FINAL PUBLISHABLE REPORT

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1 Overview

Nanomaterials and nanotechnology are widely used today, but their use may expose humans and the environment to new health and sustainability risks. To control and minimise these risks, the European Commission has mandated European standardisation bodies to develop standardised methods that can reliably characterise manufactured nanomaterials. This project has developed reference materials: i) TiO_2 and Au nanoparticles with well-defined non-spherical shape, ii) SiO_2 nanoparticles with relatively high polydispersity index, and iii) Au and SiO_2 nanoparticles with accurate particle concentrations. Based on these new reference nanoparticles, measurement procedures for improved traceable measurement of the size of non-spherical and non-monodisperse nanoparticles have been also developed and published. Finally, full algorithm sequences for nanoparticle detection and size measurement as developed on both a physical basis and by machine learning have been developed. All newly developed reference nanoparticles, measurement procedures and modelling algorithms contribute significantly to the improvement of the traceability chain, comparability and compatibility of nanoparticle size measurements to support standardisation within the framework of CEN/TC 352 “Nanotechnologies”, ISO/TC 229 ‘Nanotechnologies’, ISO/TC 24/SC 4 ‘Particle characterization’ and ISO/TC 201/SC 9 ‘Scanning probe microscopy’.

2 Need

The global nanotechnology market is predicted to reach 110 Billion US$ by 2022. Further, nanotechnology has been identified by the European Commission (EC) as one of its 5 key technologies. New and emerging uses of nanomaterials include medical and pharmaceutical applications as well as use in conductive inks, optical sensing and power delivery. However, nanotechnologies and nanoparticles may expose humans and the environment to new health and sustainability risks, which need to be reliably linked to their size, shape, concentration and chemical properties.

In order to support the increasing and safe use of nanomaterials it is essential that robust normative standards are introduced. NMIs have a key role to play in this by developing comparable and traceable measurements and instrument calibrations for real-world nanoparticles. The EC has recognised this need and therefore mandated CEN, CENELEC and ETSI (EC Mandate M/461) to develop standardisation activities regarding nanotechnologies and nanomaterials as one of the building blocks for the “safe, integrated and responsible” use of nanomaterials as outlined in the EC European Strategy for Nanotechnologies. A significant part of this strategy relates to the need for improved, traceable measurement procedures for size of real-world nanoparticles as a prerequisite for the reliable evaluation of their potential toxicity to the environment.

Three different ISO technical committees offer suitable standardisation platforms for projects on the accurate measurement of nanoparticle size and shape distribution: ISO/TC 229 for electron microscopy, ISO/TC 201/SC 9 for atomic force microscopy (AFM) and ISO/TC 24/SC 4/WG 10 for small angle X-ray scattering (SAXS). Three projects on the measurement of nanoparticle size and shape distribution using scanning electron microscopy (SEM), scanning electron microscopy in transmission mode (TSEM) and transmission electron microscopy (TEM) have been developed under ISO/TC 229, at all of them the present project have provided significant contributions. Alignment of European standardisation activities within CEN/TC 352 to the ongoing ISO standardisation projects is further necessary. The physical modelling of the output signals in electron microscopy and new reference nanoparticles with more complex shape and size distribution were also required. This is also the case both for future AFM standards on the measurement of nanoparticle size and shape distribution, which would be developed under ISO/TC 201/SC 9, and for the SAXS activities on nanoparticle size distribution in progress under ISO/TC 24/SC 4/WG 10.

The preselection by the present project of only traceable sizing methods was of special relevance. For the first time, both critical aspects, namely the lack of reference nanoparticles and signal modelling were systematically considered. The advanced data analysis software developed by the present project together with a public database with representative documented data include a robust determination of the measurement uncertainty. Last, but not least, hybrid measurement approaches contribute to a more accurate final result by data fusion, particularly for non-spherical nanoparticles.

3 Objectives

The overall objective was to improve the traceability chain, comparability and compatibility for nanoparticle size measurements to support standardisation and to ensure that these developments are fed into the standards development process within CEN/TC 352 and ISO/TC 229 and related groups. The specific objectives were:

1. To assess the performance and establish the traceability of existing nanoparticle size and characterisation methods, such as SEM, TSEM, TEM, AFM and SAXS, in terms of their sensitivity to material, shape and quantity (number, volume or mass) for representative nanoparticulate materials (i.e. metals, oxides and polymers), including analysis of the effect of material and shape parameters on size and size distribution measurements, as well as the effect of conversion of the measured signal on the particle size distribution.
2. To develop validated nanoparticle reference materials with (i) non-spherical shapes, (ii) non-monodisperse size distributions and (iii) accurate concentrations. In addition, to use such nanoparticle reference materials to evaluate measurement uncertainties for nanoparticle quantity determination (expressed as number, volume, mass or intensity) and to establish their dependence on particle size.
3. To develop improved physical models of the output signals from nanoparticle size measurement systems, that accurately account for nanoparticle material, shape and quantity. The physical models will include nanoparticle material type, shape and quantity parameters such as number, volume, and mass. The goal of the models is to improve the evaluation of nanoparticle measurement uncertainty and comparability between results of different methods.
4. To use the new physical models (from objective 3) to develop validated and traceable methods for the transfer of nanoparticle size from (certified) reference nanoparticles of spherical shape and monodisperse size distribution to other types of nanoparticles. This will include different nanoparticle shapes (such as elongated nanoparticles and platelets) as well as nanoparticles with non-monodisperse size distribution.
5. To contribute significantly to the standards development work of the technical committees CEN/TC 352 Nanotechnologies and ISO/TC 229 Nanotechnologies ensuring that the outputs of the project are aligned with their needs, communicated quickly to those developing the standards and to those who will use them, and in a form, that can be incorporated into the standards at the earliest opportunity.

4 Results

4.1 Objective 1: Towards assessment of performance and establishment of the traceability of existing nanoparticle sizing methods, such as SEM, TSEM, TEM, AFM and SAXS.

The performance of the characterisation methods SEM, TEM, TSEM, AFM and SAXS for traceable measurement of nanoparticle (NP) size, size distribution, shape and concentration in suspensions have been evaluated systematically regarding parameters including instrument calibration, sample preparation, measurement conditions and data analysis conditions starting from ideal nanoparticles (monodisperse spheres) to complex nanoparticles (polydisperse in size, non-spherical shape and different chemical compositions). Out of these possibilities and limitations of the methods the corresponding measurement uncertainties have been extracted.

A critical quantitative assessment has been done by LNE and BAM based on the results reported in the literature, other projects, on the expert knowledge available at the projects partners as well as preliminary results carried out on project nanoparticles. Following dependencies have been systematically investigated: i) Influence of nanoparticle material type/chemistry, ii) Capability of the CMs to measure NP size distribution as a function of polydispersity and shape, and iii) Capability of the CMs to convert the measured intensity signals into number-weighted size distribution. The objective has been successfully achieved.

4.1.1 Influence of nanoparticle material type on the performance of the measurement method

For electron microscopy techniques, the material type/chemistry has intrinsic influence on electron density difference of NPs with respect to background. The higher the electron density difference, the higher is the nanoparticle/substrate contrast. Consequently, the contrast improvement has a positive impact on the

robustness of the NP segmentation process (especially regarding to the smaller NPs) during the image analysis step and the accuracy of projected shape and its distribution. Further, regarding SEM, non-conductive materials lead to charging effects, which can disturb the size measurements. The height measurements performed by AFM do not depend on the NP chemical nature within the uncertainties. Similarly, there is no influence of material type other than signal intensity in SAXS measurements. When the intensity is sufficiently high, the SAXS signal intensity is not considered for the determination of the NP size, polydispersity and shape.

4.1.2 Capability of the measurement methods to measure NP size distribution as a function of polydispersity and shape

Concerning EM, the transmission mode used in SEM (i.e. TSEM or STEM-in-SEM) and TEM techniques implies that the obtained image is a 2D-projection of the NPs on a plane parallel to the substrate. Thus, in the case of non-spherical NPs, the projected sizes and shapes are strongly dependent on the orientation of the NP on the substrate. Hence, shape and its polydispersity can cause measurement errors and increase the measurement uncertainties. The project partners LNE, PTB and BAM have demonstrated that the gold nanocubes and bi-pyramidal TiO_2 measurements by EM have been impacted by the different possible NP orientations on the substrate. For instance, it is often impossible to distinguish an imperfect cube from a bad-positioned NP. By contrast, in SEM, the orientation or real shape can be estimated through surface information given by grey levels (along Z-axis) in addition to the NP projected surface. Studies in high-resolution concerning nanocubes and bi-pyramidal-shaped NPs deposited on specific substrates have been also carried out within the project.

AFM cannot be used for accurately measuring the profile width or the NP shape in general, due to the convolution between the tip and the nano-object. However, the height can be accurately measured because the convolution is zero at the NP top. Obviously, in microscopy, the term height depends on the orientation of nano-objects on the substrate. But the AFM heights determined by VSL and SMD from a population of randomly oriented nanocubes on silicon give robust information about mean side length. By contrast, the height measured on bipyramidal-shaped NPs is difficult to interpret without any knowledge of their orientation on the substrate.

Finally, PTB, BAM and CEA have demonstrated the SAXS ability to determine the size distribution of the populations of the nPSize nanoparticles is linked to a-priori knowledge of the NP shape. Furthermore, a large size polydispersity can affect the reliability of the results because any dimensions outside of the measurement range (approximatively quite well by $2\pi/q$) cannot be quantified.

4.1.3 Capability of the CMs to convert the measured intensity signals into number-weighted size distribution

AFM, TEM, SEM and TSEM are measurement methods based on direct observation and the size distribution is determined from counting and measuring nanoparticles one-by-one. Consequently, the size distribution is directly expressed in numbers. In contrast, the SAXS results can be converted into number-weighted size distribution if: (i) a distribution form is considered, (ii) the distributions are narrow, (iii) the scattering is strong (high contrast and good volume fraction).

4.1.4 Calibration procedures and assessment of main sources of measurement uncertainty

In order to demonstrate the traceability of size results for spherical shape nanoparticles two main aspects have been investigated in the project for each individual measurement method: i) calibration of the instruments and ii) establishment of the different traceability chains. In the following these points are addressed in detail for each measurement method.

4.1.4.1 SEM

The approach for calibrating the SEM instrument consists of two steps: (i) measuring a transfer standard (for instance, VLSI reference structure) and comparing the results with those obtained with a metrological AFM on the same structure for determining the pixel size, and (ii) measuring a reference nanomaterial (e.g. ERM-FD304) for adjusting two major setting parameters, accelerating voltage and working distance, which have an influence on the measurement results and depend on the NP size and material type.

The uncertainty budget (see Table 1) has been established by LNE, PTB and BAM for the mean diameter measurements of reference silica NPs: (i) resolution linked to the dimension of electron beam diameter in focal plane, (ii) calibration uncertainty on reference NPs and (iii) measurement repeatability. The standard

uncertainty (k=1) has been found to be 2.01 nm for a diameter of 23.05 nm, this representing less than 10 % of the NP diameter used in this study.

Table 1: *Errors sources and their significancy for SEM*

Error sources	Contribution
e-beam size	Significant
Sample preparation	Significant (depending on the sample)
Thresholding	Significant
Repeatability	Medium
Magnification	Medium (when following ISO standard)
Contamination	Medium
Beam damage	Minor (when taking precautions)
Orientation/adhesion on substrate	Minor (if near-spherical NP)

4.1.4.2 TSEM

The traceability relies on a 2D grating with a nominal grating pitch of 144 nm of aluminum bumps on silica (150-2D from Advanced Surface Microscopy Inc.) and a deep UV laser diffractometer which yields traceable values for the mean grating pitch. The traceability to meter is realized at the primary level in terms of the wavelength from a helium-neon laser. The diffractometer is used to determine the pitch $a_{optical}$ of the 2D grating. The latter is also measured by TSEM and a_{SEM} is determined. The pixel size is then calculated ($=a_{optical}/a_{SEM}$) as a traceable result. The leading-edge distortion is a well-known effect of SEM imaging, due to a non-constant scanning speed of the electron beam. A graph reporting the calculated pixel size as a function of scanning direction along x-axis has been used to bring out the image part impacted by the leading-edge distortion.

An Ishikawa diagram was used to illustrate the various error sources identified during the TSEM measurement. Table 2 compiled by PTB and BAM shows the parameters having significant contributions (image analysis, selection of particles, simulation/modelling, reproducibility, sample preparation); the particle contamination impacts moderately the uncertainty budget and the statistics; determination of grey levels and pixel size are minor components. The uncertainty increases linearly with the size of nanoparticles in the range from 10 to 200 nm and has been found to be between 0.5 to 2.5 nm.

Table 2: *Errors sources and their significancy for TSEM*

Error sources	Contribution
Image analysis, selection of particles	Significant
Simulation / modelling	significant
Reproductibility (incl. digitalization	significant
Sample preparation	significant
Particle contamination	medium
Statistics (thousand particles)	minor
Determination of grey levels (thresholding)	minor
Pixel size	minor

4.1.4.3 TEM

The main error sources for the TEM case are compiled by CEA and LGC in Table 3. The calibration of a TEM at different magnifications using reference samples (sample named ‘cross grating’ for low magnification, and gold or silicon crystal lattice parameter for high magnification) is proposed. Measurement uncertainties are estimated to be about 1.2 % (image of 2 k x 2 k pixels and cumulative uncertainties of length of about 24 pixels).

During the acquisition of TEM images, a bright boundary surrounding the particle can be observed as presented in Figure 1. This boundary is determined by means of a defocusing of the image by the operator when there is no focusing gradient on the particle. Thus, the contour of the particles on the image appears visually sharper because of this defocusing.

One can also note that an additional phenomenon may appear when imaging anisotropic or large particles. The white contour may be present on a portion of the particle and is related to the difference in focus because the focal plane does not pass through the entire particle. Moreover, even if the particle is almost spherical, a slight outgrowth (or deformation) above the equatorial plane and/or a slight outgrowth (or deformation) below the equatorial plane can lead to a non-focus contrast in some part of the contour.

The anisotropic particle presented in Figure 1 (left) aims, by its rather generic shape, to point out this anisotropic unequal contour contrast described in the sentence before. Indeed, Figure 1 (right) gives a schematic explanation about the different out-of-focus portion of a NP depending on their relative position regarding the focal plan and, the equatorial plan of NP (that is projected on the camera screen).

Table 3: *Errors sources and their significancy for TEM*

Error sources	Contribution
Calibration	Significant, medium, minor
Repeatability	Significant, medium, minor
Sample preparation	Significant, medium, minor
Statistics	Significant, medium, minor
Thresholding	Significant, medium, minor

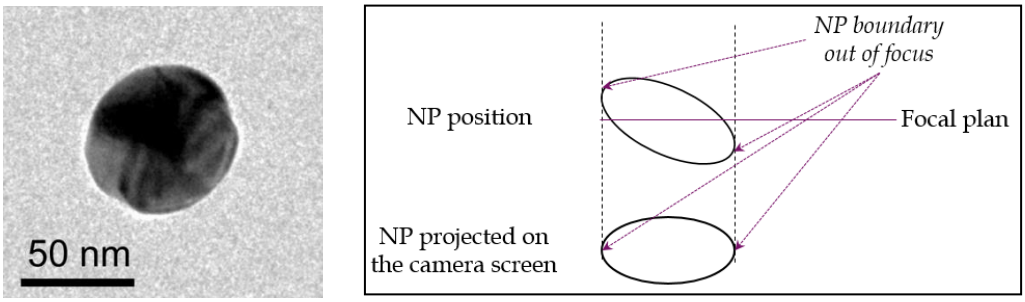

Figure 1: *Example of a nanoparticle (2nd mode of 60 nm of the SiO_2 nPSize1 sample) imaged with TEM by CEA. Note the thickness of the boundary here of 7 pixels; right: Out-of-focus parts of a NP depending on the position of its equatorial plan regarding the focal plan. The larger the particles and the greater the particle shape anisotropy are, the greater this out-of-focus effect is.*

4.1.4.4 AFM

The main error sources for the AFM case were compiled by VSL and SMD and are presented in Table 4. Along Z-axis, the AFM calibration was carried out either (i) by comparison to a physical step height standard via a calibration curve (for h >20 nm) or (ii) through a virtual height standard (for h <20 nm).

In the first case, the calibration curve of the microscope is determined by fitting the standard reference mean step height values as a function of the measured mean step height values. Then, the spherical NP dimensions are measured and related to the calibration curve.

In the second case, a piezo-actuator applies a known periodic displacement to the virtual standard or selects an appropriate physical step height close to the nominal height of the particles to be measured. The values

generated by the virtual standard are traceable by laser interferometry and the linearity of the displacement versus square wave amplitude is nearly perfect in the range from 2 pm to 20 nm.

A calibration procedure is also proposed for NP lateral measurements. A physical 2D grating can be measured in the same conditions (scan range, scan speed etc.) as the NP measurements. Then, the scale and the non-linearity are determined along X- and Y-axis. A virtual standard has been developed for lateral diameter below 20 nm by implementing a shear piezo actuator.

Finally, The AFM is calibrated for the specific height, lateral range and scan speed used in the NP measurements. The main error sources are: AFM probe, tapping force through the amplitude setpoint, scan speed, operator, image analysis, step height standard uncertainty. Regarding lateral measurements, the probe shape is determined by measuring spherical NPs with a broad range of diameters. The main error sources are: calibration of/with the virtual standard, reproducibility and analysis methods.

Table 4: *Errors sources and their significancy for AFM*

	Height	
	Contribution	**Contribution**
Error sources	**Qualitative**	**Quantitative**
Agglomeration state	Significant when overlapping	
Calibration*	Minor	<1 nm
Probe shape	Minor	0 nm
Amplitude set point*	Medium	1.4 nm
Temperature drift*	Minor	<1 nm
Substrate surface state	Unknown	-
Operator*	Minor	<1 nm
Image analysis*	Minor	<1 nm
Reproducibility	Medium	1 nm
Size dispersion	Significant	2-5 nm
Baseline roughness	Minor	<1 nm

	Lateral (width)	
	Contribution	**Contribution**
Error sources	**Qualitative**	**Quantitative**
Agglomeration state	Significant when overlapping	
Calibration*	Medium	1 nm
Probe shape	Significant	3 nm
Amplitude set point*	Significant	10 nm
Scan speed*	Minor	<1 nm
Temperature drift*	Minor	<1 nm
Substrate surface state	Unknown	-
Operator*	Minor	<1 nm
Image analysis*	Minor	<1 nm
Reproducibility	Medium	1 nm
Size dispersion	Significant	2-5 nm

4.1.4.5 SAXS

The main error sources for SAXS were compiled by PTB, BAM and CEA and are presented in Table 5. Four relevant parameters have been determined to be decisive for the size traceability: distance sample-detector, detector pixel size, photon energy and scattering intensity fraction.

A properly normalized scattering curve shows the differential scattering cross section per volume sample (absolute scattering intensity) as a function of the momentum transfer q of the photons. The correct q-scaling is calculated from a known sample-detector-distance, the beam center position on the detector as well as the photon energy. Alternatively, a known standard sample (like silver behenate) can be used.

For the calibration of the intensity, the ratio between the scattered photons and the photons in the incident beam must be calculated. This can be achieved by calibration of the detectors in use, a measurement of the direct beam with the scattering detector, or calibration with scattering standards such water or glassy carbon. Additionally, the transmitted sample length must be known.

For isotropic particle orientations, the detected 2D scattering signal consists of concentric circles such that azimuthal integration and proper normalization (by incident photon flux, exposure time, solid angle of the scattered beam, quantum efficiency of the detector, sample transmission and sample thickness) of the photons counts lead to a 1D scattering curve. After the 1D scattering curve has been generated, it can be fitted by an appropriate model function which depends on the particle shape. Details on the calibration, model fitting and physical background can be found in the ISO standard ISO 17867:2020.

***Table 5**: Errors sources and their significancy for SAXS*

Error sources	Contribution
Model Fitting	Significant
Detector pixel size	Minor
Distance sample-detector	Minor
Photon flux	Medium
Sample transmittance	Medium
Photon energy	Minor
Exposure time	Minor
Capillary thickness	Medium
Electron density contrast	Medium
Detector quantum efficiency	Medium

4.2 Objective 2: Towards development of validated nanoparticle reference materials with non-spherical shapes, non-monodisperse size distributions and accurate concentrations.

One aim of the project was to develop and validate three classes of candidate reference (test) materials (RTMs), with i) well-defined non-spherical shape, ii) relatively high polydispersity index, and iii) accurate particle concentrations.

To fulfil the requirements of the project, 11 different types of materials were prepared, see Table 6 and Figure 2. Following the initial assessment of the materials suitability, nPSize5_PT_UNITO, nPSize6_AC_UNITO (both produced by UNITO) and nPSize7_GN_CEA (produced by CEA) materials were found unsuitable for the project, due to various reasons. PT material (produced by UNITO) was deemed unsuitable due to its predominantly agglomerated nature. AC material (produced by UNITO) contained relatively high amount of impurities (other particle forms). GN material (produced by CEA) was found too heterogeneous in both the length and width for the purpose of the project. The remaining 8 candidate RTMs (produced by UNITO, CEA and LGC) were assessed for their homogeneity and stability and used for successful delivery of the associated activities within the project. The objective has been successfully achieved.

Table 6: *List of the candidate RTMs used in the project*

RTM group	Material type	Responsible partner	Material's code	Nominal particle size	Nominal Particle shape
accurate concentration	bimodal gold	LGC	nPSize1_BMG_LGC	30 nm and 60 nm	spherical
	bimodal gold	LGC	nPSize2_BMG_LGC	30 nm and 60 nm	spherical
accurate concentration /non-spherical	bipyramidal titania	UNITO/LGC	nPSize3_BPT_UNITO/LGC	60 nm lateral x 40 nm width	bipyramid
non-spherical	gold cubes	CEA	nPSize4_GC_CEA	60 nm x 60 nm x 60 nm	cube
	platelets titania	UNITO	nPSize5_PT_UNITO	10-15 nm thickness x 50-60 nm lateral	platelet
	acicular titania	UNITO	nPSize6_AC_UNITO	100 nm length /15-20 nm width; AR 5.5/6	acicular
non-monodispersed /non-spherical	gold nanorods	CEA	nPSize7_GN_CEA	10 nm width x 20-30 nm length	rod
non-monodispersed	monomodal silica, PSD<10%	CEA	nPSize10_MS_CEA	50 nm	spherical
	bimodal silica	CEA	nPSize12_BMS_CEA	30 nm and 60 nm	spherical
	bimodal silica	CEA	nPSize13_BMS_CEA	30 nm and 60 nm	spherical
	monomodal silica, PSD~20%	CEA	nPSize14_PS_CEA	50 nm	spherical

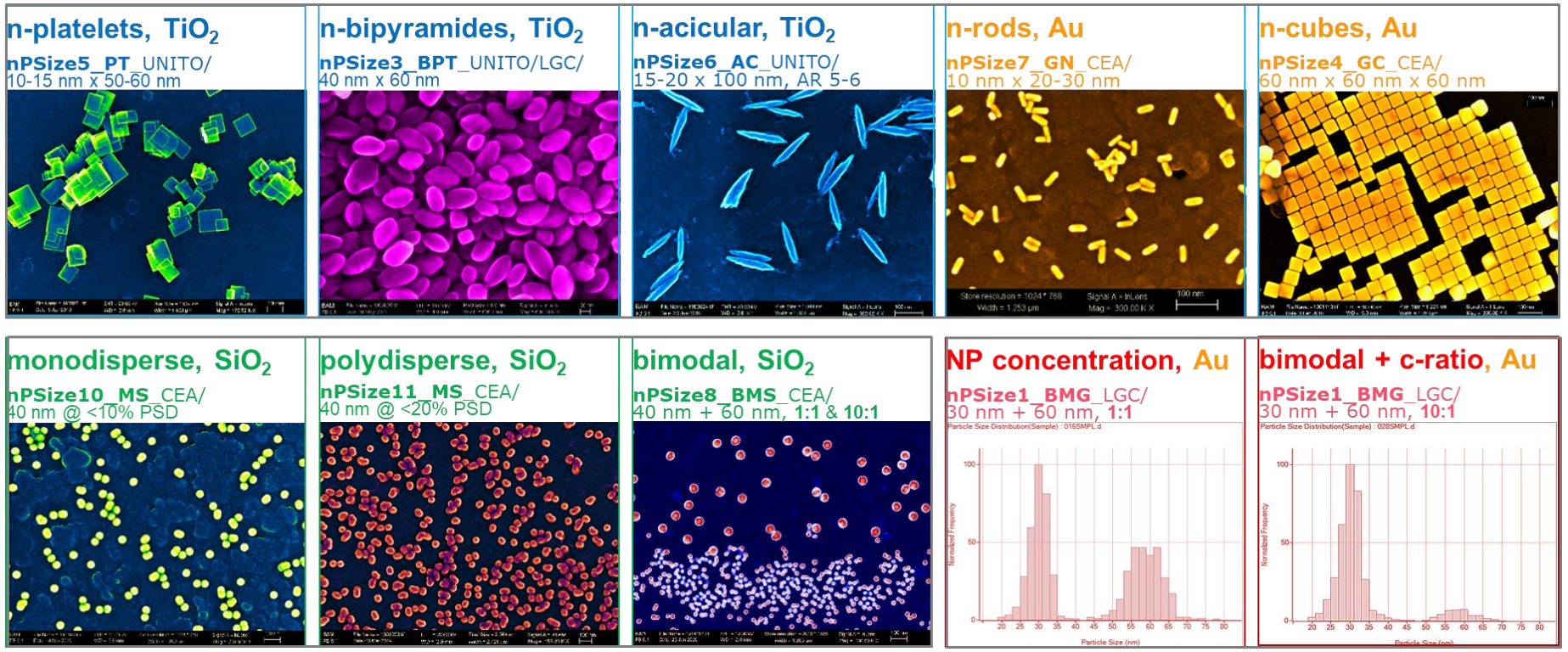

Figure 2: *Overview of the various types of nPSize materials developed and tested in the project*

In the following, the characterisation of all the project materials is presented in a relatively succinct way. The detailed results are in progress to be published jointly by the project partners.

Bimodal gold (BMG) 'nPSize1' and 'nPSize2'

The equivalent area diameter (D_{ae}) results with mean diameter, median diameter and mode as measured with TEM by the partner CEA are summarized in Table 7. Both materials (produced by LGC) were also found stable in terms of particle size and size distribution over the tested period based on the TEM measurement conducted in October 2019, January 2020 and March 2021.

***Table 7**: Summary of stability and size characterisation results for nPSize1 and nPSize2 materials by TEM*

Date	nPSize1						nPSize2					
	Mode 1			Mode 2			Mode 1			Mode 2		
	$D_{AE\ (nm)}$			$D_{AE\ (nm)}$			$D_{AE\ (nm)}$			$D_{AE\ (nm)}$		
	mean	mode	med.	mean	mode	med.	mean	mode	med.	mean	mode	med.
Mean ± u, k=1	61.9 ± 0.5	58.7± 3.7	62.0± 2.0	30.8 ± 0.5	30.8 ± 1.3	30.8 ± 0.5	62.4 ± 1.0	62.9 ± 0.5	62.4 ± 0.8	31.0 ± 0.5	32.1 ±1.1	31.3 ±0.7

As far as the number concentration of the two materials is regarded, both materials were found by spICP-MS 'frequency method' (by CEA) as stable in terms of the number-based concentration over the tested period with no significant difference observed (within k=1, being ~13 %) between measurements.

Bipyramid titania (BPT) 'nPSize3'

This type of nanoparticles (produced by UNITO) is probably the most successful one, many scientific articles on their characterisation being already published and other being in plan/progress. The stability measurements of the minimum Feret diameter ($D_{minFeret}$) and maximum Feret diameter ($D_{maxFeret}$) results with mean diameter, median diameter and mode as measured with TEM by CEA (in October 2019, January 2020 and March 2021) are summarized in Table 8.

***Table 8:** Summary of TEM stability and pre-characterisation results obtained for nPSize3 material*

Date	nPSize3					
	$D_{minFeret\ (nm)}$			$D_{maxFeret\ (nm)}$		
	mean	mode	med.	mean	mode	med.
Mean ± u, k=1	45.2 ± 0.6	44.5 ± 0.3	44.9 ± 0.5	59.3 ± 4.5	55.6 ± 7.5	58.2 ± 6.0

Gold nanocubes (GC) 'nPSize4'

This material (produced by CEA) could not be studied between unit homogeneity as only a small single batch was obtained for the purpose of the project. nPSize4 was measured with TEM in September 2019 and March 2020. The minimum Feret diameter ($D_{minFeret}$) and maximum Feret diameter ($D_{maxFeret}$) results with mean diameter, median diameter and mode are summarized in Table 9.

Table 9: *Summary of TEM stability and pre-characterisation results obtained for nPSize4 material*

Date	nPSize4					
	$D_{minFeret\ (nm)}$			$D_{maxFeret\ (nm)}$		
	mean	mode	med.	mean	mode	med.
Mean ± u, k=1	59.0 ± 0.5	57.8 ± 0.9	58.9 ± 0.5	61.8 ± 0.5	59.1 ± 1.7	61.1 ± 0.5

Monodisperse (MS) 'nPSize10', polydisperse (PS) 'nPSize12' and bimodal silica (BMS) 'nPSize13' and 'nPSize14'

Homogeneity of the nPSize10, nPSize12, nPSize13 and nPSize14 (all produced by CEA) was assessed by CEA with SAXS based on the measurements performed on 10 individual vials from each of the materials. No differences between units were seen and the materials were considered sufficiently homogeneous based on the SAXS data.

SAXS was also used by CEA to assess the materials stability upon storage. No significant differences were found in the particle size, size distribution and number concentration in the silica materials. The summary of data obtained for the nPSize10 material is shown in Table 10 as an example. Similar results were obtained for the nPSize14 material.

Table 10: *Summary of SAXS stability results obtained for nPSize10 material*

Date	Diameter (nm)	Sigma	Concentration (NP cm^{-3})
2019-01-11	50.85	1	1.29E+13
2019-11-05	50.55	1.8	1.47E+13
2020-01-29	50.91	2.27	1.31E+13
2020-06-19	50.48	1.99	1.91E+13
2021-01-06	50.54	0.79	1.68E+13
2021-03-18	50.59	2.1	1.80E+13
2021-05-20	49.74	2.1	2.00E+13
2021-08-02	50.14	2.8	1.85E+13

Silica materials were also found stable in terms of particle size and size distribution over the tested period based on the TEM measurement conducted in March 2020 and January 2021 by CEA. The equivalent area diameter (D_{ae}) results with mean diameter, median diameter and mode are summarized in Table 11.

Detailed information about the project materials gold cubes, platelets titania, acicular titania and gold nanorods can be found in Crouzier et al 2021).

Table 11: *Summary of TEM stability and pre-characterisation results obtained for nPSize10 and nPSize14 materials.*

Date	$D_{AE\ (nm)}$					
	nPSize10			nPSize14		
	mean	mode	med.	mean	mode	med.
Mean ± u, k=1	49.2 ± 1.3	49.6 ± 1.1	48.9 ± 0.8	47.7 ± 0.8	47.4 ± 0.4	47.8 ± 0.5

For the nPSize13 material, TEM thresholding was performed by CEA on 10 images for 149 labelled particles in September 2020 and on 17 images for 200 particles labelled in March 2021. In case of the nPSize13, thresholding was performed by CEA on 8 images for 127 labelled particles in September 2020 and on 14 images for 224 particles labelled in March 2021. The equivalent area diameter (D_{ae}) results with mean diameter, median diameter and mode are summarized in Table 12.

Table 12: *Summary of TEM stability and pre-characterisation results obtained for nPSize12 and nPSize13 materials.*

Date	nPSize12						nPSize13					
	Mode 1			Mode 2			Mode 1			Mode 2		
	$D_{AE\ (nm)}$			$D_{AE\ (nm)}$			$D_{AE\ (nm)}$			$D_{AE\ (nm)}$		
	mean	mode	med.	mean	mode	med.	mean	mode	med.	mean	mode	med.
Mean ± u, k=1	**29.5 ± 0.5**	**29.6 ± 0.9**	**29.6 ± 0.5**	**57.9 ± 0.5**	**57.5 ± 0.5**	**57.9 ± 0.7**	**28.9 ± 1.1**	**29.5 ± 1.1**	**29.2 ± 1.4**	**58.7 ± 0.7**	**58.8 ± 1.3**	**58.7 ± 0.8**

Summing up, the extensive homogeneity, stability and pre-characterisation data obtained in the project for the materials nPSize1, nPSize2, nPSize3, nPSize4, nPSize7, nPSize10, nPSize12, nPSize13 and nPSize14 were found to be suitable for their use as candidate RTMs.

4.3 Objective 3: Towards development of improved physical models of the output signals from nanoparticle size measurement systems, that accurately account for nanoparticle material, shape and quantity, with the final aim to improve the evaluation of nanoparticle measurement uncertainty and comparability between results of different methods.

4.3.1 SEM

To determine the equivalent diameter Deq of the particles by SEM technique, different segmentation methods can be used. However, the lack of reference particles and knowledge on dimensional properties of the electron beam introduces a high uncertainty to this measurement. These unknowns then make the modeling of the experimental beam difficult. An inverse method was thus proposed by LNE. This consists of simulating several profiles for different sets of experimental input parameters and then proceeding by identification between the experimental measurements (obtained profiles) and the library of simulated profiles.

A Monte Carlo algorithm (JMONSEL) was implemented by LNE. This includes various physical models of electron-sample interaction that can be used to model the secondary electron signal as a function of the beam position on the sample: i) Mott cross section for elastic scattering, ii) input tables provided with JMONSEL for inelastic scattering of electrons in gold, silica and silicon. In this way, the SEM signal can be simulated (cross-

sectional profile over a spherical particle) by varying various parameters such as sample geometry (particle size), chemical composition (gold or silica), and size (standard deviation) and energy of the incident Gaussian electron beam.

For all the JMONSEL simulations carried out here, a schematic view of the studied configuration is given in Figure 3 by LNE, where a sphere (silica or gold), with radius R and center coordinates (0, 0, -R) is placed on a silicon substrate assumed to be infinite. Cross-sectional profiles could be carried out by simulating the yield of secondary electrons along the particle in steps of 1 nm on 121 points (Figure 3, right).

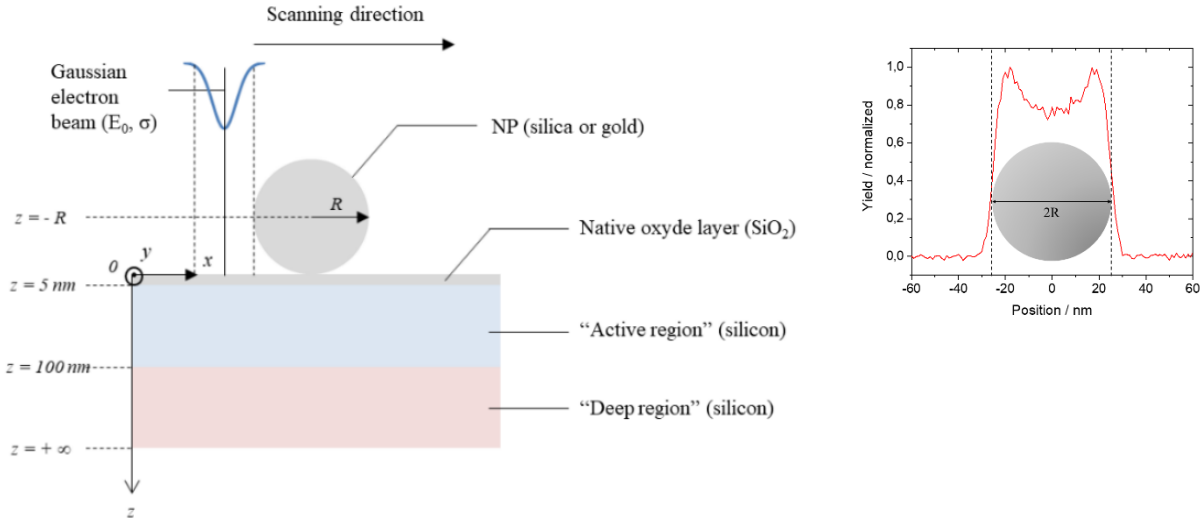

Figure 3: *left: Representation of the sample geometry used for all JMONSEL simulations; right: Cross-sectional profile (normalized) of a silica particle with a radius of 20 nm. Simulation performed with a beam size of 3 nm and an incident electron energy of 3 keV.*

From these profiles, it is possible to determine the theoretical position of the threshold to be applied on the SEM images to obtain R, the particle radius. For this, in order to match the SEM signal, expressed in grey levels, with the signal obtained on JMONSEL defined as a secondary electron yield, all the profiles were normalized.

A second algorithm was then developed by LNE in the Matlab software to determine which profile in the database best fits the measured SEM profile. With this method, two measurands are determined. The first one is the size of the assumed Gaussian electron beam represented by its standard deviation σ. The second is the radius R of the particle (output parameter) resulting from the comparison between the experimental profile and the library of simulated profiles. However, to save time, the experimental profile corresponding to signal measured at the center of the particle on the SEM image is only handled to determine the parameters σ and R associated with this profile from the library. For a refined identification of R and regarding near spherical nanoparticles, a thresholding of the image is then performed. The threshold position is then evaluated from the set of parameters (accelerating voltage, chemical composition of particle, σ and R) in the database giving the best correspondence with the experimental profile, see approach sketched in Figure 4. According to it, following sequence is concepted: 1) a particle is extracted from the SEM image and the associated thumbnail is created, 2) on this particle, the secondary electron intensity profile passing through the center of the particle is extracted, 3) the extracted profile is compared with the whole library of simulated profiles to determine R and σ, 4) these two parameters are used to determine the threshold to be applied, and 5) the dimensional parameters are calculated from the binary image. More details are available in the project publication: Crouzier et al. 2019.

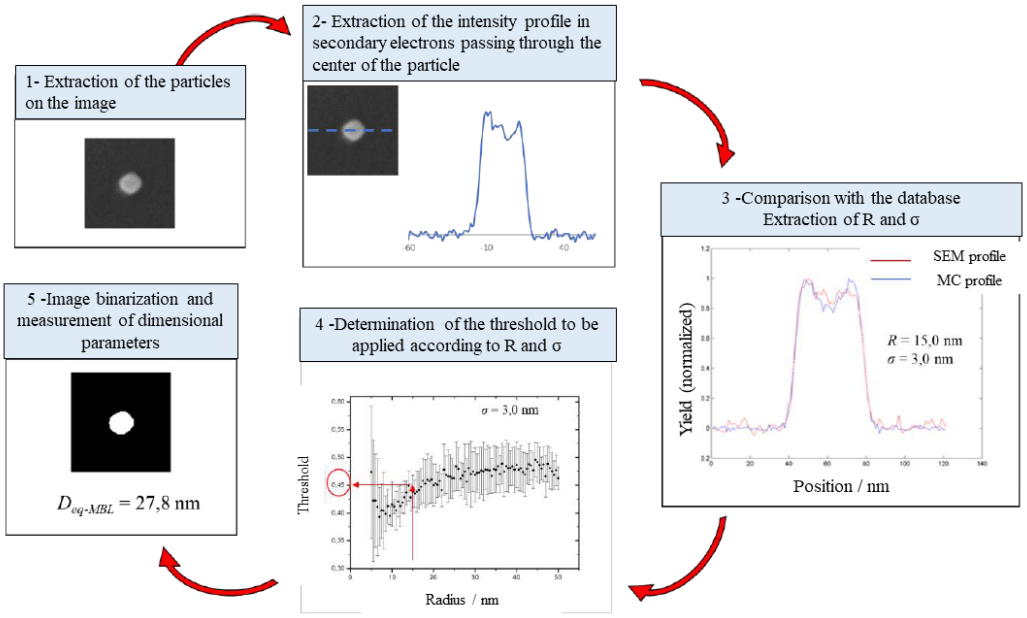

Figure 4: *Approach concepted by LNE to measure the dimensional properties of a nanoparticle with Monte Carlo simulation*.

4.3.2 TSEM

To characterize the size of a nanoparticle, the decisive parameter is the threshold of the TSEM signal at the boundary of the particle. Simulated relative threshold values are required to be able to set the threshold values of experimental grey value micrographs. TSEM images have been modelled by PTB for spherical, cylindrical, cubic, and bipyramidal nanoparticles of various materials under well-known measurement conditions.

The Monte-Carlo simulations that are used to generate artificial TSEM micrographs are based on the modeling of electron scattering in solid matter. Elastic scattering processes are modeled by Mott cross sections, which are provided as tables by ELSEPA. Inelastic scattering processes are dealt with in the framework of dielectric function theory. For the physics of electrons interacting in the three materials, gold, silica, carbon, PTB has used the most recent JMONSEL tables and for TiO2, PTB is currently developing the inelastic scatter tables. Furthermore, all accessible experimental parameters such as electron beam divergence and width, geometry and material of the nanoparticle, and detector geometry are considered in the simulations. The Monte Carlo simulation for TSEM has been implemented into the Geant4 framework using its Monte Carlo engine and its geometry/material library to consider the variety of differently shaped nanoparticles. This implementation has been validated for spherical particles by comparisons with JMONSEL, developed by NIST, for which PTB thanks John Villarrubia, NIST.

Obtaining a complete scan for a micrograph by running the Monte Carlo for each pixel is very costly in terms of computation time. Therefore, for each set of experimental parameters, the transmission yield as function of thickness is simulated by running the Monte Carlo simulation without scanning. Transmission yields are recorded whilst stepping through the material for each thickness by counting the electron trajectories passing the different thickness lines. In a subsequent and independent software, such a yield curve is input for a program (implemented in python) that generates three-dimensional particle geometries from which height maps are derived and which converts the heightmap into a yield-map using the yield curve. The finite width of the beam is included by convolving the yield-map with a Gaussian distribution representing the widened beam finally resulting in the micrograph as TSEM signal. Figure 5 illustrates this efficient simulation strategy.

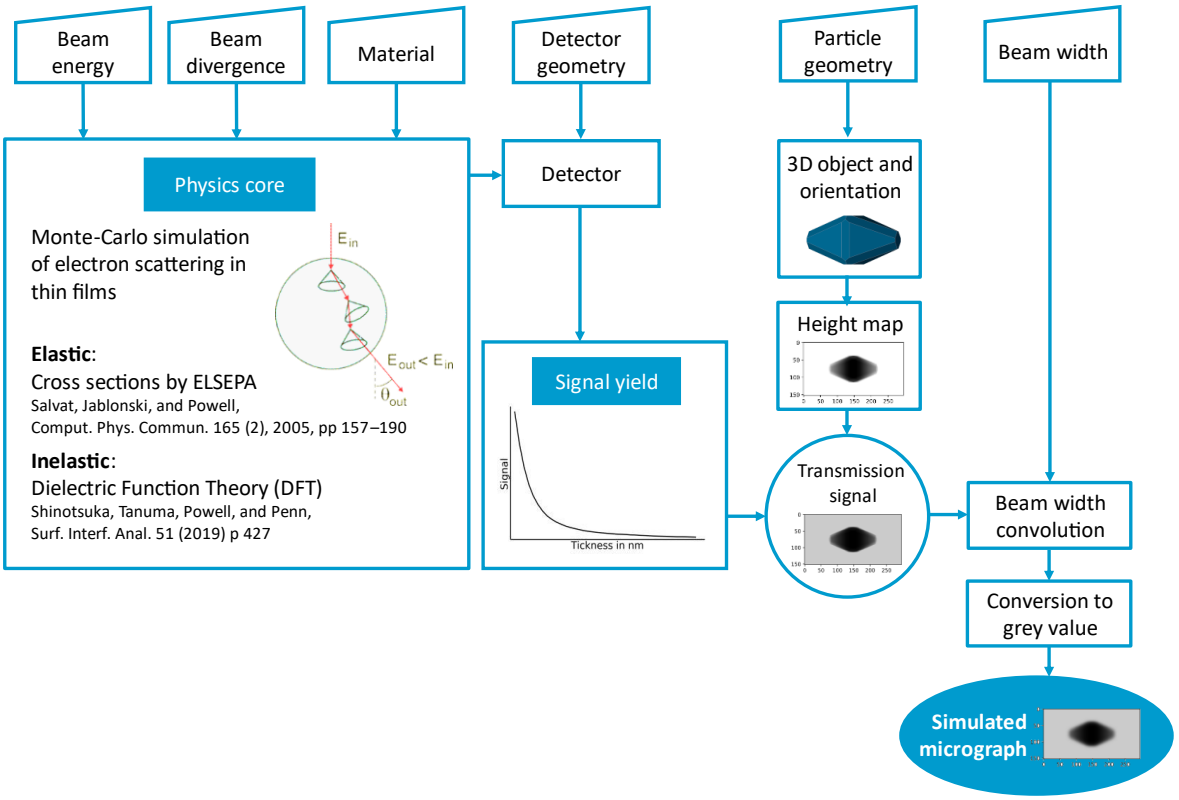

Figure 5: *Efficient simulation of TSEM micrographs by running a Monte Carlo simulation to obtain a yield curve and subsequently scanning a complex geometry of a nanoparticle*

To validate the fast efficient simulation method, relative threshold values characterizing the particle boundaries obtained by the Monte Carlo running on the center and at the boundary of the particle (for which we assume an uncertainty of about $u(S_{rel})=0.025$) are compared with the relative threshold values obtained by the efficient method as described in Figure 5. If the finitely wide and divergent beam falls on the boundary of the particle, a small fraction of those trajectories that are outside the particle enters into the particle side wall due to the divergence of the beam. This effect is visible in case of particles with straight vertical sidewalls, if the complete Monte Carlo simulation is employed, but omitted by the efficient simulation method. This effect, however, is negligible for curved particle boundaries, showing relative threshold values for cubic particles with a chamfer size of 20 % of the particle size in comparison of cubes with sharp edges.

4.3.3 AFM

Because the selected nPSize particles have nominal dimensions that are comparable to the AFM probe size, the geometry of the features in the raw measurement data are partly resulting from the probe shape. Especially the lateral dimensions are dilated by the probe shape, so particle width and shape properties in general, cannot be accurately extracted from raw AFM data without correcting for effects from the probe shape. The accuracy of the correction process has been investigated by VSL and SMD by first simulating the measurement process of a nano particle array with a spherical probe and subsequently correcting the dilated measured data with the same probe. The difference between the original and recovered field are at the sub-nanometer level demonstrating the validity of the approach. However, the selected system is nearly ideal without measurement noise and with well-known particles and probe shape.

Since the probe-sample interaction results in dilation of the actual nano particle, accurate reconstruction of the particle width requires detailed knowledge of the probe shape. The measurement process for spherical particles and the obtained profile is illustrated in Figure 6.

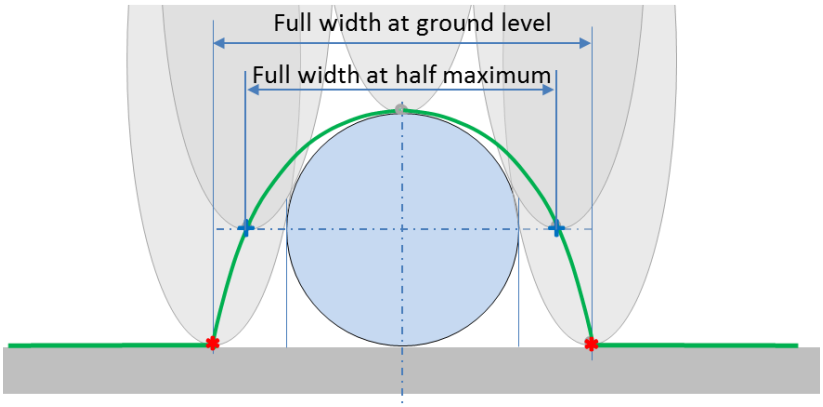

Figure 6: *Schematic view of the probe-sample interaction resulting in dilation of the particle profile (green line). The profile full width at ground level (red indicators) is dilated by the probe width at approximately half the nanoparticle height.*

The analysis of profiles to extract particle width is usually based on the measured full width a half maximum (FWHM) of the profile. However, as can be seen in Figure 6, the FWHM of the profile is not related to a clear property of the probe shape. Correction of the measured curve for the probe shape to determine the particle width is therefore not clear. In contrast, the full width at ground level provides a more accurate measurand to enable correction for the probe shape: at ground level the apparent width of the particle is dilated by the full width of the probe, estimated at approximately half the height of the particle. Therefore, correction of the measured full width at ground level with the probe width estimated at half the particle height should result in the best possible estimate for the actual particle width and should be similar to the particle height for a spherical particle. Our probe shape correction will therefore be based on the full width at ground level and the estimation of the probe width at half the height of the nanoparticle.

The measurements on the silica particles revealed a height of about 116 nm. This implies that the probe width must be determined at ~ 60 nm from the top of the probe in order to enable reconstruction of the particle width.

Measurements were performed on two types of tip characterizers on order to reconstruct the relevant part of the probe shape to enable correction of the raw NP data. The first characterizer was a sample with randomly oriented sharp structures. The measurements on this tip characterizer were analyzed by a blind reconstruction model to extract the probe shape for the two probes, Probe1 and Probe2.

From these reconstructed 3D shapes, the probe profiles along the horizontal scan direction through the maxima were calculated, as shown in Figure 7. Note that the reconstructed profiles are not sufficiently high to accurately determine the probe width at half the height of the nanoparticle, i.e. at about -60 nm. Extrapolation is possible as indicated by the red lines, but this does not seem to be an accurate method. In order to more accurately determine the probe width at about 60 nm from the top of the probe, measurements on a 1D line structure with straight edges and sufficient height were performed. The results are summarized in Table 11.

Table 11: *Results for the correction of the probe shape for the values of the full width at ground level and the full width at half maximum, compared to the measured average height of the particles*.

Probe	**Silica height**	**Probe width at half nanoparticle height**	**Silica full width at ground level**		**Silica full width at half maximum**	
			uncorrected	**corrected**	**uncorrected**	**corrected**
	/nm	/nm	/nm	/nm	/nm	/nm
Probe1	116.0 ± 2.7	81.0 ± 1.0	194.5 ± 4.4	113.5 ± 4.4	151.9 ± 3.9	70.9 ± 3.9
Probe2	115.8 ± 2.5	57.3 ± 3.1	174.3 ± 2.7	116.9 ± 2.7	135.4 ± 1.8	78.1 ± 1.8

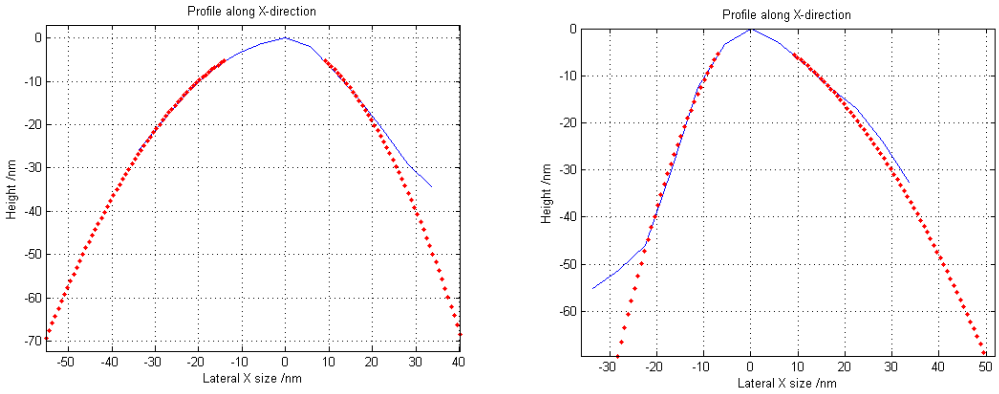

Figure 7: *Reconstructed profile of the probe along the fast scan direction for Probe1 (left) and Probe2 (right). The probe width at half the nanoparticle height was calculated by extrapolating the profiles (red).*

4.3.4 SAXS

A software tool was created by PTB to simulate the scattering from arbitrary form factors on the basis of the Debye scattering equation. It was then used to conduct a study to develop a simple protocol for the analysis of scattering curves for NP with complex shapes and a narrow size distribution. The chosen simulated shapes with increasing complexity were spheres, rods, cubes, octahedra and a hypothetical shape of a smurf.

The resulting scattering curves were then fitted with an ensemble of spheres using the Monte Carlo fitting algorithm implemented by BAM in McSAS. The resulting size distribution was then manually analyzed by a SAXS expert to guess the shape of the simulated ensemble. The expert was able to work out the simple shapes (spheres, rods, discs) from the size distribution, and correctly guessed the highly complex particles as a “complex shape,” but was unable to identify the cubes and octahedra. Based on this experience, a procedure for identifying the scattering from complex shapes was suggested, see Figure 8.The objective has been successfully achieved.

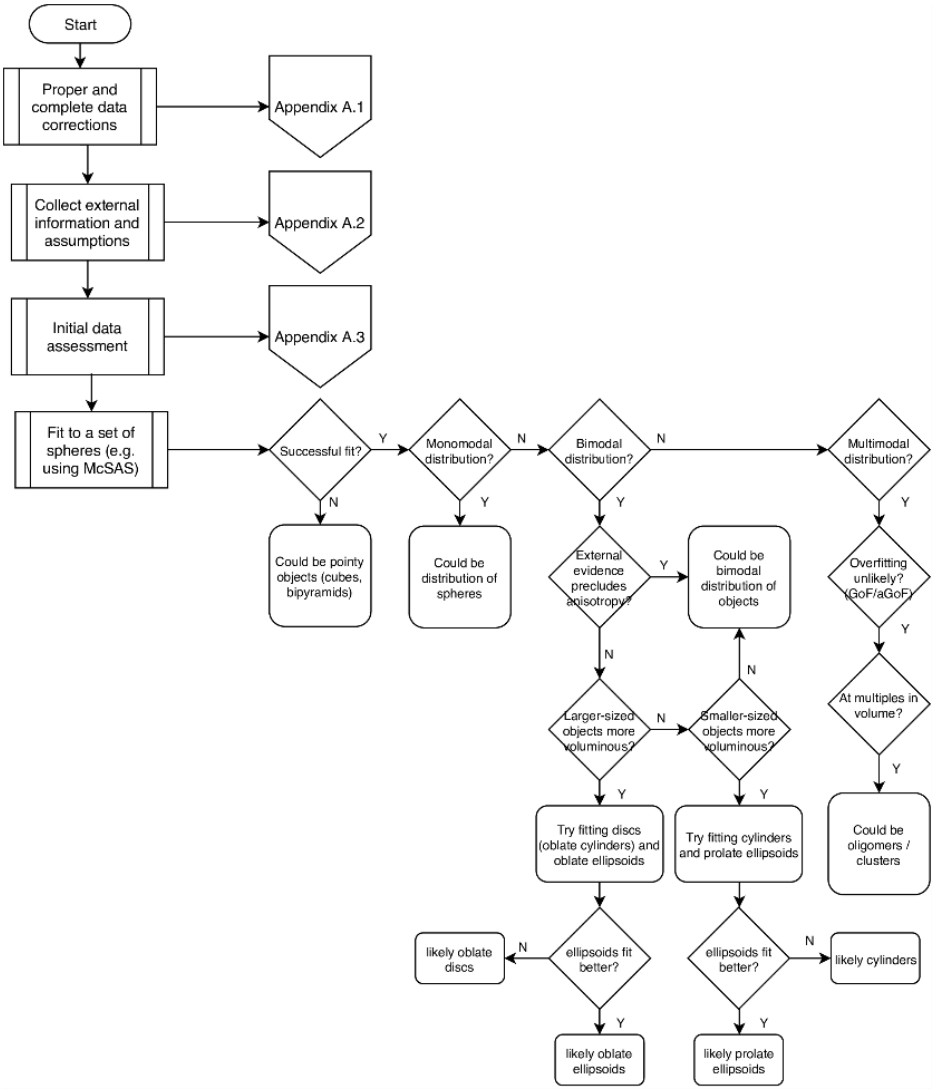

Figure 8: *Flow chart to determine the nanoparticle shape from the SAXS scattering curve, assuming narrow size distributions.*

4.4 Objective 4: Towards the use of the new physical models to develop validated and traceable methods for the transfer of nanoparticle size from (certified) reference nanoparticles of spherical shape and monodisperse size distribution to other types of nanoparticles.

4.4.1 Public database library of tagged electron microscopy images and AFM and SAXS measurement data

The image formats as used by the project partners have been established: TIFF, DM3, HDF5 and Bruker format. To label data, it was agreed that each partner uses Platypus™ software provided under the license by POLLEN. The image format for the public database is composed of TIFF image and an XML file describing information contained in the image. The public database was constructed by POLLEN using the labelled data produced by partners. The database grew gradually, following the output of labelled data produced by partners. The nPSize public database is on the Zenodo platform https://zenodo.org/communities/17nrm04-npsize/?page=1&size=20, where a dedicated nPSize project Zenodo community has been created.

The detailed description of the public database containing tagged electron microscopy (SEM, TEM and TSEM) images, AFM and SAXS measurement data can be found in the project Summary Report Description of the nPSize Public Database (Deliverable 1) in Zenodo https://doi.org/10.5281/zenodo.5789285 (Figure 9).

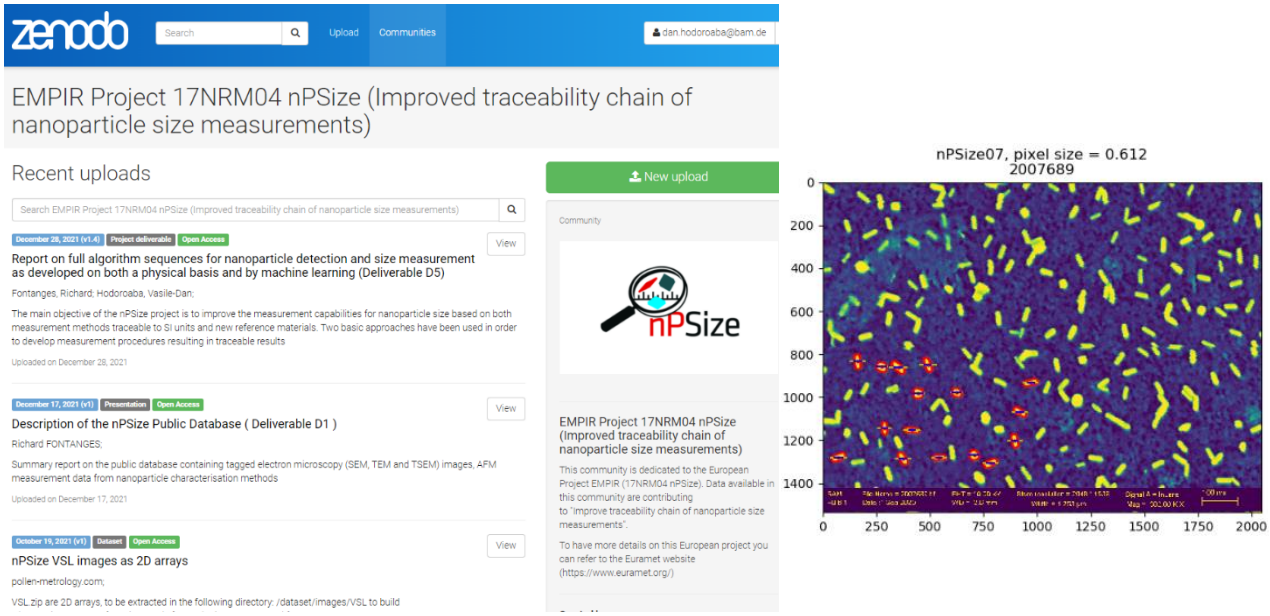

Figure 9: *left- Screenshot with the Zenodo interface showing the nPSize community and the nPSize datasets and reports created and published; right- Example with an SEM image of the nPSize7 (Au nanorods) taken by BAM as a snapshot on Zenodo after annotation.*

One purpose of the dataset is dedicated to machine learning (ML), with the Deep Learning technique as proposed by POLLEN: the data to be used for training are images with particles. Then, to build a model, one has to tell the model where the particles on each image are: these are the so-called 'annotations'. With a quantity of [image; particles], a dedicated model can be trained to automatically find the particles from any image.

In the present project, 12 different 'families' of nanoparticles have been selected by the partners and all can be found in the dataset. This tool shared with the partners during the life of the project, helps considerably in making these annotations. To be noticed is the fact that, before a model is trained, these annotations must be set manually. POLLEN's Platypus helps to manually annotate the regions of interest in the image, i.e. the contour of each particle. All the visible particles must be annotated; otherwise, the neuronal network is not able to learn finding particles (is some are annotated to be found, and other are not, then it is unclear what a particle is).

In all the project images (except for the SAXS images, which are physically different), the annotations are simply the yellow boundaries, that are referenced in (x, y) in each image. The images themselves are saved in the dataset in a format dedicated for modelling as an array of intensities (table of pixels). The objective has been successfully achieved.

4.4.2 Procedures for the validation of the performance parameters of the traceable measurement methods for complex nanoparticles

The performance parameters of the traceable measurement methods selected in this project have been validated based on the newly developed nPSize reference nanoparticles. An inter-laboratory comparison (ILC) exercise under the lead of LNE was conducted with all near-spherical, multi-modal and complex-shaped populations of nanoparticles produced by the project. The most samples were measured by all techniques; SEM, TEM, TSEM, AFM, SAXS and spICP-MS. By combining information from literature and ILC results, an overview matrix has been built by LNE and BAM compiling performances of each measurement method and includes: size range, shape, type/class of material, size distribution, polydispersity, sample state, capability for measuring constituent nanoparticles, aggregation/agglomeration state etc. Information about main sources of uncertainty and available standards were added. The complementarity of possibilities for a hybrid approach was included in the table as well. Figure 10 shows an excerpt from the overview matrix with the performance parameters attainable for non-spherical nanoparticles.

Very good
Good
Fair

			Type of method						
			COUNTING					ENSEMBLE	Comment
			SEM	TSEM	TEM	AFM	sp - ICP MS	SAXS	
Size range		1 nm -10 nm					N/A		
		10 nm -30 nm					Metal based only		
		30 nm -100 nm							
		100 nm -1 µm							
		1 µm -10 µm					>2µm only possible with specialized sample / introduction system		
		> 10 µm					only possible with specialized sample / introduction system		
Shape	Sphere	D = diameter	D_{AE}	D_{AE}	D_{AE}	h_{AFM}			
		expected σ	<1-2 nm	<1-2 nm	<1-2 nm	<2 nm	-	<2 nm	
	Sphere Multimodal	D = diameter	D_{AE}	D_{AE}	D_{AE}	h_{AFM}			spICPMS: equivalent spherical diameter (if composition and density are known)
		expected σ	<1-2 nm	<1-2 nm	<1-2 nm	<2 nm	-	<2 nm	
	Square-based bipyramid	L = length	depends on the orientation of NP deposited on substrate $D_{maxFeret}$				D_{VE} Volume equivalent diameter		Hybrid approach correlating AFM and EM for determining orientations Or combining EM and SAXS.
		expected σ	-	-	-	-			
		S = width	$D_{minFeret}$	$D_{minFeret}$	$D_{minFeret}$	h_{AFM}			
		expected σ	<2 nm	<2 nm	<2 nm	<4 nm		<7 nm	
	cubes	S = side	D_{Feret}	D_{Feret}	D_{Feret}	h_{AFM}	D_{VE}		
		expected σ	<2 nm	<2 nm	<2 nm	<8 nm			
	platelets	S = width	$D_{minFeret}$	$D_{minFeret}$	$D_{minFeret}$			not evaluated	Hybrid approach correlating AFM and EM for determining S and L by EM and H by AFM.
		expected σ	-	-	-	-			
		L = length	$D_{maxFeret}$	$D_{maxFeret}$	$D_{maxFeret}$		D_{VE}	not evaluated	
		expected σ	-	-	-	-			
		H = thickness	-	-	-	h_{AFM}	Suspension only	not evaluated	
		expected σ	-	-	-	-			
	acicular	D = section	$D_{minFeret}$	$D_{minFeret}$	$D_{minFeret}$	h_{AFM}		not evaluated	
		expected σ	-	-	-	-	D_{VE}		
		L = length	$D_{maxFeret}$	$D_{maxFeret}$	$D_{maxFeret}$			not evaluated	
		expected σ	-	-	-	-			
	nanorods	D = section	$D_{minFeret}$	$D_{minFeret}$	$D_{minFeret}$	h_{AFM}		not evaluated	spICPMS: some literature reports suggest that for high aspect ratio particles it is possible to address non-sphericity to some extent, but more research is needed in this area.
		expected σ	<2 nm	<2 nm	<2 nm	<4,5 nm	D_{VE}		
		L = length	$D_{maxFeret}$	$D_{maxFeret}$	$D_{maxFeret}$			not evaluated	
		expected σ	<2 nm	<2 nm	<2 nm	-			

Figure 10: *Excerpt from the overview matrix with experimentally validated performance parameters for SEM, TSEM, TEM, AFM, spICP-MS and SAXS for nanoparticles of non-spherical shape.*

In Figure 11 the TEM, SEM and AFM results of the $D_{MaxFeret}$ and $D_{MinFeret}$ for the nPSize4 sample (Au nanocubes) are given. The small discrepancies between the two descriptors and results between methods can be explained and will be given in detail in the planed publication (lead LNE and BAM).

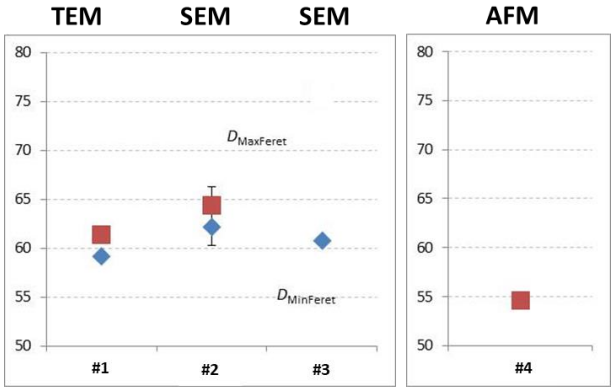

Figure 11: *TEM, SEM and AFM results of $D_{MaxFeret}$ and $D_{MinFeret}$ for the nPSize4 material (Au nanocubes). The unit of the ordinate is nm.*

The results of the nPSize ILC measurements applying the nPSize protocols on the nPSize7 material (Au nanorods) are shown in Figure 12.

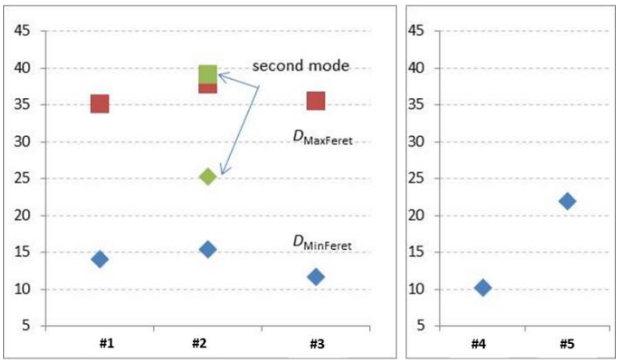

Figure 12: *SEM and TEM results of $D_{MaxFeret}$ and $D_{MinFeret}$ (left) and AFM (right) for the nPSize7 material (Au nanorods). The unit of the ordinate is nm.*

The $D_{MaxFeret}$ and $D_{MinFeret}$ for the nPSize6 sample (TiO_2 elongated nanoparticles) as measured with SEM are given in Figure 13.

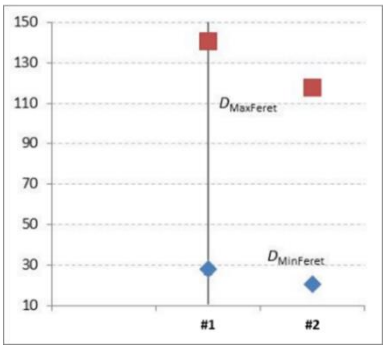

Figure 13: *SEM results of $D_{MaxFeret}$ and $D_{MinFeret}$ (in nm) for the nPSize6 material (TiO_2 acicular nanoparticles)*

As far as the measurement capabilities of non-monodisperse nanoparticles are regarded two examples are given here: in Figure 14 the results of of the bi-modal, nearly spherical nanoparticles of Au nPSize1 material; the same evaluation according to the same procedures but for the silica bimodal spherical nanoparticles nPSize12 is visualized in Figure 15.

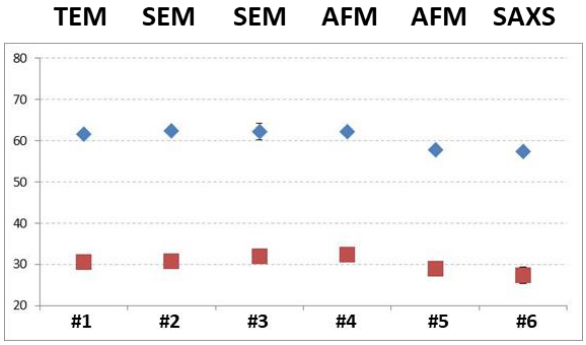

Figure 14: *SEM, TEM, AFM and SAXS results of $D_{MinFeret}$ (in nm) for the nPSize1 material (Au bi-modal nanoparticles).*

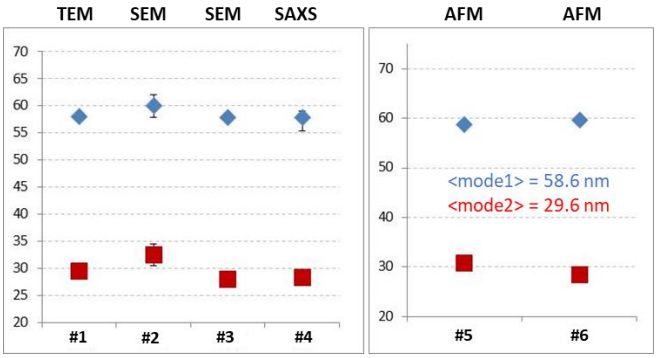

Figure 15: *SEM, TEM, AFM and SAXS results of $D_{MinFeret}$ (in nm) for the nPSize12 material (SiO_2 bi-modal nanoparticles).*

The project material nPSize3, the bipyramidal titania nanoparticles, has been also supposed to an extensive characterization according to the analysis procedures developed in the project. Due to its complexity, this material was selected as the best possible case study for testing the hybrid analysis approaches developed in nPSize, where targeted correlative (imaging) analysis makes the difference from a 2D image projection characterization to a true 3D characterization.

4.4.3 Hybrid approaches and data fusion for the most accurate assessment of complex nanoparticles

The hardest test of the nPSize measurement procedures is the accurate measurement of the most complex material, namely nPSize3, the titania bipyramides. Several papers on accurate procedures for its characterization have been published already and other are in progress. The material is very attractive for testing the limits of nanoparticle size characterization procedures. For the first time, correlative analysis approaches involving conventional measurement methods SEM and AFM (LNE), but also TSEM (BAM), SAXS (PTB) and a new method TKD (Transmission Kikuchi Diffraction, University of Science and Technology in Krakow, Poland) have been used.

One correlative approach is the imaging of the same field-of-view with the same nanoparticles by SEM and AFM. Figure 16 illustrates the highlights of this hybrid approach. Both AFM and SEM are able to identify the orientation of a particle on the substrate: AFM by the asymmetry of a linescan along the long axis of a bipyramid and SEM by tilting the sample (with stage) and 'chasing' the longest projection of the long axis of a NP. Furthermore, AFM allows to estimate the angle between two adjacent {101} faces. The measured angles are very close (below 7 %) to the value of the theoretical angle of 136.6°.

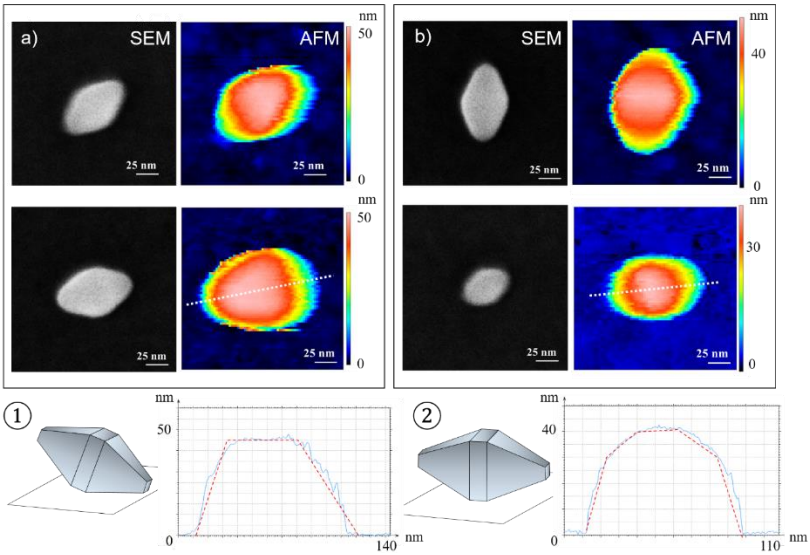

Figure 16: *AFM (colored) and SEM (grey) images of nPSize3 with two geometrical orientations on the substrate: a) with a {101} facet and b) with the long axis parallel to the substrate. For each orientation, an AFM profile along the major axis of the TiO_2 NPs (as shown by dotted lines) is displayed at the bottom.*

Very detailed results obtained after the correlative analyses with SEM and TSEM, EM with SAXS (with the NPs in liquid suspension), or EM with TKD can be found in the recently published nPSize paper dedicated exclusively to the nano-bipyramids: Crouzier *et al.* 2021.

5 Impact

The project website has been developed by BAM and is available to stakeholders at: https://www.bam.de/Content/DE/Projekte/laufend/nPSize/npsize.html. Four public workshops covering the topics 'current situation and future needs for reference nanomaterials', 'production and certification of reference nanomaterials', 'metrology for measurement of nanoparticle size by electron microscopy and atomic force microscopy' and 'Traceable characterisation of NPs (size, size distribution, concentration determination) by SAXS' were organised. A total of about 250 people from public institutes, academia, and industry, the JRC, DIN, ISO, CEN, EU and the Chief Stakeholder have participated. A new nPSize YouTube channel has been created by CEA where 12 videos mainly on measurement of NP size by SAXS have been uploaded: https://www.youtube.com/channel/UC6kdn4epvHF4OZM7T-mLXJQ/videos. 24 subscribers and about 1500 views of the published videos have been attained at the end of the project (Dec 2021). The project results were presented at international conferences including posters and talks (25 presentations). The project technical results are available in 14 peer reviewed publications. One textbook on the characterisation of nanoparticles was published. The topics cover the synthesis and characterisation of non-spherical nanoparticles, machine

learning approaches to predict final size and shape parameters of synthesized nanomaterials, accurate analysis of particle size distribution for non-spherically shaped nanoparticles by new hybrid approaches, etc.

Impact on industrial and other user communities

Representatives of manufacturers of nanomaterials attended the project's public Workshop on Reference Nanomaterials on May 14-15, 2018 organized by PTB and BAM. At the workshop the VCI (German Chemistry Industry Association) Chairman presented and discussed the needs expressed by this large industry association in terms of regulatory framework, development of measurement strategy at nanoscale, and European definition of nanomaterials. Stakeholder requirements were also systematically collected for use in the project. The consortium has links with instrument manufacturers such as Zeiss and Hitachi (manufacturers of electron microscopes) and Xenocs (a manufacturer of X-ray based devices for nanomaterial characterisation), which will support prompt uptake of the project results by industry. In addition, the project has developed under the lead of Pollen a nPSize 'community' on the Zenodo platform for a public database containing tagged electron microscopy (SEM, TEM and TSEM) images, AFM and SAXS measurement data from nanoparticle characterisation methods: https://zenodo.org/communities/17nrm04-npsize/?page=1&size=20. A Training course on Reference nanoparticles Production and Certification has taken place successfully at LGC (https://twitter.com/NML_ChemBioGC/status/1207582000313442304). Further, a fruitful virtual training course on the Metrology for Measurement of Nanoparticle Size by Imaging (EM and AFM) was organised online by VSL (https://www.vsl.nl/en/about-vsl/news/virtual-training-course-metrology-measurement-nanoparticle-size). At the Training Course 100 participants have attended from academia (many PhD students and postdocs) and industry.

Impact on the metrology and scientific communities

The project will have direct impact on several metrology committees, especially the EURAMET Technical Committee for Length (TC-L), and the CIPM Consultative Committee for Length (CCL) Working Group on Nanometrology (WG-N). The in-depth evaluation of the performance of the traceable nanoparticle sizing techniques selected in this project enhances the understanding of the physical processes involved in the signal generation and their dependence on shape and material variations. For the first time in a large research project both critical aspects, namely the lack of reference nanoparticles and signal modelling are systematically considered. Furthermore, the data fusion for hybrid methods facilitates better knowledge of nanoparticle 3D size measurements.

The project partners have disseminated the results to the scientific nanoparticle characterisation community at international conferences such as NANOSAFE 2018, Microscopy & Microanalysis 2019, Nanoscale 2019, ECASIA 2019, Microscopy & Microanalysis 2020, NANOSAFE 2020, E-MRS Spring 2021, Microscopy & Microanalysis 2021, and E-MRS Fall 2021. Following recommendation by the Chief Stakeholder, the project has been presented at the 2019 International Congress of Metrology.

Impact on relevant standards

The project has the following links between project partners and national standardisation bodies; ISO/TC 229 (BAM, DIN, LGC), CEN/TC 352 (DIN, BAM, LGC) and ISO/TC 202 (BAM, PTB). The project has been presented to DIN NA 062-08-17 AA Nanotechnologies. A case study on the analysis of the size and shape distribution of TiO_2 bipyramidal NPs by TEM under the lead of BAM, with support from UNITO and PTB has been included in the standard ISO 21363 Nanotechnologies — Measurements of particle size and shape distributions by transmission electron microscopy, recently published (August 2020). This documentary standard is of special importance as the very first full ISO standard developed under ISO/TC 229 Nanotechnologies. With support from DIN, a project liaison between the present project with BAM as a coordinator and CEN/TC 352 has been successfully contracted. The project has also provided input to OECD (Organisation for Economic Cooperation and Development) Test 110 Guideline on Particle Size and Particle Size Distribution of Manufactured Nanomaterials. The specification DIN SPEC 52407 was adapted, and the proposal was prepared by PTB with contributions from BAM to be submitted to ISO/TC 229 Nanotechnologies JWG 2 as a new work item. A contribution to ISO/TC 24 SC 4 ISO 19430 was made by PTB by drafting parts of the revised document.

Of particular impact for the standardisation nanoparticle community, two new VAMAS projects consisting of two inter-laboratory comparisons have been approved and started in November 2021 under VAMAS/TWA 34 'Nanoparticle population' having BAM as lead of both projects. Within these projects, TiO_2 nano bipyramids and two bimodal SiO_2 materials were selected as materials of choice for the measurement of complex shape nanoparticles as well as the measurement of the number concentration of bimodal nanoparticles. Sample preparation, measurement and data analysis protocols developed in the present project are offered to all

participants. If successful, the results of the ongoing VAMAS inter-laboratory studies will be published and used to extend case studies in the available standard ISO 21363 under the lead of BAM.

Longer-term economic, social and environmental impacts

It is intended that the measurement capabilities for accurate size distribution of nanoparticles developed by this project will be further transferred via normative documents, dedicated workshops and new reference nanoparticles to European large-scale manufacturers of nanoparticles, to service laboratories, and measurement instrument manufacturers. The new reference nanoparticles, improved signal modelling and data fusion offered by the project, will also contribute to metrological measurement capabilities to address the question of how to implement the EC definition of a nanomaterial. Furthermore, the project will contribute to the accurate identification of nanomaterials and nano-products and thus will provide a more reliable link to characterisation of potential toxic effect, environmental protection and safety will be improved.

6 List of publications

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This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

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