TECHNICAL SPECIFICATION

ISO/TS 23151

First edition
2021-09

Nanotechnologies — Particle size distribution for cellulose nanocrystals

Nanotechnologies — Distribution en taille des particules pour les nanocristaux de cellulose

Reference number
ISO/TS 23151:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html and www.iec.ch/national-committees.

Introduction

Cellulose nanomaterials, including cellulose nanocrystals (CNCs) and cellulose nanofibrils, are anticipated to have significant commercial impact. Cellulose nanocrystals are produced from naturally occurring cellulose, primarily from wood pulps and annual plants, by acid hydrolysis. Their production from readily available cellulose sources makes them a candidate for use as a potentially non-toxic, biodegradable and sustainable nanomaterial. The recent demonstration of the feasibility of large-scale CNC production and the availability of infrastructure for harvesting raw materials will facilitate their commercial development. CNCs and cellulose nanofibrils are produced in a number of countries on pilot, pre-commercial or commercial scales. Estimates of the market potential for cellulosic nanomaterials are as high as 35 million metric tons annually, depending on the predicted applications and the estimated market penetration[10],[11]. Standards for characterization of CNCs are required for material certification to facilitate sustained commercial and applications development.

Cellulose nanocrystals have high crystallinity and are nanorods with high aspect ratio, surface area and mechanical strength. They assemble to give a chiral nematic phase with unique optical properties and their surface chemistry can be modified to ensure colloidal stability in water and to facilitate dispersion in a variety of matrices. These properties, plus their biocompatibility, low cost and minimal toxicity, enable many potential applications. Industrial producers are working with receptor industries in various application areas, including nanocomposite materials, health and personal care products, paints, adhesives and thin films, rheology modifiers and optical films and devices. Standardization activities within ISO/TC 229 and ISO/TC 6 have focused on nomenclature and terminology, characterization methods in general and specific methods for determining surface functional groups, metal ion and dry ash content. Particle size distribution is also a key property for CNC characterization. Particle morphology and size distribution control some properties of individual CNCs and contribute in part to their organization in suspensions, dry films and matrices. These properties and chemical characteristics determine CNC colloidal stability, viscosity and self-assembly, as well as performance in applications (e.g. reinforcement of nanocomposites). Length distribution may also be used to differentiate among cellulose nanocrystal grades or products.

This document describes a method for reproducibly dispersing dry CNCs for preparation of microscopy samples, provides image acquisition protocols for atomic force and transmission electron microscopy and summarizes image analysis procedures for determining particle size distributions. The methods are compatible with analysis of CNCs as produced by several processes and can be extended to surface modified CNCs with adjustment of dispersion and sample deposition methods. The two microscopy methods provide complementary information, and both have been widely used for size analysis of CNCs.

Nanotechnologies — Particle size distribution for cellulose nanocrystals

1 Scope

This document describes methods for the measurement of particle size distributions for cellulose nanocrystals using atomic force microscopy and transmission electron microscopy. The document provides a protocol for the reproducible dispersion of the material using ultrasonication, as assessed using dynamic light scattering. Sample preparation for microscopy, image acquisition and data analysis are included.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO 21363:2020, *Nanotechnologies — Measurements of particle size and shape distributions by transmission electron microscopy*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
cellulose nanocrystal
nanocrystal predominantly composed of cellulose with at least one *elementary fibril* (3.3), containing predominantly crystalline and paracrystalline regions, with an aspect ratio of usually less than 50 but usually greater than 5, not exhibiting longitudinal splits, inter-particle entanglement, or network-like structures

Note 1 to entry: The dimensions are typically 3 nm to 50 nm in cross-section and 100 nm to several µm in length depending on the source of the cellulose nanocrystal.

Note 2 to entry: The aspect ratio refers to the ratio of the longest to the shortest dimension.

Note 3 to entry: Historically cellulose nanocrystals have been called nanocrystalline cellulose (NCC), whiskers such as cellulose nanowhiskers (CNW), and microfibrils such as cellulose microfibrils; they have also been called spheres, needles or nanowires based on their shape, dimensions and morphology; other names have included cellulose micelles, cellulose crystallites and cellulose microcrystals.

[SOURCE: ISO/TS 20477:2017]

3.2
cellulose nanofibril
cellulose nanofibre composed of at least one *elementary fibril* (3.3), containing crystalline, paracrystalline and amorphous regions, with aspect ratio usually greater than 10, which may contain longitudinal splits, entanglement between particles, or network-like structures

Note 1 to entry: The dimensions are typically 3 nm to 100 nm in cross-section and typically up to 100 µm in length.

Note 2 to entry: The aspect ratio refers to the ratio of the longest to the shortest dimensions.

Note 3 to entry: The terms "nanofibrillated cellulose", "nanofibrillar cellulose", "microfibrillated cellulose", "microfibrillar cellulose", "cellulose microfibril" and "cellulose nanofibre" have been used to describe cellulose nanofibrils produced by mechanical treatment of plant materials often combined with chemical or enzymatic pre-treatment steps.

Note 4 to entry: Cellulose nanofibrils produced from plant sources by mechanical processes usually contain hemicellulose and in some cases lignin.

Note 5 to entry: Some cellulose nanofibrils might have functional groups on their surface as a result of the manufacturing process.

[SOURCE: ISO/TS 20477:2017, 3.3.6, modified — Note 6 to entry has been deleted.]

3.3
elementary fibril
structure, originating from a single terminal enzyme complex, having a configuration of cellulose chains specific to each cellulose-producing plant, animal, algal and bacteria species

[SOURCE: ISO/TS 20477:2017, 3.2.5]

4 Abbreviated terms

AFM	atomic force microscopy
CNC(s)	cellulose nanocrystal(s)
DLS	dynamic light scattering
ILC	interlaboratory comparison
PLL	poly-L-lysine
PSD	particle size distribution
PI	polydispersity index
PVDF	polyvinylidene difluoride
TEM	transmission electron microscopy
VAMAS	Versailles project on advanced materials and standards

5 Dispersion of CNCs

5.1 General considerations

Dry CNCs are aggregated and require energy input, typically by ultrasonication, for dispersion. Previous studies have examined the sonication efficiency for CNCs derived from wood pulp by sulfuric acid hydrolysis and neutralization with sodium hydroxide which generates $-SO_3^-Na^+$ groups on the

surface[12]. The average CNC size and size distribution varied with the sample concentration even when the sonication energy divided by mass of CNC was kept constant; CNC suspensions with a mass fraction of 2 % were shown to be optimal for efficient dispersion by sonication. The protocol below has been developed using spray-dried sodium exchanged sulfated CNCs. The protocol may require optimization for freeze-dried CNCs[12],[13], CNCs produced from other cellulose biomass sources and CNCs with a different loading of sulfate half esters or other negatively charged surface groups.

A procedure for sample preparation and sonication (probe sonicator) to generate a well-dispersed CNC suspension is provided in 5.2. Bath sonication has been shown to be inadequate for dispersion of CNCs[12][14]. A protocol for analysis of CNC suspensions by DLS is provided in 5.3; general details on the use of DLS for particle size determination are available in ISO 22412[7].

Representative results illustrating changes in size (Z-average) and PI as a function of sonication energy are provided in Annex A. The Z-average is the intensity-weighted harmonic mean diameter derived from a cumulants analysis of DLS data, as described in ISO 22412[7].

The Z-average provides the equivalent hydrodynamic diameter, the diameter of a sphere that will diffuse at the same rate as the acicular CNC particle.

Although the Z-average determined by DLS is not a direct measure of CNC particle size, it provides a useful and rapid means of assessing changes in relative size for a large number of CNC suspensions. Recent developments in the use of field flow fractionation coupled with multiple detection systems for CNC analysis may provide an alternative to DLS analysis[15]. The protocols for dispersion by sonication and DLS assessment have been used by three laboratories with repeatable and reproducible results[12][15][16].

Plots of Z-average and PI as a function of sonication energy can be used to select an appropriate sonication energy for specific samples, see 5.4. This selection is a compromise between applying sufficient sonication to disperse most aggregates while ensuring that the applied sonication energy does not damage the sample.

5.2 Dispersion of CNCs by sonication

Remove dry CNC from low temperature storage and keep unopened until the sample reaches room temperature (typically several hours).

Use an analytical balance to weigh the desired amount of CNC in a polypropylene centrifuge tube. Amounts of CNC in the 50 mg to 300 mg range have been used with either 15 ml or 50 ml centrifuge tubes in this protocol for preparation of 2 % mass fraction CNC suspensions. Glass tubes can be used, although some optimization of the protocol may be required since the sonication efficiency is sensitive to a number of factors, including the probe depth and placement and the container material and geometry[17].

Add deionized water to the tube in the amount required to obtain a 2 % mass fraction suspension of CNC, close the tube cap, and shake the tube vigorously by hand for a few seconds to promote CNC dispersion. Freshly obtained deionized water (18,2 MΩ cm) filtered with a 0,22 µm filter (typically part of the purification system) is used throughout.

The optimal concentration of CNC for dispersion by ultrasonication is 2 % mass fraction; disruption of aggregates and agglomerates by sonication is less effective at lower concentrations. If suspensions of lower concentration are required, dilute the sonicated 2 % mass fraction CNC suspension with deionized water to the desired concentration.

Leave the mixture at room temperature for 24 h for the CNC to disperse. The mixture can be shaken by hand periodically to accelerate dispersion; a tube shaker may also be used.

Check the condition of the ultrasonic probe (a 6-mm probe is recommended for the volumes used here) and clean if pitting or roughness of the surface is observed.

Sonication is most effective at low temperatures. Therefore, heating of the suspension during prolonged sonication should be avoided. The temperature increase should not be more than 2 °C to 3 °C for the amounts of dry CNC and processing energy recommended in this protocol, if the probe is in good working condition, properly installed in the processor, and immersed in the suspension as recommended above. During sonication, the tube may be placed in a room temperature water bath cooled when necessary with a few ice cubes. Use of an ice bath is not recommended.

Immerse the ultrasonic probe in the suspension ensuring that the tip is centered in the tube and at least 1,3 cm both below the suspension surface and above the bottom of the tube.

Sonicate the suspension with the required energy (J/g dry CNC) at room temperature and an average power of approximately 10 W. Ensure that the suspension surface remains as flat as possible and no excessive aerosoling or bubbling is observed. If excessive aerosoling, bubbling, or suspension surface fluctuation is observed, adjust the probe position immediately. Cover the tube to minimize loss of suspension due to aerosoling.

The energy transfer efficiency may be measured calorimetrically[17] to ensure that the applied energy is reliable and does not change with time. Knowledge of the sonication energy is necessary for comparisons between laboratories.

Remove the sample from the ultrasonic processor, and store for a short period of time at room temperature (≈ 21 °C to 22 °C) or refrigerate (≈ 5 °C) for longer term storage.

NOTE This protocol has been tested with 50 mg to 300 mg dry CNC; preparation of suspensions with larger amounts of CNC can require optimization of sonication conditions.

5.3 Dynamic light scattering assessment of dispersions

Set up the instrument as recommended in the manual.

Information on the importance of cell cleanliness and handling and proper technique for preparing and transferring suspensions for DLS measurements is available in ISO/TR 22814[8].

It is good laboratory practice to verify the operability of a DLS instrument by measuring a reference nanomaterial (for which DLS data is available) to obtain Z-average and PI. Gold, silica and polystyrene nanoparticles with diameter <100 nm are in the same size range as most CNC samples. For larger CNCs, a reference material with diameter above 100 nm may be used. The use of a reference material from a source qualified under ISO guidelines[1] is recommended. The measured Z-average and PI should be within the quoted uncertainty for the reference material. It is important to note that instrument operability as verified using a reference material does not mean that a Z-average value obtained for acicular CNCs is a quantitative or accurate measurement of diameter.

Dilute the 2 % mass fraction CNC suspensions to 0,1 % using deionized water, and then add 1 ml of 10 mmol/l NaCl solution to 1 ml of 0,1 % mass fraction CNC suspension to obtain 2 ml of 0,05 % mass fraction suspension in 5 mmol/L NaCl. The 0,05 % suspension shall be analyzed within several hours of preparation and shaken vigorously before transfer to the DLS cell. Filter the sample through a 0,45 µm PVDF membrane syringe filter and discard the first several drops before adding the required volume to the DLS cuvette. Ensure that there are no bubbles in the cell.

Place the cuvette in the instrument and equilibrate at the desired temperature. The time required for equilibration will vary depending on the difference between the target temperature and the ambient temperature. The equilibration time can be verified by measuring the temperature for an equivalent volume of water under the same conditions. Adjust the scattering intensity using the instrument software. Measure each sample three times with each measurement consisting of the average of a number of runs (e.g. 10 runs of 10 s each).

Use the cumulants method to obtain the three-measurement average value and standard deviation for Z-average and PI for the sample.

NOTE Different instrument optical configurations are available. The use of forward/backward scattering and the scattering angle will affect the measured Z-average.

5.4 Determination of optimal sonication energy

Sonicate CNC suspensions with varying energies and measure the DLS Z-average and PI for each sample as described in 5.3. Plot Z-average and PI against sonication energy.

Select the optimal sonication energy for production of a well-dispersed suspension from a region of the curve where the measured Z-average and PI change slowly with increasing energy. An example plot is shown in Annex A.

To ensure reproducibility, measure a minimum of three replicate, independently prepared samples sonicated with the selected optimal sonication energy.

6 Sample preparation for microscopy

6.1 General considerations

There are a number of general considerations that apply to the preparation of CNCs deposited on a suitable support for either AFM or TEM. The first consideration is the importance of ensuring that a representative sample is used. When starting with dry CNCs, it is important to verify that the sample is well-mixed prior to weighing a sub-sample for dispersion. It is recommended to prepare dispersions from three sub-samples in order to confirm that the preparation of the dispersion by sonication is reproducible. Sonicate each of the three samples with the required sonication energy and then measure the DLS Z-average and PI as described in 5.3. Changes in Z-average of less than 5 % indicate reproducible dispersion of the sample. Alternatively, the entire sonication curve can be measured for each of the sub-samples.

A second consideration is the agglomeration of the CNCs in the initial suspension. Reduction (but not complete removal) of aggregates and agglomerates in solution can be accomplished by sonication and filtration.

The third consideration is the selection of an appropriate support or TEM grid and a deposition method that minimizes agglomeration of particles, while maximizing the number of individual particles that can be analyzed per image. The use of a positively charged support or grid is in principle useful for immobilization of negatively charged CNCs. Further details for AFM and TEM are noted in 6.2 and 6.3, respectively.

A final factor is the number of independently prepared samples that should be imaged and the number of particles that must be analyzed. Imaging multiple samples will provide information on reproducibility of the sample deposition process and its possible impact on the CNC size distribution. The number of individual particles (n) analyzed for each sample must be sufficiently large that the parameters which define the size distribution (e.g. mean and standard deviation for a normal distribution) can be determined with the desired level of uncertainty. As a general guideline the uncertainty is inversely proportional to the square root of n for normal distributions; an analysis of the effects of sample size on measurement uncertainty for log normal distributions can be found in ISO 13322-1:2014, Annex A[4]. The number of particles required will increase with increasing polydispersity of the sample. Recommended starting points in a number of studies range from 200 particles to 1 000 particles. ISO 21363 recommends analysis of 500 particles as a starting point and this has been adopted for the ILCs for AFM and TEM of CNCs that are summarized in Annexes C and D.

Although automation of AFM and TEM image analysis can be used successfully for a number of spherical and high contrast nanomaterials (see ISO 21363 and references cited therein for TEM examples), there are currently no reliable methods for automation of image analysis for CNCs.

Representative methods for sample deposition are outlined below.

NOTE Some optimization of sample concentrations and amounts can be required for specific CNC samples.

6.2 AFM sample preparation

Most AFM imaging of CNCs has employed mica as the support, typically coated with a thin layer of poly-L-lysine (PLL) [14],[18]. This surface coating is preferable to bare mica since electrostatic effects help to immobilize the CNCs thereby minimizing particle agglomeration and possible artifacts due to movement of particles during imaging. Other substrates have been used occasionally; see ISO/TR 19716[5] for additional details. The procedure below employs positively charged PLL coated mica and uses spin coating for deposition. This deposition procedure provides more reproducible samples (area to area particle density) than incubation methods and is designed to maximize the number of particles per image while minimizing agglomeration and aggregation [16],[19].

Prepare a suspension of CNCs in water as described in Clause 5. Dilute 500-fold with deionized water.

Prepare a PLL-coated slide by adding an aliquot of 0,01 % mass fraction PLL solution to a freshly cleaved mica substrate (e.g. 40 µl for 12 mm diameter mica and 200 ul for 2,54 cm × 2,54 cm mica). Place the mica with PLL solution in a covered petri dish for 10 min. Rinse the mica substrate with deionized water five times and dry in a nitrogen stream.

Pipette the freshly diluted CNC suspension onto the center of a freshly prepared PLL-mica substrate that is mounted in the spin coater; volumes of 40 µl and 200 µl are adequate for 12 mm diameter and 2,54 cm × 2,54 cm mica, respectively. Ensure that the suspension covers most of the substrate area. Spin the mica substrate at 4 000 rpm for 25 s with an acceleration rate of 2 000 rpm/s. Air dry the sample and store in a desiccator under a positive pressure of nitrogen prior to imaging.

Some optimization (amount and concentration of CNC suspension, spin coating speed and time) of the above procedure can be required, depending on the sample dispersion and aggregation level of the initial sample.

NOTE Samples can also be prepared by incubating an aliquot of CNC suspension (≈ 80 µl of 0,001 % mass fraction CNC for 2,54 cm × 2,54 cm mica) on PLL-coated mica for 2 min, washing 5 times with deionized water and drying under nitrogen. Typically the level of agglomeration will be higher and the area-to-area reproducibility lower for samples prepared by incubation than for those prepared by spin coating [16],[20].

6.3 TEM sample preparation

Sample preparation for TEM has been described in several recent reviews [14],[21]-[23]. The following procedure is typical of many literature studies and has been employed to characterize a reference material and samples for an interlaboratory comparison (see Annex D).

Prepare a suspension of CNCs in water as described in Clause 5. Dilute the suspension ≈100-fold with deionized water and vortex-mix for 5 s.

Plasma clean (2 min) a carbon film covered copper grid (e.g. 200 mesh, Ted Pella 01840-F). Deposit 10 µl of CNC suspension on the grid, leave for 4 min and then wick away excess liquid with a filter paper. Wash the sample by adding one drop of deionized water to the grid and wicking with a filter paper after several seconds.

Stain the sample by depositing 10 µl of filtered (0,22 µm PVDF filter) 2 % mass fraction uranyl acetate solution on the grid and leaving for 4 min. Immerse the grid in deionized water, remove the sample and air dry for at least 1 h prior to installation in the microscope.

NOTE After uranyl acetate staining the grid can be washed by adding one drop of deionized water and wicking with filter paper, rather than immersion in water.

7 Atomic force microscopy

7.1 General

Atomic force microscopy is used to measure the PSD for length and height for CNCs. Lateral dimensions derived from AFM images are influenced by tip-particle convolution. Due to the high aspect ratios

of CNCs, the effect of convolution is proportionally smaller for length. However, the magnitude of the broadening due to tip-particle convolution is comparable to the CNC width and therefore has a significant effect on width measurements. Measurement of width by AFM is not recommended unless a correction for convolution effects is applied[24]. Imaging conditions shall be optimized to ensure that the minimum possible imaging force is used to prevent compression of the particles. Size measurements shall only be derived from areas that have not previously been scanned.

7.2 Instrumentation and accessories

The following instruments and accessories can be used to image CNCs by atomic force microscopy and measure the particle size distribution:

— AFM capable of high-resolution imaging in contact and intermittent contact mode;

— AFM probes for both contact and intermittent contact imaging in air;

— either calibration grids or nanoparticle reference materials, or both;

— image analysis software.

7.3 Microscope calibration

Dimensional calibration of the microscope shall be verified prior to CNC imaging unless the calibration records indicate that this is not necessary. The frequency of microscope calibration depends on the type of instrument and its stability, the purpose of the measurements and potential changes in ambient operating conditions. Calibration, if necessary, shall be carried out according to the manufacturer's instructions. General guidance for calibration of height and lateral dimensions for AFM is provided in ISO 11952[3] and a more practical guide for users is currently under development[25]. The use of multiple standards that cover the appropriate x-y and z-scales for CNC imaging and that have certified values and uncertainty and metrological traceability are preferred. Typical calibration standards include step height standards (z-scale) and 2D lateral measurement standards that have equidistant structures with defined features with a fixed spacing (x-y scale).

7.4 Data acquisition

Select an appropriate tip for intermittent contact mode imaging and install in the AFM. CNCs have been imaged with cantilevers varying in spring constant from $k \approx 40$ N/m to $k < 10$ N/m and give comparable results provided that care is taken to minimize the imaging force.

Select initial scan parameters and tune the cantilever resonance.

Install the sample and engage the tip and adjust parameters for intermittent contact mode imaging. Adjust scan rate, gains and setpoint as needed to obtain optimal trace and retrace tracking. Record several large size images (5 µm × 5 µm or 10 µm × 10 µm) to verify the overall morphology and homogeneity of the sample.

Prior to collecting images for analysis, image one or more sample areas (1 µm × 1 µm or smaller) with multiple setpoint values in order to determine the minimum imaging force that can be used. Plots of height for 10 or more individual CNCs as a function of the ratio between the amplitude setpoint (A_{sp}) and the free amplitude (A_0) can be used to determine the minimum imaging force that allows for stable imaging and to estimate the uncertainty contribution in the height measurements due to variation of applied force as a result of amplitude. Alternatively, plots of height versus applied force can be used. Examples of both approaches are shown in Annex B.

Acquire a series of 1 µm × 1 µm AFM images with a minimum resolution of 512 pixels × 512 pixels, 0,8 Hz to 1,0 Hz scan rate, and Z-piezo range of 1 µm to 2 µm. Collect images in different regions close to the centre of the substrate avoiding areas previously imaged. Collect a sufficient number of images to provide the required number of individual CNCs for size measurement, considering the factors outlined

in 6.1. Typically, an average of ≈ 25 individual CNCs/image can be analyzed for 1 µm × 1 µm AFM images using the sample preparation protocol provided in 6.2.

NOTE 1 The resolution is approximately 2 nm/pixel for 1 µm × 1 µm images with 512 pixels × 512 pixels. Assuming a 1-pixel measurement error, this pixel size will give a relative uncertainty of approximately 1,3 % for a 50 nm long CNC. For CNC suspensions with a large fraction of short (< 50 nm) particles, it can be desirable to increase the resolution (although at the cost of added data acquisition time) by scanning smaller areas or using 1 024 pixels × 1 024 pixels.

NOTE 2 Optionally the cantilever can be calibrated by acquiring a thermal tune spectrum to determine the resonance frequency and quality factor; the spring constant can be determined using the Sader method[26].

NOTE 3 The image quality can deteriorate after recording a number of images, either due to changes in tip sharpness or contamination. In such cases it is necessary to use multiple tips to record a sufficient number of images to analyze the required number of individual CNC particles.

The relative humidity has been reported to change the height of CNCs [27]. It is recommended either to record the relative humidity or to image in a humidity-controlled environment, or both.

7.5 Image analysis

Flatten images using a first-order polynomial fit using the AFM software, after excluding CNCs using threshold masking. Save the flattened images for size analysis using appropriate software, such as Gwyddion 2.35[1)], Scanning Probe Image Processor (SPIP™ by Image Metrology A/S)[2)] or microscope software.

For each image, measure the length and height for all individual particles. Analyze adjacent particles only if the separation between them is clearly established in the contact or near-contact areas. Exclude aggregated particles, particles crossing or touching an edge of the image, particles <25 nm long, particles crossing each other and particles with imaging artifacts.

Measure the particle length as the longest distance from one end of the CNC to the other. Use a standard approach for measuring the particle height; for example, measure the maximum height along the long axis used to measure the length. Ensure that random noise spikes are excluded when measuring the maximum height.

Record height and length data for all particles and save the image with analyzed particles numbered, which can be useful post-analysis if any anomalous data are detected.

An example of an AFM image analysis procedure using Gwyddion is provided in Annex C.

NOTE The identification of individual particles is challenging due to the irregular shape of some CNC particles and the fact that the height can vary across the length of the particle.

8 Transmission electron microscopy

8.1 General

Transmission electron microscopy is used to measure the particle size distribution for CNC length and width but does not provide information on the particle height. Adequate contrast requires staining, typically with uranyl acetate; other staining methods have also been used [21].

1) Gwyddion 2.35 is the trade name of a product supplied by the Czech Metrology Institute. It is a free and open source software, available at: http://gwyddion.net/.This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of the product named.

2) SPIP™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of this product.

8.2 Instrumentation and accessories

— Transmission electron microscope with accelerating voltage between 80 kV and 300 kV.

— Calibration grids or nanoparticle reference materials.

8.3 Microscope calibration

Verify the validity of the calibration prior to imaging the sample unless calibration records for the laboratory indicate that this is not necessary. If necessary, calibrate the microscope using a suitable calibration standard according to the manufacturer's instructions. Perform the calibration using similar operating conditions and magnification to those that will be used for the CNC sample. Suitable calibration standards include: MAG*I*CAL (EMS), gold lattice spacings, line gratings and gold or silica nanoparticles. A calibration standard with a certified value and uncertainty and a metrological traceability statement is preferred. Detailed guidance on TEM calibration is provided in ISO 21363 and ISO 29301[9].

8.4 Data acquisition

Set up the microscope as recommended by the manufacturer and optimize parameters in accordance with ISO 21363. The TEM instrument parameters must be selected to provide high quality images with good contrast between background and particles. High magnification and high accelerating voltages are usually preferred.

The sample preparation procedure in 6.3 has been optimized to minimize aggregated CNCs while ensuring a reasonable number of individual particles in the field of view. Nevertheless, the sample staining and CNC distribution is likely to be heterogeneous, requiring acquisition of larger scale images (e.g. 2 µm × 2 µm) at various grid positions to identify areas that have adequate staining and CNC density.

After identifying an area with appropriate staining and CNC density, acquire images at ≈30 000 magnification, such that the resolution is ≤ 0,3 nm/pixel. Ensure reproducible calibration settings by performing lens normalization at the selected magnification for imaging. Use an image recording time that is short enough to minimize stage drift but long enough to provide sufficient contrast between particle and background.

Record a sufficient number of micrographs to analyze the required number of particles (see 6.1 for guidance) and ensure that multiple areas (a minimum of three) of the grid are imaged.

NOTE A pixel size of ≈ 0,3 nm/pixel and a 1-pixel measurement error will give a relative uncertainty of ≈ 1,5 % for a 6-nm wide particle.

8.5 Image analysis

Analyze images using software such as ImageJ[3] or Digital Micrograph[4] as outlined below.

Open an image and check for issues with contamination or artifacts; exclude images with such issues. Adjust the brightness and contrast and set the measurement scale.

Select single particles and measure their length and width. All single particles in each image should be analyzed. Exclude aggregated CNCs, particles with imaging artifacts or contamination and CNCs touching an edge of the image. Analyze particles crossing one another only if they cross at an angle in the approximate range of 30° to 60° and there is a clear indication that they are individual particles.

3) ImageJ is a free and open source software, available at: https://imagej.nih.gov/ij/.This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of the product named.

4) Digital Micrograph is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of this product.

Particles crossing at an angle outside the specified range are selected for analysis only if the separation between the particles is clearly established in the contact or near-contact areas.

Measure the length in a straight line between the ends of the particle, independent of any asymmetry or curvature in the particles. Measure the width of the particles at the midpoint of their length unless the particle is clearly asymmetric; measure the width of asymmetric particles at the widest point.

An example of an image analysis procedure using a semi-automated ImageJ macro is provided in Annex D.

Record width and length for each particle and save analyzed images with particles marked.

9 Data analysis

9.1 General

There are a variety of statistical methods that can be applied to particle size data. These methods can help to

a) assess the robustness of data within one measurement and the repeatability and reproducibility of multiple measurements,

b) fit the particle size data to a reference model, and

c) calculate grand statistics or consensus values for multiple measurements.

One common approach uses the analysis of variance (ANOVA) to assess whether there is a difference between the mean value of the descriptor for one dataset and the grand mean for all data sets or to evaluate differences between the means values of descriptors for two datasets. Bivariate analysis can be used for a pair-wise comparison of descriptor distributions. Non-parametric tests such as Kolmogorov-Smirnov can be used to test for differences between cumulative distributions without prior knowledge of the best distribution model for the dataset. Particle size distribution functions (e.g. normal, log normal, skew normal, Weibull, gamma) can be fitted to data using a variety of methods such as moment-matching, maximum likelihood or Bayesian methods and will give uncertainties for the fit parameters (e.g. for the mean and the standard deviation of these distributions). These methods have been applied to analysis of particle size data for a number of nanomaterials; representative examples are provided in ISO 21363.

9.2 Assessment of data quality

The assessment of individual images to check for contamination, imaging artifacts, etc., is typically done prior to, or as part of the particle size analysis. With an entire data set in hand it is useful to examine the data set by, for example, plotting histograms or using box plots (see examples in Annexes D and E). This examination can indicate extreme points in a single data set. By examining the saved images with analyzed particles one can verify whether there is an error in either analysis or particle selection for such points. Note however, that such examinations should not be used to discard data points, unless an obvious artifact or technical error is detected.

When multiple data sets are recorded (e.g. multiple samples or repeat measurements on the same sample), pairwise comparison of data sets (e.g. using the Kolmogorov-Smirnov test) can also be useful. This approach can be used to evaluate repeatability and to make comparisons between two different sets of conditions (e.g. images recorded with two different resolutions). However, data sets should not be discarded on the basis of a statistical test alone, although experimental artifacts or technical errors may justify exclusion of some data.

9.3 Fitting distribution models to data

CNC size measurements typically measure particle length and either width (TEM) or height (AFM) and calculate aspect ratios as summarized in ISO/TR 19716[5]. Most literature data report the mean

value usually with the standard deviation as a measure of the breadth or width of the size distribution. However, this approach makes the implicit assumption that the data can be adequately fit to a normal distribution which is not usually the case. In a few cases, log normal distributions which are more appropriate for the asymmetric (skewed to larger values) distributions typically observed for CNCs have been employed[24],[28],[29]. A recent TEM standard (see ISO 21363) recommends testing normal, lognormal and Weibull distributions whereas other recent work has employed other approaches such as combinations of Gaussian distributions to better describe particle size distributions with complex shapes[30]. The entire data set, rather than the binned data, should be used for fitting to distribution models. Various methods can be used to optimize the fit of the data and to provide evaluation of the fit quality. The standard error (or relative standard error) for the fit parameter provides a measure of the quality for that parameter.

The ILC data in Annexes C and D are assessed using an alternate approach for data fitting to deal with the complex shapes and relatively large variation in distribution width and asymmetry observed for data sets from different laboratories. In this case data sets are fit to a skew normal distribution which is characterized with three parameters: the location (a measure of central tendency such as mean or median), the scale (a measure of the distribution width, such as standard deviation) and a shape factor (which measures the skewness or asymmetry of the distribution). The final consensus distribution is obtained by combining the distributions from individual laboratories and fitting a skew normal distribution to the pooled data using Bayesian methods[31]. This provides an estimate of the location, scale and shape and their respective uncertainties for the consensus distribution.

9.4 Measurement uncertainty

The ISO/IEC Guide 98-3:2008[2] approach to uncertainty involves the following steps:

a) definition of the measurand;

b) identification of all relevant sources of uncertainty;

c) calculation of the combined (pooled) uncertainty;

d) estimation of the expanded uncertainty at a specific confidence level.

Sources of uncertainty are generally classified in two categories. Type A components are evaluated by statistical methods, such as those from repeated measurements. Type B components are evaluated by other methods and include factors such as calibration errors, temperature variation and instrument/method related uncertainties. Typical Type B components for AFM and TEM are summarized below.

Type B uncertainty components for AFM include the following: tip-sample interactions that compress the sample, leading to an error in height; calibration errors for z (height) and/or x,y (lateral measurements); tip convolution effects for lateral size measurements; uncertainty in determination of particle height due to selection of maximum height and background level (which is influenced by substrate flatness); pixel error for lateral measurements; sample preparation (which can be included in repeatability, reproducibility measurements); subjectivity in selection of individual particles for size analysis; use of multiple AFM probes due to probe deterioration after collecting a number of images.

Type B uncertainty components for TEM include the following: calibration errors; stage or beam drift; focus; pixel resolution; threshold level; background flatness; subjectivity in collection of individual particles for size analysis; sample preparation (which can be included in repeatability, reproducibility measurements); effects due to particle staining.

For a limited number of experiments carried out in one laboratory, the uncertainty is typically assessed by:

$$u_c = (u_{rep}^2 + u_t^2 + u_{cal}^2 + u_{other}^2)^{1/2}$$

where

u_{rep} is the repeatability;

u_t is the trueness;

u_{cal} is the calibration;

u_{other} is the various other Type B uncertainties that can be estimated.

For assessing overall uncertainty from an ILC study an approach in which the final consensus distribution is modeled to obtain fit parameters and uncertainties is summarized in Annexes C and D. Alternatively, ISO 21363 recommends that the coefficient of variation be calculated for each fitted parameter and used to estimate the measurement uncertainty.

10 Test report

10.1 Atomic force microscopy

10.1.1 General information

— Laboratory.

— Author of report.

— Date of report.

— The international standard used, including its year of publication (i.e. ISO/TS 23151:2021).

10.1.2 Sample

— Sample ID, date received.

— Source of CNC (manufacturer, method), type of sample (dry, powder).

— Sample treatment (dispersion method, dilution).

— Deposition (suspension concentration, substrate type size and coating, deposition method).

10.1.3 Data acquisition

— AFM instrument (manufacturer, model number).

— Scanner type or range.

— Operation mode (e.g. open or closed loop, tapping)

— Imaging conditions (temperature, vibration or acoustic isolation).

— Operator.

— Calibration (standard, method, date of most recent).

— Data acquisition date(s).

— AFM probe (type or manufacturer, cantilever, spring constant, tip dimensions or radius of curvature).

— Scan size, number of pixels.

— Scan speed.

— Other parameters (e.g. free amplitude in air, set point, gains).

— Test of imaging force (include graph of height vs amplitude ratio for selected particles).

— Any deviations from the procedure.

— Any unusual featues observed.

— Image file names.

10.1.4 Image analysis

— Image processing (e.g. flattening).

— Software or method.

— Analyzed image files.

— Number of particles analyzed.

— Statistical analysis methods.

10.2 Transmission electron microscopy

10.2.1 General information

— Laboratory.

— Author of report.

— Date of report.

— The international standard used, including its year of publication (i.e. ISO/TS 23151:2021).

10.2.2 Sample

— Sample ID, date received.

— Source of CNC (manufacturer, method), type of sample (dry, powder).

— Sample treatment (dispersion method, dilution).

— Deposition (suspension concentration, grid type, deposition method, staining).

10.2.3 Data acquisition

— TEM instrument (manufacturer, model number).

— Acceleration voltage.

— Calibration (standard, procedure, date of most recent).

— Field of view.

— Image magnification.

— Pixel size.

— Operator.

— Data acquisition date(s).

— Other parameters.

— Any deviations from the procedure.

— Any unusual featues observed.

— Image file names.

10.2.4 Image analysis

— Software or method.

— Analyzed image files.

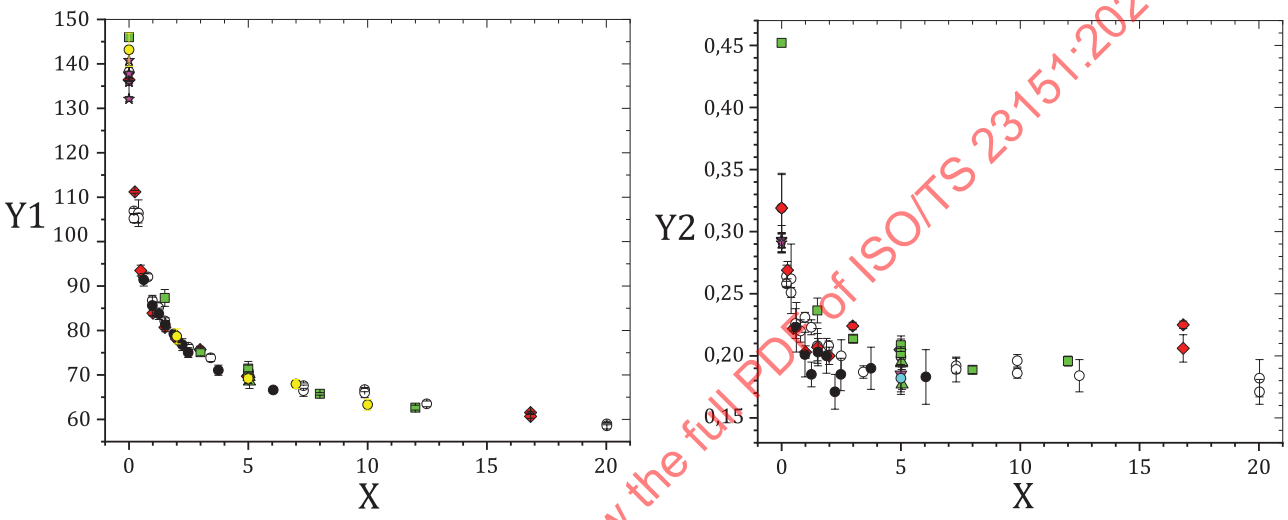
— Number of particles analyzed.

— Statistical analysis methods.

Annex A
(informative)

Assessment of CNC dispersions

Figure A.1 presents plots of Z-average and polydispersity index measured by DLS as a function of sonication energy divided by CNC mass.



a) Z-average as a function of sonication energy divided by mass of CNC

b) Polydispersity index as a function of sonication energy divided by mass of CNC

Key

X sonication energy (kJ/g)

Y1 Z-average

Y2 PI

NOTE Measurements are for a 2 % mass fraction suspension; individual experiments were carried out by multiple people over a period of approximately 1 year and each experiment is shown with a symbol of a different shape or color[16]. Error bars correspond to one standard deviation of three DLS measurements for each sample. The error bars for most of the Z-average points are obstructed by the symbols.

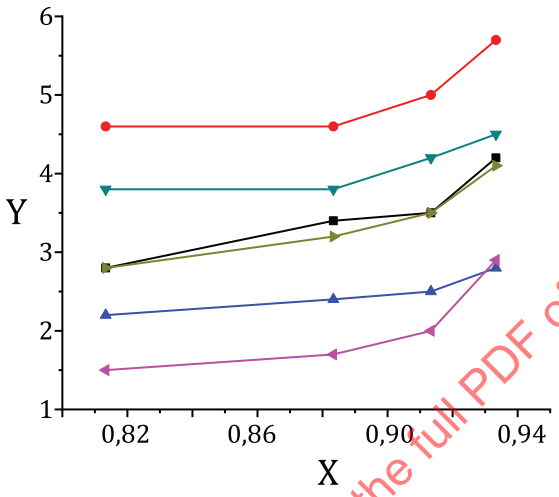
SOURCE Reference [16], reproduced with permission under crown copyright.

Figure A.1 — DLS measurements as a function of sonication energy divided by mass of CNC

Annex B
(informative)

Assessment of applied imaging force

Figure B.1 presents plots of measured CNC height as a function of the ratio of the amplitude setpoint and the free amplitude.



Key

X amplitude setpoint/free amplitude

Y height (nm)

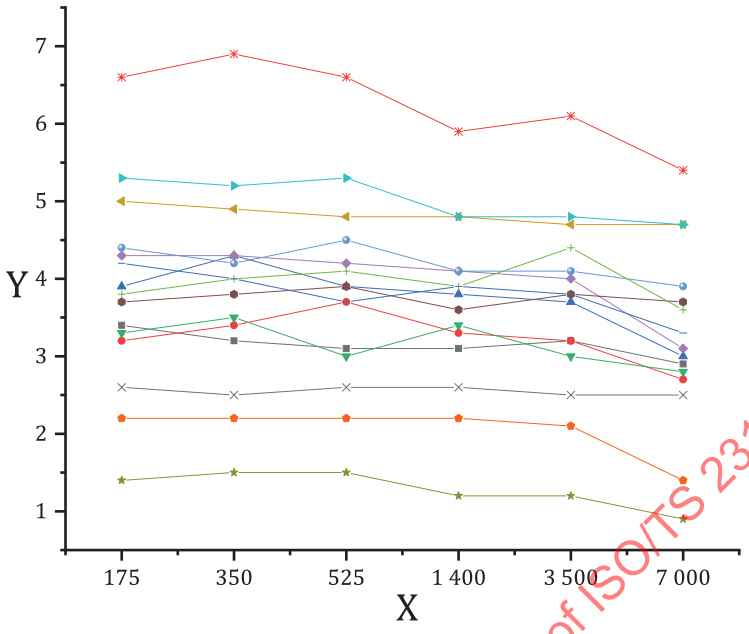
NOTE Data were obtained using a JPK NanoWizard II[5)] in intermittent contact mode with a silicon AFM tip (HQ:XSC11/AL BS, MikroMasch; typical radius 8 nm, spring constant 2,7 N/m).

SOURCE Reference [16]. Adapted with permission under crown copyright.

Figure B.1 — Plots of measured CNC height as a function of the ratio of the amplitude setpoint and the free amplitude

5) JPK NanoWizard II is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of this product.

Figure B.2 presents plots of measured CNC height as a function of the applied force.



Key

X force (pN)

Y height (nm)

NOTE Data were obtained using a Bruker AFM in PeakForce QNM®[6] mode with ScanAsyst-Air probes, 0,4 N/m (nominal spring constant), 2 nm (nominal radius). Each colored symbol/line provides data for one CNC.

SOURCE Reference [35]. Adapted with permission under crown copyright.

Figure B.2 — Plots of measured CNC height as a function of the applied force

6) PeakForce QNM® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IEC of this product.

Annex C
(informative)

Interlaboratory comparison results: AFM

C.1 Background and objectives

AFM is frequently used to measure length and height for CNCs. This interlaboratory comparison was conducted under VAMAS Technical Working Area 34 (nanoparticle populations) and aimed to test AFM image acquisition and image analysis protocols. The ILC used a CNC reference material (CNCD-1) released by the National Research Council Canada and was organized by the NRC. The CNC was produced by sulfuric acid hydrolysis of softwood pulp followed by neutralization with NaCl, generating sulfate half ester groups on the surface. Full characterization details are available [16],[32]. Ten participating institutions from Asia, Australia, Europe and North America returned data sets; there were three universities, one industry and six government laboratories, four of which are National Metrology Institutes. Not all laboratories had prior experience with imaging CNCs. The ILC was comprised of two phases: the first phase with a limited number of participants was designed to test image analysis protocols using a set of AFM images provided by the piloting lab. The second phase involved sample preparation by the piloting laboratory, image acquisition and analysis by participants and final analysis of the data by NRC staff. This Annex summarizes the ILC method and results; full details have been published in Reference [33].

C.2 Phase one

C.2.1 General

Six AFM images were sent to three participants for analysis using Gwyddion software. The Gwyddion protocol (an abbreviated version is given in C.2.2) is based on drawing line profiles through the long axis of the particle for all individual CNCs in a single image. Height and length are then measured sequentially for each particle. This reduces data analysis time relative to one-at-a-time manual measurement of CNC profiles to extract length and height data. The significant level of aggregation makes it impractical to use an automated analysis approach. The selection of CNCs followed the recommended procedure in 7.4.

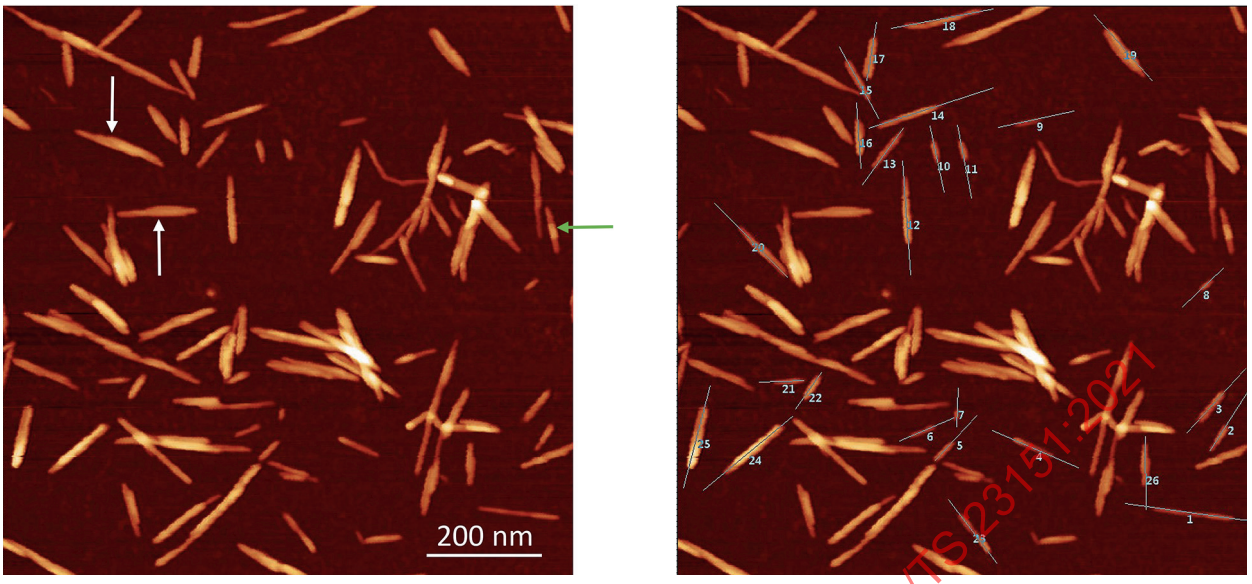
C.2.2 Gwyddion analysis procedure

Open the “Extract profiles” function to open the profile window; set thickness of 3 pixels, linear interpolation, number lines and separate profiles and deselect fixed resolution.

Draw a profile along the long axis of a CNC particle; extend the profile beyond the ends of the particle in order to establish a background level. Repeat for all analysable CNCs and save the image. An example image before and after particle selection is shown in Figure C.1.

Click “Apply” in the profiles window to open each profile in a new window; each profile is identified by a number corresponding to the numbered CNC in the image. Select “Measure distance in graph” and measure the height as the difference between the vertical displacement at the highest region of the profile, (ignoring any single point spikes typically on the edge) and the adjacent background and record the height value. Measure the length using the “Intersections” option. The length of the CNC is determined by the distance between the two intersections of the line profile with the baseline. Repeat the length and height measurement for each profile in the image and record the data.

Repeat the above procedure for all images.



a) AFM image displayed in Gwyddion before drawing profiles for all individual CNCs

b) AFM image displayed in Gwyddion after drawing profiles for all individual CNCs

NOTE The two CNCs marked with a white arrow appear to be laterally aggregated particles when viewed on a larger scale; the CNC marked with a green arrow has been excluded because it is not clear whether this is a single particle or there is a small particle on top of a larger particle.

SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.1 — AFM images of CNCs displayed in Gwyddion

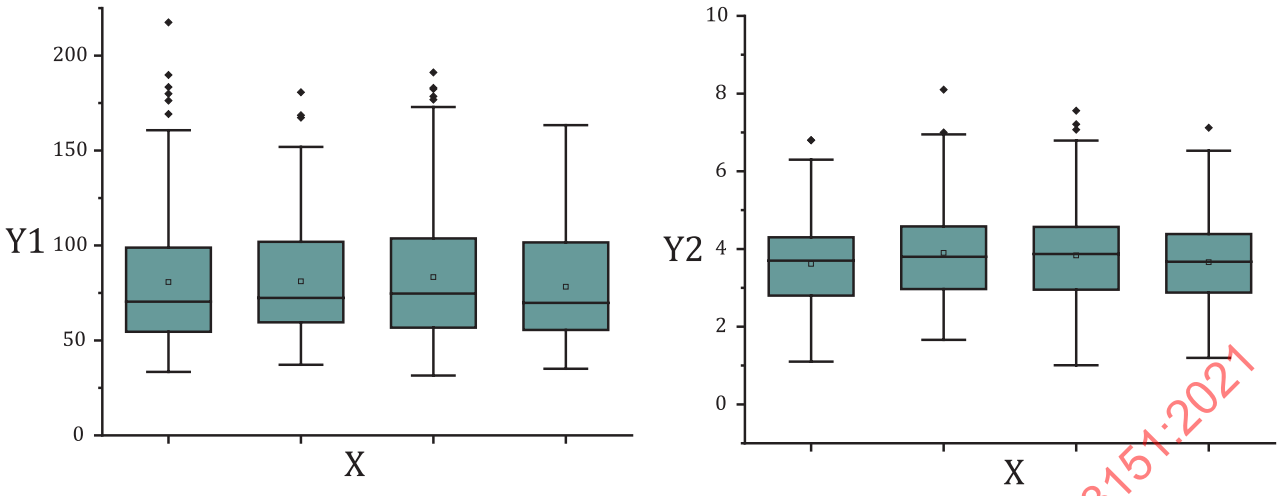
C.2.3 Results

Two laboratories returned an analysis of the data. These data along with data measured by two analysts as part of the characterization of CNCD-1 were compared. The four data sets for length and height are displayed as box plots in Figure C.2. Although participants selected and analyzed different numbers of CNCs (n = 87, 140, 146, 198), the results are very similar. Kolmogorov-Smirnov analysis indicates that only 1 of the possible pairs of laboratories is different for length (Lab 1 analyst 2, Lab 3) and none are different for height. Despite the limited number of data sets, this suggests that variability in selection and analysis does not introduce a large uncertainty in the final result.

C.3 Phase two

C.3.1 General

In phase two, participating laboratories imaged CNC samples prepared by the piloting laboratory and analyzed the images using the data acquisition and analysis protocols provided. The following data were submitted: an excel template with general information on the instrument and operating conditions; the length and height data for all analyzed particles; histograms for length, height and aspect ratio with mean and standard deviation for each; copies of the original image files and the processed images with particles numbered.



a) Box plot for length data for AFM image analysis tests

b) Box plot for height data for AFM image analysis tests

Key

X laboratory code (left to right): lab 1 analyst 1, lab 1 analyst 2, lab 2, lab 3

Y1 length (nm)

Y2 height (nm)

NOTE The bottom and top of the colored boxes represent the 25th and 75th percentiles (the middle 50 % of the data); the mean and median are shown as an open square and solid line, respectively. The vertical bars are 1,5 times the interquartile range, QR (from Q1 – 1,5 IQR to Q3 + 1,5 QR), with points falling outside this range shown as filled diamonds.

SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.2 — Box plots for length and height data for AFM image analysis tests

C.3.2 Sample preparation

The piloting laboratory prepared 2 % mass fraction CNC dispersions using the standard sonication protocol (see 5.2)[16], and deposited CNCs on poly-lysine coated mica (see 6.2). Either 12-mm diameter or 2,54 cm × 2,54 cm mica slides were used, depending on the participant's choice. Two samples were sent to each participating laboratory; the second sample was a backup in case of issues. At least one sample from each batch was imaged at NRC to verify sample quality prior to shipping samples to participants. Tests showed that the CNC sample did not deteriorate if stored in a clean dry environment for up to five weeks.

C.3.3 Data acquisition and image analysis

The protocol requested that participants image the samples within two weeks of receipt. They were instructed to calibrate the AFM prior to imaging according to the manufacturer's recommendations and the participating laboratory's standard practice, unless the instrument use and calibration records indicated that the previous calibration was adequate. Intermittent contact mode imaging was recommended. Several larger scale images were to be recorded to verify sample quality before recording a series of 1 µm × 1 µm images. Participants were instructed to verify that the imaging force used was the minimum that allowed for stable imaging as outlined in 7.3.

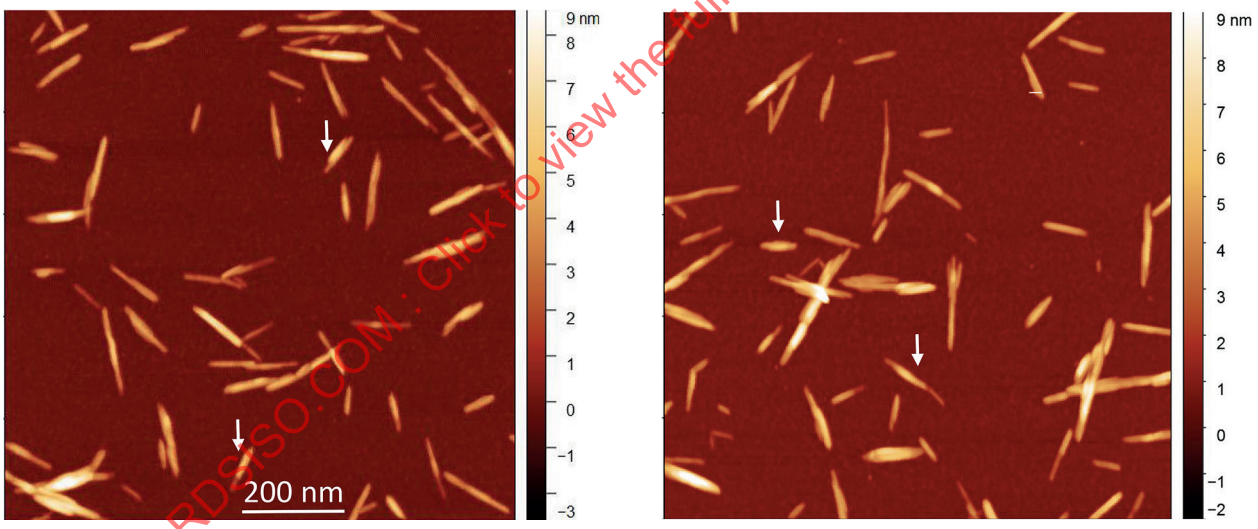
The participating laboratories used instruments from four manufacturers and all used intermittent contact mode as requested. Probes with a range of spring constants (0,4 N/m to 42 N/m) and tip geometries and radius (nominal radii of 2 nm, 7 or 8 nm and <10 nm) were employed. The image size and number of pixels gave the minimum resolution (1,95 nm/pixel) requested for all but one lab which used 0,98 nm/pixel. Six data sets had ≥500 CNCs analyzed as requested; four data sets had 126, 355,

409 and 441 analyzed CNCs. Typically, the participants reported the time required as the limiting factor for the number of CNCs imaged. Most laboratories used multiple probes in order to image the required number of CNCs, but four used a single probe for all images.

Image analysis was done using the Gwyddion procedure provided to the participants with two exceptions; the latter laboratories used Nanoscope software and open source software (FiberApp)[34].

C.3.4 Data analysis

The data sets were reviewed to ensure that all requested information was provided, and images were reviewed to assess the particle selection; individual follow-up with participants was used to obtain missing information and clarify details. Representative images are shown in Figure C.3. Initial examination of histograms, box plots and cumulative distribution plots for the various data sets indicated that the shape and width of the particle distributions varied significantly; this is illustrated by the quantile plots shown in Figure C.4. Initial attempts to fit the data sets to standard distribution models (e.g. normal, log normal, Weibull, gamma) that have been used for other studies indicated that there was not a single two-parameter distribution that provided acceptable fits for all the data sets for either length or height. Since it is impractical to choose a distribution model that provides the best result for each data set we have chosen to employ a flexible three-parameter distribution. Individual data sets were fit to a skew normal distribution as shown for several examples in Figure C.5. This provides three parameters: the mean, the standard deviation (a measure of the distribution width) and a shape factor, α, (a measure of the distribution skewness). Unlike the normal or lognormal distributions, the skew normal distribution allows one to accommodate a wide variation of shapes for particle size distributions; it collapses to a normal distribution when $\alpha = 0$.

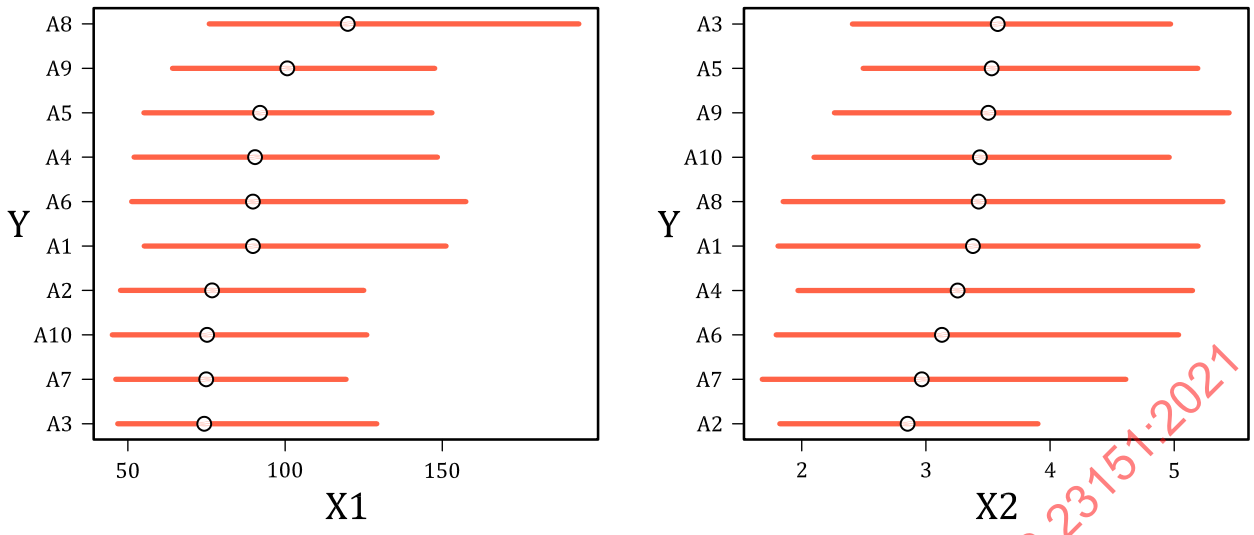


a) Representative AFM image for lab A4 **b) Representative AFM image for lab A7**

NOTE The arrows show features that appear to be more than one particle.

SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.3 — Representative AFM images



a) Plot comparing the 10 data sets for AFM length

b) Plot comparing the 10 data sets for AFM height

Key

X1 length (nm)

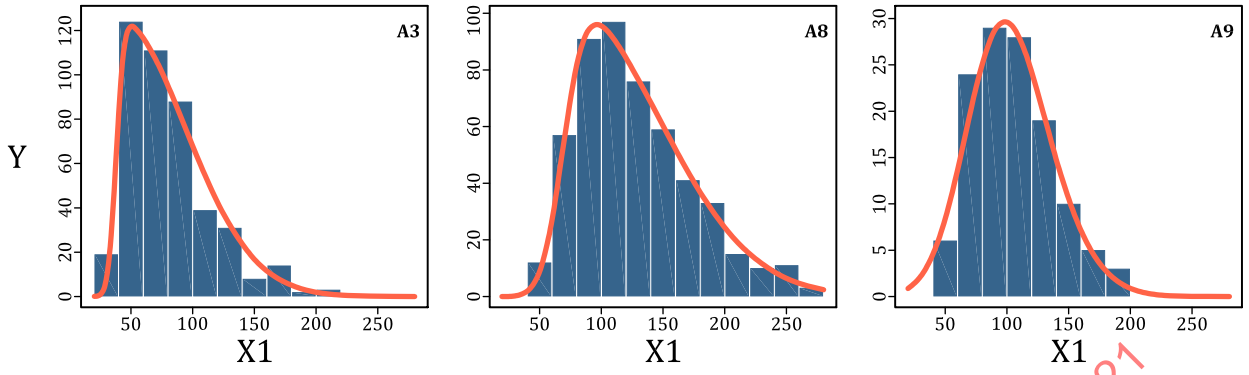
X2 height (nm)

Y laboratory code

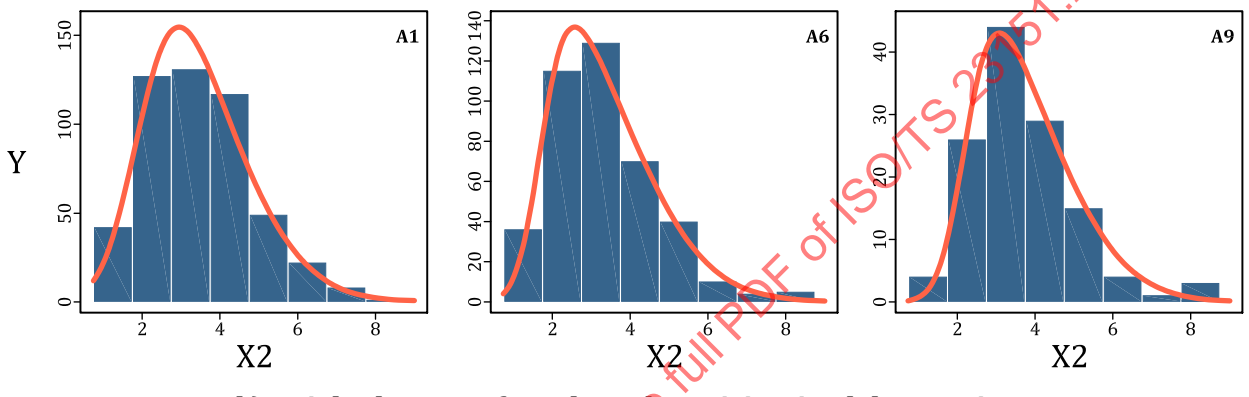
NOTE The red lines represent 10 % to 90 % of the data with the 50 % value (median) indicated with a circle. The laboratories are arranged in order of increasing value of the median.

SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.4 — Plots comparing the 10 data sets for AFM length and height



a) Length data sets for selected participating laboratories



b) Height data sets for selected participating laboratories

Key

X1 length (nm)

X2 height (nm)

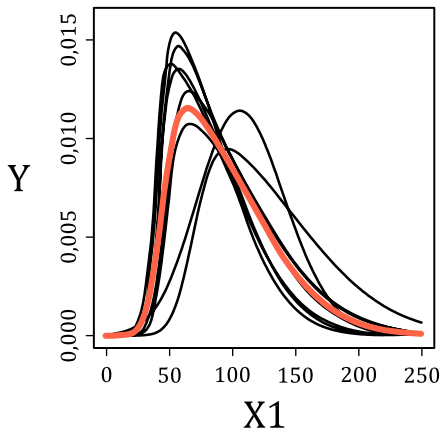
Y frequency

NOTE The raw data is shown as histograms with the skew normal fits overlaid in red. The laboratory code is shown on each image.

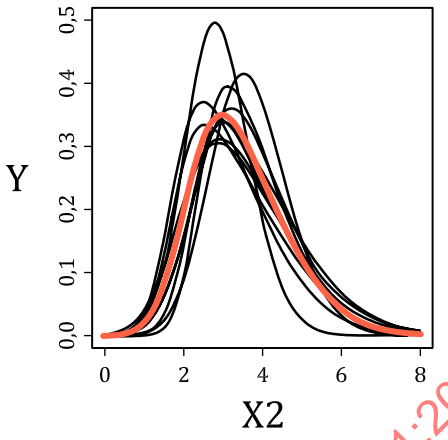
SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.5 — Length and height data sets for selected participating laboratories

The dispersion between laboratories was also modelled as a skew normal distribution. The consensus distribution was obtained by pooling the skew normal distributions representing the individual laboratory results. An equal number of random samples (N = 1 000) were drawn from each of the laboratory skew normal distributions and were placed into the pool. The statistical model of the entire interlaboratory study (for each measurand) is therefore summarized by three parameters for each laboratory along with an additional three parameters describing the distribution of consensus values, the grand mean, scale, and shape. Figure C.6 provides a comparison of the individual height and length distributions for each laboratory and the final skew normal consensus distribution (in red). The values and associated uncertainties, as obtained using Bayesian methods, are provided in Table C.1. The dispersion between the mean laboratory results is also provided as a measure of variability between the ten laboratories.



a) Comparison of the consensus distribution (red) and the individual skew normal fits for each data set (black) for AFM length



b) Comparison of the consensus distribution (red) and the individual skew normal fits for each data set (black) for AFM height

Key

X1 length (nm)

X2 height (nm)

Y probability density

SOURCE Reference [33], reproduced with the permission under crown copyright.

Figure C.6 — Comparison of the consensus distribution (red) and the individual skew normal fits for each data set (black) for AFM length and height

Table C.1 — Skew normal distribution parameters for the consensus distributions for CNC length and height from AFM measurements

		Mean nm	Standard deviation nm	Shape factor α
Length	**Consensus value (standard uncertainty)**	94,5 (0,4)	39,6 (0,3)	6,4 (0,3)
	Dispersion (standard uncertainty)	15,0 (0,6)		
Height	**Consensus value (standard uncertainty**	3,44 (0,01)	1,21 (0,01)	2,7 (0,1)
	Dispersion (standard uncertainty)	0,28 (0,02)		

Analysis of the various data sets demonstrated that differences between probes and deterioration of the probe with continued imaging are significant contributing factors to the variability in mean length between laboratories. Tests of applied imaging force indicate that it is possible to image without compressing the CNCs. Although the number of CNCs needed to obtain a reliable data set varies with operating conditions, analysis of 250 CNCs to 300 CNCs is adequate if the various parameters are carefully controlled.

Annex D
(informative)

Interlaboratory comparison results: TEM

D.1 Background and objectives

TEM is one of the most widely used methods for size analysis of CNCs. This interlaboratory comparison was conducted under VAMAS Technical Working Area 34 (nanoparticle populations) and aimed to test TEM image acquisition and image analysis protocols. The ILC used a CNC reference material (CNCD-1) released by the National Research Council Canada and was organized by the NRC. The CNC was produced by sulfuric acid hydrolysis of softwood pulp followed by neutralization with NaCl, generating sulfate half ester groups on the surface. Full characterization details are available in References [16] and [32]. Ten participating institutions from Asia, Europe and North America returned data sets; there were three universities, one industry and six government laboratories. Not all laboratories had prior experience with imaging CNCs. The ILC was comprised of two phases: the first phase with a limited number of participants was designed to test image analysis protocols using a set of TEM images provided by the piloting lab. The second phase involved sample preparation by the piloting laboratory, image acquisition and analysis by participants and final analysis of the data by NRC staff. This Annex summarizes the ILC method and results and full details have been published in Reference [31].

D.2 Phase one

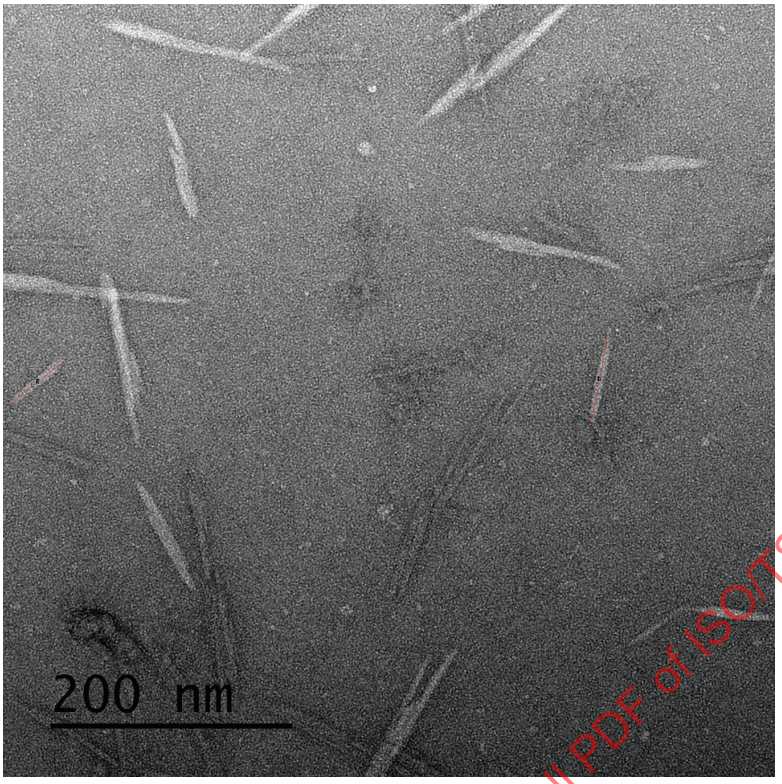
D.2.1 General

A set of 30 TEM images was sent to four participants for analysis, with a request to use a custom ImageJ macro provided by the piloting laboratory. The images were collected as part of the characterization of CNCD-1, the material used to prepare samples for phase two of the ILC. The ImageJ macro allows for automatic sequential opening of a set of images and manual measurement of length and width profiles, and saves the data and images with analyzed CNCs and profiles marked (an abbreviated version of the procedure is provided in D.2.2). All individual CNCs in each image were to be analyzed using the guidelines provided in 8.4. Initial tests using either the manual or polygon outlining tool in ImageJ (previously used for several ILCs summarized in ISO 21363) did not yield appropriate values for minFeret, possibly due to issues with applying these tools to tapered particles.

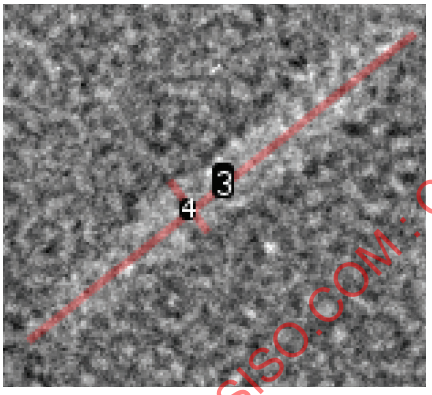
D.2.2 ImageJ analysis procedure

The ImageJ macro uses the 'next file' option which requires .Tiff file format and allows the user to sequentially open and analyse each image in a specific folder. The measurement data in pixels are stored as a comma separated value (.CSV) file and the images are saved with the profiles marked. The macro also allows the user to exit and continue analysing an image set at a later time.

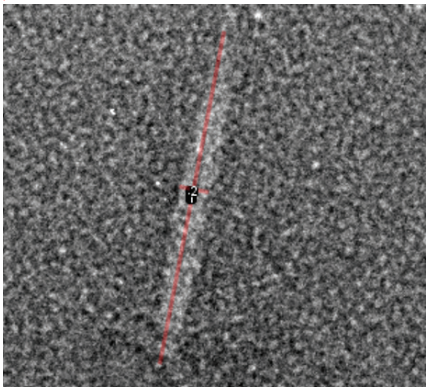
The analyst is prompted to open an image and can adjust the contrast and brightness for improved visualization. A profile is then drawn through the long axis of the first particle to be analysed; after clicking to record the length, a line profile appears perpendicular to the profile marking the length and the user can adjust the position and length and click to record the data (in a CSV file). This process is repeated for each particle in the image, zooming in or out as needed for visualization of individual CNCs. The annotated image is then saved. An example of a saved image file is shown in Figure D.1, a), with Figures D.1, b) and c) showing the zoom for two CNCs.



a) TEM image with two measured CNCs marked with red lines



b) Magnifed image of a CNC particle with length and width marked with red lines

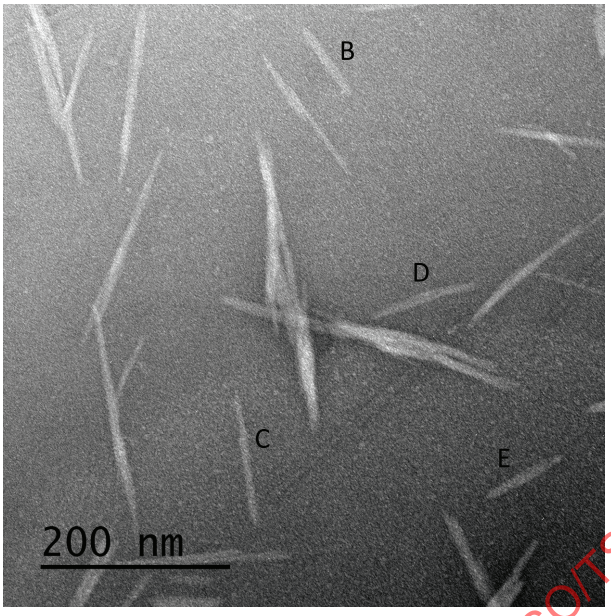


c) Magnified image of a CNC particle with length and width marked in red

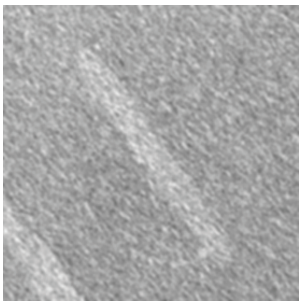
SOURCE Reference [31], reproduced with the permission of the authors.

Figure D.1 — Analyzed TEM image with magnified images of two measured CNCs

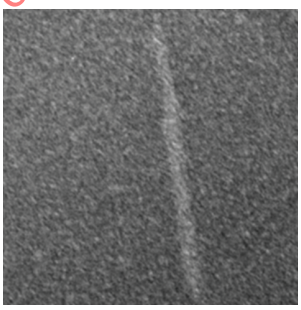
The above procedure is repeated for all images in the data set. Figures D.2 and D.3 provide examples of images with analyzable CNCs (see D.2) and particles that should not be analyzed (see D.3).



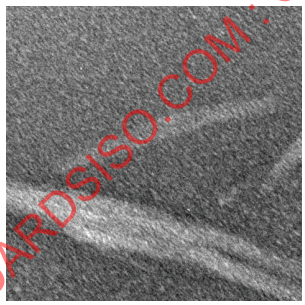
a) TEM image of CNCs



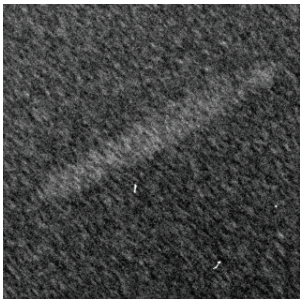
b) Magnified image of an analysable particle



c) Magnified image of an analysable particle



d) Magnified image of an analysable particle (top feature)



e) Magnified image of an analysable particle

NOTE The crossing particles on the left edge of Figure D.2, a) have not been analyzed.

SOURCE Reference [31], reproduced with the permission of the authors.

Figure D.2 — TEM image (a) with zooms [b) to e)] of four analyzable CNC particles