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# Cellulose nanocrystals from native and mercerized cotton

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**Abstract:** Nanocelluloses occur under various crystalline forms that are currently being selectively used for a wide variety of high performance materials. In the present study, two cellulose fibers (CF-I) were mercerized by alkaline treatment (CF-II) without molar mass variation (560,000 g/mol) and both were acid hydrolyzed, forming cellulose nanocrystals in native (CNC-I) and mercerized (CNC-II) forms. This study focuses on the detailed characterization of these two nanoparticle morphologies (light and neutron scattering, TEM, AFM), surface chemistry (zetametry and surface charge), crystallinity (XRD, <sup>13</sup>C NMR), and average molar mass coupled to chromatographic techniques (SEC-MALLS-RI, A4F-MALLS-RI), revealing variations in the packing of the crystalline domains. The crystal size of CNC-II is reduced by half compared to CNC-I, with molar masses of individual chains of 41,000 g/mol and 22,000 g/mol for CNC-I and CNC-II, respectively, whereas the same charged surface chemistry is measured. This study gives an example of complementary characterization techniques as well as results to help decipher the mechanism involved in mercerization.

**Keywords:** cellulose nanocrystals, mercerization, cellulose II, biobased nanoparticles, nanostructure.

## 1. Introduction

Cellulose is a linear homopolysaccharide of D-glucopyranose units connected by  $\beta(1-4)$  glycosidic bonds (Habibi, Lucia, and Rojas 2010; Moon et al. 2011; Nishiyama 2009). It is stabilized by an inter- and intramolecular complex network of hydrogen bonds and van der Waals interactions.

According to the association type, cellulose exists in six crystalline forms called cellulose I, II, III-I, III-II, IV-I and IV-II (Kroon-Batenburg, Bouma, and Kroon 1996). Cellulose I corresponds to fibrillary native cellulose with parallel oriented chains. The other forms are obtained by conversion of type I by chemical and/or thermal treatments (Atalla and VanderHart 1999; Gardner and Blackwell 1974; Nishiyama, Langan, and Chanzy 2002). Cellulose I can undergo an irreversible transition into a more thermodynamically stable crystalline form, cellulose II, by two distinct processes: regeneration or mercerization. Mercerization involves intracrystalline swelling of the cellulose in concentrated aqueous NaOH where the limit concentration depends on the temperature between 8-15%, with lower temperatures that allow transformation at lower concentrations (Duchemin 2015; Warwicker 1967) and where chains change their orientation from original parallel chains

43 of cellulose I to antiparallel chains (~~opposite polarity~~) (Fink and Philipp 1985; Kolpak,  
44 Weih, and Blackwell 1978; Stipanovic and Sarko 1976). The mechanism of mercerization  
45 has long been studied. An interdigitation mechanism was first proposed by Okano and  
46 Sarko (Okano and Sarko 1985) . NaOH is absorbed, converting cellulose I into a swollen  
47 structure in which all contacts between adjacent chains are removed. Once NaOH has  
48 been removed by washing with water, a bi-oriented cellulose II structure is obtained (P.  
49 Langan, Nishiyama, and Chanzy 1999; Paul Langan, Nishiyama, and Chanzy 2001).

50 Nishiyama et al. (Nishiyama, Kuga, and Okano 2000) proposed a molecular  
51 association in Na-Cellulose where van der Waals' interaction is the driving force of the  
52 formation of cellulose II. The effect of mercerization on crystallinity was investigated for  
53 different cellulose sources (J. F. Revol, Dietrich, and Goring 1987). All cellulose II  
54 obtained had a narrow range of crystallinity and, a constant crystal size. The crystallinity  
55 index for the mercerized celluloses remained in a narrow range of 0.50-0.66, whereas it  
56 varied from 0.41 to 0.95 for the native cellulose. The crystal size was approximately  
57 constant for the mercerized celluloses, from 3.4 nm to 4.4 nm, whereas it varied from  
58 2.9 nm to 15.4 nm in native celluloses. The result is that in the case of highly crystalline  
59 cellulose, mercerization reduces crystallinity and crystal size, whereas in the case of low  
60 crystallinity cellulose, mercerization increases crystallinity and the size of the crystal.  
61 These trends would not be expected if the conversion of cellulose I to cellulose II was  
62 simply a change in conformation of the chain or arrangement of atoms. These results  
63 are more in line with the idea that mercerization involves a complete destruction of the  
64 structure of cellulose I by separation of the molecular chains, followed by the reforming  
65 of the crystalline structure in the form of cellulose II. These results are consistent with  
66 the hypothesis that mercerization involves a mixture of adjacent and antiparallel  
67 cellulose microfibrils (Okano and Sarko 1985).

68 Type II cellulose nanocrystals have already been obtained from acid hydrolysis  
69 (Sebe, Ham-Pichavant et al. 2012) or after mercerization of fibers (Neto et al. 2016) .  
70 Sèbe et al. (Sèbe et al. 2012) prepared CNC samples using nine different conditions  
71 involving H<sub>2</sub>SO<sub>4</sub> at concentrations varying from 62 to 66% and up to 120 min. One of  
72 them led to CNC-II only (not a mixture of CNC-I and CNC-II). The resulting nanocrystals  
73 (CNC-II) were found to be smaller than CNC-I and were ribbon-shaped with rounded tips  
74 and larger crystallites, whereas Neto et al. (Neto et al. 2016) described CNC-II as being  
75 shorter (from 240 nm to 132 nm) and broader (from 15 nm to 19 nm), with identical  
76 thickness (around 4 nm), and with an increased crystallinity from 56% to 68%. For Li et  
77 al. (Li et al. 2018), the mercerized CNCs were even much smaller (19 nm in length and 11  
78 nm in width) with ellipsoid shapes.

79 CNCs are predicted to have a major impact in the coming years, and variability will  
80 be a key of this development. Recent reviews show the interest of the selective  
81 modification of the reducing end (Heise et al. 2021; Tao et al. 2020) of CNC-I. A growing  
82 interest is now focused on CNC-II with the hemiacetal form at the two extremities. A  
83 precise control of their various forms is therefore of great importance but the transition  
84 mechanism is still a matter of debate. In order to better understand the mechanism  
85 involved in mercerization, it is consequently of interest to compare different packs of  
86 data produced using both different and similar hydrolyses. But also to compare the  
87 results obtained from different technics. For example, the ratio of crystalline regions to  
88 total fibrils of cellulose (the crystallinity index) is usually investigated by X-ray diffraction  
89 and solid-state <sup>13</sup>C-NMR experiments (Park et al. 2010; Zugenmaier 2008). The first one  
90 is based on the detection of a diffraction plane, which considers structuration of several  
91 glucose residues. The second one is based on the variation of the chemical shift  
92 associated with the angles of the glycosidic bond. These two techniques that observe  
93 cellulose at two different scales are quite complementary. In the present study, native  
94 (CF-I) and mercerized (CF-II) cotton fibers are both hydrolyzed using the same sulfuric  
95 acid hydrolysis process, leading to CNC-I and CNC-II. A full set of complementary

96 techniques is described and used to precisely characterize the morphology, molar mass,  
97 structure, surface charge and degree of polymerization of both nanocrystals.  
98

## 99 2. Materials and Methods

100 **Materials:** The native cotton cellulose fibers were obtained from Buckeye Technology  
101 Inc., USA. All reactants had a purity of above 95% and were acquired from Sigma Aldrich  
102 and used without further purification. Ultrapure water was produced with the Milli-Q  
103 reagent system (18.2 M $\Omega$  cm, Millipore Milli-Q purification system).  
104

105 **Cellulose sample preparation:** Native cotton cellulose fiber (CF-I) was mercerized  
106 (CF-II) according to a protocol similar to that described by Neto et al. (Neto et al. 2016).  
107 Ten grams of CF-I were introduced into 300 mL of 20 wt% NaOH and mechanically  
108 stirred for 5 h at 25°C. The mixture was washed several times with distilled water in  
109 order to remove the NaOH solution, and then dried at 40°C for 48 h. This conversion was  
110 carried out with a yield of 100%.  
111

112 **Preparation of cellulose nanocrystals (CNC-I and CNC-II):** Both CNCs were prepared  
113 by hydrolysis with sulfuric acid according to the method of Revol et al. (J.-F. Revol et al.  
114 1992) with minor modifications. Briefly, cellulose nanocrystals (CNC-I and CNC-II) were  
115 prepared under the same conditions from fibers (CF-I and CF-II, respectively) using  
116 sulfuric acid hydrolysis at 64% at 68°C under stirring for 20 min. After hydrolysis, the  
117 suspensions were washed by centrifugation, dialyzed to neutrality against Milli-Q water  
118 for 2 weeks, and deionized using mixed bed resin (TMD-8). The final dispersion was  
119 sonicated for 10 min, filtered and stored at 4°C. The yield was 64% and 40% for CNC-I  
120 and CNC-II, respectively.  
121

### 122 **Cellulose sample characterization**

123 **X-ray Diffraction.** The determination of crystalline type, crystallinity index and crystal  
124 size of the different samples was performed by X-ray Diffraction (XRD) analysis using a  
125 Bruker D8 Discover diffractometer (Karlsruhe, Germany) equipped with a VANTEC 500  
126 2D detector. X-ray radiation, CuK $\alpha$ 1 ( $\lambda = 0.15406$  nm), produced in a sealed tube at 40  
127 kV and 40 mA, was selected and parallelized using crossed Göbel mirrors and collimated  
128 to produce a beam of 300 or 500  $\mu$ m in diameter. The suspensions of nanocrystals were  
129 freeze-dried and then pressed at room temperature to obtain dense pellets, while the  
130 fibers were used as such. The diffraction patterns were recorded for 10 min over a range  
131 from 3° to 40° ( $2\theta$ ). The recorded intensity was normalized by the total peak area to  
132 eliminate the influence of the thickness variation and the absorption coefficient of the  
133 samples. The X-ray crystallinity index ( $CI_{XRD}$ ) was estimated from the crystalline to  
134 amorphous areas using Origin (v8.0891) software.  
135

136 **Solid-state NMR CP-MAS.** The NMR experiments were carried out on an Avance III-400  
137 MHz spectrometer (Bruker; France) operating at 100.62 MHz for  $^{13}C$ , equipped with a  
138 double-resonance H/X CP-MAS 4-mm probe for CP-MAS (Cross-Polarization Magic Angle  
139 Spinning) solid-state experiments. The samples were wetted and spun at 12,000 Hz at  
140 room temperature.

141 CP-MAS spectra were acquired with a contact time of 1.5 ms and over an accumulation  
142 of 2048 scans separated by a recycling delay of 10 s. The carbonyl carbon was set to  
143 176.03 ppm through external glycine calibration. NMR spectra deconvolution was  
144 performed using PeakFit<sup>®</sup> (v.4.11) software (Systat Software, Inc., USA). Peak chemical  
145 shifts were assigned according to (Larsson et al. 1999; Newman and Davidson 2004). The

146 NMR crystallinity index of CF and CNC was calculated according to (Larsson et al. 1999;  
147 Zuckerstätter et al. 2013).

148

149 **Conductometry.** The hydrolysis of the cellulose with sulfuric acid makes it possible to  
150 obtain a colloidal suspension of the nanometric-sized crystals with SO<sub>3</sub><sup>-</sup> charges on their  
151 surface. The measurement of the quantity of charges on the CNC surface charge was  
152 performed by conductometric titration with a 0.001 M NaOH solution using a TIM900  
153 titration manager and a CDM230 conductimeter equipped with a CDC749 conductivity  
154 cell.

155

156 **Zeta Potential** ( $\zeta$ -potential).  $\zeta$ -potential experiments were performed with a Malvern  
157 NanoZS instrument. All measurements were made at a temperature of 20°C with a  
158 detection angle of 12.8°. CNC dispersions of 1 g/L at pH = 7 were prepared at 20°C and  
159 filtered by 5  $\mu$ m. Each sample was measured a total of five times. The confidence  
160 interval (error) presented is the standard deviation of samples measured in triplicate.

161

162 **Asymmetrical flow field-flow fractionation coupled to Multi-Angle Laser Light**  
163 **Scattering and Refractive Index** (A4F-MALLS-RI) detection. An AF4 instrument was  
164 coupled with two online detectors: a MALLS instrument (DAWN Heleos II) fitted with a  
165 K5 flow cell and a GaAs laser ( $\lambda = 663$  nm), and a refractometric detector operating at  
166 the same wavelength (Optilab T-rEX) from Wyatt Technology (Santa Barbara, CA, USA).  
167 The AF4 instrument consisted of an AF4 channel (275 mm-long), a 350- $\mu$ m-thick spacer  
168 and a regenerated cellulose membrane with a nominal cut-off of 10 kDa (Millipore,  
169 Bedford, MA, USA). The refractive index increment  $dn/dc$  was 0.146 mL/g, a value  
170 classically used for glucans in water (**Paschall and Foster 1952**). The AF4 channel flow,  
171 cross flow, sample injection and focus flow were controlled with a Wyatt Eclipse AF4  
172 flow chassis, a pump and an autosampler from ThermoFisher Scientific (Waltham, MA,  
173 USA). CNC dispersions of 0.5 g/L in water were prepared at 20°C and systematically  
174 freshly sonicated (amplitude 5, 8 s, 2 on/1 off) before being injected. Each sample was  
175 measured a total of two times. The weight and number-average molar masses ( $\bar{M}_w, \bar{M}_n$ )  
176 and the polydispersity ( $\bar{M}_w/\bar{M}_n$ ) of CNCs were determined with Wyatt ASTRA<sup>®</sup> software  
177 (v. 6.1.4) with Zimm extrapolation of order 1.

178

179 **Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering and**  
180 **Refractive Index** (SEC-MALLS-RI) detection. The determination of molar mass  
181 distribution of chains of cellulose in DMAc/LiCl was carried out at room temperature  
182 using an OMNISEC system (Malvern). The size exclusion chromatography (SEC)  
183 (OMNISEC Resolve, Malvern) system was coupled with a multi-angle laser light  
184 scattering detector (MALLS, Malvern) and OMNISEC Reveal devices (Malvern). The SEC  
185 columns used were Viscotek Tguard, LT4000L, LT5000L and LT7000L. The mobile phase  
186 used for SEC was N,N-dimethylacetamide (DMAc) (HPLC grade) containing lithium  
187 chloride (LiCl) (0.9% v/w), that had been filtered through 0.6- $\mu$ m polypropylene  
188 prefilters. This eluant was chosen because it solubilizes cellulose without significant  
189 depolymerization during the dissolution process or during storage at room temperature  
190 for long periods (Dupont and Harrison 2004; Yanagisawa and Isogai 2005). Calculation of  
191 weight- and number-average molar masses ( $\bar{M}_w, \bar{M}_n$ ) and polydispersity ( $\bar{M}_w/\bar{M}_n$ ) of  
192 samples were performed with a  $dn/dc$  value of 0.136 mL/g (Hasani et al. 2013) and  
193 determined with OMNISEC software (v.10.30) with Zimm extrapolation of order 2.  
194 Cellulose was solubilized in the DMAc/LiCl (9% v/w) (Dawsey and McCormick 1990;  
195 Medronho and Lindman 2015) via solvent exchange steps H<sub>2</sub>O/Met-OH/DMAc CF-I and  
196 CF-II and H<sub>2</sub>O/Et-OH/DMAc for CNC-I and CNC-II.

197 For fibers, 100 mg (dry content) of CF-I and CF-II were washed with 30 mL methanol, and  
198 the excess of methanol was removed by filtration on fritter n° 3. This step was repeated

199 three times. The recovered pellet was washed three times with 30 mL of DMAc for  
200 solvent exchange, and the excess of DMAc was removed by filtration on fritter n° 3.  
201 After solvent exchange steps, 10 mL of DMAc/LiCl (9% v/w) were added to the vial  
202 containing the sample and allowed to stir magnetically at 4°C for dissolution.

203 For CNCs, the samples in the form of aqueous suspensions were freeze-dried. The dry  
204 extract obtained (approximately 20 mg) was washed with ethanol, and the excess of  
205 ethanol was removed by centrifugation (2220 g for 15 min at 20 °C) (Hasani et al. 2013).  
206 This step was repeated twice and the material was then put in DMAc for solvent  
207 exchange under magnetic stirring at room temperature overnight. The excess of DMAc  
208 was removed by centrifugation (2220 g for 15 min at 20°C). After the solvent exchange  
209 steps, 2 mL of DMAc/LiCl 9% (v/w) were added to the vial containing the sample and  
210 allowed to stir magnetically at 4°C for dissolution.

211 The final concentration of the samples was 10 g/L. The dissolution was stopped by the  
212 addition of pure DMAc. The final concentration of samples in DMAc/LiCl (0.9% v/w) was  
213 1 g/L. Before injection, the samples were filtered through a 0.45- $\mu$ m  
214 polytetrafluoroethylene (PTFE) membrane filter.

215

216 **Transmission Electron Microscopy (TEM).** Droplets of CNC suspensions at 0.8 g/L were  
217 deposited on freshly glow-discharged carbon-coated microscope grids (200 mesh, Delta  
218 Microscopies, France) for 2 min. The excess liquid was removed by filter paper,  
219 negatively stained with an aqueous solution of phosphotungstic acid at 10 g/L for 2 min  
220 and dried just before TEM observation. We used a JEOL type transmission electron  
221 microscope (JEM-1230) operating at a voltage of 80 keV. The average dimensions  
222 (length and width) of the CNCs were determined from TEM image analysis of  
223 approximately 350 particles using ImageJ software.

224

225 **Atomic Force Microscopy (AFM).** To determine the average thicknesses of the  
226 nanocrystals, the suspensions were diluted to 0.05 g/L and then deposited on mica  
227 substrates. The measurements were carried out at room temperature by an Innova AFM  
228 (Bruker) using a monolithic silicon tip (TESPA, Bruker, spring constant  $k = 42$  N/m,  
229 frequency  $f_0 = 320$  kHz). Image processing was performed with WSxM 5.0 software.

230

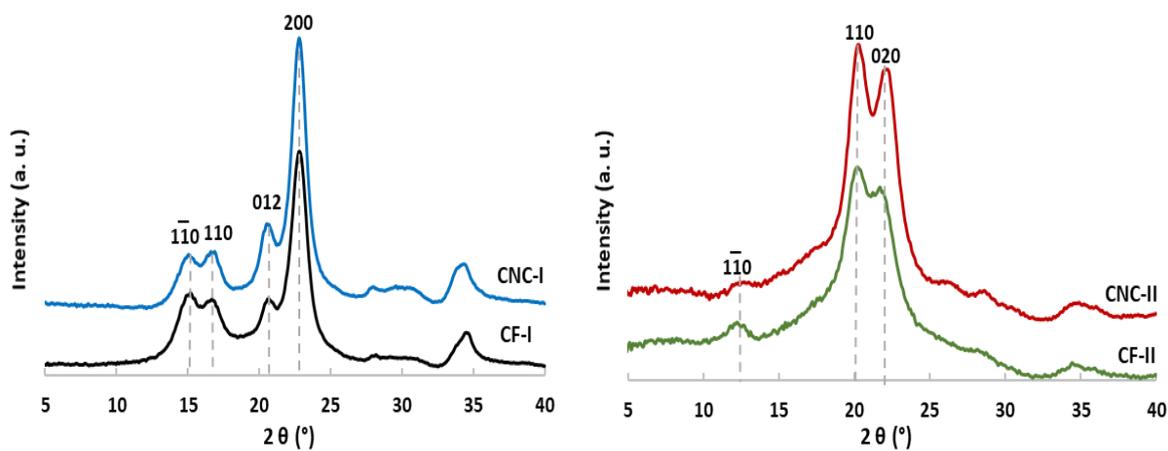
231 **Small Angle Neutron Scattering (SANS) experiments.** SANS experiments were carried  
232 out at room temperature using the small-angle PA20 and PAXY diffractometers at the  
233 Laboratoire Léon Brillouin (CEA/CNRS) in Saclay (France). Three configurations were  
234 used for PA20, covering a Q range from 0.0006 and 0.44  $\text{\AA}^{-1}$  (6  $\text{\AA}$  at 1.1 m, 6  $\text{\AA}$  at 8 m,  
235 and 15  $\text{\AA}$  at 17.5 m), where Q is the wave vector ( $Q = 4\pi \sin \theta/2$ , where  $\theta$  is the  
236 scattering angle and  $\lambda$  is the neutron wavelength), and four configurations for PAXY,  
237 covering a Q range from 0.002 and 0.5  $\text{\AA}^{-1}$  (5  $\text{\AA}$  at 1 m, 5  $\text{\AA}$  at 3 m, 8.5  $\text{\AA}$  at 5 m and 15  $\text{\AA}$   
238 at 6.7 m). CNC dispersions of 2 g/L in 2 mM NaCl were prepared at 20°C and then  
239 extensively dialyzed against D2O to obtain the best possible contrast as well as to  
240 reduce the incoherent scattering as much as possible, and then systematically freshly  
241 sonicated for 10 s and loaded in quartz cells (Hellma) with small path lengths (1 and 2  
242 mm). To determine the CNC dimensions, the data were fitted with Sasview software.  
243 Several fitting models were tried using the form factor of a parallelepiped with a  
244 rectangular section, averaged over all space orientations, and constituting a perfectly  
245 fitting model of the rod-like CNCs (Cherhal, Cousin, and Capron 2015). Aggregation  
246 experiments in solution were performed on suspensions at 2 g/L of CNC-I and CNC-II in  
247 2, 50 and 100 mM NaCl. The suspensions were measured after sonication.

248

### 249 3. Results

#### 250 3.1 Structural description

251 The XRD patterns of native, mercerized and hydrolyzed cotton samples are shown in Fig.  
252 1.



253  
254

255

256 **Figure 1.** X-ray diffraction patterns of cotton fibers in native (CF-I) and mercerized CF-II forms and  
257 their respective hydrolyzed cellulose nanocrystals in the native (CNC-I) and mercerized (CNC-II)  
258 forms, and cross-sections of elementary crystallites deduced from the analysis of peak  
259 broadening (the indexing of corresponding lattice planes is described in Supporting  
260 Information).

261

262 The diffraction patterns of CF-I and CNC-I are typical of cellulose I with the presence  
263 of diffraction peaks at 15.1°, 16.9°, 20.7° and 22.8°, corresponding to (1-10), (110),  
264 (012/102) and (200) crystallographic planes, respectively. After mercerization, the  
265 crystallinity index ( $Cl_{XRD}$ ) of CF-II decreased. For the mercerized sample, CF-II and CNC-II  
266 at 12.3°, 20.0° and 21.7° corresponded to the (1-10), (110) and (020) reflections,  
267 respectively (Duchemin 2015; Isogai et al. 1989; Nishiyama, Kuga, and Okano 2000),  
268 whereas traces of cellulose I residuals can be recognized at 15.1° and 16.9° (Fig. 1). This  
269 allomorphic modification was achieved without loss in mass (Table 1). XRD peak analysis  
270 (see values in SI) allowed representation of the crystals (Fig. 1). The (1-10) and (110)  
271 crystalline planes have interplane dimensions of 0.61 nm and 0.54 nm, respectively  
272 (Goussé et al. 2002; Sugiyama, Vuong, and Chanzy 1991). Similarly, for CNC-II, the  
273 distances for (1-10) and (110) are 0.72 nm and 0.44 nm, respectively (Kolpak, Weih, and  
274 Blackwell 1978; P. Langan, Nishiyama, and Chanzy 1999; Sèbe et al. 2012).

275

276

277 After sulfuric acid hydrolysis of the fibers, the XRD results showed an increase of  
 278 the crystallinity index ( $CI_{XRD}$ ). For the native form, 64% of the cellulosic material was  
 279 recovered after hydrolysis, whereas the  $CI_{XRD}$  only increase by 5% (from CF-I to CNC-I).  
 280 The hydrolysis then affects amorphous as well as crystalline domains.  
 281 Considering fibers, all the material was recovered after mercerization (yield of 100%).  
 282 However, after acid hydrolysis, only 40% of the initial material was recovered, while the  
 283  $CI_{XRD}$  increased by 30% (from CF-II to CNC-II). Mercerization leads to fibers that are more  
 284 susceptible to acid hydrolysis, probably due to lower organization. Moreover, as can be  
 285 observed in other studies (French 2014; Neto et al. 2016), mercerization drastically  
 286 reduces crystallinity as well as the crystal dimensions of the cotton.  
 287

288

289 **Table 1:** Weight fraction (yield) recovered after treatment, crystallinity index (CI) calculated from  
 290 XRD ( $CI_{XRD}$ ), Mean CI calculated from solid-state NMR ( $^{13}C$  CP-MAS) spectra ( $CI_{NMR}$ ).  
 291 Deconvolution of the C4 region of  $^{13}C$  CP-MAS spectra.

Samples	Yield (%)	$CI_{XRD}$ (%)	$CI_{NMR}$ (%)	Deconvolution of the C4 region		
				crystalline	paracrystalline intermediary domain	amorphous
CF-I	-	60	67	25%	42%	26% Acc + 7% inAcc
CNC-I	64	65	75	36%	39%	25%
CF-II	100	40	72	58%	14%	28%
CNC-II	40	70	85	74%	11%	15%

292

293

294 Figure 2 shows the  $^{13}C$  CP-MAS NMR spectra of CF-I and CF-II and confirms the  
 295 mercerization process with the two peaks at 88.1 and 86.9 ppm in the CF-II spectrum  
 296 that are characteristic of type II cellulose (Ibbett, Domvoglou, and Fasching 2007;  
 297 Newman and Davidson 2004). CF-I had a  $CI_{NMR}$  of 67%, and this crystallinity increased  
 298 after acid hydrolysis. For CF-II, this  $CI_{NMR}$  increased up to 72% after mercerization and up  
 299 to 85% after subsequent hydrolysis (CNC-II preparation).

300 The signals in the 86-92 ppm region that refer to crystalline domains were further  
 301 decomposed. This deconvolution analysis discriminates an "in-core" ordered region  
 302 from a "paracrystalline" organization described as having an intermediate order  
 303 between amorphous and crystalline cellulose (Zuckerstätter et al. 2013) (Fig. 2).  
 304 According to this analysis, original CF-I is characterized by a  $CI_{NMR}$  of 67% composed of  
 305 25% of a pure crystalline domain and 42% of a so-called paracrystalline domain (Table  
 306 1). The remaining 33% are divided into 26% of accessible and 7% of inaccessible  
 307 amorphous domains.

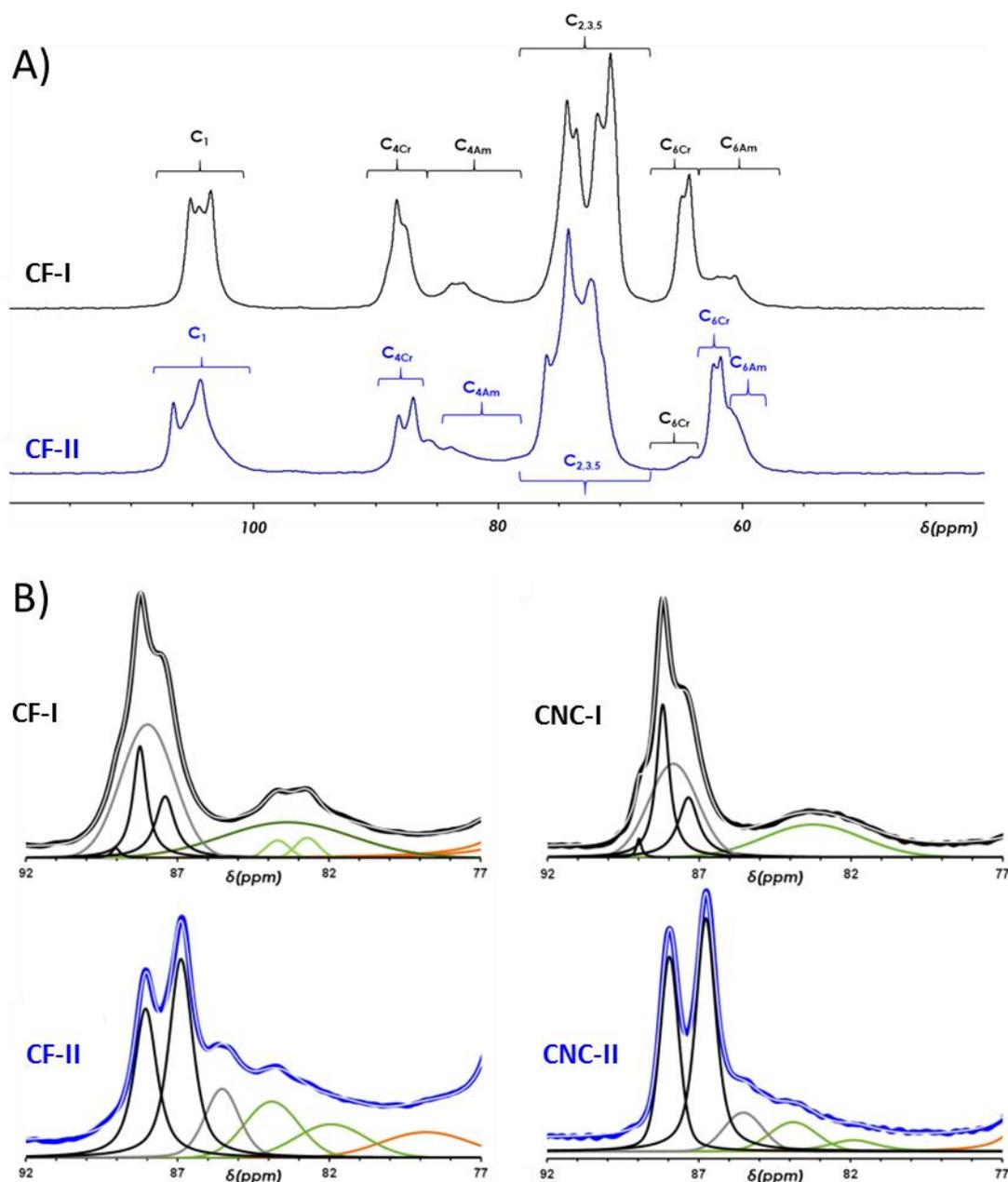
308 After acid hydrolysis, an increase in the relative area of crystalline peaks at 86-92  
 309 ppm is observed, and the  $CI_{NMR}$  increased in accordance with XRD results. However, the  
 310 selective analysis of crystalline and paracrystalline structures shows that the  
 311 paracrystalline organization is only slightly decreased. The increase in crystallinity  
 312 between CF-I and CNC-I is then correlated with a loss of the amorphous part, since the  
 313 paracrystalline domains are much less affected. According to the model proposed by  
 314 Larsson and also used by Wickholm, paracrystalline domains are structures surrounding  
 315 nanocrystals in the nanofibers and are less accessible than amorphous domains. After

316 hydrolysis, the amorphous domain, visible in the 80-86 ppm region, shows only one  
317 remaining peak.

318 After mercerization, a typical spectrum of cellulose II revealed the allomorphic  
319 transition. However, the  $^{13}\text{C}$  NMR spectrum of CF-II shows a signal characteristic of  
320 crystalline C6 of cellulose I representing about 4% of the total C6 signal. This residual  
321 crystalline cellulose I-type conformation results from an ineffective penetration of NaOH  
322 in crystalline domains; they are potentially dispersed in a random way, as proposed by  
323 Kim et al. (Kim et al. 2006).

324 The mercerization process of the nanofibers results in a slight increase of  $\text{C}_{\text{I-NMR}}$   
325 from CF-I to CF-II (Table 1), which is contradictory with XRD results. Simultaneously, a  
326 slight decrease of the amorphous contribution from 33% to 28% is observed, and only  
327 one peak is observed that refers to only one amorphous type domain. Compared to this,  
328 the so-called paracrystalline region, which usually refers to structures surrounding  
329 cellulose I nanocrystals, undergoes a sharp decrease from 42% to 14%. The origin and  
330 structure of such a state is still not clear (Bregado et al. 2019; Larsson et al. 1999),  
331 except that it is intermediate (in terms of mechanical properties, hydrogen bonding and  
332 chain ordering) between crystalline and amorphous cellulose. After mercerization, a  
333 peak is clearly visible at 85.5 ppm (Fig. 2), referring to that imperfect crystalline region  
334 (or, similarly, to an ordered amorphous region). Such a peak was previously observed  
335 and attributed to partially ordered cellulose (Ibbett, Domvoglou, and Fasching 2007).  
336 The result is that only one type of the amorphous structure remains in a slightly reduced  
337 amount, whereas a large part of the paracrystalline-I structure that presumably  
338 surrounds the crystalline domains formed by mercerization is lost.

339 Acid hydrolysis of the mercerized cellulose occurs with a loss of mass (yield: 40%)  
340 but without much change in the peak attributed to the intermediate structure. The  
341 same trend is then observed for both CNC-I and CNC-II. This was already reported by  
342 (Wickholm et al. 2001). It implies that acid hydrolysis removes amorphous regions,  
343 contrary to the mercerization process that strongly impacts paracrystalline/intermediate  
344 domains. The same fraction of 4% of cellulose I observed in CF-II was recovered in the  
345 CNC-II sample.



346

347 **Figure 2.** (A)  $^{13}\text{C}$  CP-MAS NMR spectra of CF-I and CF-II, and (B) deconvolution of the C4 region of  
 348 CF-I, CF-II, CNC-I and CNC-II NMR spectra with crystalline forms (black), paracrystalline (gray) and  
 349 amorphous (green).

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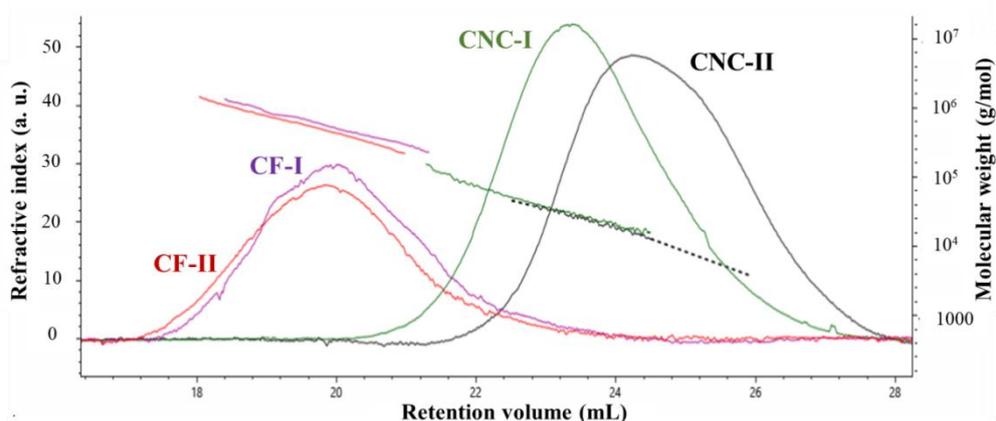
351 However, the results obtained by XRD and NMR are controversial. The loss of  
 352 crystallinity observed by XRD after mercerization is not observed by NMR (Table 1). In  
 353 solid-state NMR, ~~considering only the C4 region,~~ chemical shifts are influenced by the  
 354 conformation of carbon atoms in glycosidic chains bonds, which may be involved in a  
 355 crystalline, paracrystalline or amorphous structure. For XRD analysis, beyond crystallite  
 356 orientation, it is the crystal lattice that is directly identified. It is therefore easy to  
 357 imagine that parts of chains may have conformations related to those of crystal lattices  
 358 without having a dimension that allows XRD to identify them as such, explaining a higher  
 359 value of CI by NMR. The variations observed can then be linked to the ability of each  
 360 technique to detect imperfect organizations. NMR assumes that all the carbons involved  
 361 are in crystalline structure which considers very short-scale. It analyzes crystalline and

362 paracrystalline organizations in the so-called  $Cl_{NMR}$ , and distinguishes these forms from  
 363 the amorphous domain with signals shifted to lower ppm values. In contrast, XRD  
 364 analysis requires longer scale organization since the presence of paracrystalline  
 365 organizations is included in the widening peaks attributed to amorphous domains.

366 As a result, a major modification during mercerization comes from this  
 367 intermediate state that is reformed in smaller amounts after swelling in NaOH and the  
 368 recrystallization process. In addition, mercerization leads to more crystalline domains  
 369 that seem to be more discontinuous than the former. Such structures are not fully  
 370 detected by XRD analysis but assumed by NMR to be globally crystalline. Furthermore,  
 371 only one amorphous peak is visible after mercerization by NMR, implying only one type  
 372 of amorphous area. This might reveal a more homogeneous but less organized system,  
 373 with more imperfections, which is also in accordance with the increased susceptibility to  
 374 acid hydrolysis of CF-II. After hydrolysis, imperfections are removed and highly  
 375 crystalline particles are recovered, as detected by both XRD and NMR analyses.  
 376

### 377 3.2 Molar mass characterization

378 In order to follow the process at a molecular level, the native and mercerized fibers  
 379 were dissolved in DMAc/LiCl and injected into a SEC-MALLS-DRI device. This experiment  
 380 made it possible to determine the molar mass ( $M_w$ ) distribution of individual cellulosic  
 381 chains. It may also determine whether the process that involved NaOH at a high  
 382 concentration had an impact on the glucosidic chain length. The size exclusion  
 383 fractionation mode implies that larger molecules are the first to elute. Both fibers were  
 384 found to have an average molar mass of 560,000 g/mol with a low polydispersity (Table  
 385 2). Even just a slight shift to higher retention volumes seems to indicate more flexibility  
 386 of CF-I. However, it is demonstrated here that mercerization treatment of native  
 387 cellulose fibers through NaOH swelling does not induce any molecular disruption.  
 388



389  
 390 **Figure 3.** Dissolution profiles of samples obtained by SEC-MALLS-DRI. The two nanofibers (CF-I in  
 391 purple and CF-II in red) are eluted at low retention volumes, whereas the nanocrystals are eluted  
 392 at higher elution volumes (CNC-I in green and CNC-II in black).

393  
 394 **Table 2.** Weight-average molar masses ( $\bar{M}_w$ ), polydispersity ( $\bar{M}_w/\bar{M}_n$ ) and degree of  
 395 polymerization (DP) of individual chains of cellulosic fibers (CF-I and CF-II) and cellulose  
 396 nanocrystals (CNC-I and CNC-II) solubilized in DMAc/0.9% LiCl.

Samples	$M_w$ (g/mol)	$M_w/M_n$	$DP_w$	$DP_n$
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CF-I	565,000 ± 47,000	1.3	3487	2683
CF-II	556,000 ± 43,000	1.3	3432	2640
CNC-I	41,000 ± 1,000	1.2	253	210
CNC-II	22,000 ± 1,000	1.2	135	112

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Similarly, both CNCs were solubilized in DMAc/9% LiCl (v/w) for Mw distribution determination. They logically appear to have a larger retention volume compared to the fibers (Fig. 3), indicating a significant decrease in the hydrodynamic volume of the chains. The acid hydrolysis of the fibers led to a clear decrease of the Mw, from 560,000 g/mol for both fibers, down to 41,000 g/mol for CNC-I and to 22,000 g/mol for CNC-II (Table 2). Contrary to mercerization that did not affect the chain length, the degree of polymerization (DP) of CNC-II is about half as low as CNC-I after the hydrolysis. Furthermore, the Mw distribution curves of CNC-II were shifted to lower retention volumes but superimposed on a large domain, illustrating the same proportion in occupied volume. In other words, CNC-II is similar in conformation but smaller.

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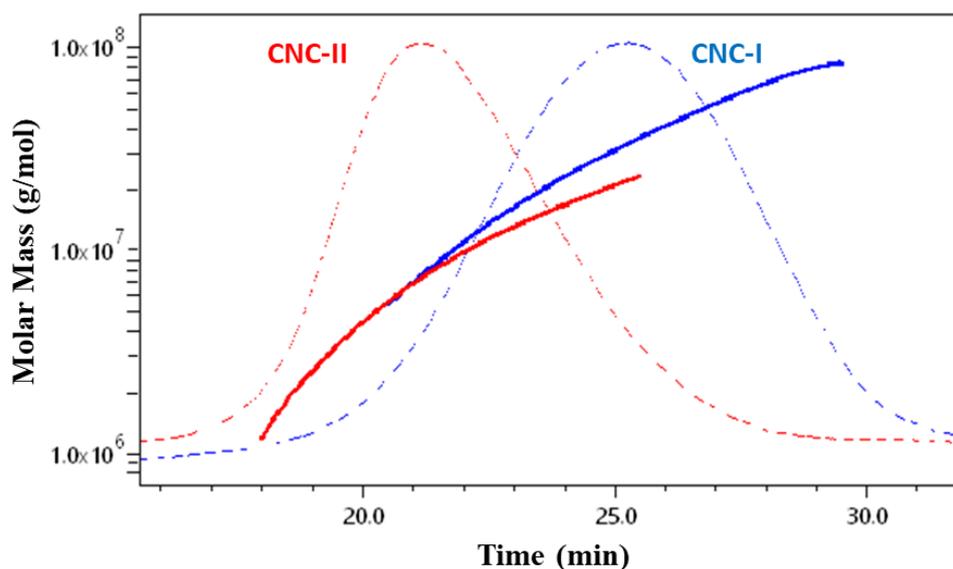
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Simultaneously, Mw distributions of the CNCs directly in suspension in water (without a solubilization step) were obtained using A4F-MALLS-DRI analysis (Fig. 4). Since the fractionation is carried out by a cross-flow device, the smaller molecules were the first to elute. The shift to a lower elution time for CNC-II compared to CNC-I confirmed the lower hydrodynamic volumes of CNC-II. The Mw measured were also much lower (Table 3), with 36.106 g/mol and 11.106 g/mol for CNC-I and CNC-II, respectively. These values are in agreement with the results found by the SEC-MALLS-DRI device.



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**Figure 4.** Distribution of molar masses of suspensions of CNC-I (blue) and CNC-II (red) in water, and RI signal (dotted curves).

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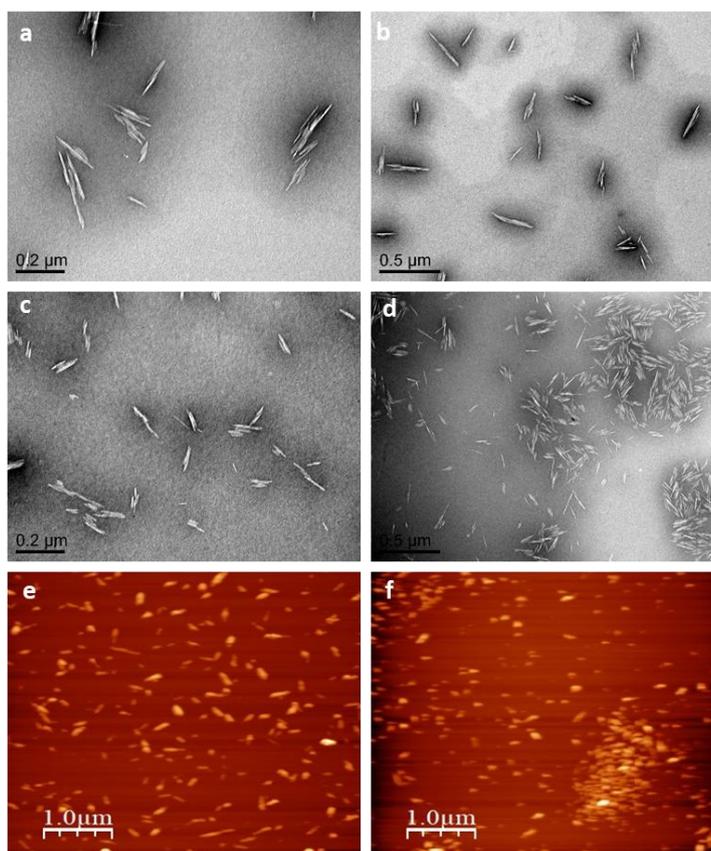
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When dividing the molar mass obtained in crystalline form from both CNCs (Table 3) to that of their individual chains (Table 2), the packing appeared to decrease from 878 to 500 chains for CNC-I and CNC-II, respectively. This is a very high value compared to the dimensions of the elementary CNCs, revealing that some aggregation still remains.

426 However, it clearly appears that the mercerized CNCs are two to three times smaller in  
 427 length and packing. The result is that the crystalline domains in NF-II are shorter, with a  
 428 DP of less than half of those in NF-I.  
 429

### 430 3.3 Characterization of cellulose nanocrystal morphology

431 The morphology of native and mercerized CNCs was characterized and compared  
 432 by TEM, AFM and SANS. Figure 5 shows TEM and AFM images of native and mercerized  
 433 CNCs. Both CNCs are in the form of rigid rods with shorter CNC-II. The average lengths of  
 434  $118 \pm 65$  nm and  $65 \pm 22$  nm were determined for CNC-I and CNC-II, respectively (Table  
 435 3). This is in accordance with previous results (Neto et al. 2016). When selecting  
 436 individual CNCs, in order to measure elemental nanocrystals, it was found that CNC-I  
 437 and CNC-II have the same individual width of  $7 \pm 3$  nm. More surprisingly and differently  
 438 from what was previously reported by (Neto et al. 2016), the average thicknesses found  
 439 by AFM were  $6.0 \pm 2.4$  nm and  $3.4 \pm 1.5$  nm for CNC-I and CNC-II, respectively (Table 3).  
 440 The thickness reduced by half of its value is clearly observable.



441  
 442 **Figure 5.** TEM images of CNC-I (a,b) and CNC-II (c,d) and AFM images of CNC-I (e) and CNC-II (f).

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445 **Table 3.** Weight-average molar masses ( $M_w$ ) and polydispersity ( $M_w/M_n$ ) of CNC-I and CNC-II  
 446 dispersed in water determined by A4F-MALLS-DRI; average dimensions determined from the  
 447 SANS curve, TEM images and AFM images.

Samples	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	Length (nm)	Width (nm)	Thickness (nm)
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	(10 <sup>6</sup> g/mol)		SANS	TEM	SANS	TEM	SANS	AFM
CNC-I	36 ± 1	1.5	175 ± 25	118 ± 65	21 ± 1	7 ± 3	6.5 ± 0.5	6.0 ± 2.5
CNC-II	11 ± 1	1.5	75 ± 25	65 ± 22	22 ± 2	7 ± 3	3.5 ± 0.5	3.4 ± 1.5

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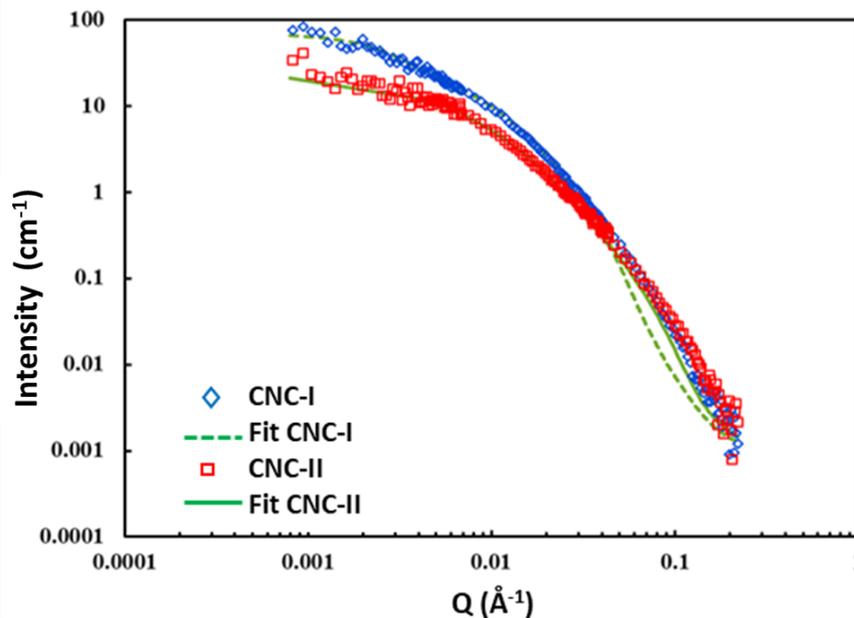
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The validation of these results was carried out in suspensions of CNCs in water at 2 mM NaCl by the fit of the curves obtained by small angle neutron scattering (SANS) using the parallelepiped form factor (Figure 6). This measurement allows analysis in dilute suspensions without a drying step. CNC-I shows a higher intensity at low  $q$ , revealing a higher  $M_w$ , and crosses the profile of CNC-II at intermediate  $Q$ . For both samples, the best fit obtained confirmed length and thickness values obtained by microscopy. Even if some individual CNCs must be present in suspension, a best fit is obtained for an average width of 21 nm for both samples, corresponding to an average of three to four elementary laterally associated crystals, as already measured (Cherhal, Cathala, and Capron 2015; Elazzouzi-Hafraoui et al. 2007). The lateral association is then not modified during the mercerization process. The elementary cotton-based CNC-I is generally viewed with a squared cross-section. CNC-II then appears with a rectangular cross-section. The values are in agreement with the results found by A4F-MALLS-DRI and SEC-MALLS-DRI devices.



464

465

**Figure 6.**  $I = f(Q)$  SANS curves of suspensions of CNC-I and CNC-II in water at 2 g/L in 2 mM NaCl.

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### 3.4. CNC surface charge density

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Hydrolysis with sulfuric acid is known to graft anionic sulfate half esters ( $OSO_3^-$ ) on to the surface of the CNCs. The same surface charge density is obtained for both CNCs as indicated by the sulfate content of 0.27% and the zeta-potential values of  $-42$  mV for

472 both CNC-I and CNC-II (Table 4). This implies the same susceptibility of both fiber  
 473 surfaces to acid treatment.

474

475 **Table 4.** Sulfur content (S), surface charge density (SC) and zeta potential of CNC-I and CNC-II.

Samples	S (%)	SC (mmol/g)	$\zeta$ -potential (mV)
CNC-I	0.278 ± 0.09	0.087 ± 0.03	- 42.3 ± 2.7
CNC-II	0.271 ± 0.03	0.085 ± 0.01	- 41.9 ± 1.9

476

## 477 4. Discussion

478 **Table 5:** Comparison with other studies in length (L), width (W), thickness (T) and crystallinity  
 479 index (CI).

	Sèbe et al. (2012)				Neto et al. (2016)				Haouache et al. (present work)			
	L	W	T	CI	L	W	T	CI XRD/NMR	L	W	T	CI XRD/NMR
CNC-I	246	-	5.9	-	240	15	3.8	56/50	175/118	21	6	65/75
CNC-II	153	6.3	4.2	-I	132	19	5.2	68/63	75/65	22	3.5	70/85

480

481 Comparing these results with previous ones (Table 5), Sèbe et al. (Sèbe et al. 2012)  
 482 prepared CNC samples using nine different sulfuric acid conditions. This resulted in a  
 483 new type of preparation of cellulose II that led to shorter CNCs with rounded tips and  
 484 larger crystallites but a lower degree of order. This morphology is very different from  
 485 our mercerized samples. Since we used the same process as Neto et al. (Neto et al.  
 486 2016), the results are more similar. These two studies reveal that the nanocrystals are  
 487 shorter and preserve lateral associations after mercerization, known as an average of  
 488 trimer associations. However, the thickness was half as much after mercerization,  
 489 whereas we confirmed by several technics a decrease by half in our experiment.

490

491 On the basis of our results, we can determine the average amount of chains per  
 492 elementary crystal in several ways.

493 Using the number-average molar mass ( $M_n$ ) given by A4F-MALLS-RI, and dividing  
 494 these values by 3, we obtain an average molar mass of  $8 \cdot 10^6$  g/mol for the elementary  
 495 CNC-I, and  $2.4 \cdot 10^6$  g/mol for the elementary CNC-II (Table 6). These results, together  
 496 with those obtained by SEC/MALLS, make it possible to determine the number of  
 497 cellulosic chains in an elementary crystal: 235 chains per elementary CNC-I and 133  
 498 chains per elementary CNC-II. This is large compared to theoretical calculations based on  
 499 crystal dimensions.

500

501 Another calculation considers the CNC section obtained from microscopy and the  
 502 interchain dimensions. The average CNC thickness is 6.5 nm and 3.5 nm for CNC-I and  
 503 CNC-II, respectively. The (1-10) and (110) interplane dimensions in CNC-I are 0.61 nm  
 504 and 0.54 nm, respectively (Goussé et al. 2002; Sugiyama, Vuong, and Chanzy 1991).  
 505 Similarly, interplane dimensions for CNC-II are 0.72 nm and 0.44 nm for (1-10) and (110),  
 506 respectively (Kolpak, Weih, and Blackwell 1978; P. Langan, Nishiyama, and Chanzy 1999;  
 Sèbe et al. 2012). Considering that CNC is completely crystalline, this leads to

507  $7 \times 6.5 / 0.61 \times 0.54 = 162$  cellulose chains per elementary CNC-I and  $7 \times 3.5 / 0.72 \times 0.44 = 77$   
 508 cellulose chains for CNC-II.

509 Calculating average crystalline dimensions from XRD analysis (see Fig. 1), we obtain  
 510  $4.3 \times 6.2 / 0.61 \times 0.54 = 80$  cellulose chains per elementary CNC-I and  $2.9 \times 5.5 / 0.72 \times 0.44 = 50$   
 511 cellulose chains for CNC-II.

512 Considering these different results, the microscopy should overestimate the crystal  
 513 dimension; overestimating also the number of chains per elementary crystals. On the  
 514 other hand XRD results taking into account the effective crystalline part may not take  
 515 into account defaults and surface effects underestimating the number of chains per  
 516 elementary crystals. The effective value should then be somewhere in between that we  
 517 can estimate at 120 chains/elementary crystals for CNC-I, and 60 chains/elementary  
 518 crystals for CNC-II.

519 We couldn't find values to compare such results with other works, regardless of the  
 520 calculation method, about half of the former number of chains per elementary  
 521 nanocrystal is recovered after mercerization. The chains are presumably mixed in the  
 522 global fiber by interdigitation and during crystallization rearrangement on shorter  
 523 distances with smaller crystals packing less chain. However, they seem more  
 524 homogeneously distributed along the fiber.

525 This may indicate that all the chains of the fibril are redistributed during  
 526 mercerization, forming a globally more regular fiber, but composed of smaller, more  
 527 discontinuous and bi-oriented crystallites.

528  
 529

530 **Table 6.** Number-average molar mass ( $\bar{M}_n$ ) of CNCs, elementary nanocrystals and individual  
 531 chains, and the number of chains per individual CNC.

CNCs	$\bar{M}_n$ of CNCs (g/mol)	$\bar{M}_n$ of elementary nanocrystals (g/mol)	$\bar{M}_n$ of individual chains (g/mol)	Number of chains/elementary crystals (from $\bar{M}_n$ )	Number of chains/elementary crystals (from microscopy)	Number of chains/elementary crystals (from XRD)
CNC-I	$24 \pm 1 \cdot 10^6$	$8 \cdot 10^6$	$34,000 \pm 1,000$	235	162	82
CNC-II	$7 \pm 1 \cdot 10^6$	$2.4 \cdot 10^6$	$18,000 \pm 1,000$	133	77	50

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## 536 5. Conclusions

537 Using identical acid hydrolysis on native and mercerized NFs, a panel of techniques  
 538 is used to show that the mercerization treatment does not degrade cellulosic chains  
 539 (Mw of 560,000 g/mol) but instead limits the resistance to acid (yield of 64% and 40%  
 540 for CNC-I and CNC-II, respectively) and impacts the resulting CNCs. The thickness and  
 541 length of nanocrystals are reduced, preserving the lateral average association  
 542 corresponding to a trimer (three elementary nanocrystals), and resulting in molar  
 543 masses of 40,000 g/mol and 11,000 g/mol for CNC-I and CNC-II, respectively. By probing  
 544 the internal structure, we were able to show more intermediary structures between

545 ordered and amorphous domains. In addition, the two distinct (accessible/inaccessible)  
546 amorphous domains that are detected in cellulose I are not detected in mercerized  
547 form, even before acid hydrolysis. This occurs with unchanged surface charge density  
548 but a reduction of the crystal thickness by half. Finally, mercerization has a major impact  
549 on crystal organization with a much lower chain packing per nanocrystal. Compared to  
550 previous works, this article includes additional values notably on molar masses and  
551 proposed various comparative techniques. We hope this analysis can further help  
552 researchers to characterize their own samples.  
553

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## 685 **Figure captions:**

686 **Figure 1.** X-ray diffraction patterns of cotton fibers in native (CF-I) and mercerized CF-II forms and  
687 their respective hydrolyzed cellulose nanocrystals in the native (CNC-I) and mercerized (CNC-II)  
688 forms, and cross-sections of elementary crystallites deduced from the analysis of peak  
689 broadening (the indexation of corresponding lattice planes is described in Supporting  
690 Information).

691 **Figure 2.** (A) <sup>13</sup>C CP-MAS NMR spectra of CF-I and CF-II, and ( B) deconvolution of the C4 region of  
692 CF-I, CF-II, CNC-I and CNC-II NMR spectra with crystalline forms (black), paracrystalline (gray) and  
693 amorphous (green).

694 **Figure 3.** Dissolution profiles of samples obtained by SEC-MALLS-DRI. The two nanofibers (CF-I in  
695 purple and CF-II in red) are eluted at low retention volumes, whereas the nanocrystals are eluted  
696 at higher elution volumes (CNC-I in green and CNC-II in black).

697 **Figure 4.** Distribution of molar masses of suspensions of CNC-I (blue) and CNC-II (red) in water,  
698 and RI signal (dotted curves).

699 **Figure 5.** TEM images of CNC-I (a,b) and CNC-II (c,d) and AFM images of CNC-I (e) and CNC-II (f)

700 **Figure 6.**  $I = f(Q)$  SANS curves of suspensions of CNC-I and CNC-II in water at 2 g/L in NaCl 2 mM

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## 703 **Table Captions:**

704 **Table 1:** Weight fraction (yield) recovered after treatment; crystallinity index (CI) calculated from  
705 XRD (CIXRD); mean CI calculated from solid-state NMR (<sup>13</sup>C CP-MAS) spectra (CINMR); and  
706 deconvolution of the C4 region of <sup>13</sup>C CP-MAS spectra.

707 **Table 2.** Weight-average molar masses ( $\bar{M}_w$ ); polydispersity ( $\bar{M}_w/\bar{M}_n$ ); and degree of  
708 polymerization (DP) of individual chains of cellulosic fibers (CF-I and CF-II) and cellulose  
709 nanocrystals (CNC-I and CNC-II) solubilized in DMAc/0.9% LiCl.

710 **Table 3.** Weight-average molar masses ( $M_w$ ) and polydispersity ( $M_w/M_n$ ) of CNC-I and CNC-II  
711 dispersed in water determined by A4F-MALLS-DRI; and average dimensions determined from the  
712 SANS curve, TEM images and AFM images.

713 **Table 4.** Sulfur content (S), surface charge density (SC) and zeta potential of CNC-I and CNC-II.

714 **Table 5.** Number-average molar mass ( $\bar{M}_n$ ) of CNCs, elementary nanocrystals and individual  
715 chains, and the number of chains per individual CNC

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