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Transmission Electron Microscopy for the Characterization of Cellulose Nanocrystals

Madhu Kaushik, Carole Fraschini, Grégory Chauve, Jean-Luc Putaux and Audrey Moores

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http://dx.doi.org/10.5772/60985

Abstract

Cellulose nanocrystals (CNCs) are high aspect ratio nanomaterials readily obtained from cellulose microfibrils via strong acid hydrolysis. They feature unique properties stemming from their surface chemistry, their crystallinity, and their three-dimensional structure. CNCs have been exploited in a number of applications such as optically active coatings, nanocomposite materials, or aerogels. CNC size and shape determination is an important challenge and transmission electron microscopy (TEM) is one of the most powerful tools to achieve this goal. Because of the specifics of TEM imaging, CNCs require special attention. They have a low density, are highly susceptible to electron beam damage, and easily aggregate. Specific techniques for both imaging and sampling have been developed over the past decades. In this review, we describe the CNCs, their properties, their applications, and the need for a precise characterization of their morphology and size distribution. We also describe in detail the techniques used to record quality images of CNCs. Finally, we survey the literature to provide readers with specific examples of TEM images of CNCs.

Keywords: cellulose nanocrystals, transmission electron microscopy, particle size, characterization, size distribution, sample preparation

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1. Introduction

1.1. Native cellulose and the production of cellulose nanocrystals

Cellulose, the most abundant biopolymer on Earth, has been extensively used by man, in the form of macro- and microstructures, as a traditional resource in many aspects of daily life, notably to produce textiles and papers. This polymeric material is biosynthesized by a wide variety of living species such as plants, animals, bacteria, and some amoebas. Glucose is polymerized by enzymes in a continuous fashion. The resulting cellulose chains are homopolymers of β-1,4-linked anhydro-D-glucose units which associate to form microfibrils, further assembled into macrofibers and fibers. Crystalline and disordered regions alternate along the microfibrils (Figure 1a) [1-4].

![Figure 1. (a) Structural hierarchy of the cellulose fiber components from the tree to the anhydroglucose molecule (SEM image of wood cell structure: courtesy of D. Dupeyre, CERMAV); b) preparation of nanocrystals by selective acid hydrolysis of the disorganized regions of cellulose microfibrils (TEM image of cotton CNCs: courtesy of CERMAV).](image)

In the late 1940s, cellulose crystallites were isolated for the first time by chemical treatment of a cotton substrate in hot concentrated sulfuric acid [5]. Soon after, Rånby showed that stable colloidal suspensions of negatively charged cellulose particles could be obtained [6, 7]. During
the extraction process, the cellulosic fibrous structure is broken down in the presence of concentrated sulfuric acid (other mineral acids can be used such as HCl). After diffusion of the acid within the substrate, the glycosidic linkages in disordered regions, more accessible and reactive, are preferentially broken. An additional mechanical or ultrasound treatment results in the release of rodlike cellulose crystallites, so-called cellulose nanocrystals or CNCs (Figure 1b). Consequently, as the hydrolysis proceeds, the degree of polymerization of the cellulose macrostructures decreases, while the crystallinity of the nonsoluble particles increases [8].

1.2. Cellulose nanocrystal properties

In the early 1950s, detailed characterizations of the shape and size of various CNCs (cotton, ramie [9], and bacterial cellulose [10]) were proposed from transmission electron microscopy (TEM) images. Since then, CNCs have been produced from a wide (and expanding) variety of sources [11] such as wood (Figure 2a), cotton (Figure 2b), bamboo (Figure 2c) [12], bacteria (Figure 2d) [13], algae (Figure 2e), tunicates (Figure 2f) [14], eucalyptus [12], spruce bark [15], corncob [16], etc. The CNCs are rodlike objects with a length typically ranging from 50 to 1000 nm and a width varying from 3 to 50 nm (Table 1). CNCs thus have high length-to-width (aspect) ratios (10-100) [17-22]. Their morphology depends on the cellulose source and the conditions of preparation (type and concentration of acid, acid-to-cellulose ratio, reaction time, and temperature) (Figure 2). The nanoscopic features of the resulting CNCs considerably influence their colloidal and macroscopic properties such as suspension rheology, phase separation concentration, liquid crystal behavior, orientation under electric or magnetic field, and mechanical reinforcement ability in nanocomposites [14, 16].

CNCs can be prepared in different forms. First, CNCs can be manipulated in the form of acidic aqueous suspensions resulting from the strong acid hydrolysis of microfibrils or as neutral suspensions after neutralization. Subsequent surface modifications can be achieved via chemical treatment [23, 24]. Alternatively, different drying methods may be used to afford a fluffy material, with the aspect of white sugar candy [25].

In 1959, Marchessault et al. revealed that the chemical reaction of sulfuric acid with hydroxyl groups at the surface of cellulose CNCs formed sulfate ester groups, resulting in electrostatic repulsions between the particles and ensuring colloidal stability. Moreover, the authors showed that CNCs could self-organize into liquid crystalline phases [26].

The sulfuric acid hydrolysis of cellulose has remained confined to academic research laboratories until a big step was made in the mid 1990s, when it was shown that CNCs were efficient reinforcing fillers in latex-based polymer matrices, opening a new potential market for this high-end material [14, 27]. Since then, CNCs have become intensively studied with an accelerating rate of publications released each year [17-22]. Beside mechanical properties, colloidal properties of CNCs in suspension generated a series of studies investigating their ability to form liquid-crystal phases. The colloidal suspensions of CNCs spontaneously organize into a chiral nematic phase above a certain critical concentration. As a consequence, CNCs have been used to produce iridescent and birefringent films [28-31], chiral mesoporous silica [32, 33] and carbon [34], gold nanoparticle films with chiral plasmonic properties [35], enantioselective amino acid hydrolysis catalysts [36], hydrogels [37], and aerogels [38].
Moreover, the high surface area and controllable surface chemistry of CNCs make them a unique support for metal nanoparticles (NPs) [39]. CNC supported NPs, including Pd [40] and Au [41], were used as nanocatalysts. Pd NPs distributed onto CNCs were used for hydroge‐nation and carbon-carbon bond coupling reactions [40, 42-44], while the Au counterparts were used for 4-nitrophenol reduction [45]. Ag NPs were also deposited onto CNCs and the resulting material featured antibacterial activity [46].

Figure 2. TEM images of negatively stained preparations of CNCs of various origins: a) wood (courtesy of G. Chauve, FPInnovations); b) cotton (courtesy of F. Azzam, CERMAV); c) bamboo (courtesy B. Jean, CERMAV; d) *Gluconacetobacter xylinus* (courtesy of H. Bizot, INRA); e) *Glaucocystis* (courtesy of Y. Nishiyama, CERMAV); f) *Halocynthia papillosa* (courtesy of A. Osorio-Madrazo, A. Ludwig University of Freiburg).

This large panel of high-end applications strongly encouraged industry to achieve large-scale production of CNCs. The forest-based industry in North America, Northern Europe, and Japan is currently looking into renewing and reinventing itself to extend its activities and guarantee its survival, while renewable materials are being increasingly appealing as fossil-based material replacement. In addition, CNCs are inherently safe, practically nontoxic materials [47]. Nanocellulose-based, value-added materials definitely constitute promising vectors to “turn wood into gold” and revive the forest-based industry. These combined factors led to the opening of the first commercial plant by CelluForce Inc. (Windsor, Quebec, Canada), producing 1 ton of CNCs per day [48].
Several review articles and books have been published over the last few years which cover in detail the various aspects of the CNC features and applications [17, 18, 20, 21, 49].

1.3. Need for CNC characterization

CNCs possess appealing properties in direct relation with their three-dimensional (3D) structure, including well-defined shape, size, and aspect ratio. From an industrial perspective, it is essential to collect reliable data on CNCs, especially for quality control, toxicology assessment, R&D and applications.

1.3.1. Quality control

The large-scale production of CNCs became a reality when CelluForce Inc. started up its demonstration plant. Getting reliable, fast, and accurate measurement of the particle size is a key to guarantee a consistent production of high-quality CNCs. Several methods exist (vide infra) to determine the size, size distribution, and shape of CNCs. So far, they are typically used in academic studies and provide a fairly consistent understanding of the material structure. However, for the moment, no systematic and streamlined method exists for size determination and evaluation of polydispersity for anisometric nano-objects such as CNCs. This question is particularly acute for large-scale quality control, for instance, in pilot plant production and beyond.

1.3.2. Toxicology assessment

Manufactured nanomaterials have recently caused societal concerns about their possible adverse effects on health and safety. Properties of nanomaterials typically differ from those of their parent bulk materials because of their larger surface area, leading to a greater activity, their smaller size, resulting in their ability to cross natural barriers, and intrinsic effects caused by nanometric size, including electronic and plasmonic effects. Since such particles may have a negative effect on biological systems and ecosystems, their toxicological risks must be evaluated and an accurate description of the product in terms of dimensions, chemistry, and toxicity is required by the authorities. CNCs have extensively been evaluated using standard ecotoxicological and mammalian test protocols and have, to date, been shown to be practically nontoxic in each of the individual tests [47]. In addition, CNCs have recently obtained regulatory approval under Canada’s New Substances Notification Regulations (NSNR) for unrestricted use in Canada and is the first organic nanomaterial to be added to Canada’s domestic substance list.

1.3.3. R&D and applications

To maximize the reinforcing or liquid crystal behavior potential of CNCs, the particles have to be as well-dispersed as possible, especially in nanocomposite applications. An aspect that can lead to aggregation/agglomeration and then affect the further redispersion of the particles is mainly the final drying stage. Drying is a critical process for the large-scale industrial production of CNCs. Aqueous suspension leads to bacterial contamination and precludes...
long-term storage. In addition, the cost associated to the transportation of suspensions containing up to 90% water is not economically viable. Various drying processes can be used such as freeze-drying, supercritical drying, spray-drying, oven drying, and air drying. Spray-drying methods are used in the industrial production of CNCs. The resulting solid, hornified macrostructure may be difficult to further redisperse, even in water. Neutralization of the acidic sulfate ester groups prior to drying helps with redispersion [25].

1.3.4. Challenges regarding CNC particle size measurement

The aspect ratio of CNCs dictates the percolation threshold, a key parameter to control mechanical properties in nanocomposites. Knowing the size and morphology of CNCs with precision plays a key role in the development of numerous applications where these features directly impact the properties of the final product. CNCs prepared by sulfuric acid hydrolysis of cellulose substrates are rigid, acicular-shaped and highly crystalline nanoparticles. The geometrical dimensions of these rodlike crystallites vary exceedingly with the source of cellulose (Table 1) and with the hydrolysis reaction conditions. For example, CNCs extracted from wood are 3-7 nm in width and 100-200 nm in length, while CNCs derived from tunicate are 10-20 nm in width and 500-2000 nm in length [14]. These values are indicative as different reaction conditions result in different sizes and size distributions. Indeed, size distributions were published as early as 1944 for ramie and cotton CNCs [50]. Lists of the different sizes of CNCs extracted from various sources can be found in recent reviews [17, 18, 20, 21] and in Table 1. The size distribution may have an impact on the properties and thus the applications of CNCs. For instance, it has been shown that polydispersity influenced the phase separation behavior of liquid crystalline suspensions [51, 52]. CNCs are usually obtained as colloidal aqueous suspensions and the production process induces batch-to-batch particle size variability. The rheological properties of the suspension and the state of individualization of the particles are strongly affected by external parameters such as nanoparticle concentration, pH, ionic strength, temperature, or the presence of an additional compound or impurities [53-55]. The stability of the suspension is due to the electrostatic repulsion forces created by the negatively charged sulfate ester groups located at the surface of the crystals. More generally, their propensity to agglomerate is driven by their surface chemistry. For example, the addition of electrolyte screens the surface charges of the particles, reducing the electrostatic repulsion that prevents CNCs from agglomerating, which results in either an increase or a decrease of the measured particle size by photon correlation spectroscopy (PCS) depending on the amount of electrolyte introduced. Since CNC particles do not undergo swelling or compaction upon the addition of electrolyte, this increase/decrease of the measured particle size is a pure artifact driven only by the laws of physics and thermodynamics. Consequently, for a given sample, particle size variability depends on the sample preparation conditions and the measurement techniques.

Particle size is a good indicator of the quality of the CNC dispersion but the direct observation of nanoparticles remains a challenge and high-resolution direct imaging or light scattering techniques are required.
1.4. Microscopy and spectroscopy techniques used for CNC characterization

As previously mentioned, CNCs come in a wide variety of length, width, and shape depending on the cellulose sources. The cross sections of CNCs also display a variety of shapes, e.g., square, rectangular, or parallelogram, that are dictated by the arrangements of enzymatic terminal complexes extruding cellulose chains during the biosynthesis. As shown in different cases, the edges of the crystals can be eroded during the acid hydrolysis yielding hexagonal or octagonal cross sections exposing small surface area of hydrophobic (200) planes [56].

Many instruments are commonly used for the determination of particle size distribution (PSD) and particle dimension(s), each instrument detecting size through its own physical principle. Numerous techniques based on microscopy, light interactions, electrical properties, sedimentation, sorting, and classification allow access to particle size. Depending on the technique, the results are more or less accurate and are related to the nanoparticle shape and its physico-chemical features such as chemical composition, heterogeneity, topography, surface charge density, dispersing medium, viscosity, etc. The morphology of CNCs can be accurately revealed either by microscopic methods including transmission electron microscopy (TEM), cryo-TEM, atomic force microscopy (AFM), field emission gun scanning electron microscopy (FEG-SEM), or by scattering techniques such as polarized and depolarized light scattering (DLS and DDLS, respectively) and small- and wide-angle neutron or X-ray scattering (SANS, WANS, SAXS, and WAXS, respectively). Each of these techniques has its own advantages and limitations in their application to the study of CNCs. Consequently, care should be taken while comparing particle size data obtained from different techniques.

Light scattering techniques rely on the interaction of light with the hydrodynamic volume of a particle. With anisometric nanoparticles, the response varies as a function of the orientation of each individual particle. The collected data thus need to be mathematically processed to extract meaningful information. Typically, for acicular particles, such as CNCs, light scattering techniques have not been able to match the precision of microscopy. Microscopy, on the other hand, provides direct images of individual particles and allows characterization of its morphology and size (length, width and thickness). Microscopy techniques which have the nanometer scale resolution capabilities to image CNCs are electron microscopy and AFM. Electron microscopy enables the direct observation of the dimensions (i.e., length and width) of a given particle. AFM provides information on morphology, surface topography, mechanical properties, and adhesion of CNCs under ambient conditions [15, 16, 57, 58]. While AFM provides reliable information on the thickness of the particles deposited on a flat substrate, the lateral resolution is limited by the convolution of the tip end, whose size and curvature are significantly larger than the dimensions of the nano-objects. However, this so-called tip-broadening effect can sometimes be deconvoluted [59]. On the contrary, TEM images provide good nanometric (and often subnanometric) lateral resolution, allowing to rapidly screen a large population of particles, thus avoiding major sampling issues. However, as TEM images are projections of the objects along the incident beam direction, it may be difficult to accurately measure the particle thickness. Moreover, the low density of CNCs calls for the use of staining methods. These limitations may be overcome by some of the recent developments described in the following sections, including low-dose microscopy and 3D imaging. When possible, the
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Figure 13. TEM images of patches of Pd onto CNCs recorded using a DDD camera: a) the white arrows point at Pd plates located on the surface of isolated CNCs. The black arrows point at larger and darker NPs that are not physically linked to CNCs; b) close-up view of the area indicated by the box on the left and revealing Pd patches (reprinted with permission from [44]. Copyright 2015 American Chemical Society).
3.3. TEM of CNC-polymer nanocomposites

Due to their nanoscale dimensions, high surface area, low density, and high mechanical strength, CNCs have been incorporated as reinforcing component into various polymer matrices: styrene-butyl acrylate [14, 27] polylactic acid [116, 117], poly(vinylalcohol) [118], polyurethane [119, 120], epoxy [121], and polyvinylacetate [100, 122]. In order to explain the variation in mechanical properties, these bulk materials have often been characterized by SEM after fracturing, allowing to visualize the distribution of CNCs that emerge at the fracture surface. However, SEM remained limited to the characterization of the topography of the fracture surfaces. TEM was also used to visualize the CNCs inside the material via preparation by ultramicrotomy of ultrathin sections of the composites. Figure 14 shows examples of images of nanocomposites of CNCs in matrices of styrene-butyl acrylate [14] and polyurethane [120]. CNCs can be visualized using their intrinsic diffraction contrast [14] but, as they are very sensitive to beam damage, their contrast rapidly decreases to the point where they become hardly visible with the polymer matrix due to the lack of density difference. Additional staining of the matrix can be used, for instance with OsO₄, creating a negative staining effect revealing the embedded CNCs [121].

![TEM images of ultrathin sections of nanocomposite materials incorporating CNCs as reinforcing component: a) tunicate CNCs in a matrix of styrene-butyl acrylate – diffraction contrast in bright field mode (reprinted with permission from [14]. Copyright 1995 American Chemical Society); b) thin cryosection of a cotton CNC/polyurethane composite observed under cryo-TEM conditions (reprinted with permission from [120]. Copyright 2011 American Chemical Society)](image)

3.4. 3D imaging of CNCs and polymer-wrapped CNCs

Electron tomography (described in § 2.3.3) has only very recently begun to be used to characterize cellulose-based architectures or individual particles. Ciesielski et al. have recently published ET reconstructions of treated plant cell walls, allowing to get 3D information on the microfibril morphology [123]. Majoinen et al. studied the morphology of individual CNCs embedded in vitreous ice. Using cryo-TEM conditions, they recorded tilt series and reconstructed tomograms from pristine CNCs (Figure 15a) and from CNCs wrapped up with dendronized polymers (Figure 15b). This 3D visualization of the CNCs revealed the presence of an helicoidal twist along the rodlike particles. These twists have been proposed to be significant in explaining the cholesteric liquid crystalline assembly [18, 19] and their use in chiral templating [32, 33]. More recently, tomograms of ice-embed-
ded Pd modified CNCs were also useful to gain insight into the nature of the material under close-to-catalysis conditions [44].

Figure 15. Electron tomography reconstitution of initial (a) and dendronized (b) polymer-wrapped cotton CNCs (reprinted with permission from [62]. Copyright 2014 American Chemical Society).

4. Conclusion and perspectives: Challenges and solutions in imaging CNCs

CNCs have been extensively studied and many reports can be found in the literature. The particle shape and size vary as a function of the cellulose source and the methodology used to produce the CNCs. In all cases, the CNC populations are polydisperse, featuring wide and asymmetrical size distributions. An accurate knowledge of the CNC dimensions and size distribution is crucial to characterize the properties of individual particles and their assemblies in suspension, in dry films or after incorporation into matrices. For instance, morphology and size will influence the phase separation behavior and the formation of liquid crystalline organizations or the percolation properties in nanocomposites. The development of chemical modifications strategies also requires a good knowledge of the accessible surface. In addition, CNCs have recently become a new type of industrial nanomaterial with numerous exciting properties, and an accurate characterization is important to optimize processes and control their reproducibility.

TEM is a key imaging technique to achieve this goal, thanks to the development of specific techniques to observe beam-sensitive polymers. Cellulose is without doubt one of the most beam-sensitive materials that can be observed by TEM. Consequently, special care must be taken to record reliable images of CNCs (low dose, low temperature, additional staining). In this article, we have described several sample preparation procedures and observation techniques adapted to the recording of images of CNC populations and have carried out reliable size distribution analyses. New approaches have recently been proposed, taking advantage of technical improvements of the microscopes, such as a better control of the stage movements, the use of highly sensitive digital cameras, and automatized corrections of several optical aberrations, allowing to record electron tomography image series and reconstruct the volume of the specimens. Although these sophisticated approaches are not routinely used yet
in the field of cellulose science, they will certainly rapidly develop. In addition, at a time when the research on CNCs is booming and the range of commercial applications is expanding, TEM imaging will continue to play a central role. Automated particle measurement procedures using TEM image analysis software will be needed to streamline quality control.

Acknowledgements

The TEM images in Figures 3, 8, and 9 were specifically recorded for this chapter by J.-L. Putaux on a Philips CM200 microscope operating at an accelerating voltage of 200 kV and equipped with a TVIPS TemCam F216 camera. We thank the Electron Microscopy Platform of Institut de Chimie Moléculaire de Grenoble for granting access to this equipment.

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