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Two-step immobilization of metronidazole prodrug on TEMPO cellulose nanofibrils through thiol-yne click chemistry for in situ controlled release

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Abstract

Nowadays, drug encapsulation and drug release from cellulose nanofibrils systems are intense research topics, and commercial grades of cellulose nanomaterials are currently available. In this work we present an ester-containing prodrug of metronidazole that is covalently bound to cellulose nanofibrils in aqueous suspension through a two-step immobilization procedure involving green chemistry principles. The presence of the drug is confirmed by several characterization tools and methods such as Raman spectroscopy, elemental analysis, Dynamic Nuclear Polarization enhanced NMR. This technique allows enhancing the sensitivity of NMR by several orders of magnitude. It has been used to study cellulose nanofibrils substrates and it appears as the ultimate tool to confirm the covalent nature of the binding through thiol-yne click chemistry. Moreover, the ester function of the immobilized prodrug can be cleaved by specific enzyme activity thus allowing controlled drug release.

Keywords

Cellulose nanofibrils, drug release, click chemistry, DNP-NMR

Introduction

Among cellulosic nanomaterials, cellulose nanofibrils (CNF) are considered as one of the best bio-sourced starting platform material for the development of unique systems. The tremendous

31 enthusiasm in the scientific community for this material arises from the general characteristics of
32 cellulose nanomaterials such as widespread availability, biodegradability, biocompatibility, excellent
33 mechanical properties and tunable surface chemistry (Kargarzadeh et al., 2018). Today, such CNF can
34 be used in several and various applications (i.e. paper and packaging (Aulin et al., 2010; Bardet & Bras,
35 2014; Boufi et al., 2016; Lavoine et al., 2015), electronics (Hoeng et al., 2016), composites (Dufresne
36 et al., 2013) medical and cosmetics (Halib et al., 2017; Jorfi & Foster, 2015; Laurén, 2018; Lin &
37 Dufresne, 2014) industries) as reinforcement agent, rheology modifier or nanostructured networks
38 and membranes. Among identified CNF grades, TEMPO oxidized CNF (CNF-t) has enhanced features
39 such as its chemical reactivity together with its specific rheological properties, both explained by the
40 presence of carboxylic acid and aldehydes moieties along the cellulose nanofibers.

41 Industrial production of CNF-t is under development in different places of the globe. Pioneer research
42 investigations on TEMPO oxidation of cellulose by the group of Professor Akira Isogai in Japan (Isogai
43 et al., 2011; Saito et al., 2006) led to the apparition of CNF-t production facilities while in Europe and
44 North America, small companies are starting to sell CNF-t. The growing production facilities come along
45 with novel and promising applications of CNF-t such as the biomedical field. A couple of reviews deal
46 with the use of CNF-t (together with other cellulose nanomaterials) for biomedical application and
47 describe the potential of this material for the design of new innovative healthcare systems (Du et al.,
48 2019; Jorfi & Foster, 2015; Lin & Dufresne, 2014; Syverud, 2016). CNF-t draws specific attention since
49 it is possible to produce it in an ultrapure form with low endotoxin content making them suitable for
50 wound healing when freeze-dried into aerogels: low cytotoxicity was also confirmed in addition with
51 really high water holding capacity (Nordli et al., 2016). CNF-t were also investigated to be used as drug
52 carriers. The ability to deliver Ibuprofen through the skin from CNF-t-Ibuprofen gel formulations was
53 assessed *in-vitro* and *in-vivo*. CNF-t systems were able to match with commercial references while
54 including five times lower drug loading proving a more efficient and optimized use of drug (Celebi et
55 al., 2016). Aerogels of different kinds of cellulose nanofibrils suspensions mixed with beclomethasone
56 dipropionate (BDP) nanoparticle coated with hydrophobin proteins were also produced through freeze
57 drying. CNF-t based systems showed a sustained drug release. The CNF three-dimensional structure
58 was tunable by varying the freeze drying parameters allowing for a controlled release of the drug (Valo
59 et al., 2013).

60 The antibacterial metronidazole is used to fight diseases concerning different organs and especially in
61 gastrointestinal tract and reproductive system, mainly against anaerobic micro-organisms. Its
62 mechanism of action involves the penetration into the anaerobic bacteria, its chemical transformation
63 (reduction) by proteins to an active product that will cleave DNA strands, and thereby inhibits the
64 replication mechanism thus preventing cell growth (*Metronidazole*, n.d.; Pubchem, n.d.). Since the

65 1980's, metronidazole has been successfully used with cellulose derivatives tableting compounds such
66 as hydroxypropyl methyl cellulose (Campos-Aldrete & Villafuerte-Robles, 1997), methyl cellulose (Itiola
67 & Pilpel, 1986) and cellulose acetate phthalate (Gates et al., 1994). Up to our knowledge, only a very
68 few publications deal with the use of CNF-t and metronidazole. CNF-t hydrogels were used as matrix
69 for encapsulation of 6 different active principle ingredients, including metronidazole. Researchers
70 investigated the effect of freeze drying and subsequent rehydration on rheological properties and the
71 drug release profiles. Results suggested that the CNF-t hydrogels were successfully rehydrated and
72 release profiles were equivalent, before and after freeze drying, confirming their potential for
73 controlled release applications (Paukkonen et al., 2017). These uses of metronidazole relied only on
74 physical adsorption of the drug onto the cellulosic substrates. Micro and nano-sized three dimensions
75 structures of the developed systems allowed for a diffusion-controlled release mechanism.

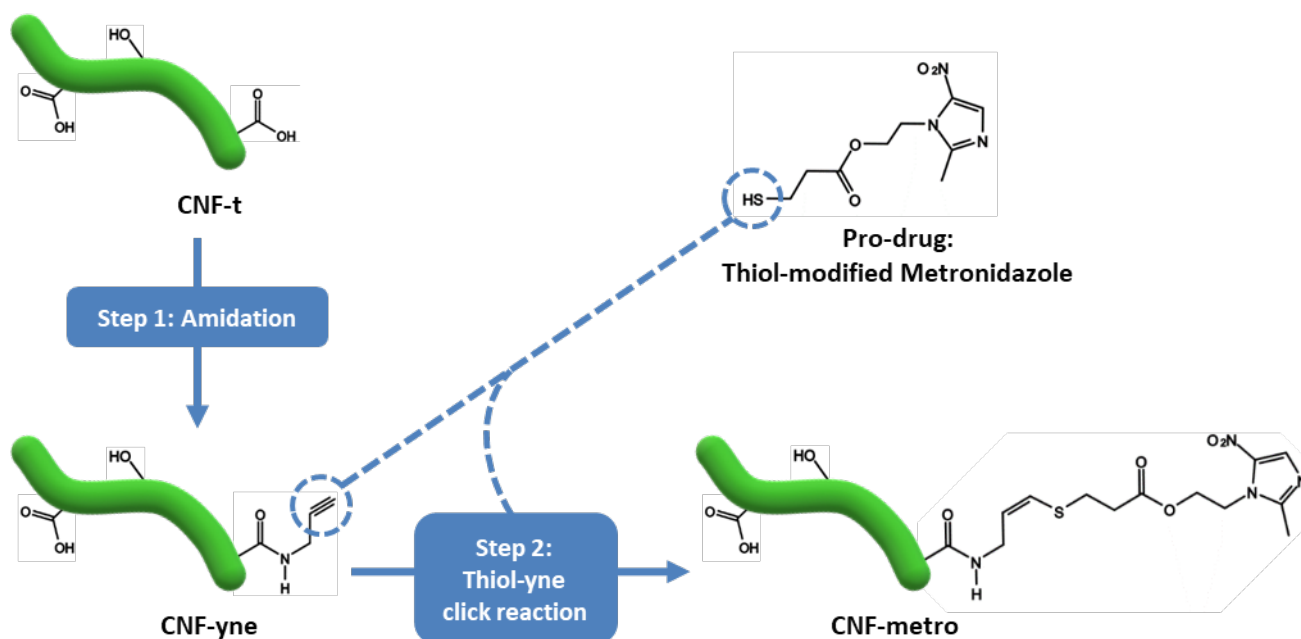
76 The purpose of this work is to prepare an enhanced CNF based drug vehicle that can be integrated in
77 innovative implantable medical devices with controlled drug release properties. This requires a
78 complementary anchoring strategy of the drug to the cellulosic substrates in order to achieve in situ
79 controlled drug release. For this purpose, metronidazole was chosen as drug model as it offers two
80 advantages. First, its chemical versatility with the pending hydroxyl group makes easy to design a new
81 prodrug that will contain a cleavable ester linker necessary to obtain the in situ release, and a reactive
82 thiol end-group for anchoring to the CNF. Secondly, as mentioned already, metronidazole is of first
83 choice for anaerobic conditions, those that are met in the targeted implantation site. The presence of
84 ester link will allow the liberation of the drug by ester-specific enzymes (present in fat tissues and on
85 infection sites (Schakenraad et al., 1990)) on the targeted site of treatment.

86 On the CNF side, efficient chemical modification is required to bind the end-group of the prodrug. It
87 should be noted that surface modification of CNF is still an intense and challenging field of research.
88 The very large specific area of CNF, together with their high amounts of reactive hydroxyls (along with
89 carboxylic acids and aldehydes groups in the case of CNF-t) lead to a wide range of strategies for
90 surface modifications. Reactions such as etherification, esterification, silylation and amidation have
91 been performed to covalently bind small molecules or polymer chains to CNF as described in relevant
92 reviews (Habibi, 2014; Missoum et al., 2013; Rol et al., 2018). Characterization of resulting modified
93 CNF substrates is often challenging and calls for cross characterizations, covering both chemical and
94 physical approaches, and also functionality evaluation. The use of sustainable materials such as CNF
95 goes hand in hand with the increasing need for low-environmental impact processes and green
96 chemistry procedures for CNF surface functionalization. In this context, click chemistry is of particular
97 interest. Sharpless et al. introduced in 2001 the concept of click chemistry that gathers reactions that
98 are efficient, stereospecific, modular, requiring simple conditions (not sensible to oxygen and water),

99 and results quickly in very high yields while involved reagents must be readily available (Kolb et al.,
100 2001). Click chemistry was seldom used to modify cellulose nanofibrils. CuAAC and thiol-ene click
101 chemistry were applied to CNF films in order to immobilize fluorescent compounds (Navarro et al.,
102 2015; Pahimanolis et al., 2011). So far, only two publications mention the use of thiol-yne click
103 chemistry with CNF. These researcher's aim was to tailor the wettability of CNF films surface to develop
104 fluidic channels for biosensing (Guo et al., 2018; Solin et al., 2019). In the present work, CNF was
105 modified with a two-step chemical strategy: in the first part an alkyne group was introduced through
106 amidation, while in the second step, the thiol-yne chemistry was used to bind the prodrug to the
107 intermediate alkyne-CNF, as described in Figure 1.

108 To the best of our knowledge, this work constitutes the first example of such a sophisticated strategy
109 towards active CNF based materials in situ release. Furthermore, the use of dynamic nuclear
110 polarization NMR (DNP-NMR), a recent hyperpolarization technique that allows enhancing the NMR
111 signal sensitivity by several orders of magnitude, was the ultimate tool, for the second time for our
112 team (Kumar et al., 2020), to confirm the success of the chemical functionalization applied to cellulose
113 nanofibrils. Its unique sensitivity allows the identification and quantification of any trace of compound,
114 thus overcoming the challenging modified CNF characterization.

115



116

117 **Figure 1: General multistep immobilization procedure of thiol modified metronidazole prodrug on CNF-t. Note that thiol-**
118 **yne click reaction can give a double addition of thiol compound. However, due to steric hindrance, the second addition is**
119 **less likely to happen so that only the “one-addition” product is depicted.**

120

121 **Experimental procedure**

122 **Materials**

123 The cellulose nanofibrils (CNF) suspensions were provided by the Centre Technique du Papier (CTP,
124 Grenoble, France). A first suspension referred as CNF-e was produced by a 2h enzymatic pre-treatment
125 of a pre-refined (40°SR) bleached birch pulp followed by homogenization: 3 passes at 1500 bars in an
126 Ariete homogenizer from GEA. The second suspension, which is referred as CNF-t, was also produced
127 by the Centre Technique du Papier (CTP, Grenoble, France). The TEMPO mediated oxidation was
128 performed on a pre-refined (40°SR) bleached bisulfite pulp provided by TEMBEC following classical
129 procedure developed by A. Isogai's team in the end of 2000s (Isogai et al., 2011; Saito et al., 2006).
130 The cellulose fiber pulp concentration was set at 1.5 wt% and the pre-treatment was done at pH 10
131 for 2h and involved NaBr, NaClO and the TEMPO reagent. The oxidized pulp was then subjected to the
132 high pressure homogenizer (GEA Niro Soavi) to produce the CNF-t suspension.

133 Propargyl-amine (CAS: 2450-71-7), propargyl-bromide (CAS: 106-96-7), N-(3-dimethylaminopropyl)-N'-
134 ethylcarbodiimide hydrochloride (EDC, CAS: 25952-53-8), N-hydroxysuccinimide (NHS, CAS: 6066-82-
135 6), sodium hydroxide (NaOH, CAS: 1310-73-2), and hydrogen chloride (HCl, CAS: 7647-01-0) 4-
136 (dimethylamino)pyridine (DMAP, CAS: 1122-58-3), N,N-Dicyclohexylcarbodiimide (DCC, CAS: 538-75-
137 0), trifluoroacetic acid (TFA, CAS: 76-05-1), triisopropylsilane (TiPS, CAS: 6485-79-6) were purchased
138 from Sigma Aldrich and used as received. Isopropanol (CAS: 67-63-0) was purchased from ACROS
139 Organics. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, >98%, CAS: 51805-45-9) was purchased
140 from ThermoFisher Scientific. Biocompatible photoinitiator lithium phenyl(2,4,6-
141 trimethylbenzoyl)phosphinate (LAP, >98%, CAS: 85073-19-4) was purchased from Tokyo Chemical
142 Industry. The biradicals 15-[[[(7-oxyl-3,11-dioxa-7-azadispiro[5.1.5.3]hexadec-15-yl)carbamoyl][2-
143 (2,5,8,11-tetraoxatridecan-13-ylamino)]-[3,11-dioxa-7-azadispiro[5.1.5.3]hexadec-7-yl]]oxidanyl
144 (AMUpol) was obtained from ICR (Aix-Marseille University, UMR7273, France). Deionized water was
145 used for every experiment. Carboxylesterase 2 human (expressed in baculovirus infected BTI insect
146 cells) was purchased from Sigma Aldrich and used as received. Tris(hydroxymethyl) aminomethane
147 (TRIS, >99.8% CAS: 77-86-1) was purchased from Euromedex.

148 **Alkylation of CNF-t through amidation**

149 The CNF-t suspension concentration was decreased from 1.5wt% to 0.4wt% in order to be easily
150 stirrable. Deionized water was added before homogenization with an IKA Ultra-Turrax high shear
151 mixer (China) for 1 minute at 10 000 rpm. The pH of the suspension was then adjusted to 4 under
152 magnetic stirring using a 0.5 M HCl solution.

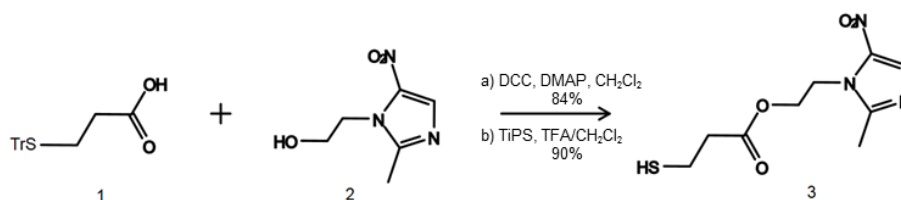
153 A solution of the coupling agents EDC and NHS was prepared in deionized water. After complete
154 dissolution the solution was added to the suspension of CNF-t. A molar ratio of 4 equivalents of EDC
155 and NHS for 1 equivalent of carboxyl group of CNF-t was used. The mixture was magnetically stirred
156 for 30 min at room temperature in order to activate the carboxyl groups of the CNF-t. The pH was
157 maintained at 4 during the reaction with 0.5M HCl or NaOH solution droplets addition. This pH favors
158 the EDC carbocation availability and acid form of the carboxyl groups that are both required for a better
159 reaction.

160 The pH was then increased to 8.5 for the second part of the reaction i.e. the amine addition. A solution
161 of propargyl amine was prepared in deionized water and added to the mixture. Again, a molar ratio of
162 4 equivalents for 1 equivalent of carboxyl groups of CNF-t was used. The mixture was magnetically
163 stirred for 72h at room temperature and the pH was kept at 8.5 with 0.5M HCl or NaOH solution
164 droplets addition.

165 After 72h of reaction, the mixture was washed by centrifugation and dialysis. First, the reaction was
166 quenched by decreasing the pH to 2-2.5 with 0.5M HCl solution. Then, centrifugation – re-dispersion
167 cycles were applied to the suspension: centrifugations were operated during 10 minutes at 20 000 g
168 (about 11 100 rpm) and re-dispersions were done in acidic water (pH 2-2.5) in order to remove all the
169 non-covalently bound chemicals (EDC, NHS and free amine). In between each centrifugation cycle, re-
170 dispersions were done using high shear mixer (Ultra-turrax, IKA) for 1 min. A total of 6 centrifugation
171 – re-dispersion operations were performed to ultimately isolate the alkyne modified CNF-t, referred
172 as CNF-yne. The last dispersion was done in neutral water.

173 The last step of purification consisted in a dialysis of the CNF-yne suspension against neutral water
174 with 6-8 kDA MWCO membranes (Spectra/Por® 1 Standard RC Tubing, SPECTRUM) for at least 5 days
175 under slow magnetic stirring and renewal of the medium twice a day.

176 Synthesis of Metro-SH compound



177

178 **Scheme 1: Synthesis path of metronidazole-thiol (3) from Metronidazole (2) and S-trityl-3-mercaptopropionic acid (1)**

179 The prodrug approach is a new way to investigate drug anchoring strategy to CNF. A specific prodrug
180 need to be designed. S-Trityl-3-mercaptopropionic acid (1) was synthesized according to a procedure

181 designed by Sharma et al (Sharma et al., 2008). This product **1** (1.05 g, 3 mmol), metronidazole **2** (467
182 mg, 2.73 mmol) and DMAP (24 mg, 0.2 mmol) were dissolved in 30 ml of CH₂Cl₂ at 0°C. DCC (618 mg,
183 3 mmol) was added in the solution after 15 minutes. The reacting mixture was stirred during 3 h at
184 room temperature, then filtered and concentrated under vacuum. The crude product was purified by
185 flash chromatography on silica gel with CH₂Cl₂ as eluent to give the protected compound as a white
186 solid (1.15 g, 2.3 mmol, 84 %). The tritylated compound was dissolved at 0°C in 30 ml of CH₂Cl₂ and 15
187 ml of TFA. After 10 minutes, TiPS (1 ml, 4.87 mmol, 2 equiv.) was added to the solution that was
188 warmed up to room temperature and stirred for 1 h. The solution was concentrated under vacuum
189 and the TFA was co-evaporated twice with methanol. The residue was washed three times with cold
190 cyclohexane and dried under vacuum. The amorphous solid was purified by flash chromatography on
191 silica gel with CH₂Cl₂/MeOH : 97/3 as eluent to give the compound **3** (**Erreur ! Source du renvoi**
192 **introuvable.**) as a rosy amorphous solid. (568 mg, 95%). The full liquid ¹H and ¹³C NMR characterization
193 of the intermediate product and prodrug **3** are available in the supporting information (SI) on Figure S
194 1 and Figure S 2.

195

196 Immobilization of Metro-SH on CNF substrates through thio-yne click chemistry

197 The prodrug metro-SH **3** (62.5 mg, 0.25 mmol) was dissolved in a 15 g mixture of deionized water and
198 ethanol (50:50 v/v ratio). To this solution, 5 ml of deionized water containing a 5% molar ratio relative
199 to the thiol groups of **3** of tris-(2-carboxyethyl)phosphine (TCEP) (3.1mg, 0.0125 mmol), was added to
200 prevent the air oxidation of the thiol into disulfure, and lithium phenyl(2,4,6-
201 trimethylbenzoyl)phosphinate (LAP), a biocompatible photoinitiator required to activate the thiol-yne
202 click reaction, was introduced at a quantity of 0.01wt% relative to the CNF-yne mass (0.02mg, 6.8E⁻⁵
203 mmol). Then the resulting mixture was added to the CNF-yne suspension. The whole mixture was
204 transferred to a UV transparent two necked balloon and put under magnetic stirring. The suspension
205 was exposed to UV radiation with a UV lamp (Fire Edge™ FE300, Phoseon Technology USA, wavelength
206 345-385nm) and a nitrogen flux was used to remove the dioxygen from the free volume of the balloon.
207 The UV-exposition and nitrogen flux were maintained for 4 h. The modified CNF (CNF-Metro), was
208 purified by several centrifugation – re-dispersion steps as described before: three steps of washing
209 with water/ethanol mixture (50/50 v/v) and two steps with deionized water only. Washed suspensions
210 were further purified by dialysis against deionized water under slow magnetic stirring for 5 days with
211 daily renewal of dialysis medium in order to completely remove non-covalently bound compounds.
212 Dialysis membranes were 6-8 kDA MWCO membranes (Spectra/Por® 1 Standard RC Tubing,
213 SPECTRUM). CNF-Metro suspensions were stored in the fridge at 5°C before characterization. The

214 corresponding CNF films were produced by solvent casting in Teflon molds and let dry overnight in an
215 oven at 40°C, these films were used for solid state characterization.

216 Characterization methods and tools

217 Atomic Force Microscopy

218 AFM images were recorded on a Dimension icon® (Bruker, USA). A $7,5 \cdot 10^{-4}$ wt% suspension was
219 prepared by several dilution of the gel using high shear mixer Ultra-Turrax (IKA). A drop of this
220 suspension was deposited on freshly cleaved mica plate before drying overnight under fumehood at
221 room temperature. The acquisition was performed in tapping mode using a silica coated cantilever
222 (OTESPA® 300 kHz – 42 N/m, Bruker, USA). Zones of $3,3 \cdot 3,3 \mu\text{m}^2$ were analyzed.

223 FTIR

224 FTIR spectra of the films were obtained on a Perkin Elmer Spectrum One spectrometer (Waltham,
225 Massachusetts, USA) in attenuated total reflectance (ATR) mode. At least 5 measurements of 16 scans
226 with a resolution of 2cm^{-1} between 600 and 4000cm^{-1} on each side of the CNF films were performed.
227 Spectra were normalized at 1110cm^{-1} , the approximate wavenumber of C-O-C bonds in cellulosic
228 substrates. The most representative spectra were chosen for discussion.

229 RAMAN

230 Raman spectra were recorded on a Renishaw (INVIA, UK) spectrometer equipped with a 1200
231 lines/mm grating and a CCD detector. Excitation wavelength was 785 nm and the beam power was
232 adjusted to avoid sample degradation. The incident beam was focused on the samples through an x50
233 ultra-long working distance objective which resulted in a spot size of an approximate diameter of 2
234 μm . The spectra were recorded between 0 and 3600cm^{-1} .

235 Conductometric titration and DO/DS calculation

236 The carboxylic content of the CNF-t suspension was measured before and after the first step of
237 functionalization by conductometric titration. The amount of CNF-t suspension corresponding to 50
238 mg of dry material was precisely diluted to 200 ml with de-ionized water. Magnetic stirring and high
239 shear mixer Ultra-Turrax (IKA) were used to produce a homogeneous dispersion. The pH was
240 decreased around 2.5 with 0.1 M HCl to turn every remaining carboxylate moieties of CNF surface into
241 the acid form of the carboxylic acids, and the volume of added HCl solution was recorded. The titration
242 was done with a 0.01 M NaOH solution, which precise concentration was measured with 3 colorimetric
243 titrations before the titration of the CNF suspensions. The conductivity of the suspension was recorded
244 after stabilization all along NaOH addition and added volumes of NaOH were reduced when the curve
245 indicated changes in the slopes to improve the assessment of the transitions. The titration curves

246 typically display three regions, a first slope showing a decrease of the conductivity corresponding to
247 the neutralization of remaining strong acid, a plateau where the weak acid groups are titrated
248 (carboxylic groups of CNFs) and a last part where conductivity increases when all acids are titrated.

249 The carboxylic group content X_{ox} on CNF-t can be calculated with equation (1) and is expressed in
250 micro-mole of carboxylic group per gram of dry CNF ($\mu\text{mol/g}$).

251

$$252 \quad (1) \quad X_{ox} = \frac{C \times (V_{eq1} - V_{eq2})}{m}$$

253 The precise concentration of the NaOH solution is C . The CNF dry quantity is m . The two NaOH solution
254 equivalent volumes V_{eq1} and V_{eq2} are extracted at the edge of the plateau on the titration curve by
255 calculating the intersections of the first (decreasing slope) and third (increasing slope) part of the curve
256 with the plateau.

257 The degree of oxidation (DO) represent the number of carboxylic groups per anhydroglucose unit
258 (AGU) on oxidized CNF. It can be calculated with equation (2) according to Da Silva Perez et al. (da Silva
259 Perez et al., 2003), and uses common terms C , m and equivalent volumes.

$$260 \quad (2) \quad DO = \frac{162 \times C \times (V_{eq1} - V_{eq2})}{m - 36 \times C \times (V_{eq1} - V_{eq2})}$$

261 The value of 162 (g/mol) is the molar mass of the AGU. The value 36 (g/mol) is the difference between
262 the molar mass of the carboxylate form of the carboxylic groups including the sodium counter ion (198
263 g/mol) and the molar mass of the AGU (162 g/mol). This term arises from the partial replacement of
264 primary hydroxyl groups by carboxylate groups during the TEMPO mediated oxidation process.

265 All titrations were repeated at least three times.

266 Elemental analysis

267 CNF films were prepared by solvent casting from CNF suspensions. The suspensions were poured into
268 Teflon molds and evaporated overnight in an oven at 40°C. Elemental Analysis was performed on a
269 vario Micro Cube device from Elementar. Carbon, Hydrogen, Nitrogen, Sulfur and Oxygen mass
270 proportion of CNF films were measured. Films pieces of 4 to 7 mg were weighted on a *micro-balance*.
271 An average of four measurements was made for each sample.

272 Conventional NMR analyses

273 Liquid ^1H and ^{13}C NMR analyses of the new prodrug were performed at ICMG (FR2607) Chemistry
274 Nanobio Platform, Grenoble. The results are detailed in SI.

275 Solid state ^{13}C nuclear magnetic resonance (^{13}C ssNMR) analyses were performed at the « Institute for
276 Nanoscience and Cryogenics (INAC) » in the «French Alternative Energies and Atomic Energy
277 Commission (CEA) » at Grenoble, on a Bruker AVANCE400 spectrometer. Acquisition, data treatment
278 and peaks deconvolution were done using the LINUX TopSpin 3.2 software. Samples were placed in 4
279 mm ZrO_2 rotors. All spectra were recorded using a combination of cross-polarization (CP), high power
280 proton decoupling and magic angle spinning (CP/MAS). ^{13}C NMR spectra were acquired at 298 K, with
281 a 4 mm probe operating at 100.13 MHz. The chemical shift values were measured with respect to TMS
282 via glycine as a secondary reference with the carbonyl signal set to 176.03 ppm. MAS was performed
283 at 14 kHz. The number of scans was 15 000 with a recycle delay of 2 s and CP time of 1.5 ms.

284 Dynamic Nuclear Polarization – NMR (DNP-NMR)

285 DNP experiments were performed on a 263 GHz/400 MHz AVANCE III Bruker DNP system at the « École
286 Polytechnique Fédérale de Lausanne (EPFL) ». The spectrometer is equipped with a low temperature
287 MAS probe and a 263 GHz gyrotron capable of outputting ca. 5-10 W of CW microwaves. The probe
288 was configured in double mode $^1\text{H}/^{13}\text{C}$. The sweep coil of the main magnetic field was optimized so
289 that microwave irradiation gave the maximum positive proton DNP enhancement for AMUPol (a
290 biradical polarizing agent). Proton and carbon DNP enhancements were determined by comparing the
291 intensity of their respective spectra acquired with and without microwave irradiation. For DNP
292 experiments, 30 mg of sample is impregnated with 16 μl of 10 mM AMUPol in $\text{D}_2\text{O}/\text{H}_2\text{O}$ (9/1 v/v). The
293 DNP sample is then packed in a 3.2 mm sapphire rotor and capped with a Teflon plug and zirconia cap.
294 The filled DNP rotor was then spun at room temperature in the spinning station up to 12.5 kHz before
295 being inserted into the pre-cooled (ca. 100 K) 3.2 mm low temperature MAS NMR probe, where the
296 sample is frozen within a few seconds.

297 For ^{13}C NMR experiments, the recycle delays were 3 or 4 s. The ^1H $\pi/2$ pulse length used for the variable
298 amplitude CP experiments was 2.5 μs to afford 100 kHz ^1H decoupling using SPINAL-64 (Fung et al.,
299 2000). The contact time was typically 5 ms. The MAS frequency used is 12.5 kHz.

300 For the 1D and 2D INADEQUATE experiments the corresponding sequences are those of TOPSPIN, the
301 NMR software proposed by Bruker Corporation. The 2D ^{13}C - ^{13}C INADEQUATE is the version proposed
302 by Lesage et al. adding DNP conditions (Cadars et al., 2007; Lesage et al., 1999). Tau delay,
303 synchronized with the rotation, was optimized and set to 4 ms. The number of scans was set to 64 and

304 the number of experiments in the indirect dimension to 128 and the recycling delay to 3s leading to
305 an experiment time in the range of 8 h.

306 Drug release in enzymatic medium

307 In order to confirm the controlled drug activation, the enzymatic release of metronidazole was
308 assessed from the suspension of CNF-metro. It was induced by the addition of a human
309 carboxylesterase 2 (hCE2), inspiring from a recent work with similar enzymatic release strategy (Bliman
310 et al., 2018). This enzyme favors the cleavage of the ester bond that binds metronidazole to the CNF-
311 metro and prodrug arm. CNF-metro suspension was centrifuged at 20 000 g for 10 min and re-
312 dispersed in TRIS buffer (pH 7.4, 50 mM). This operation was repeated 2 times. A volume of 10 ml of
313 CNF-metro suspension was poured into a balloon and heated at 37°C. The commercial enzyme solution
314 was diluted to 1 mg/ml with TRIS buffer and 2 ml of this solution was introduced in the CNF-metro
315 suspension. The mixture was maintained under magnetic stirring and at 37°C for 48 h. In parallel, a 10
316 ml CNF-metro suspension without the enzyme (also re-dispersed in TRIS buffer) was exposed to the
317 same condition, acting as a reference. Aliquots were collected from both balloons in order to assess
318 the influence of the enzyme addition on the release of the metronidazole. 500 µl aliquots were
319 collected at predetermined time intervals and centrifuged at 14 000g for 10 min and filtrated with
320 syringe filter (0.45 µm, polyamide/nylon, CHROMAFIL AO-45/3 MACHEREY NAGEL) before
321 characterization. Reverse-phase high performance liquid chromatography (HPLC) was performed with
322 a micro-bondapak-C18 analytical column (Waters Associates) to track the presence of metronidazole
323 in the aliquot. A Waters chromatographic system was used, with two M-510 pumps and photodiode
324 array detector Waters 996 using Millenium 32 software. A linear gradient from 10 to 100 % methanol
325 in H₂O pH 2.5 (phosphoric acid), 1 ml/min flow rate, was used.

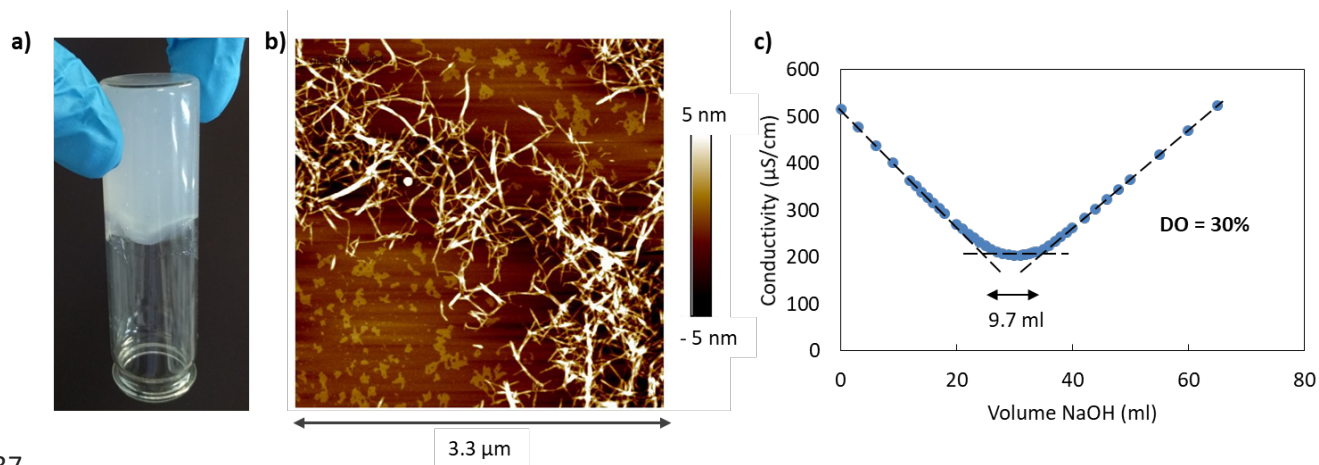
326 **Results and discussions**

327 Characterization of CNF-t starting materials

328 Considering the numerous commercially available CNF types and the wide variety of lab-scale
329 produced CNF, it is of high importance to precisely describe the properties and characteristics of the
330 CNF that are used in this work. Cellulose nanofibrils suspensions are often a mixture of nanoscale
331 particles, microfibrillated fibers, aggregates and residual fibers. The objective is to understand the
332 starting material and be able to draw relevant conclusions from the presented results.

333 The CNF-t suspension that was produced by the CTP has the appearance of a thick transparent gel at
334 1.6wt% concentration as depicted on Figure 2 . AFM images of the low concentrated CNF-t suspension

335 were also recorded in order to confirm the nanosize morphology of cellulose nanofibrils, as depicted
336 on **Erreur ! Source du renvoi introuvable.b**.



337

338 **Figure 2: CNF-t starting material characterization: a) picture of the thick 1.6 wt% gel, b) an AFM height sensor image of a**
339 **15.10⁻⁵ wt% suspension and c) results of the conductometric titration (DO: degree of oxidation)**

340 In order to assess the chemical reactivity of the CNF-t suspension, conductometric titration was
341 performed to measure the degree of oxidation (DO) and initial carboxylic acid group content X_{ox} . A
342 degree of oxidation of 30% and quantity of 1.7 mmol/g \pm 0.1 of carboxylic acid group was detected,
343 which confirmed the high surface charge arising from TEMPO-mediated oxidation.

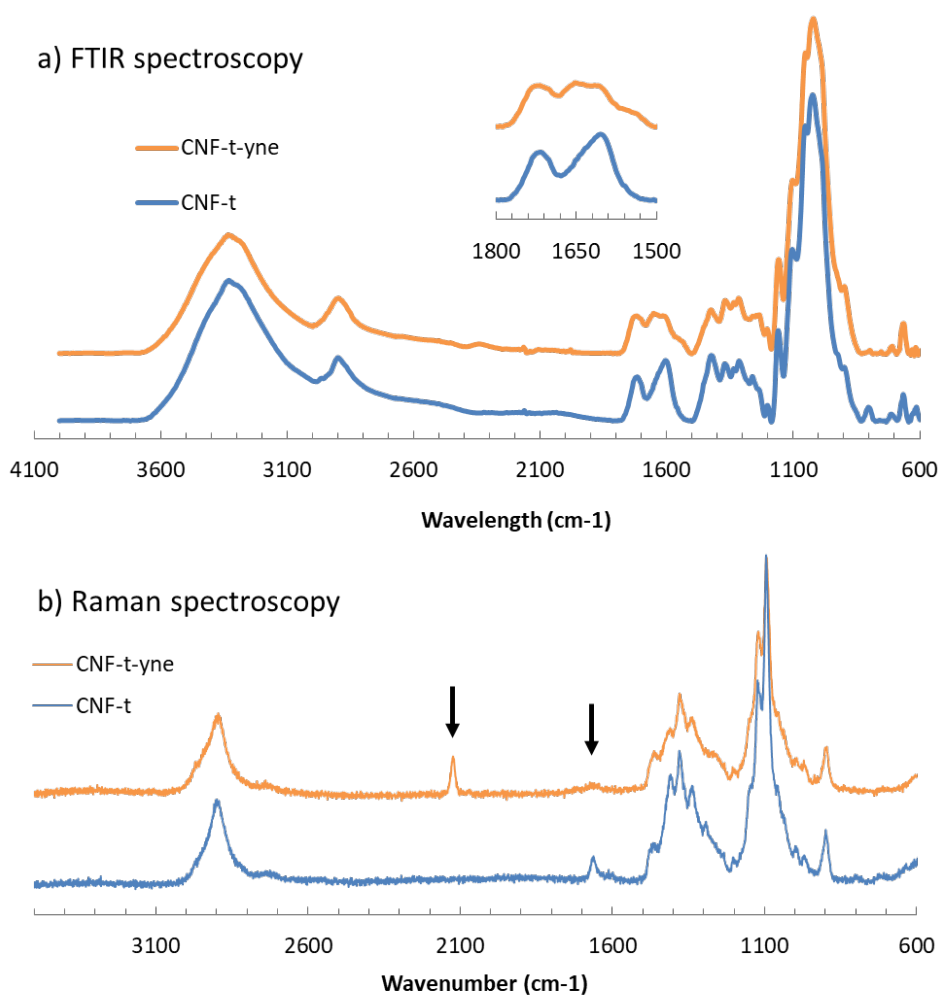
344 First step: Introduction of alkyne function through amidation

345 The first reaction of the two-step immobilization procedure involves the CNF-t gel, on which an
346 amidation reaction was performed with propargyl amine to tether pending alkyne groups. After
347 purification steps, characterization of the grafting was made, either by analyzing the resulting CNF-yne
348 suspension or CNF-yne dry films produced by solvent casting. Characterization techniques allowed to
349 confirm the presence of the propargyl amine and/or the covalent nature of the bond between CNF-t
350 and propargyl amine. We will first present the data that indicate the presence of alkyne groups, and
351 then discuss the data confirming the covalent nature of the bond and the success of the amidation.

352 Spectroscopies

353 Chemical structure of the resulting CNF-yne dry films was investigated through FTIR spectroscopy in
354 ATR mode. The surface of the films was then analyzed in order to reveal new chemical bonds. **Erreur !**
355 **Source du renvoi introuvable.a** shows FTIR spectra of CNF-t, CNF-yne and CNF-Metro. The three
356 spectra showed classical peaks for cellulose backbone: stretching of O-H and C-H bonds around 3300
357 cm^{-1} and 2900 cm^{-1} , C-O-C glycosidic bond stretching at 1160 cm^{-1} and C-O bonds at 1100, 1050 and
358 1030 cm^{-1} (Johan Foster et al., 2018). CNF-t IR spectrum also showed two clear peaks at 1602 cm^{-1} and
359 1715 cm^{-1} that were respectively associated with C=O of carboxylate and carboxylic acid groups. The

360 pH of the CNF-t suspension was adjusted to 3 before producing films for analysis thus explaining the
361 presence of carboxylic acid group signal. CNF-yne also showed the same peaks but with a lower
362 intensity. Moreover, another peak arising around 1655 cm^{-1} was attributed to the C=O bond of amide
363 I function, and a weak signal around 1550 cm^{-1} was due to the C-N bond of amide I function (Hoeng
364 et al., 2015). These data qualitatively proved the successful amidation reaction. Recent papers on
365 amidation of CNF-t showed similar behavior in FTIR experiments and based their main conclusions on
366 this characterization tool (Lavoine et al., 2017). In our case, as the shifts were limited, complementary
367 characterizations were performed.



368

369 **Figure 3: Spectroscopy results for a) FTIR-ATR and b) Raman spectra of CNF-t and CNF-yne samples**

370 While FTIR spectroscopy relies on the study of absorption or transmission of light on a wide range of
371 wavelength, Raman spectroscopy relies on the study of the inelastic scattering of a specific wavelength
372 light beam. This technique is complementary to the FTIR spectroscopy because some chemical bonds
373 are much more active in Raman spectroscopy and could hardly be detected with FTIR spectroscopy
374 and vice versa, especially when the proportion of the expected bond is low. This is the case of alkyne
375 bonds, which appeared to be more sensitive to Raman spectroscopy than in FTIR (Mangiante et al.,

2013). The Raman spectroscopy spectra are shown on **Erreur ! Source du renvoi introuvable.** Both spectra showed characteristic peaks of cellulosic materials: C-H stretching at 2900 cm^{-1} and C-O bridges around 1100 cm^{-1} (Agarwal, 2017). On CNF-t spectrum, the peak at 1660 cm^{-1} referred to the C=O of carboxylic acid groups. A clear signal appearing at 2120 cm^{-1} was attributed to the C \equiv C bond on the grafted propargyl-amine moiety (Mangiante et al., 2013). This signal was not detected on the ATR-FTIR spectra. Moreover, the signal of the C=O of the carboxylic acid was slightly flatten indicating its partial conversion to amide group. This Raman spectroscopy analysis further confirmed the success of the amidation.

384 Conductometric titration results

385 After the amidation reaction with propargyl amine, the remaining carboxylic group were also
386 quantified by conductometric titration and the residual degree of oxidation DO_{res} was calculated with
387 equation (3) (detailed in SI) by integrating the molecular weight of the propargylamine and the same
388 previous terms as it was done before on cellulosic nanomaterials (Hoeng et al., 2015).

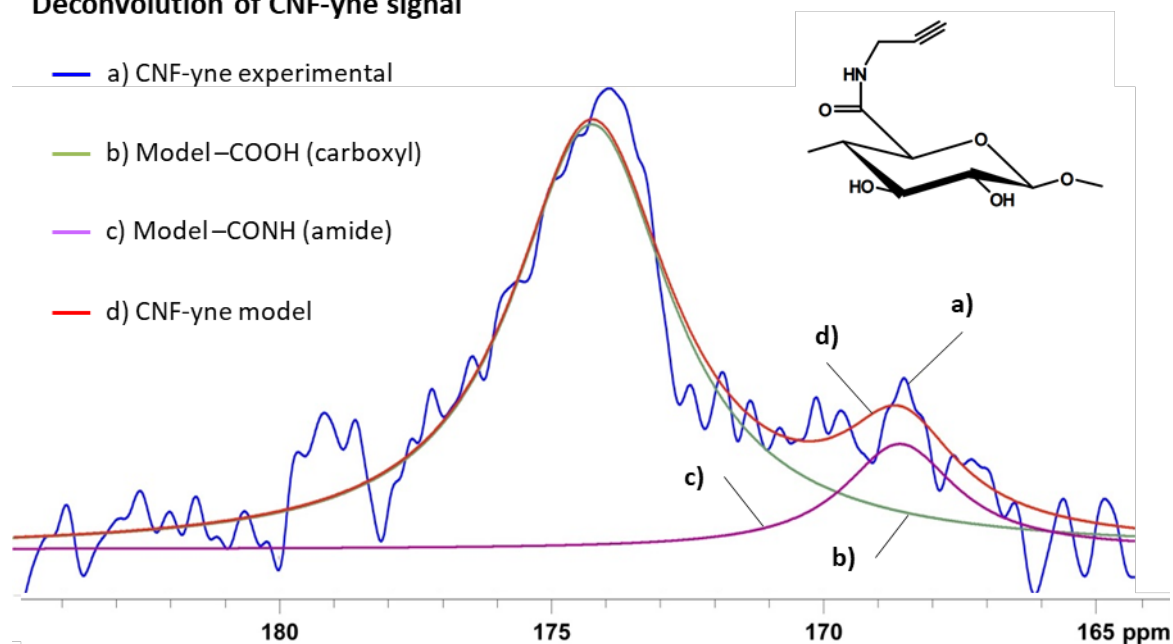
389 A value of 9% of residual carboxylic acid content DO_{res} was obtained. According to equation (4)
390 (detailed in SI), a proportion of 69% of initial carboxylic acid groups converted to amide function. This
391 first quantitative result indicates that more than two-third of the carboxylic acid moieties reacted to
392 become amide function. From the values of X_{ox} and $\%COOH_{\text{conv}}$ obtained with equations (3) and (4), it
393 was possible to calculate with equation (5) a degree of substitution based on conductometric titration
394 measurements DS_{cond} (detailed in SI). The degree of substitution based on conductometric titration
395 was calculated with equation (5) and revealed to be $DS_{\text{cond}} = 0.19$, which is in accordance with
396 commonly reported results for this kind of heterogeneous chemical grafting (Missoum et al., 2013; Rol
397 et al., 2018).

398 Solid state ^{13}C NMR

399 It was also used to further identify the composition of CNF-t and CNF-yne. The full signals of solid state
400 ^{13}C NMR are available in SI and display the expected peaks for cellulose based material. The discussion
401 here will focus on the 165-180 ppm region where carboxyl signals are usually detected. Deconvolutions
402 of the ^{13}C NMR signals for CNF-t and CNF-yne are showed on Figure 4. The peak at 174 ppm was
403 associated with the carbon of the carboxylic acid groups of CNF-t (Montanari et al., 2005). This peak
404 was slightly shifted on CNF-yne signal, indicating a change of chemical environment due to the
405 amidation reaction. A deconvolution technique was applied to unveil the contributions that explain
406 this shift. After deconvolution, the experimental CNF-yne signal displayed on Figure 4d can be
407 simulated by a combination of carboxylic acid and amide model contributions. The overlap obtained

408 was as high as 98.72%, confirming the formation of the amide function on the CNF-t and the successful
409 covalent immobilization of alkyne function on CNF.

Deconvolution of CNF-yne signal



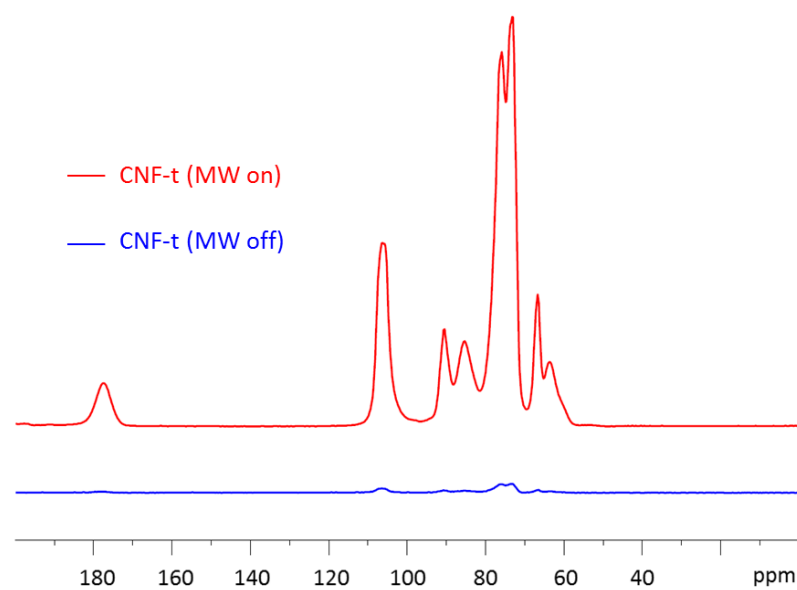
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411 **Figure 4: Deconvolution of solid state ^{13}C NMR signal for CNF-yne, a) the experimental signal, b) and c) the model**
412 **carboxyl and amide signals respectively and d) the calculated signal after deconvolution based on b) and c)**

413

414 Dynamic nuclear polarization enhanced NMR (DNP-NMR)

415 This hyperpolarization method can increase sensitivity of solid state NMR by several orders of
416 magnitude (Rossini et al., 2013). As a proof of its potential, Figure 5 compares the spectra of CNF-t
417 without and with the microwave (MW) irradiation that allows the dynamic nuclear polarization
418 transfer. The acquisition time of such signals is also very fast in comparison with conventional solid
419 state NMR. When several hours are necessary to increase signal to noise ratio in conventional solid-
420 state NMR, only minutes-long acquisition of DNP enhanced NMR result in sharp and fine signals. As a
421 matter of fact, in this example we could reach a 55 fold enhancement of signal to noise ratio. On a
422 practical point of view in order to reach similar signal to noise ratio without DNP technique it would
423 have been necessary to multiply the number of accumulated transients by 3000. This is the reason why
424 2D ^{13}C INADEQUATE experiments in the solid phase become possible in natural abundance.



425

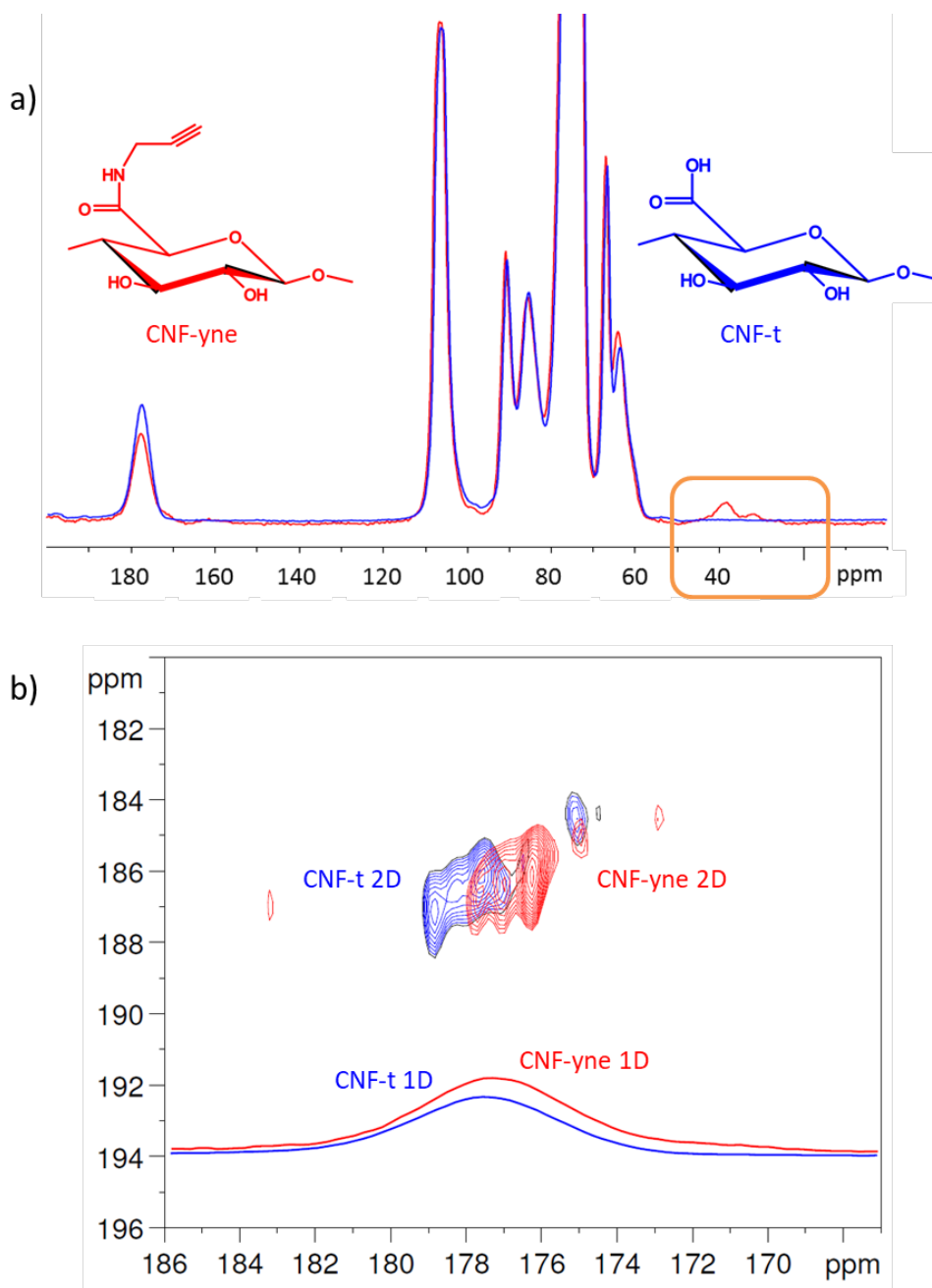
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Figure 5: ^{13}C DNP-NMR spectra for CNF-t, with (red) and without(blue) microwave irradiation(μwave)

427

428 The first result arising from DNP enhanced NMR was a further confirmation of the success of the
 429 amidation. Well defined signals appeared on CNF-yne spectra of Figure 6a. Two peaks around 32 and
 430 38 ppm were attributed to the aliphatic $-\text{CH}_2-$ carbons introduced with propargylamine immobilization
 431 on CNF-t. These peaks were barely visible on conventional solid-state NMR. The carbons involved in
 432 the alkyne function are not visible since they are supposed to be located around 70-79 ppm, which
 433 overlaps with the C2, C3 and C5 of cellulose backbone.

434 Moreover, as explained above, DNP enhanced NMR allows the acquisition of 2D INADEQUATE ^{13}C - ^{13}C
 435 analysis with ^{13}C natural abundance. For instance, this analysis was used to study the region of carbons
 436 involved in carbonyls of CNF-t and CNF-yne samples. Figure 6b shows the shift in the signal indicating
 437 the presence of a new type of carbon. This result confirmed the deconvolution treatment described
 438 above regarding the apparition of a signal due to the amidation reaction. This further proves the
 439 success of the amidation reaction.



440

441 **Figure 6: a) ^{13}C DNP-NMR spectra of CNF-t and CNF-yne. Aliphatic $-\text{CH}_2-$ carbons arises from CNF-yne signal and b) 2D ^{13}C -**
 442 **^{13}C inadequate DNP-NMR analysis of CNF-t and CNF-yne. The shift indicates the apparition of a carbon involved in an**
 443 **amide function.**

444 Second step: thiol-yne click chemistry reaction

445 The second step of the grafting strategy involved a click reaction to anchor the thiol-modified Metro-
 446 SH to the CNF-yne. As depicted in Figure 1, the CNF-metro product displays only one molecule of thiol-
 447 modified drug per residue while the alkyne group is supposed to bind up to two thiol containing
 448 molecules by thiol-yne click reaction. However, considering the strong steric hindrance and the
 449 heterogeneous conditions of the chemistry that happened here, the binding of two molecules on the

450 alkyne group is probably strongly disfavored. We therefore depicted the result of the single addition
451 even if double addition was not completely ruled out.

452 Elemental analysis

453 Carbon, hydrogen, nitrogen and sulfur mass proportion were measured for each sample. Elemental
454 composition is available for the first step amidation reaction. However, the amount of nitrogen arising
455 from the new amide function competes with the amount of nitrogen contained in potential residual
456 adsorbed coupling agents EDC/NHS and reaction by-products such as EDC-urea and NHS-ester, despite
457 thorough washing steps (Fujisawa et al., 2011; Kumar et al., 2020). The detection of nitrogen on CNF-
458 yne, compared to CNF-t, supports the presence of the propargyl amine and its alkyne function at best.
459 Nevertheless, when it comes to discuss the outcome of the thiol-yne click chemistry, elemental analysis
460 provided more robust information, especially for sulfur atoms since they are introduced for the first
461 time in the chemical sequence. Considering the amount of nitrogen and sulfur atoms that were
462 theoretically introduced at this second step, the confirmation of the presence of the Metro-SH on the
463 CNF-yne was clearly set. In fact on Table 1, a significant increase of nitrogen content in comparison
464 with the CNF-yne was detected, going from 1.15 % \pm 0.05 to 1.42 % \pm 0.03. Moreover, in the second
465 step, no nitrogen-containing chemical agents were introduced confirming that the increase of this
466 element is only due to the presence of Metro-SH. In parallel, sulfur was also detected at 0.37% \pm 0.01
467 while it was not detectable on CNF-yne. Also, the final ratio between the quantity of nitrogen and
468 sulfur atoms must be close to 4 according to the chemical structure of CNF-Metro. The elemental
469 analysis results gave a ratio of $1.42/0.37 = 3.8$, which roughly fits with the expected result. The
470 progressive increase of nitrogen and the detection of sulfur both confirmed the immobilization of the
471 Metro-SH onto the CNF.

472 **Table 1: Elemental analysis results for carbon, hydrogen, nitrogen and sulfur mass proportion of the various CNF samples**

	% C	<i>sd</i>	% H	<i>sd</i>	% N	<i>sd</i>	% S	<i>sd</i>
CNF-t	37.04	<i>0.02</i>	5.34	<i>0.18</i>	< 0.10	-	< 0.10	-
CNF-yne	40.59	<i>0.13</i>	5.69	<i>0.22</i>	1.15	<i>0.05</i>	< 0.10	-
CNF-metro	40.99	<i>0.04</i>	6.02	<i>0.09</i>	1.42	<i>0.03</i>	0.37	<i>0.01</i>

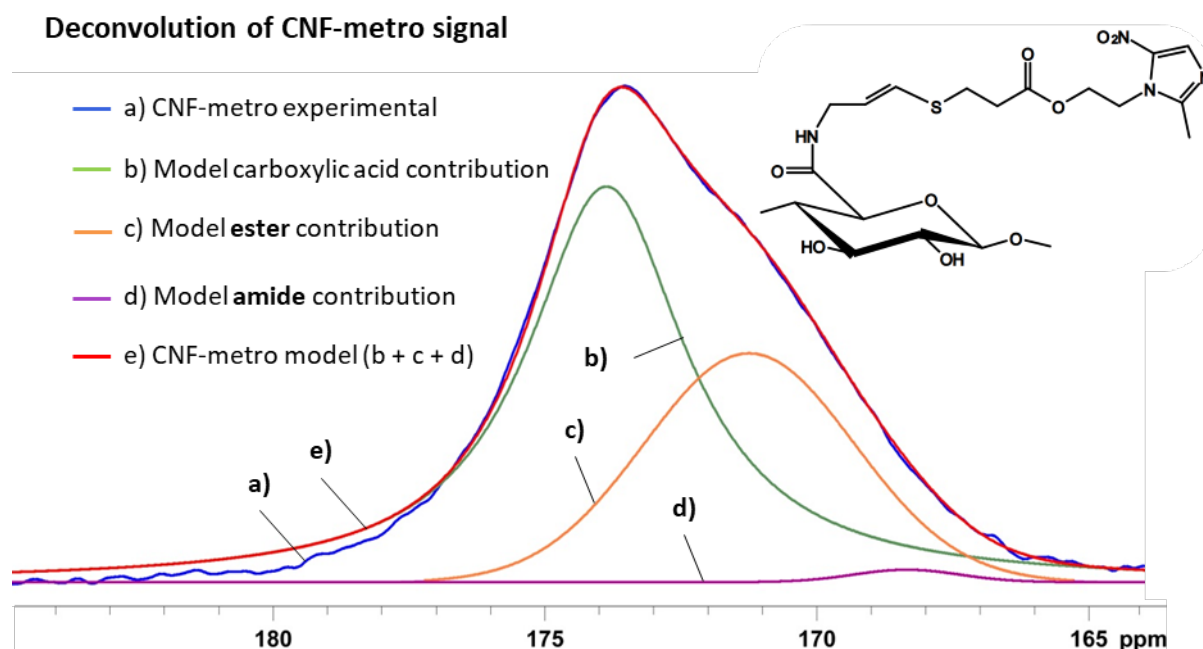
473

474

475 Solid state ^{13}C NMR

476 ^{13}C NMR was used to further identify the composition of CNF-metro sample. On **Erreur ! Source du**
477 **renvoi introuvable.**4, in the region corresponding to carbons involved in C=O around 170 ppm, we
478 noticed a difference of the shape of the CNF-yne signal suggesting a new chemical environment. The
479 deconvolution technique was used again to clarify this result. The CNF-metro model signal (red)
480 displayed on Figure 7 perfectly fits with the low signal to noise experimental signal of CNF-metro (blue).
481 A combination of model carboxylic acid, amide and ester contributions allowed the overlap to reach
482 98.9%, confirming indirectly the presence of an ester function. The thiol-modified drug is the only
483 compound that contained such a chemical function. This is a complementary proof that the
484 immobilization of the modified metronidazole on the cellulosic substrate was successful.

485



486

487 **Figure 7: Deconvolution of solid state ^{13}C NMR signal for CNF-metro, a) the experimental signal, b), c) and d) the model**
488 **carboxyl, ester and amide signals respectively and e) the reconstructed model signal based on b), c) and d)**

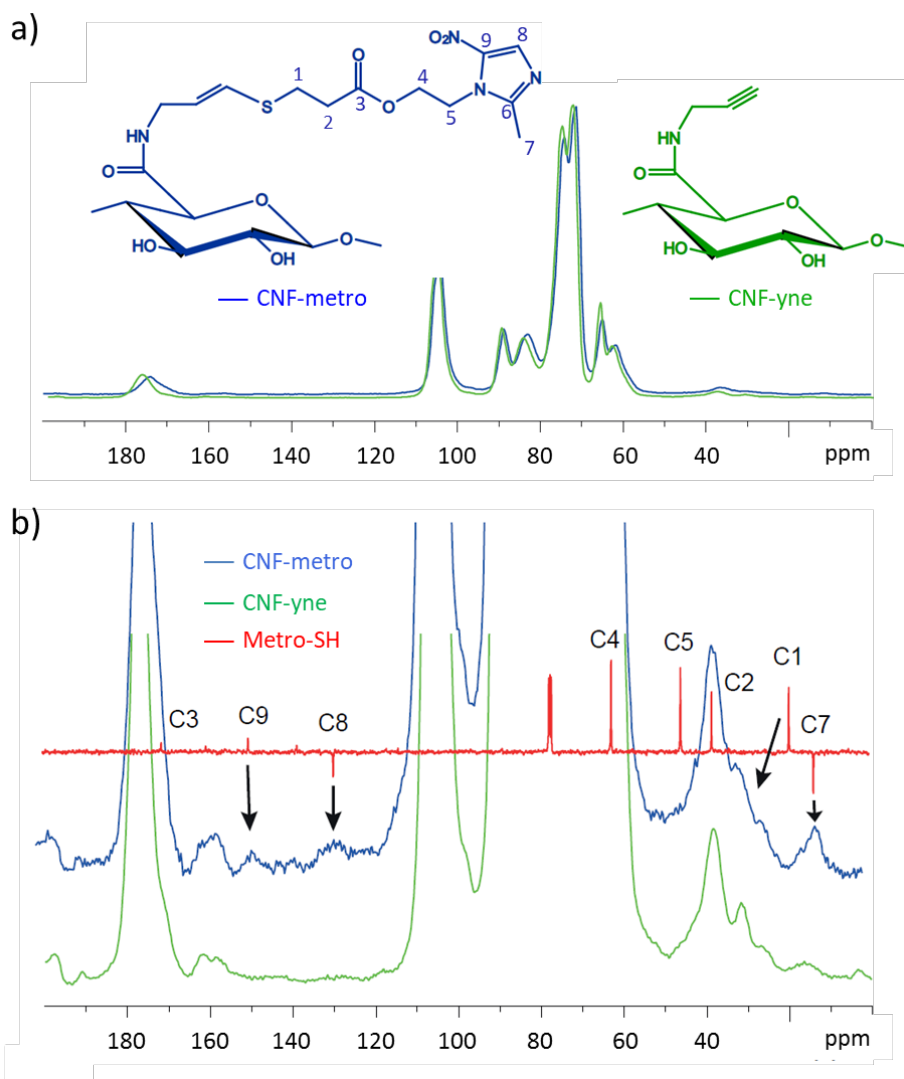
489

490 Dynamic nuclear polarization enhanced NMR (DNP-NMR)

491 CNF-metro was also analyzed with DNP-NMR. Similarly to the previous conventional NMR signal
492 discussed above, a shift can be noticed in the carbonyls region around 175 ppm on Figure 8a, which
493 suggests the same modification of chemical environment for CNF-metro. The analysis provided access
494 to the superfine signals hidden in the noise of conventional solid state NMR as clearly displayed on
495 Figure 8b. In order to perform the attribution of each new detected peak in CNF-metro signal, the

496 liquid NMR spectrum of thiol-modified metronidazole was added in red. A JMOD sequence was used
497 to facilitate the attribution of carbons involved in -CH- and -CH₃- functions since they appear with
498 opposite phase compared to CH₂ signals. The peaks that arise around 12 ppm and 128 ppm were thus
499 easily attributed to C7 and C8. Moreover, C9 carbon matches the peak of CNF-metro at 150 ppm. These
500 peaks indicate again the immobilization of the prodrug molecule on the CNF-metro samples. However,
501 the nature of the grafting was still to be confirmed since both covalently bound prodrug and adsorbed
502 prodrug can be present.

503 Finally, the C1 signal at 21 ppm on Metro-SH red signal on Figure 8b was not visible on the spectrum
504 of CNF-metro, despite the enhanced sensitivity of DNP. In parallel, we can observe a wider signal for -
505 CH₂- in comparison with CNF-yne spectrum between 25 and 45 ppm. The C1 signal has been shifted
506 to higher chemical shifts and this is fully consistent with the thiol-yne reaction that replaced the proton
507 on the sulfur atom by an -CH=CH- group. These results are a good indication of the covalent nature of
508 the binding of the prodrug molecule to the alkyne groups of the CNF substrate through thiol-yne click
509 chemistry. This study also highlighted the unprecedented potential of DNP-NMR to detect very small
510 differences in solid material functionalization. This technique allowed us to unambiguously confirm
511 the two-step covalent immobilization of the prodrug on cellulose nanofibrils substrate in aqueous
512 media.



513

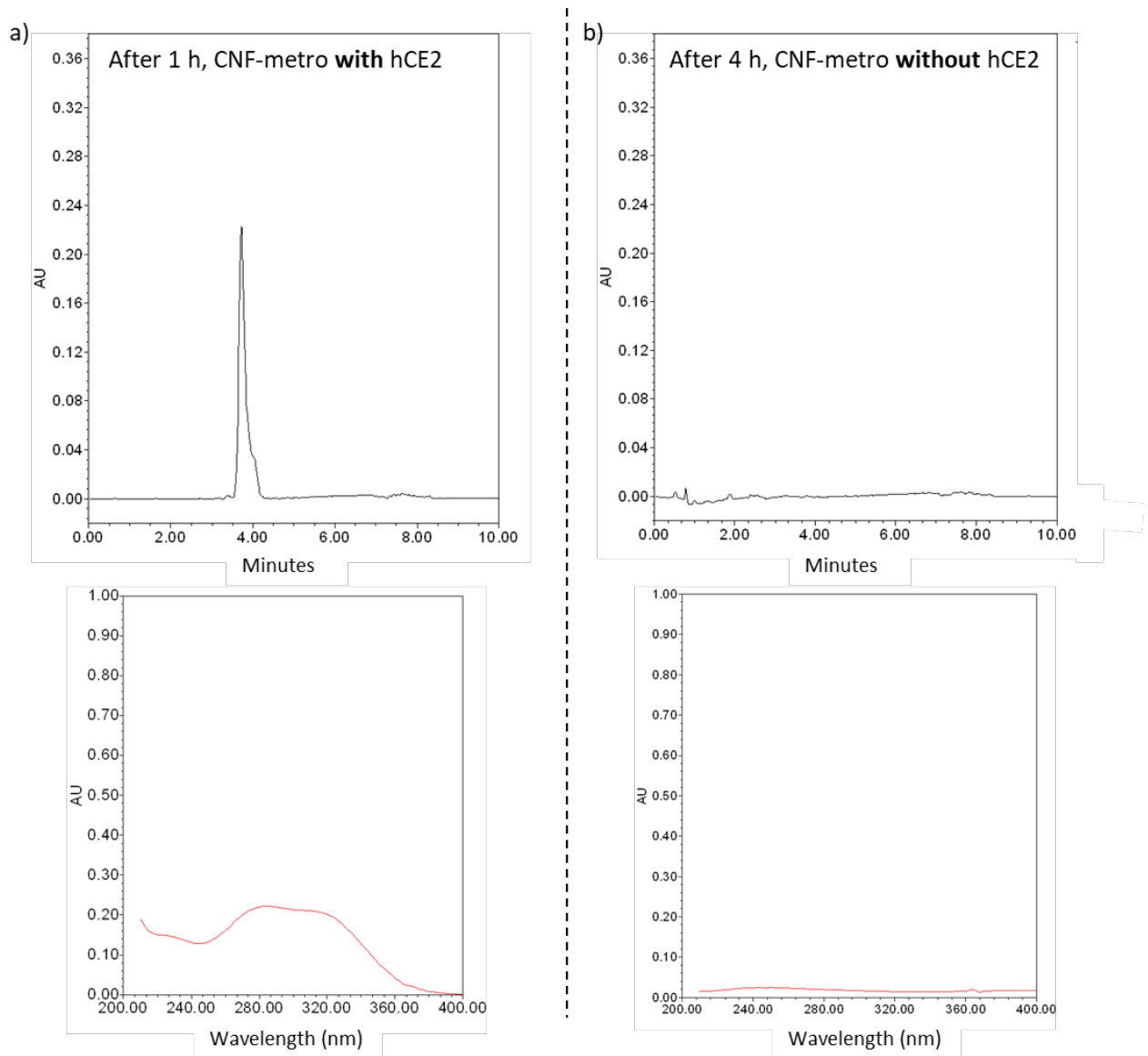
514 **Figure 8: a) DNP enhanced ^{13}C NMR of CNF-yne and CNF-metro samples and b) the same signals completed with the**
 515 **liquid ^{13}C NMR spectrum of the prodrug Metro-SH containing the metronidazole molecule, a JMOD sequence was used to**
 516 **give negative -CH- and -CH₃- signals**

517

518 Proof of concept: esterase catalyzed in situ drug release

519 The release of metronidazole under enzymatic stimuli was analyzed. Indeed, infection sites have
 520 important enzymatic activity that may be useful for prodrug activation through ester bond cleavage.
 521 Two suspensions of CNF-metro were maintained at 37°C for 48 h under magnetic stirring. Human
 522 carboxylesterase 2 was added in one of the two suspensions for releasing the metronidazole grafted
 523 on CNF substrate, through the cleavage of the ester function. Aliquots of the suspensions were
 524 analyzed by HPLC analysis. After 1 h of agitation, a clear signal with a 3.7 minute retention time (RT)
 525 was obtained with the suspension that contains the enzyme, as illustrated on Figure 9a. By comparison
 526 with the HPLC data of metronidazole itself (results not shown), this signal (both RT and UV-Visible
 527 maximum at 283.9 nm) can be attributed to the release of the metronidazole. The CNF-metro

528 suspension that was gently stirred in the absence of the enzyme was also analyzed with HPLC.
529 However, even after 4 h, no trace of the metronidazole was detected as confirmed by both the
530 chromatogram and UV spectrum of Figure 9b.



531

532 **Figure 9: HPLC chromatograms (diode-array detector) (top) and associated UV spectra (bottom) of CNF-metro**
533 **suspensions reacted a) with, and b) without the presence of carboxylesterase human 2 (hCE2)**

534 This preliminary studies would deserve more investigation however the main outcome is that CNF-
535 metro suspension, when exposed to hCE2 enzyme, is able to release metronidazole molecule in one
536 hour. This result validates our starting hypothesis of controlled release of metronidazole from CNF
537 under enzymatic activity.

538

539 **Conclusion**

540 In this work, a multistep immobilization procedure was proposed to graft thiol modified metronidazole
541 prodrug onto TEMPO oxidized cellulose nanofibrils. The first step was an amidation reaction between
542 carboxylic acid groups of CNF-t mainly confirmed by Raman spectroscopy, Solid state NMR and
543 especially DNP enhanced NMR, with 2D analysis. Thus, an alkyne function was introduced at the
544 surface of CNF. This key intermediate alkyne CNF opened the way to a range of chemical
545 functionalization such as the thiol-yne reaction with thiol containing metronidazole prodrug described
546 here, or a Michael-type addition of a suitably thiol- or amine-substituted prodrug. The presence of the
547 prodrug was then confirmed by elemental analysis, conventional solid-state NMR and, more
548 importantly DNP enhanced NMR that appeared as the ultimate tool to confirm the covalent nature of
549 the grafting of the prodrug onto CNF substrates. These results further expand the strength of DNP
550 enhanced NMR in the field of material characterization. Finally, the in situ drug release, arising from
551 the presence of the cleavable ester function, was validated with the human carboxylesterase 2
552 medium, thus confirming that the presence of the CNF material did not prevent the enzyme activity.
553 Following this work, the modified CNF system can be implemented in the design of innovative medical
554 devices. Inspiring from commercial collagen-based implantable meshes, a composite of the prodrug
555 modified CNF and collagen has been prepared to obtain an in situ triggered release of the drug (data
556 not shown). The prototypes shows efficient preliminary antibacterial activity against anaerobic
557 bacteria, confirming the particular potential of CNF-based medical devices in implantable applications.

558 **Supporting information**

559 The following data may be found in the Supporting Information file

- 560 • Conductometric titration of CNF-yne
- 561 • Detailed calculation for DO_{res} , $\%COOH_{conv.}$ and DS_{cond}
- 562 • Liquid 1H and liquid ^{13}C NMR methods
- 563 • Prodrug characterization
- 564 • Solid state ^{13}C NMR on CNF-t, CNF-yne and CNF-metro
- 565 • 2D INADEQUATE for amidation confirmation

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575 **References**

- 576 Agarwal, U. P. (2017). Raman Spectroscopy of CNC-and CNF-Based Nanocomposites. *Handbook of*
577 *Nanocellulose and Cellulose Nanocomposites*, 609–625.
- 578 Aulin, C., Gällstedt, M., & Lindström, T. (2010). Oxygen and oil barrier properties of microfibrillated
579 cellulose films and coatings. *Cellulose*, *17*(3), 559–574. [https://doi.org/10.1007/s10570-009-](https://doi.org/10.1007/s10570-009-9393-y)
580 [9393-y](https://doi.org/10.1007/s10570-009-9393-y)
- 581 Bardet, R., & Bras, J. (2014). Cellulose Nanofibers and Their Use in Paper Industry. In K. Oksman, A. P.
582 Mathew, A. Bismarck, O. Rojas, & M. Sain, *Materials and Energy* (Vol. 5, pp. 207–232). WORLD
583 SCIENTIFIC. https://doi.org/10.1142/9789814566469_0013
- 584 Bliman, D., Demeunynck, M., Leblond, P., Meignan, S., Baussane, I., & Fort, S. (2018). Enzymatically
585 Activated Glyco-Prodrugs of Doxorubicin Synthesized by a Catalysis-Free Diels–Alder Reaction.
586 *Bioconjugate Chemistry*, *29*(7), 2370–2381.
587 <https://doi.org/10.1021/acs.bioconjchem.8b00314>
- 588 Boufi, S., González, I., Delgado-Aguilar, M., Tarrès, Q., Pèlach, M. À., & Mutjé, P. (2016). Nanofibrillated
589 cellulose as an additive in papermaking process: A review. *Carbohydrate Polymers*, *154*, 151–
590 166. <https://doi.org/10.1016/j.carbpol.2016.07.117>
- 591 Cadars, S., Sein, J., Duma, L., Lesage, A., Pham, T. N., Baltisberger, J. H., Brown, S. P., & Emsley, L. (2007).
592 The refocused INADEQUATE MAS NMR experiment in multiple spin-systems: Interpreting
593 observed correlation peaks and optimising lineshapes. *Journal of Magnetic Resonance (San*
594 *Diego, Calif.: 1997)*, *188*(1), 24–34. <https://doi.org/10.1016/j.jmr.2007.05.016>

595 Campos-Aldrete, M. E., & Villafuerte-Robles, L. (1997). Influence of the viscosity grade and the particle
596 size of HPMC on metronidazole release from matrix tablets. *European Journal of*
597 *Pharmaceutics and Biopharmaceutics*, 43(2), 173–178. <https://doi.org/10.1016/S0939->
598 6411(96)00004-5

599 Celebi, D., Guy, R. H., Edler, K. J., & Scott, J. L. (2016). Ibuprofen delivery into and through the skin from
600 novel oxidized cellulose-based gels and conventional topical formulations. *Int. J. Pharm.*,
601 514(1), 238–243. <https://doi.org/10.1016/j.ijpharm.2016.09.028>

602 da Silva Perez, D., Montanari, S., & Vignon, M. R. (2003). TEMPO-Mediated Oxidation of Cellulose III.
603 *Biomacromolecules*, 4(5), 1417–1425. <https://doi.org/10.1021/bm034144s>

604 Du, H., Liu, W., Zhang, M., Si, C., Zhang, X., & Li, B. (2019). Cellulose nanocrystals and cellulose
605 nanofibrils based hydrogels for biomedical applications. *Carbohydrate Polymers*, 209, 130–
606 144. <https://doi.org/10.1016/j.carbpol.2019.01.020>

607 Dufresne, A., Thomas, S., & Pothan, L. A. (2013). *Biopolymer Nanocomposites: Processing, Properties,*
608 *and Applications*. John Wiley & Sons.

609 Fujisawa, S., Okita, Y., Saito, T., Togawa, E., & Isogai, A. (2011). Formation of N-acylureas on the surface
610 of TEMPO-oxidized cellulose nanofibril with carbodiimide in DMF. *Cellulose*, 18(5), 1191.
611 <https://doi.org/10.1007/s10570-011-9578-z>

612 Fung, B. M., Khitrin, A. K., & Ermolaev, K. (2000). An Improved Broadband Decoupling Sequence for
613 Liquid Crystals and Solids. *Journal of Magnetic Resonance*, 142(1), 97–101.
614 <https://doi.org/10.1006/jmre.1999.1896>

615 Gates, K. A., Grad, H., Birek, P., & Lee, P. I. (1994). A New Bioerodible Polymer Insert for the Controlled
616 Release of Metronidazole. *Pharmaceutical Research*, 11(11), 1605–1609.
617 <https://doi.org/10.1023/A:1018913921956>

618 Guo, J., Filpponen, I., Johansson, L.-S., Heißler, S., Li, L., Levkin, P., & Rojas, O. J. (2018). Micro-patterns
619 on nanocellulose films and paper by photo-induced thiol–yne click coupling: A facile method

620 toward wetting with spatial resolution. *Cellulose*, 25(1), 367–375.
621 <https://doi.org/10.1007/s10570-017-1593-2>

622 Habibi, Y. (2014). Key advances in the chemical modification of nanocelluloses. *Chem. Soc. Rev.*, 43(5),
623 1519–1542. <https://doi.org/10.1039/C3CS60204D>

624 Halib, N., Perrone, F., Cemazar, M., Dapas, B., Farra, R., Abrami, M., Chiarappa, G., Forte, G., Zanconati,
625 F., Pozzato, G., Murena, L., Fiotti, N., Lapasin, R., Cansolino, L., Grassi, G., & Grassi, M. (2017).
626 Potential Applications of Nanocellulose-Containing Materials in the Biomedical Field.
627 *Materials*, 10(8), 977. <https://doi.org/10.3390/ma10080977>

628 Hoeng, F., Denneulin, A., & Bras, J. (2016). Use of nanocellulose in printed electronics: A review.
629 *Nanoscale*, 8(27), 13131–13154. <https://doi.org/10.1039/C6NR03054H>

630 Hoeng, F., Denneulin, A., Neuman, C., & Bras, J. (2015). Charge density modification of carboxylated
631 cellulose nanocrystals for stable silver nanoparticles suspension preparation. *J. Nanoparticle*
632 *Res.*, 17(6). <https://doi.org/10.1007/s11051-015-3044-z>

633 Isogai, A., Saito, T., & Fukuzumi, H. (2011). TEMPO-oxidized cellulose nanofibers. *Nanoscale*, 3(1), 71–
634 85. <https://doi.org/10.1039/C0NR00583E>

635 Itiola, O. A., & Pilpel, N. (1986). Tableting characteristics of metronidazole formulations. *Int. J. Pharm.*,
636 31(1), 99–105. [https://doi.org/10.1016/0378-5173\(86\)90218-8](https://doi.org/10.1016/0378-5173(86)90218-8)

637 Johan Foster, E., J. Moon, R., P. Agarwal, U., J. Bortner, M., Bras, J., Camarero-Espinosa, S., J. Chan, K.,
638 D. Clift, M. J., D. Cranston, E., J. Eichhorn, S., M. Fox, D., Y. Hamad, W., Heux, L., Jean, B., Korey,
639 M., Nieh, W., J. Ong, K., S. Reid, M., Renneckar, S., ... Youngblood, J. (2018). Current
640 characterization methods for cellulose nanomaterials. *Chem. Soc. Rev.*, 47(8), 2609–2679.
641 <https://doi.org/10.1039/C6CS00895J>

642 Jorfi, M., & Foster, E. J. (2015). Recent advances in nanocellulose for biomedical applications. *Journal*
643 *of Applied Polymer Science*, 132(14), n/a-n/a. <https://doi.org/10.1002/app.41719>

644 Kargarzadeh, H., Mariano, M., Gopakumar, D., Ahmad, I., Thomas, S., Dufresne, A., Huang, J., & Lin, N.
645 (2018). Advances in cellulose nanomaterials. *Cellulose*, 25(4), 2151–2189.
646 <https://doi.org/10.1007/s10570-018-1723-5>

647 Kolb, H. C., Finn, M. G., & Sharpless, K. B. (2001). Click Chemistry: Diverse Chemical Function from a
648 Few Good Reactions. *Angewandte Chemie International Edition*, 40(11), 2004–2021.
649 [https://doi.org/10.1002/1521-3773\(20010601\)40:11<2004::AID-ANIE2004>3.0.CO;2-5](https://doi.org/10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5)

650 Kumar, A., Durand, H., Zeno, E., Balsollier, C., Watbled, B., Sillard, C., Fort, S., Baussanne, I., Belgacem,
651 N., Lee, D., Hediger, S., Demeunynck, M., Bras, J., & De Paëpe, G. (2020). The surface chemistry
652 of a nanocellulose drug carrier unravelled by MAS-DNP. *Chemical Science*, 11(15), 3868–3877.
653 <https://doi.org/10.1039/C9SC06312A>

654 Laurén, P. (2018). *Biomedical applications of nanofibrillar cellulose (Ph.D. thesis)*.
655 <https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwjOtPzOjsPFAhWuTxUIHbgSAZAQFjAAegQIBhAC&url=https%3A%2F%2Fhelda.helsinki.fi%2Fbitstream%2Fhandle%2F10138%2F235619%2FBiomedic.pdf%3Fsequence%3D1%26isAllowed%3Dy&usg=AOvVaw3GZ5igbi7YuuPcEB1TFcgA>

659 Lavoine, N., Bras, J., Saito, T., & Isogai, A. (2017). Optimization of preparation of thermally stable
660 cellulose nanofibrils via heat-induced conversion of ionic bonds to amide bonds. *Journal of*
661 *Polymer Science Part A: Polymer Chemistry*, 55(10), 1750–1756.
662 <https://doi.org/10.1002/pola.28541>

663 Lavoine, N., Desloges, I., Manship, B., & Bras, J. (2015). Antibacterial paperboard packaging using
664 microfibrillated cellulose. *J. Food Sci. Technol.*, 52(9), 5590–5600.

665 Lesage, A., Bardet, M., & Emsley, L. (1999). Through-Bond Carbon–Carbon Connectivities in Disordered
666 Solids by NMR. *Journal of the American Chemical Society*, 121(47), 10987–10993.
667 <https://doi.org/10.1021/ja992272b>

668 Lin, N., & Dufresne, A. (2014). Nanocellulose in biomedicine: Current status and future prospect.
669 *European Polymer Journal*, 59, 302–325. <https://doi.org/10.1016/j.eurpolymj.2014.07.025>

670 Mangiante, G., Alcouffe, P., Burdin, B., Gaborieau, M., Zeno, E., Petit-Conil, M., Bernard, J., Charlot, A.,
671 & Fleury, E. (2013). Green Nondegrading Approach to Alkyne-Functionalized Cellulose Fibers
672 and Biohybrids Thereof: Synthesis and Mapping of the Derivatization. *Biomacromolecules*,
673 *14*(1), 254–263. <https://doi.org/10.1021/bm3016829>

674 *Metronidazole: MedlinePlus Drug Information*. (n.d.). Retrieved August 30, 2018, from
675 <https://medlineplus.gov/druginfo/meds/a689011.html>

676 Missoum, K., Belgacem, M. N., & Bras, J. (2013). Nanofibrillated Cellulose Surface Modification: A
677 Review. *Materials*, *6*(5), 1745–1766. <https://doi.org/10.3390/ma6051745>

678 Montanari, S., Roumani, M., Heux, L., & Vignon, M. R. (2005). Topochemistry of Carboxylated Cellulose
679 Nanocrystals Resulting from TEMPO-Mediated Oxidation. *Macromolecules*, *38*(5), 1665–1671.
680 <https://doi.org/10.1021/ma048396c>

681 Navarro, J. R. G., Conzatti, G., Yu, Y., Fall, A. B., Mathew, R., Edén, M., & Bergström, L. (2015). Multicolor
682 Fluorescent Labeling of Cellulose Nanofibrils by Click Chemistry. *Biomacromolecules*, *16*(4),
683 1293–1300. <https://doi.org/10.1021/acs.biomac.5b00083>

684 Nordli, H. R., Chinga-Carrasco, G., Rokstad, A. M., & Pukstad, B. (2016). Producing ultrapure wood
685 cellulose nanofibrils and evaluating the cytotoxicity using human skin cells. *Carbohydrate*
686 *Polymers*, *150*, 65–73. <https://doi.org/10.1016/j.carbpol.2016.04.094>

687 Pahimanolis, N., Hippi, U., Johansson, L.-S., Saarinen, T., Houbenov, N., Ruokolainen, J., & Seppälä, J.
688 (2011). Surface functionalization of nanofibrillated cellulose using click-chemistry approach in
689 aqueous media. *Cellulose*, *18*(5), 1201–1212. <https://doi.org/10.1007/s10570-011-9573-4>

690 Paukkonen, H., Kunnari, M., Laurén, P., Hakkarainen, T., Auvinen, V.-V., Oksanen, T., Koivuniemi, R.,
691 Yliperttula, M., & Laaksonen, T. (2017). Nanofibrillar cellulose hydrogels and reconstructed
692 hydrogels as matrices for controlled drug release. *International Journal of Pharmaceutics*,
693 *532*(1), 269–280. <https://doi.org/10.1016/j.ijpharm.2017.09.002>

694 Pubchem. (n.d.). *Metronidazole*. Retrieved August 30, 2018, from
695 <https://pubchem.ncbi.nlm.nih.gov/compound/4173>

696 Rol, F., Belgacem, M. N., Gandini, A., & Bras, J. (2018). Recent advances in surface-modified cellulose
697 nanofibrils. *Progress in Polymer Science*. <https://doi.org/10.1016/j.progpolymsci.2018.09.002>

698 Rossini, A. J., Zagdoun, A., Lelli, M., Lesage, A., Copéret, C., & Emsley, L. (2013). Dynamic Nuclear
699 Polarization Surface Enhanced NMR Spectroscopy. *Accounts of Chemical Research*, *46*(9),
700 1942–1951. <https://doi.org/10.1021/ar300322x>

701 Saito, T., Nishiyama, Y., Putaux, J.-L., Vignon, M., & Isogai, A. (2006). Homogeneous Suspensions of
702 Individualized Microfibrils from TEMPO-Catalyzed Oxidation of Native Cellulose.
703 *Biomacromolecules*, *7*(6), 1687–1691. <https://doi.org/10.1021/bm060154s>

704 Schakenraad, J. M., Hardonk, M. J., Feijen, J., Molenaar, I., & Nieuwenhuis, P. (1990). Enzymatic activity
705 toward poly(L-lactic acid) implants. *Journal of Biomedical Materials Research*, *24*(5), 529–545.
706 <https://doi.org/10.1002/jbm.820240502>

707 Sharma, K. S., Durand, G., Giusti, F., Olivier, B., Fabiano, A.-S., Bazzacco, P., Dahmane, T., Ebel, C., Popot,
708 J.-L., & Pucci, B. (2008). Glucose-Based Amphiphilic Telomers Designed to Keep Membrane
709 Proteins Soluble in Aqueous Solutions: Synthesis and Physicochemical Characterization.
710 *Langmuir*, *24*(23), 13581–13590. <https://doi.org/10.1021/la8023056>

711 Solin, K., Orelma, H., Borghei, M., Vuoriluoto, M., Koivunen, R., & Rojas, O. J. (2019). Two-Dimensional
712 Antifouling Fluidic Channels on Nanopapers for Biosensing. *Biomacromolecules*, *20*(2), 1036–
713 1044. <https://doi.org/10.1021/acs.biomac.8b01656>

714 Syverud, K. (2016, spring). *SciFinder—Potential of cellulose nanofibrils in tissue engineering—ACS*
715 *abstract*. [https://scifinder-cas-org.gaelnomade.ujf-](https://scifinder-cas-org.gaelnomade.ujf-grenoble.fr/scifinder/view/scifinder/scifinderExplore.jsf)
716 [grenoble.fr/scifinder/view/scifinder/scifinderExplore.jsf](https://scifinder-cas-org.gaelnomade.ujf-grenoble.fr/scifinder/view/scifinder/scifinderExplore.jsf)

717 Valo, H., Arola, S., Laaksonen, P., Torkkeli, M., Peltonen, L., Linder, M. B., Serimaa, R., Kuga, S.,
718 Hirvonen, J., & Laaksonen, T. (2013). Drug release from nanoparticles embedded in four
719 different nanofibrillar cellulose aerogels. *European Journal of Pharmaceutical Sciences*, *50*(1),
720 69–77. <https://doi.org/10.1016/j.ejps.2013.02.023>

721