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1 **Adsorption versus Grafting of Poly(N-** 2 **Isopropylacrylamide) in aqueous conditions on the** 3 **Surface of Cellulose Nanocrystals**

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13 **ABSTRACT**

14 This study proposes a grafting strategy of thermo-sensitive amine-terminated oligomers of
15 Poly(N-**Isopropylacrylamide**) (Pnipam₂₅₀₀) onto the surface of Cellulose Nanocrystals (CNCs).
16 Pnipam₂₅₀₀ grafting in aqueous condition via peptidic coupling was explored to obtain CNC
17 hydrogel with thermo-reversible aggregation and new colloidal properties. A discussion
18 between grafting vs adsorption /presence of the Pnipam₂₅₀₀ is proposed. A large range of
19 experimental techniques was used to investigate the properties of the CNC decorated with
20 polymer and to confirm the grafting. Elemental analysis, infrared spectroscopy, solid state
21 NMR and conductometric titration of washed CNC-g-Pnipam₂₅₀₀ demonstrate that at least a
22 part of Pnipam₂₅₀₀ was covalently bonded with CNC. A thermo-reversible aggregation was
23 observed by Dynamic Light Scattering experiments and thermo-sensitive behavior is
24 observed by rheological experiments. **For grafted polymer the viscosity increases from 0.008**
25 **to 40 Pa·s at low shear rate when the LCST is reached, whereas, in the case of polymer**
26 **adsorption, the viscosity increases only from 0.002 to 0.3 Pa·s.** This thermo-reversible, bio-
27 based and biocompatible system paves the way for the design of injectable hydrogel and
28 biomedical nanocomposite materials.

29 **Keywords:** Cellulose nanocrystals; Poly(N-**Isopropylacrylamide**); surface functionalization;
30 thermo-sensitive

31 **1. Introduction**

32 The increasing interest to use biodegradable, renewable, non-toxic and sustainable material
33 has led to the increasing use of nanoscale cellulose in advanced materials. Among such
34 nanoscale cellulose, cellulose nanocrystals (CNCs) are obtained by acid hydrolysis and are
35 rigid rod-shaped particles with dimensions ranging from 3 to 30 nm in cross-section and
36 from 100 to 500 nm in length. Known since the 50's (Rånby, 1951), they exhibit several
37 interesting properties including excellent mechanical strength, high aspect ratio, low density
38 and an ability to self-organize into liquid crystalline phases (Habibi, 2014; Klemm et al., 2011;
39 Moon et al., 2011; Revol et al., 1992). Besides, this treatment also introduces sulfate ester
40 groups on the surface of CNC which induces a colloidal stability in aqueous media. Owing to
41 their specific physical properties and high aspect ratio (depending on the source), CNC are
42 used in several domains (Oksman et al., 2014) like environmentally friendly nanocomposites
43 (Dufresne, 2013; Eichhorn et al., 2010; Siqueira et al., 2010a), biomedical applications
44 (Camarero Espinosa et al., 2016; Domingues et al., 2014; Endes et al., 2016; Jorfi and Foster,
45 2015; Klemm et al., 2011; Lin and Dufresne, 2014; Naseri et al., 2016), coatings and printed
46 electronics (Gicquel et al., 2017; Hoeng et al., 2016; Li et al., 2013), hydrogels and aerogels
47 (De France et al., 2016, 2017; Eichhorn et al., 2010). Furthermore, CNCs are not only
48 produced at laboratory scale but are now industrially available in high quantities (Chauve
49 and Bras, 2014).

50 New properties can even be implemented to CNCs by modifying the numerous hydroxyl
51 groups at their surface and several reviews focus on the chemical surface modification of
52 CNC with molecule or polymers (Eyley and Thielemans, 2014; Habibi, 2014; Tang et al.,
53 2017). Two main approaches can be used to covalently attach polymer chains on the surface
54 of CNCs, the "grafting-from" or the "grafting-to" methods. The "grafting-from" allows
55 highest grafting polymers length chains on CNCs surfaces and highest grafted polymer
56 diversity. For the grafting from approach, the surface is first functionalized with an initiator
57 monolayer. Then, the polymerization is carried out on the surface-initiated, resulting on the
58 growth of the polymer brush directly from the surface (Alosmanov et al., 2017; Dong and
59 Roman, 2007; Follain et al., 2010; Kan et al., 2013; Wu et al., 2015; Zoppe et al., 2010).
60 However, the "grafting-from" method induces the presence of free homopolymers and
61 generally uses toxic solvents or reagents.

62 In the “grafting-onto” strategy, a greener process can be tested. Presynthesized and well
63 characterized polymer chains are reacted with cellulose hydroxyl groups or surface
64 carboxylate groups generated by TEMPO oxidation. Nonetheless, steric hindrance can
65 prevent optimal grafting and limit the grafts surface density. As examples of the “grafting-
66 onto” process, Araki et al. and Kloser et al. respectively grafted epoxy-terminated
67 polyethylene glycol (PEG) and polyethylene oxide (PEO) on CNC to create sterically stabilized
68 system (Araki et al., 2001; Kloser and Gray, 2010; Mangalam et al., 2009).

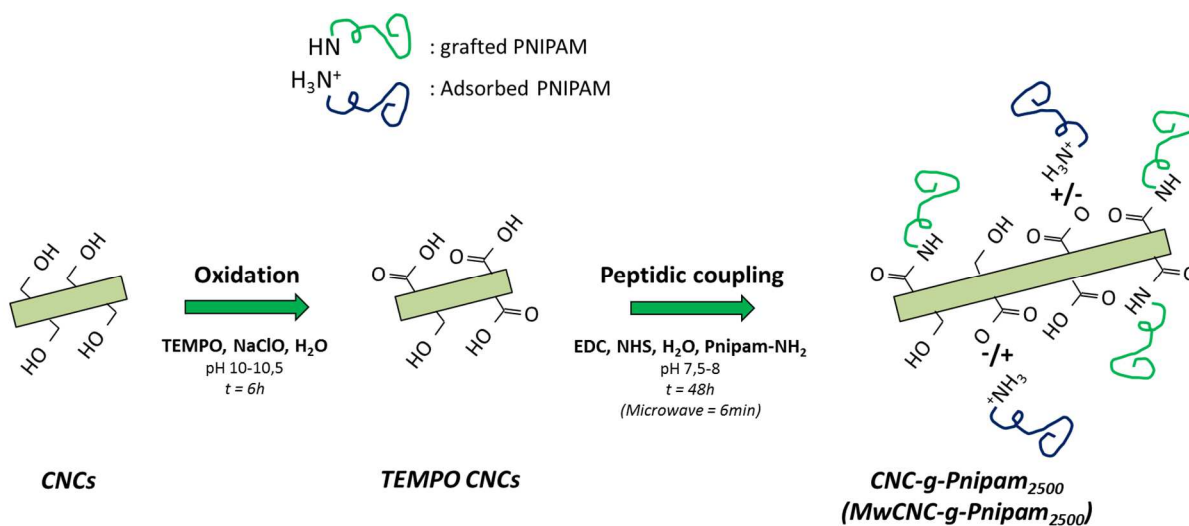
69 In this study, the “grafting-onto” strategy is used. The aim is to create a covalent amide bond
70 between a primary amine-terminated thermo-sensitive polymer and carboxylated CNC as
71 shown in **Figure 1**. In the literature, this approach has been successfully applied by coauthors
72 to the grafting of copolymers of ethylene oxide and propylene oxide , referred to as
73 Jeffamine polyetheramines (Huntsman Corporation) on TEMPO oxidized CNC (Azzam et al.,
74 2010, 2016). First, CNC are oxidized and then reacted with amine-terminated polymers using
75 a carbodiimide as amidation agent. Such reactions can be performed in an organic solvent or
76 in aqueous media (Azzam et al., 2010, 2016) but requires long reaction times and washing
77 steps. As reported in the literature, the realization of stimuli-sensitive CNC complex
78 represents an interesting way to design smart hydrogel and materials, in particular in the
79 field biomedical applications (Jorfi and Foster, 2015; Naseri et al., 2016).

80 In this work, we have focused on the peptidic grafting of one of the most common thermo-
81 sensitive polymer Poly(N-**isopropylacrylamide**) also called PNIPAM. This polymer exhibits a
82 lower critical solution temperature (LCST) at 32°C. Below the LCST, the polymer chains are in
83 good solvent conditions and adopt a swollen coil conformation with highly hydrated chains.
84 Above the LCST, the polymer becomes hydrophobic and turns into a collapsed globule (Lai et
85 al., 2013; Pelton, 2010; Plunkett et al., 2006; Schild, 1992). PNIPAM shows a LCST close to
86 physiological temperature whatever almost irrespective of its molecular weight (Okahata et
87 al., 1986), and has a huge potential in biomedical applications (Alosmanov et al., 2017; Chen
88 et al., 2014; Hebeish et al., 2014). **Its bio-compatibility and non-toxicity were discussed in a
89 recent review by Lanzalaco et al. (Lanzalaco and Armelin, 2017) and detailed in vivo
90 experiments with cartilage formation (Cho et al., 2004), ocular integration (Cao et al., 2007;
91 Lima et al., 2016), subcutaneous tissues (Ohya et al., 2004), cell sheets (Lin et al., 2012) or
92 drug release (Das et al., 2015).**

93 In the literature, the “grafting-from” method has mainly been used to graft this thermo-
 94 sensitive chains (Alosmanov et al., 2017; Hemraz et al., 2014; Zoppe et al., 2010, 2011; Zubik
 95 et al., 2017). Zoppe and co-workers (Zoppe et al., 2010, 2011) grafted PNIPAM brushes from
 96 CNC via SET-LRP, in which they control exactly the density and the length of brushes
 97 depending on the ratio of initiator and molecular ratio of NiPAAm monomer. However, such
 98 strategy needs toxic solvent and to the best of our knowledge, no previous works propose
 99 “grafting-onto” of PNIPAM on CNCs.

100 Meanwhile, more and more studies **prove** the possibility of high adsorption of poly-
 101 electrolyte or oligomers on the surface of CNC using various techniques like QCM or SPR
 102 (Reid et al, 2017; Bensefelt et al, 2016). In some case, it seems even possible to have
 103 irreversible adsorption and no desorption is observed in spite of multiple washing steps.

104 In this paper, we compare the surface functionalization of CNCs with amine-terminated
 105 thermosensitive PNIPAM chains using either simple adsorption or peptidic coupling. The new
 106 systems are then characterized by conductometric titration, infrared spectroscopy, solid
 107 state NMR and elemental analysis. Dynamic light scattering (DLS) and transmission electron
 108 microscopy (TEM) are used to describe the structural modification of the nanoparticles.



109

110 **Figure 1:** Schematic representation of chemical modification on CNCs presents in this work

111 2. Materials and Methods

112 2.1. Materials

113 Colloidal suspension of ~12 wt.% Commercial Cellulose Nanocrystals (CNCs) was purchased
114 from UMaine process Development Center (University of Maine, USA). They have been
115 produced from wood pulp using sulfuric acid hydrolysis process and have been never freeze-
116 dried. The dry matter was measured using a moisture analyzer (Ohaus® MB-35, Sigma-
117 Aldrich, USA). Poly(N-Isopropylacrylamide) with amine terminated was purchased from
118 Sigma-Aldrich (USA). The molecular weight is about 2500 g·mol⁻¹, and the LCST is close to
119 32°C. For the TEMPO oxidation, following chemicals are purchased from Sigma-Aldrich:
120 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO, 2564-83-2), Sodium hypochlorite (NaClO, 12
121 wt.%, 7681-52-9) and Sodium bromide (NaBr, 7647-15-6). For the peptidic grafting,
122 chemicals are purchased from Sigma-Aldrich: N-(3-Dimethylaminopropyl)-N'-
123 ethylcarbodiimide hydrochloride (EDC, 25952-53-8) and N-Hydroxysuccinimide (NHS, 6066-
124 82-6). Other chemicals were purchased from Sigma-Aldrich. Distilled water was used for all
125 experiments.

126 2.2. Carboxylation of cellulose nanocrystals by TEMPO oxidation

127 CNCs were subjected to TEMPO-mediation oxidation using a previously reported procedure
128 (Habibi et al., 2006). 11 g of CNC were dispersed in 730mL of distilled water. Then, this
129 suspension was exposed to an ultrasonic dispersive energy of 5 kJ per gram of dry CNC using
130 a 250 Watt sonication probe (Sonifier S-250A, Branson, USA) at 50% of maximum energy
131 during 15 minutes in this case. To prevent burning of CNC during the dispersion, the
132 suspension was placed in a crystallizer full of ice. TEMPO (323 mg, 2.07 mmol) and NaBr
133 (3.564 mg, 34.61 mmol) were dissolved in 250mL of deionized water by magnetic stirring
134 and slowly added to the CNC suspension. Then, 66 g (0.12M) of NaClO suspension was added
135 dropwise to the suspension to start the oxidation. The mixture was stirred for 3 h at room
136 temperature. The pH condition of the suspension was maintained between 10 and 10.5 by
137 addition of sodium hydroxide solution (NaOH) at 0.5M during the reaction. Reaction was
138 then quenched by the addition of ethanol (40 ml), the suspension color turned from yellow
139 to white. The resulting CNC were washed with hydrochloric acid (HCl) at 0.5M, to decrease
140 the pH to 1-2, at least three times by centrifugation (10 000 rpm, 30 min). After the last

141 centrifugation, the oxidized CNC were re-dispersed in distilled water using the minimum
142 volume to recover all CNC. This suspension was dialyzed against distilled water at least one
143 week until a neutral pH was obtained (membrane 6-8 kDa). TEMPO CNCs were stored in the
144 fridge at constant neutral pH to allow the CNC to be in their carboxylate form.

145 **2.3. Polymer grafting by peptidic coupling in water**

146 Grafting of amine-terminated PNIPAM₂₅₀₀ on TEMPO CNCs was performed by peptidic
147 coupling in water according to protocol of Bulpitt and Aeschlimann (Bulpitt and
148 Aeschlimann, 1999). The reaction was performed at ambient temperature, and started with
149 a ~1 wt.% TEMPO CNCs suspension, N_{COOH} is the mole of carboxyl unit measured by
150 conductometry. This suspension was exposed to an ultrasonic dispersive energy using a
151 250 Watt sonication probe (Sonifier S-250A, Branson, USA) at 50% of maximum energy
152 during 80 seconds per dry grams of CNC to remove any aggregates. The pH was adjusted to
153 4.5 (CNC-COOH configuration) with 0.5M HCl. Then, 2mL of EDC solution was added to the
154 suspension (N_{EDC} : mol per carboxyl group) and stirred during 30 min. Then, 2mL of NHS
155 solution was added (N_{NHS} : mol per carboxyl group) and stirred during 30 min. The pH was
156 then adjusted to 7.5-8.0 before the addition of 10mL of an aqueous suspension containing
157 PNIPAM-NH₂ (N_P : mol per carboxyl group). In accordance with the protocol of Azzam et al.
158 (Azzam et al., 2010), all results were obtained with $N_{EDC} = N_{NHS} = N_P = 4 \times N_{COOH}$. The reaction
159 lasted 48 h at room temperature under stirring while maintaining the pH at 7.5-8.0 by
160 addition of 0.5M NaOH or 0.5M HCl. The reaction was quenched by the decreasing of the pH
161 at 1-2 by addition of 0.5M HCl and the resulting suspension was dialyzed against distilled
162 water to remove excess of reagents at least seven days with exchange of water regularly
163 until neutral pH is obtained. PNIPAM₂₅₀₀ grafted CNC will be referred to as CNC-g-Pnipam₂₅₀₀.

164 **2.4. Conductometric and pH-metric titrations: Carboxyl content**

165 The carboxyl content of oxidized CNC and grafted CNC were determined by conductometric
166 titration coupled with pH-metric titration. About 15 mg of CNC was suspended in 200 mL of
167 distilled water and treated by ultrasonic bath for 5min to remove gas and increase the
168 dispersion. The pH of the suspension is adjusted to acidic condition (pH = 3) with 0.1 M HCl
169 to replace the sodium counter-ions by protons. The suspension was then titrated with
170 0.2 mL increment of 0.01 M NaOH. The titration curves exhibit two turns: the first one

171 corresponds to the NaOH neutralization of the strong acid due to the excess of HCl, and then
172 follows by the neutralization of the weak acid related to the carboxyl content.

173 Degree of Oxidation (DO) of oxidized CNC is the number of primary hydroxyl groups that
174 have been oxidized into carboxyl groups per AnhydroGlucose Unit (AGU). It was calculated
175 according to the following **Equation 1** from Da Silva Perez et al. (Da Silva Perez et al., 2003):

$$176 \quad DO = \frac{162 \times C \times (V_{eq2} - V_{eq1})}{m - 36 \times C \times (V_{eq2} - V_{eq1})} \quad (1)$$

177 Where 162 (g·mol⁻¹) corresponds to the molar mass of an AGU, C (mol·L⁻¹) is the exact
178 concentration of the NaOH solution, m (g) is the weight of the oven-dried sample, 36 in
179 g·mol⁻¹ corresponds to the difference between the molecular weight of an AGU (162 g·mol⁻¹)
180 and that of the sodium salt of a glucuronic acid moiety (198 g·mol⁻¹), and V_{eq1} and V_{eq2} are
181 the equivalent volumes of NaOH on the bends during the titration. To minimize errors,
182 titrations were reproduced at least three times and an average value was used for the
183 discussion.

184 **Equation 2** gives the calculation of the oxidation charge concentration (X_{ox}), in $\mu\text{mol}\cdot\text{g}^{-1}$:

$$185 \quad X_{ox} = \frac{C \times (V_{eq2} - V_{eq1})}{m} \quad (2)$$

186 Where C (mol·L⁻¹), m (g), V_{eq1} and V_{eq2} are the same as previously.

187 After grafting by peptidic coupling, a part of the carboxyl groups is consumed in the reaction
188 with the amine-terminated polymer. A new oxidation rate X_{res} (in $\mu\text{mol}\cdot\text{g}^{-1}$), corresponded to
189 the residual carboxyl content, was calculated after conductometric titration as given by the
190 following **Equation 3**:

$$191 \quad X_{res} = \frac{C \times (V_{eq4} - V_{eq3})}{w} \quad (3)$$

192 Where C (mol·L⁻¹) is the exact concentration of the NaOH solution, w (g) is the pure dry CNC
193 weight in total samples weight of the oven-dried sample and V_{eq3} and V_{eq4} are the equivalent
194 volumes of NaOH on the post-grafting titration.

195 Finally, the percentage of carboxyl groups which has been substituted ($\%COOH_{\text{grafted}}$) was
196 calculated according to the equation **Equation 4**, and represents the peptidic coupling yield:

$$197 \quad \%COOH_{\text{grafted}} = \frac{X_{ox} - X_{res}}{X_{ox}} \times 100 \quad (4)$$

198 **2.5. Fourier transform infra-red spectroscopy (FTIR)**

199 Infrared spectra of oxidized CNC and grafted CNC were performed using a Perkin-Elmer
200 spectrum 65 (PerkinElmer, USA). Prior to avoid the superposition of the carbonyl band with
201 the one of the water, CNC suspensions were acidified to pH 3. Samples were dried during 2 h
202 in an oven at 80°C and reduced in powder form prior to analysis. KBr pellets containing 1
203 wt.% solid cellulose sample were prepared. At least spectra of two samples (to check the
204 reproducibility) per type of CNC were recorded in transmission mode with 16 scans in the
205 400-4000 cm^{-1} wavenumber range.

206 **2.6. Elemental analysis (EA)**

207 Elemental analysis was carried out by the Analysis science institute of the “centre national
208 de la recherche scientifique”, Lyon, France. The Carbon, Hydrogen, Oxygen, Sulfur and
209 Nitrogen contents for CNC, TEMPO CNC, CNC-g-Pnipam₂₅₀₀ and MwCNC-g-Pnipam₂₅₀₀ were
210 measured independently. The obtained results from elemental analysis were used to
211 determine the Degree of Oxidation after TEMPO oxidation (DO_{EA}) (**Equation 5**):

$$212 \quad DO_{EA} = \frac{72.07 - \%C1 \times 162.14}{\%C1 \times 35.93} \quad (5)$$

213 Where %C1 corresponds to the relative carbon content in oxidized sample and 72.07,
214 162.14, 35.93 are respectively the molecular weight of carbon in AGU, molecular weight of
215 an AGU, the difference between the molecular weight of an AGU and an oxidized AGU with
216 sodium salt.

217 **2.7. Solid-State NMR**

218 NMR experiments were performed with a Bruker Avance DSX 400 MHz spectrometer
219 operating at 100.6 MHz for ^{13}C , using the combination of cross-polarization, high-power
220 proton decoupling and magic angle spinning (CP/MAS) methods. The spinning speed was set
221 at 12 kHz. The ^1H radio frequency field strength was set to give a 90° pulse duration at 2.5 μs .

222 The ^{13}C radio frequency field strength was obtained by matching the Hartman-Hahn
223 conditions at 60 kHz. Recording at least 2000 transients with contact time and recycle delay,
224 respectively, of 2 ms and 2 s represented standard conditions. The acquisition time was set
225 at 30 ms and the sweep width at 29400 Hz. The position and width of the lines were
226 maintained constant throughout a series of samples. The area corresponding to the
227 integration of the C_1 signal was used as an internal standard and set to one.

228 **2.8. Transmission Electron Microscopy (TEM)**

229 Drops of about 0.001 wt.% of CNC suspension were deposited onto glow-discharged carbon-
230 coated TEM grids. After 2 minutes, the liquid in excess was absorbed with filter paper, and
231 prior to drying, a drop of Urany-Less (Delta Microscopies, France) was deposited on the
232 specimen. Then, after 2 minutes, the solution in excess was adsorbed and the grid was dried
233 under room temperature. The sampling was observed using a Philips CM200 (FEI, USA)
234 operating at 200 kV.

235 This protocol was adapted to account for the LCST of the grafted thermo-sensitive polymer
236 ($\sim 32^\circ\text{C}$). In a first case, TEM specimens were prepared at room temperature. In a second
237 case, TEM specimens were prepared with CNC-g-Pnipam₂₅₀₀ suspension and Urany-Less
238 solution both preheated at 50°C .

239 **2.9. Dynamic light scattering (DLS)**

240 DLS measurements were performed on Malvern Nano ZS instrument (Malvern instruments,
241 United Kingdom). Samples were diluted in deionized water at 10^{-2} wt.% and the conductivity
242 was adjusted to $500 \mu\text{S}\cdot\text{cm}^{-1}$ by addition of NaCl solution. All measurements were made at a
243 well-controlled ($\pm 0.05^\circ\text{C}$) temperature at a backscattering detection angle of 173° . Non-
244 negative least squares analysis (NNLS) was performed to achieve the intensity size
245 distribution (corresponding to hydrodynamic diameter z^*) from the analysis of the
246 correlation function on the Malvern DTS software. For each samples, the final data in this
247 paper represent an average of at least 3 acquisitions with 10 measurements.

248 **2.10. Atomic Forces Microscopy (AFM)**

249 CNCs were imaged using AFM (Dimension icon[®], Bruker, USA). All suspensions were
250 previously diluted to 10^{-4} wt.% and a drop of 0.2 mL was deposited onto a freshly cleaved

251 mica plate. Samples were dried overnight at room conditions. The samples were
252 characterized in tapping mode using a silica coated cantilever (OTESPA® 300 kHz - 42 N/m,
253 Bruker, USA). Scans of $10 \times 10 \mu\text{m}^2$ and $3.3 \times 3.3 \mu\text{m}^2$ were performed to analyze the
254 dimensions of CNC. At least 4 images per sample were obtained. In order to extract the
255 length and height dimensions, measurements of about 200 particles were performed by
256 using the ImageJ software to obtain a representative average size.

257

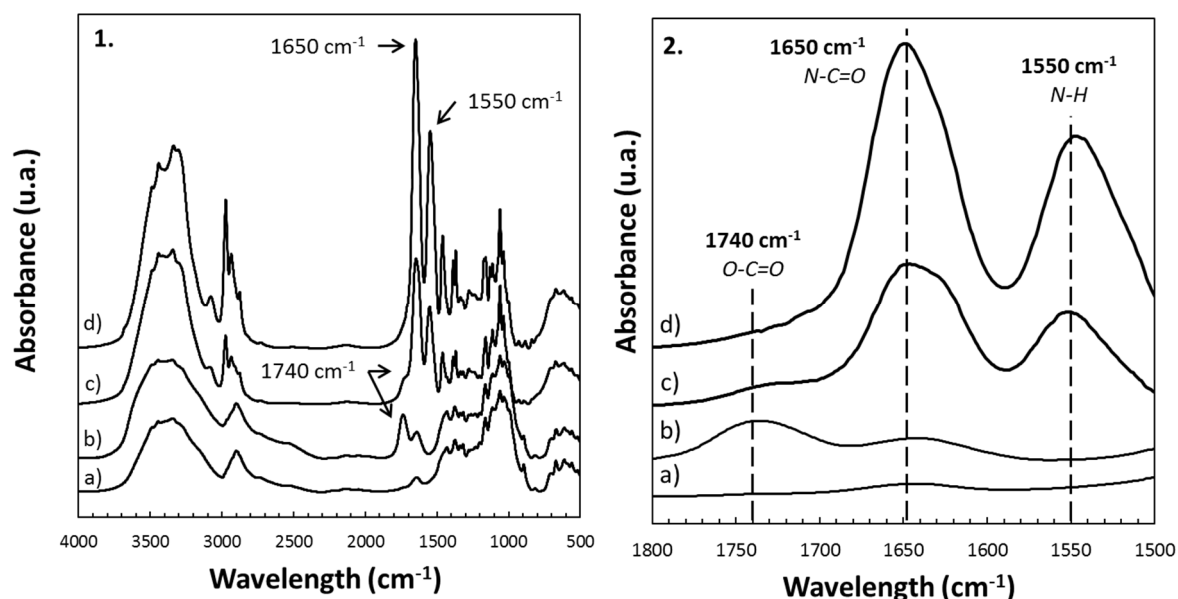
258 3. Results and Discussion

259 3.1. Grafting of amine terminated Pnipam₂₅₀₀ onto TEMPO CNCs

260 Aqueous commercial CNC suspensions were previously carboxylated using TEMPO oxidation
261 process. By conductometric titration (**Figure S1a – Support information**) and **Equation 1**, a
262 degree of surface oxidation DO of 0.25 (mol/mol of anhydroglucose unit) and an oxidation
263 rate X_{ox} of 1450 ($\mu\text{mol}\cdot\text{g}^{-1}$) were obtained.

264 Aqueous TEMPO CNCs were then grafted with amine terminated Pnipam₂₅₀₀ via peptidic
265 coupling. **Figure S1b (Support information)** reports conductometric titration. After
266 calculation with **Equation 2** and **Equation 3**, the residual carboxyl content (X_{res}) is 1010
267 ($\mu\text{mol}\cdot\text{g}^{-1}$) and the percentage of carboxyl groups which has been substituted by amine
268 groups ($\%COOH_{\text{grafted}}$) is 30 %.

269 Nevertheless, these results are indirect proof of the grafting and would be similar in case of
270 adsorption. FTIR spectroscopy and Elemental Analysis were performed to confirm the
271 presence of the polymer chains onto the CNC. **Figure 2** shows FTIR spectra of (**Figure 2a**)
272 regular CNC, (**Figure 2b**) TEMPO CNCs, (**Figure 2c**) TEMPO CNCs mixed with Pnipam₂₅₀₀ and
273 (**Figure 2d**) CNC-g-Pnipam₂₅₀₀. All samples have been extensively washed before any
274 characterization using dialysis.



275

276 **Figure 2:** 1. Infrared spectra of a) sulfated commercial CNCs, b) TEMPO CNCs c) TEMPO CNCs mixed
277 with Pnipam₂₅₀₀ and d) Pnipam₂₅₀₀ grafted onto CNCs: CNC-g-Pnipam₂₅₀₀. Each spectrum is normalized
278 at 1110 cm⁻¹. 2. Infrared Spectra zoom on characteristic peaks.

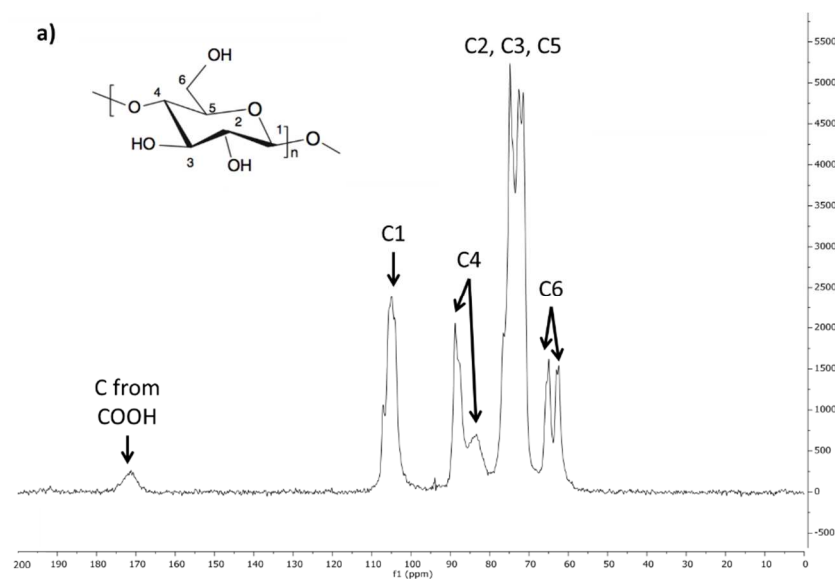
279 Clear changes occur in each steps of the chemical modification. By comparing to the
280 commercial CNCs, the spectra of the TEMPO CNCs exhibits a high absorption band at
281 1740 cm⁻¹. This peak is characteristic to the acidic form of the carboxylic acid function
282 (COOH), which validated the oxidation of CNCs. In the case of a peptidic grafting, peaks
283 characteristic of the amide bonding appear in two absorption bands. Unfortunately, in our
284 case such amide bonds are also present in the polymer and cannot prove the grafting but
285 mainly the presence or not of the polymer. The first band at 1650 cm⁻¹ can be attributed to
286 the amide bond (amide I absorption band) but also to the residual water adsorbs on the
287 cellulose (or polymer). Furthermore, at 1550 cm⁻¹, another absorption peak characteristic of
288 the N-H bond exists (called amide II band). On the **Figure 2c**, referring to Pnipam₂₅₀₀ only
289 mixed with TEMPO CNCs without coupling agent and following similar washing steps, the
290 two characteristic peaks of amine are present (1650 and 1550 cm⁻¹). They prove the
291 presence of Pnipam₂₅₀₀ in the suspension of CNCs even without grafting and after the
292 washing steps. This result was surprising and we have first checked the efficiency of our
293 washing procedure. By dialysis of pure Pnipam₂₅₀₀ during 7 days, we have checked that most
294 of the polymer (ab. 80-85%) pass through the membrane and is eliminated during the
295 dialysis. The 15-20% still present seems to be non-solubilized / aggregated polymer or
296 impurities nanoparticles (like catalyst) present in this commercial grades, as proved by some
297 DLS measurement showing some aggregates or impurities of about 200nm dimensions. So as
298 most of the Pnipam₂₅₀₀ is eliminated, the **Figure 2c** confirms the presence of oligomer in the
299 suspension. We can suppose this due to the aggregate and to the physically adsorbed
300 oligomer onto the CNC surface. This adsorption might be due to the hydrogen bonds and has
301 been investigated more in details in recent study ([Gicquel, 2017](#)).

302 However, the presence of a small peak at 1740 cm⁻¹ (e.g. COOH characteristic pic) indicates
303 that still some carboxylic ends are available after adsorption and washing. In the case of the
304 grafted sample (**Figure 2d**), the two characteristic peaks of the amine bond are present
305 (1650 and 1550 cm⁻¹). Even if it is not quantitative proof, the intensities of these peaks are
306 largely superior to those one of Pnipam₂₅₀₀ adsorbed on CNCs. Besides, as previously said
307 PNIPAM-NH₂ contains N-H bonds in the repeating NIPAM monomer. The molecular weight

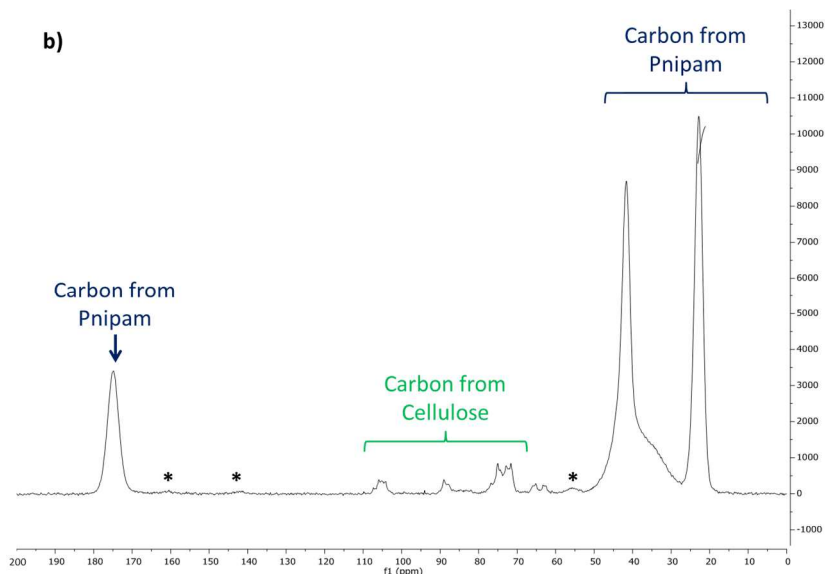
308 $\sim 2500 \text{ g}\cdot\text{mol}^{-1}$ corresponds to ~ 22 time of monomer (~ 22 amine groups) and one amine on
309 the termination. So, with a superior intensity compared to **Figure 2c**, more Pnipam₂₅₀₀ are
310 present on the CNCs' surface which might be due to a different polymer configuration on the
311 surface. In this case, the grafting efficiency is also revealed thanks to the disappearance of
312 the 1740 cm^{-1} peak which probably reveals the consumption of CNC's carboxyl groups in the
313 reaction with the amine-terminated polymers. This disappearance might be due also to
314 overlapping with peak at 1650 cm^{-1} . Probably one part of Pnipam₂₅₀₀ is adsorbed and one
315 part is grafted in this case but it is difficult to separate the amount of grafted and adsorbed
316 polymer on CNCs.

317 So this FTIR analyses proves the presence of Pnipam₂₅₀₀ into oxidized CNCs suspension. Such
318 oligomer **stays** in the suspension in spite of washing steps but also that when grafting is
319 performed a higher quantity seems present and all carboxylic group disappears.

320



321



322

323 **Figure 3:** ^{13}C Solid-state NMR spectra of A. TEMPO CNCs and B. CNC-g-Pnipam₂₅₀₀. Stars (*) correspond
 324 to the spinning side band of Pnipam₂₅₀₀ at 10 kHz (results not shown). *Insert figure shows chemical*
 325 *structure related to C₁-C₆ of cellulose.*

326 **Figure 3** shows the ^{13}C solid-state NMR of TEMPO CNCs and CNC-g-Pnipam₂₅₀₀. In the case of
 327 TEMPO CNCs (**Figure 3a**), carbon contribution of the cellulose were clearly identified (Atalla
 328 and VanderHart, 1999; Montanari et al., 2005). The region between 60 and 70 ppm
 329 correspond to the C₆ carbon of the AGU, and between 70 and 80 ppm to the carbon
 330 contribution of the C₂, C₃ and C₅. C₄ contribution is the region between 83 and 88 ppm. The
 331 C₁ carbon appears at 105 ppm. The area corresponding to the integration of the C₁ signal
 332 was used as an internal standard and set to one. The contribution of the carboxyl group is
 333 located at 174 ppm (Montanari et al., 2005). The **Figure 3b** represents the NMR spectra after
 334 the grafting of Pnipam₂₅₀₀ on CNCs (after dialysis washing steps). On this spectrum, the
 335 contribution of the carbon from the polymer is very high related to the internal standard on
 336 C₁ (from 20 to 36 times the integration of the C₁ signal). The peptidic grafting creates a new
 337 link between carbon and nitrogen (i.e. a new amide). As already said, this link is also present
 338 inside the NIPAM monomer of the polymer chain, so it is difficult to separate the
 339 contribution of the carbon amide occurring after the grafting from the carbon amide in
 340 polymer contribution. The disappearance of the peak at 174 ppm (related to COOH) replaced
 341 by a strong peak at 176 ppm (related to -CONH-) could be due to the grafting but also to
 342 solid "dilution" due to the presence of Pnipam₂₅₀₀ and high amount of amide bounds. The
 343 **Figure 3b** reveals indeed the presence in huge amount of PNIPAM on the CNCs surface after
 344 several washing steps. These values are in accordance with a presence of Pnipam₂₅₀₀ on

345 TEMPO CNCs surface, but cannot confirm if it is grafted or adsorbed. A comparison with only
 346 mixed Pnipam₂₅₀₀ would not help due to this dilution but could give us more quantitative
 347 information. For such quantitative data, we have preferred using elemental analysis.

348 3.2. Quantitative analysis of Pnipam₂₅₀₀ onto CNCs

349 Further evidence of the grafting of Pnipam₂₅₀₀ from CNCs was supported by the elemental
 350 analysis of extensively washed CNC and used to quantify the amount of polymer present on
 351 the surface. **Table 1** shows the experimental atomic composition (C, H, N, O and S) of CNCs,
 352 TEMPO CNCs, CNC-g-Pnipam₂₅₀₀ and CNCs mixed with Pnipam₂₅₀₀.

353 **Table 1:** Atomic composition determined by Elemental Analysis (standard deviation is considered to
 354 be related to equipment at 0.1 % for each value).

	<i>Experimental values</i>					<i>Corrected values</i>	
	% C	% H	% N	% O	% S	% C	%O
CNCs	40.2	6.0	< 0.1	49.7	0.9	44.44	49.37
TEMPO CNCs	37.0	5.9	< 0.1	52.2	0.8	40.91	51.80
CNC-g-Pnipam ₂₅₀₀	53.8	8.9	8.9	24.3	0.3	59.50	27.11
TEMPO CNCs + Pnipam ₂₅₀₀	53.9	8.8	9.1	24.2	0.2	59.69	24.04

355 In the case of pure cellulose, theoretical values of the weight fraction of carbon are 44.44%
 356 and 49.37% for oxygen, and theoretical ratio of oxygen-to-carbon is close to 1.11. Therefore,
 357 experimental value presents a ratio about 1.24 for CNC. It is well known that the difference
 358 between the theoretical and the experimental values in the case of the carbon content is
 359 probably due to sulfate content, the impurities present in the sample and experimental
 360 error. To incorporate this difference, the experimental values reported in **Table 1** are
 361 corrected with the product between the experimental value obtained for a given material
 362 and the ratio of the theoretical to experimental value for non-grafted nanoparticles. This
 363 strategy has already been used to determine the degree of substitution (Missoum et al.,
 364 2012; Siqueira et al., 2010b).

365 In the case of TEMPO CNCs, the DO_{EA} calculated from the **Equation 5** gives an oxidation rate
 366 of about 0.38 which is close and in the same range than conductometry titration value
 367 (i.e. DO = 0.25) if we consider the standard deviation of E.A. From these results, it is possible

368 to estimate the surface density of the carboxyl content and to calculate the maximum
 369 amount possible of polymers which can be grafted. After a TEMPO oxidation, carboxylic
 370 groups are mainly on the surface of CNCs (Isogai et al., 2011). With a DO of about 0.25 and
 371 assuming that the surface of an AGU is 1 nm², it corresponds to a density ~25 COOH every
 372 100 nm², assuming that most of oxidation occurs at the surface of CNC. **CNC dimensions**
 373 **a x b x c are 150 x 10 x 10 nm³ (Table 2).** As the surface of CNC is 6000 nm², 1500 COOH ends
 374 are available on the surface. The maximum grafting molecule will be with 1500 polymer
 375 chains per CNC. Referring to the conductometric titration, ~30 % carboxyl groups were
 376 substituted by amine groups. It corresponds to ~450 polymer chains grafted on the surface
 377 of CNCs.

378 The presence of a high amount of nitrogen content in the CNC-g-Pnipam₂₅₀₀, extensively
 379 washed confirms that Pnipam₂₅₀₀ is still present after grafting on TEMPO CNCs. This value is
 380 equivalent to the quantity of Pnipam₂₅₀₀ of 5 wt.% using ratio of %C/%N with following
 381 Equation 6 and 7.

$$382 \quad \%C = \frac{[(1 - \%Pnipam_{2500}) \times M_C \times 6] + [\%Pnipam_{2500} \times (M_C \times 134)]}{M_{tot}} \quad (6)$$

$$383 \quad \%N = \frac{\%Pnipam_{2500} \times M_N \times 23}{M_{tot}} \quad (7)$$

384 Where (1 - %Pnipam₂₅₀₀), M_C, 6, %Pnipam₂₅₀₀, 134, M_N, 23 and M_{tot} are respectively the
 385 CNC mass, carbon molecular weight, number of carbon in AGU, mass of Pnipam₂₅₀₀, number
 386 of carbon in Pnipam₂₅₀₀, Nitrogen molecular weight, number of nitrogen in Pnipam₂₅₀₀ and
 387 total mass. To evaluate the number of CNCs in a suspension, the density of cellulose (1.59)
 388 and dimension of CNC (a x b x c = 150 x 10 x 10 nm³) were used.

389 Based on the presence of ~4 E¹⁶ CNCs in 0.95 g of suspension (1g – 5% of Pnipam₂₅₀₀) and
 390 ~1200 E¹⁶ Pnipam₂₅₀₀ chains in 0.05 g of suspension, this value of 5 wt.% means ~300
 391 polymer chains on the surface of TEMPO CNCs. This value shows that all the COOH have not
 392 been grafted. But it is difficult to say if it is grafted or adsorbed. Indeed, unfortunately similar
 393 amount of Pnipam₂₅₀₀ is present when only adsorption and washing occurs.

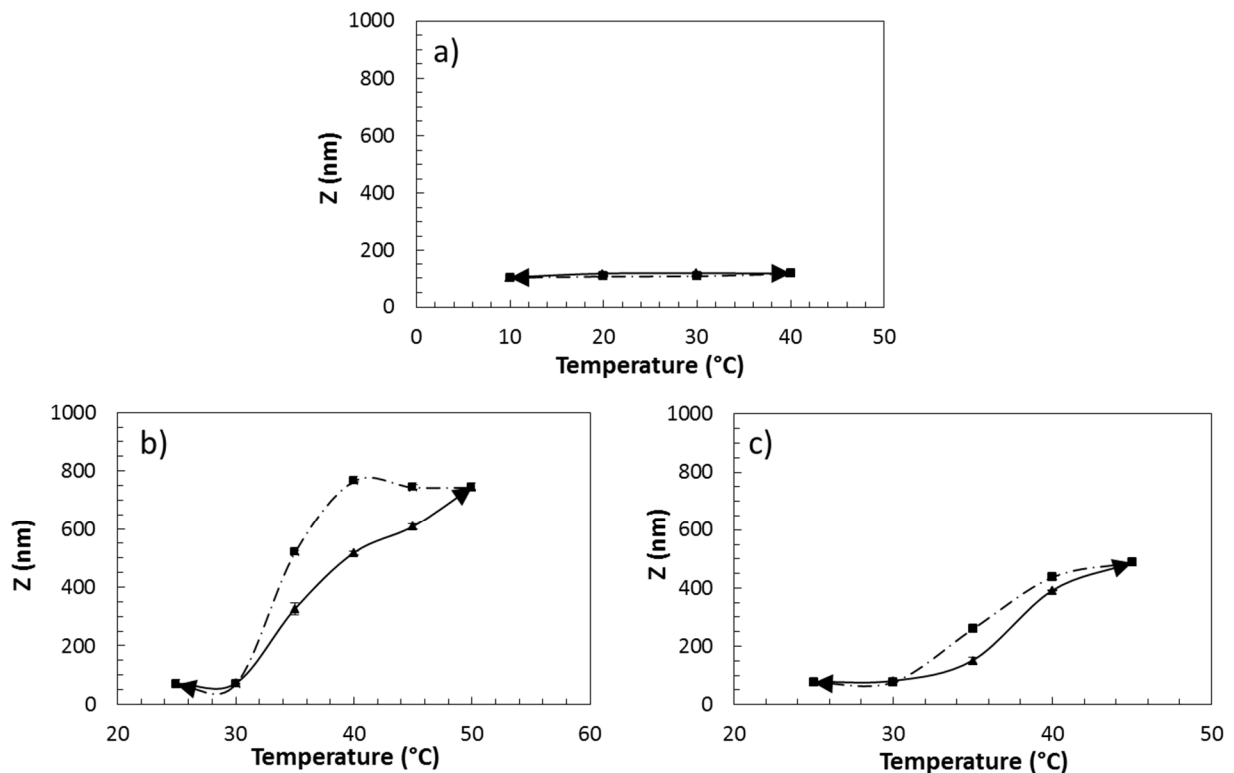
394 If we consider only adsorption, the surface of adsorbed polymer chains should be
 395 characterized by a mushroom regime, in which they do not interact together, in the case of

396 moderate polymer densities. The size of the polymer is in the order of the gyration radius,
397 which is 1.5 nm, given by $R_g = 0.022 \times M_w^{0.54}$ (Kubota et al., 1990) where M_w is the
398 polymer molecular weight, *i.e.* 2500 g·mol⁻¹. To be in mushroom regime, each polymer needs
399 to be at a distance higher than twice time the R_g . The CNC size $a \times b \times c$ are
400 150 x 10 x 10 nm³, equivalent to an area about 6000 nm². The surface occupied by a polymer
401 is 7 nm² ($\pi \times R_g^2$), so on a CNC the maximum of adsorbed polymer is ~860 polymer chains.
402 This value is in the range of grafting calculation. So in both case, adsorption and grafting,
403 similar amount of polymer chain can be present on CNC surface.

404 As a conclusion, it is difficult to have a direct proof of the grafting vs adsorption/presence
405 with techniques we have used. Our assumption is that similar amount of Pnipam₂₅₀₀ is at the
406 surface of CNC but in the case of adsorbed polymer, all polymer is present as aggregate in
407 the suspension or at the surface whereas when it is grafted, one part is grafted on the
408 surface and another part is adsorbed or present as aggregate.

409 Indirect methods have then been performed to check if we have a difference between
410 grafted and adsorbed system. Furthermore, the elemental analysis gave us an idea of the
411 ratio of Pnipam₂₅₀₀ and CNC. This has been confirmed by weight measurement of
412 suspensions. Indeed, after grafting, all samples are washed directly with dialysis, so we are
413 sure that CNCs stays (does not pass through dialysis membrane). So, knowing the initial CNCs
414 mass and just by measuring the final mass, we can calculate the quantity of Pnipam₂₅₀₀ which
415 is still present (adsorbed or grafted or both). In our case this weight measurement gives a
416 ratio of 25% of CNC and 75% of PNIPAM after adsorption and after grafting. This value will
417 be considered in DLS characterization (Figure 4).

418 **3.3. Thermosensitive behavior of CNCs decorated with Pnipam₂₅₀₀**



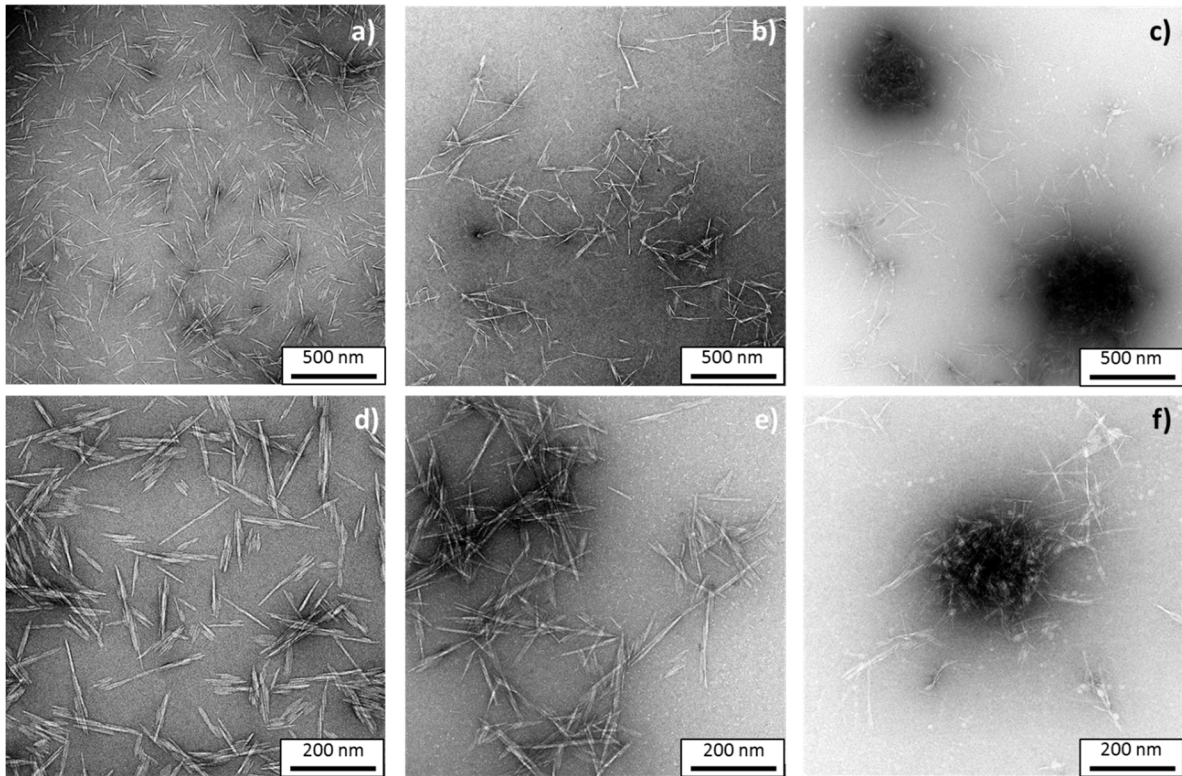
419 **Figure 4:** DLS curve for (a) TEMPO CNCs at 0.1 wt.%, (b) CNC-g-Pnipam₂₅₀₀ at 0.1 wt.% (ratio
 420 estimated at 25% CNC and 75% Pnipam₂₅₀₀) and (c) TEMPO CNCs + Pnipam₂₅₀₀ (ratio at 25% TEMPO
 421 CNCs and 75% Pnipam₂₅₀₀). Full line: temperature increases and dot line: Temperature decreases.
 422

423 In **Figure 4**, the hydrodynamic diameter of CNC-g-Pnipam₂₅₀₀ and TEMPO CNCs + Pnipam₂₅₀₀
 424 suspension and CNCs raw materials were measured as function of temperature. For
 425 TEMPO CNCs (**Figure 4a**), temperature has no clear influence on the size of the particles.
 426 Referring to the literature (Fujishige et al., 1989; Wu, 1998), PNIPAM shows sensitivity to the
 427 temperature. In works of Kubota (Fujishige et al., 1989; Kubota et al., 1990; Senff and
 428 Richtering, 1999; Wu, 1998) and Wu (Wang and Wu, 1999; Wu, 1998), under the LCST
 429 (~34°C), PNIPAM polymer presents coil conformation with hydrodynamic diameter about
 430 100 nm. In our case the measurement of DLS was difficult due to the presence of impurities
 431 (catalyst nanoparticles or other) detected with an initial DLS value of 500nm which is not
 432 possible for a so short oligomer. Then above the LCST, the polymer turns in globule
 433 conformation and presents a hydrodynamic diameter about 20 nm. For the grafting sample
 434 CNC-g-Pnipam₂₅₀₀ (**Figure 4b**), under the LCST the hydrodynamic diameter is low and
 435 constant (close to the CNC size as it is a short oligomer). After 30°C, an increase in size was
 436 observed (750 nm), probably due to aggregates formation. When the sample is cooling back
 437 to 20°C, the hydrodynamic diameter decreases and, at T < 30°C the system recovers a low

438 and constant value similar to the one measured during the heating process. This
439 phenomenon is considered reversible, even if a 5°C hysteresis is observed. This result is very
440 positive and confirms indirectly that at least a part of CNC is grafted mainly when it is
441 compared with only adsorbed Pnipam₂₅₀₀ (**Figure 4c**). In this case, the aggregation is limited
442 compared to grafted molecule, and the maximum collapse system size is close to 480 nm (vs
443 610 nm for the grafted CNC at same Temperature). The difference is even higher when
444 temperature decrease, i.e. 480 for adsorbed and 750nm for grafted CNC. Nonetheless, the
445 thermo-reversible behavior is similar in the case of grafted or adsorbed Pnipam₂₅₀₀. So the
446 thermo-stimuli properties of CNC-g-Pnipam₂₅₀₀ and TEMPO CNCs + Pnipam₂₅₀₀ are different.
447 This confirms indirectly that we don't have only presence or adsorption in the first case.

448 The grafting of Pnipam₂₅₀₀ polymer chain on the surface of CNCs gives a thermo-reversible
449 behavior to the suspension, as proved by the thermo-reversible aggregation in DLS data. The
450 behavior of a PNIPAM polymer was previously explained in detail (Lai et al., 2013). In this
451 study, when the temperature is below the LCST of Pnipam₂₅₀₀, grafted chains are under good
452 solvent conditions. Steric repulsion forces between particles are maximal, and dispersion
453 with individual objects appears. For temperature higher than the LCST, the polymer is in
454 poor solvent condition in water, resulting in individual collapsed and CNC-g-Pnipam₂₅₀₀ are
455 aggregated together. This process is reversible and generates a hysteresis effect. Similar
456 behavior **was** observed with another thermosensitive polymer grafted onto CNC (Azzam et al
457 2016).

458 **3.4. CNCs and Pnipam₂₅₀₀ grafted onto CNCs (CNC-g-Pnipam₂₅₀₀)**
 459 **characterization**



460
 461 **Figure 5:** TEM images of a)-d) Cellulose Nanocrystals (CNCs) before any treatment and b)-e) CNC-g-
 462 Pnipam₂₅₀₀ deposited on TEM grid at 20°C and c)-f) CNC-g-Pnipam₂₅₀₀ deposited at 50°C. Pictures are
 463 representatives of each sample.

464 As confirmed in **Figure 5a** and **5d**, CNC have a rod-like shape. Size measurements on TEM
 465 images show an average length of 150 ± 30 nm and a section of 10 ± 5 nm. These values are
 466 comparable with the literature data (Habibi et al., 2010).

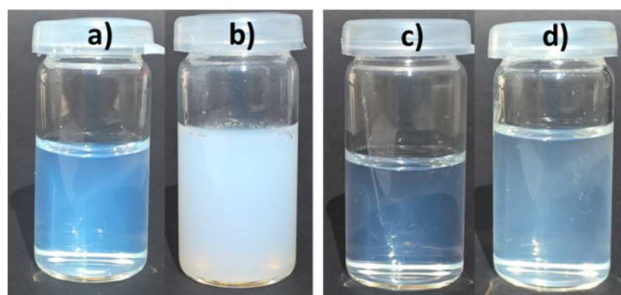
467 **Table 2:** Physical and chemical properties of CNCs from university of Maine.

Properties	Tools	Value	S.D.
Morphology	AFM & TEM	Length (nm)	150 (30)
		Thickness (nm)	10 (5)
		Aspect ratio	15 -
Chemical	EA	Sulfur content ($\mu\text{mol}/g_{\text{CNC}}$)	305 (15)
Physicochemical	Calculation based on EA & TEM	Surface charge density (e/nm^2)	0.46 (0.02)

468

469 In details in **Table 2**, the sulfur content estimated by elemental analysis is 305 $\mu\text{mol}/\text{g}_{\text{CNC}}$.
470 The surface charge density is approximately 0.46 e/nm^2 , as calculated using TEM and EA
471 data.

472 **Figure 5** presents TEM images of negatively stained CNC (**Figure 5a** and **5d**), CNCs decorated
473 with Pnipam₂₅₀₀ under LCST condition (**Figure 5b** and **5e**) and CNC-g-Pnipam₂₅₀₀ after LCST
474 (**Figure 5c** and **5f**). By comparison between **Figure 5d** and **Figure 5e**, the morphology of the
475 rod-like particles is unchanged after peptidic grafting. Under LCST, Pnipam₂₅₀₀ grafted CNCs
476 seems to be well-dispersed. The individualization of nanocrystals can be attributed to the
477 presence of polymer chains on the surface, which generate entropic repulsion forces
478 between nanoparticles. Nonetheless, polymer chains are not visible on TEM images, due to
479 their tiny size and a low surface density (hinder a detectable contrast in TEM stained grid).
480 After LCST (**Figure 5c** and **5f**), CNC-g-Pnipam₂₅₀₀ seems to be packed together and shows a
481 'bird-nest' configuration, due to the collapse Pnipam₂₅₀₀ on the surface of TEMPO CNCs.
482 These pictures are in agreement with Pnipam₂₅₀₀ grafting and thermal aggregation of the
483 system when the temperature is above the polymer LCST. Nonetheless, the system does not
484 present CNC-g-Pnipam₂₅₀₀ packed by 3-4 CNCs (as previously published by Azzam et al.
485 (Azzam et al., 2016)) but a huge aggregate without well-defined size.

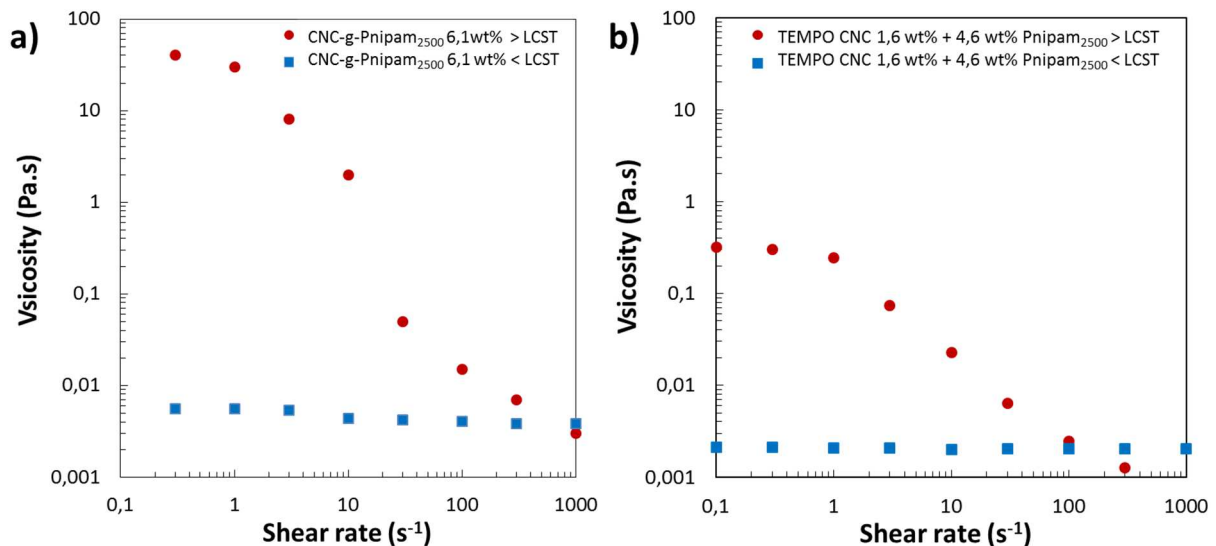


486
487 **Figure 6:** Pictures of cellulose nanocrystals suspension: a) oxidized CNC without salt, b) TEMPO CNCs
488 with 1M NaCl, c) CNC-g-Pnipam₂₅₀₀ without salt and d) CNC-g-Pnipam₂₅₀₀ with 1M NaCl. Each
489 suspension has a concentration of 2 wt.%.

490 An interesting property acquired by the presence of polymer chains (adsorbed or grafted) on
491 the CNC surface is the colloidal stability with presence of electrolyte. **Figure 6** shows the
492 behavior of TEMPO CNCs and polymer grafted CNCs when NaCl is added. Colloidal
493 suspension of TEMPO CNCs (**Figure 6a**) immediately precipitates after addition of 1 M NaCl
494 and forms a turbid suspension (**Figure 6b**). At the opposite, Pnipam₂₅₀₀ grafted on CNCs
495 suspension (**Figure 6c**) prevent any precipitation and remain stable the suspension in

496 presence of electrolyte (**Figure 6d**). Steric stabilization caused by the presence of polymer
497 (grafted or adsorbed) was already reported in literature (Araki et al., 2001; Azzam et al.,
498 2010; Kloser and Gray, 2010).

499 **Figure 7** presents the rheological behavior of CNC-g-Pnipam₂₅₀₀ and TEMPO
500 CNCs + Pnipam₂₅₀₀ under and above the PNIPAM LCST. System concentrations are 6.1 wt.%
501 (estimated ratio is 25% of CNCs and 75% of Pnipam₂₅₀₀). For grafted polymer (**Figure 7a**) the
502 viscosity increases from 0.008 to 40 Pa·s at low shear rate when the LCST is reached.
503 Nonetheless, in the case of polymer adsorption exclusively (**Figure 7b**), the viscosity
504 increases only from 0.002 to 0.3 Pa·s when the LCST is reached. This observation shows a
505 difference in kinetics of aggregation whereas similar amount of polymer is present. This
506 confirms once again indirectly that there is a difference between these two CNCs materials.



507
508 **Figure 7:** flow curves of a) CNC-g-Pnipam₂₅₀₀ at 6.1 wt.% and b) TEMPO CNCs at 1.6 wt.% +
509 Pnipam₂₅₀₀ at 4.6 wt.% (final concentration 6.2 wt.%). Each graph presents the system flow
510 curve under (blue square) and above (red dot) the PNIPAM LCST.

511 This peptidic grafting is promising and can modify CNCs suspension properties by using
512 water based grafting or adsorption. One drawback could be attributed to the low quantity of
513 grafting (similar to adsorption) and to the time duration of such treatment.

514

515 **4. Conclusions**

516 In this study, thermo-sensitive Pnipam₂₅₀₀ were successfully adsorbed and grafted (or both)
517 onto oxidized cellulose nanocrystals by a green peptidic coupling. Results demonstrate the
518 presence and the formation of the polymer onto the nanocrystals after multiple washings.
519 The polymer density was sufficient to create a steric stabilization of the CNC-g-Pnipam₂₅₀₀ in
520 high ionic strength which prevents the flocculation. In spite of several methods, it has been
521 difficult to distinguish the adsorbed from the grafted CNCs. However indirect methods show
522 clearly a difference between the two samples indicating that of CNC-g-Pnipam₂₅₀₀ has at
523 least one part which is grafted. Thanks to this sufficient density, the thermo-reversible
524 behavior of the polymer allows us to realize a thermo-reversible suspension composed with
525 nanoparticles, CNCs, and biocompatible polymers, Pnipam₂₅₀₀. CNCs give percolation
526 network properties to the system and the polymer, a thermosensitive behavior, observed in
527 rheology **measurement**. These results open the road to biomedical applications such as
528 injectable hydrogel for reparative surgery and esthetic surgery.

529

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540

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