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Nanotechnologies — Particle size distribution for cellulose nanocrystals

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

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Contents

Page

Foreword

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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).

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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