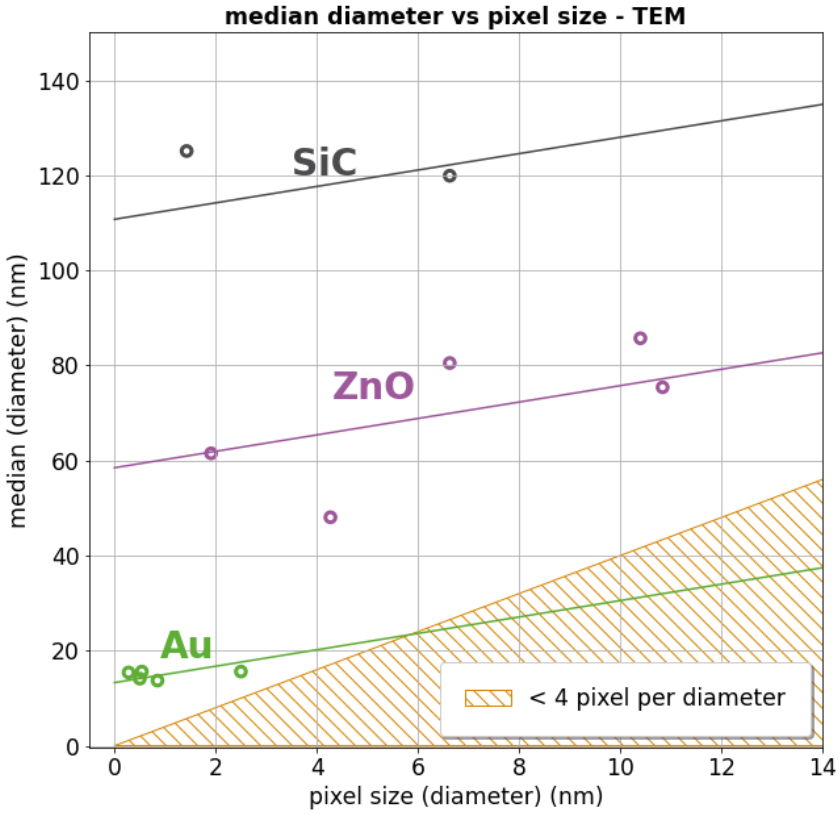
Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

3.2.5 Influence of the pixel size

According to the TG PSD the fibre size determination requires a minimum resolution of 4 pixels per diameter. This requirement for the microscopic images is intended to prevent the image resolution from distorting the result of the diameter determination. With the help of a linear fixed effects model, it was investigated whether, despite this requirement for image resolution, an influence of the pixel size on the measured median of the diameter could be detected. The linear fixed effects model calculates regression parallels for each nanomaterial. This means that the Y-axis intercept is optimised individually for each nanomaterial. However, the slope is the same for all nanomaterials.

For SEM, no significant influence of the pixel size used could be proven. However, a dependence between pixel size and diameter was found in the TEM results because the 95% confidence interval [0.139, 4.708] for the slope of the parallels is exclusively positive. Figure 15 illustrates the results of the TEM investigation in a graph plotting diameter vs. pixel size. The dependence found can be traced back to the results for ZnO.

Figure 15: Influence of the pixel size used on the measured median diameter for TEM.



Source: Own research, Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

3.2.6 Impact of the interlaboratory comparison on the Test Guideline

Based on the results of the ILC, the measurement instructions of the draft TG PSD for SEM were validated without restrictions. However, with respect to the measurement results of the long fibrous nanomaterials SiC and ZnO using TEM, such as the large measurement uncertainties, the significant deviations from the SEM measurements and the confirmed influence of the pixel size, the application range of the TEM in the draft TG PSD needed to be revised.

In consultation with the ILC participants it was discussed and decided to lower the upper limit of applicability of TEM for fibres, so that SEM is now the only valid method for size determination of fibrous nanomaterials with a mean length > 5µm and a distribution width $\sigma_g > 1.5$[3].

As a consequence of a clear overestimation of the length and diameter of Au nanorods by an institute due to a systematic neglect of short fibres, a paragraph in the fibre part of the TG PSD was added. In this paragraph it is pointed out that fibres with an aspect ratio of 3 should also be included in the statistical evaluation.

[3] σ_g is the scientific notation for the geometric standard deviation of the size distribution.

4 Conclusion

The project toward the OECD test guideline determining particle size distributions between 1-1000 nm will fill an important gap by facilitating for the first time the determination of particles in this size range under mutual acceptance of data. To register and to physically describe nanomaterials particle size distribution is an important parameter. This parameter will allow comparable test conditions between different labs for example in vitro or in vivo studies, assess environmental behaviour and mobility in air and water. To achieve the acceptance of this test guideline by the OECD it was necessary to conduct an international inter laboratory comparison.

The international ILCs conducted for near spherical particles and fibres summarised above, showed the strengths and limitations of the evaluated test methods. The results were internationally discussed during several telephone conferences and it was agreed which methods could become part of the new TG on Particle Size Distribution. Based on this information the new TG was drafted and also internationally discussed at virtual meetings of the JEG in November and December 2020. A revised draft will be submitted to the OECD WNT for commenting by mid 2021 and subsequently freely published.

The “Development of a specific OECD Test Guideline on Particle Size and Particle Size Distribution of Nanomaterials” showed that different views and terminologies by OECD members lead to different understanding of the draft TG. The discussion of the draft TG with the international colleagues proofed to be essential to improve the understanding and acceptance of the newly developed TG.

Beyond the end of the project, BAM and BAuA will finalise the draft TG PSD for submission to the OECD WNT, as mentioned above. Along with the draft the Validation report and the Response to Comments document from the JEG commenting in late 2020 will be submitted. At this time the draft TG PSD will be publically available through the OECD chemicals program website. After receiving further comments and requests for adaptation and clarification of the draft TG those will be answered and a final draft will be submitted for adoption and final publication by the WNT.

5 List of references

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