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1 **Electrostatic interactions regulate the physical properties of gelatin-cellulose**
2 **nanocrystals nanocomposite films intended for biodegradable packaging**

3
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16
17 **Abstract**

18 Cellulose nanocrystals (CNCs)-reinforced gelatin (Gel) films are appealing
19 candidates for biodegradable packaging. However, tailoring the physical properties of
20 Gel/CNCs films by control of pH and film-forming drying temperature continues
21 unstudied. Here, we described the influence of pH on the physical properties of
22 Gel/CNCs films covering different CNCs contents. The interactions between CNCs and
23 Gel were studied by assessing the ζ -potential of Gel/CNCs suspensions under acidic
24 (pH 3), Gel isoelectric point (pI, pH 6) and alkaline (pH 8) conditions. pH 3 promotes
25 the electrostatic attraction, while pH 8 favors the electrostatic repulsion in the Gel-
26 CNCs pair, increasing the suspension viscosity in both cases. The addition of 0.5 wt%
27 CNCs decreased the water vapor permeability (WVP) of the Gel/CNCs films by 68%
28 under electrostatic attractive forces and by 39% at the gelatin pI. The addition of 5 wt%
29 CNCs at pH 3 resulted in the formation of complex coacervates, which decreased the
30 mechanical properties and increased the WVP of Gel/CNCs films. Increasing pH above
31 the Gel pI remarkably increased the gelatin renaturation as triple helices, which was
32 found to be key for increasing by 152% and 56% the Young's modulus and tensile
33 strength, respectively, of the Gel/CNCs films with 0.5 wt% CNCs. Film-forming drying
34 temperatures had an inverse effect on the triple helix content, and, consequently, on the
35 physical properties of the Gel/CNCs films. These findings denote that modulating pH,
36 CNCs amount, and drying temperature is a suitable strategy for tailoring the properties

37 of nanocellulose-reinforced gelatin films for an extended range of food packaging
38 applications.

39

40 **Keywords:** Gelatin, cellulose nanocrystal, electrostatic interaction, mechanical
41 properties.

42

43 **1. Introduction**

44

45 The growing plastic production has led to a global waste disposal crisis.
46 Nowadays, researches are focused on solving this problem by obtaining biodegradable
47 packaging. Biopolymers, including proteins and polysaccharides, are the main natural
48 resources employed to develop biodegradable films (Esteghlal, Niakousari, & Hosseini,
49 2018). Proteins are natural polyelectrolytes that have attracted great interest due to their
50 unique properties (Al-Tayyar, Youssef, & Al-hindi, 2020; Moustafa, Youssef, Darwish,
51 & Abou-Kandil, 2019), in particular, their ability to form self-assembled structures,
52 such as ionic complexes obtained by the interactions with polysaccharides carrying
53 opposite charges (Derkach, Kuchina, Kolotova, & Voron'ko, 2020). These structures are
54 promising for the creation of new materials applicable in pharmacy, medicine
55 (Vuillemin, Michaux, Muniglia, Linder, & Jasniewski, 2019; Zhao, Skwarczynski, &
56 Toth, 2019), and food packaging (da Silva, de Oliveira, Simas, & Riegel-Vidotti, 2020;
57 Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015).

58

59 Gelatin is a protein obtained by the partial hydrolysis of collagen at controlled
60 temperature and pH conditions (Joshi, Rawat, & Bohidar, 2018). This protein is cheap,
61 commercially available, biodegradable, in addition to presenting good processability
62 and film-forming properties (Wang et al. 2014). The mechanical behavior of gelatin gels
63 and films is related to the regeneration of gelatin as crystalline triple helices. Gioffrè et
64 al. (2012) investigated the pH effect on the mechanical properties of pigskin type A
65 gelatin films and observed that pH values higher than 9 and lower than 5 considerably
66 reduced the triple helix content and, consequently, the mechanical properties of the
67 films.

67

68 Gelatin films are moisture-sensitive and exhibit low water vapor barrier and
69 mechanical resistance under high relative humidity due to the hydrophilicity of the
70 polypeptide chains (Gómez-Guillén et al. 2009). A suitable approach to overcoming
71 these limitations of gelatin films is the use of cellulose nanocrystals (CNCs) as nano-
sized fillers (Hosseini & Gómez-Guillén, 2018). CNCs are one of the most studied

72 polysaccharide-based nanomaterials in polymer nanocomposites (Espino-Pérez,
73 Domenek, Belgacem, Sillard, & Bras, 2014; Noorbakhsh-Soltani, Zerafat, & Sabbaghi,
74 2018). CNCs are suitable for such purposes due to their large elastic modulus (~143
75 GPa) biodegradability and abundance (Dufresne, 2018) (Habibi, Lucia, & Rojas, 2010).

76 Several reports have described a joint synergistic effect between gelatin and
77 CNCs toward the formation of percolated networks stabilized by hydrogen bonding.
78 George & Siddaramaiah (2012) showed that the use of CNCs, obtained from bacterial
79 cellulose, at a weight fraction of 4%, maximized the tensile strength, tensile modulus,
80 and stiffness of gelatin films. Santos et al. (2014) demonstrated that tilapia gelatin films
81 had a high stiffness and good ductility up to a CNCs loading of 5 wt%. Above this
82 concentration, the mechanical properties tended to reach a plateau. Mondragon et al.
83 (2015) showed that cellulose nanofibers (CNFs) and CNCs improved the oxygen gas
84 barrier properties of gelatin films and that these nanocelluloses did not significantly
85 improve the tensile properties of the films when used at weight fractions $\leq 10\%$. More
86 recently, Leite et al. (2020) demonstrated that adding 0.5 wt% CNCs increased the
87 tensile strength and Young's modulus of gelatin films obtained by continuous casting
88 by 77 % and 48 %, respectively.

89 To the best of our knowledge, no research has been published on the reinforcing
90 effect of CNCs on gelatin films under different types of electrostatic interactions. The
91 knowledge of these intermolecular interactions is crucial for understanding the structure
92 of Gel/CNCs films, as well as for the development of packaging films with tunable
93 physical properties. Furthermore, there is no report that examines the structure and
94 mechanical properties of Gel/CNCs films as influenced by the casting temperature. This
95 work aimed to investigate the effect of CNCs, pH, and drying temperature on the triple
96 helix content, mechanical, thermal, and barrier properties of gelatin films obtained by
97 bench casting.

98

99 **2. Materials and methods**

100 2.1. Materials

101

102 Bovine gelatin (Bloom Strength-190) was kindly supplied by Gelco Gelatinas do
103 Brazil Ltda (Pedreira, SP). CNCs isolated from wood pulp by sulfuric acid hydrolysis
104 and with crystallinity index of 82% (Fig. S1) and delivered as a dried powder were
105 purchased from Cellulforce Inc. (Windsor, Québec, Canada). Glycerol and ethanol were

106 purchased from Across Organics (USA). All chemicals were analytical grade and were
107 used as purchased.

108

109 2.2. Methods

110

111 2.2.1. Preparation of nanocomposite films

112

113 The nanocomposite films were prepared by solvent casting. The gelatin
114 (Gel) powder was hydrated in distilled water (10 g/100 g) at 24 °C for 5 min and heated
115 at 60 °C under stirring for 15 min. The Gel solution pH was adjusted to 3, 6, and 8 with
116 3M HCl or NaOH. Glycerol (2 g/100 g of protein) was added to the Gel solution under
117 stirring for 5 min. The obtained Gel/glycerol solution was mixed with the CNCs
118 suspension at desired contents (0 wt%, 0.5 wt%, and 5.0 wt%, on a dry gelatin mass
119 basis) and stirred for additional 5 min. The final film-forming solutions (FFS) (45 mL)
120 were cast on glass plates (40 cm× 25 cm), covered with a polyester film (Mylar®,
121 DuPont, Brazil) to facilitate film peeling off after drying in an oven at 25, 35 and 80 °C
122 for 24 h. Film samples were conditioned at 50% relative humidity (RH) in a ventilated
123 climatic chamber and 25 °C for at least 24 h prior to any testing.

124

125 2.3. Characterizations

126

127 2.3.1. Determination of Zeta potentials (ζ -potentials)

128

129 ζ -potentials of aqueous CNCs dispersion, pure Gel solution and Gel/CNCs FFS
130 obtained at pH 3, 6 and 8 (0.5 wt% and 5.0 wt% CNCs on a dry gelatin mass basis)
131 diluted to 0.25 wt% were analyzed at 25 °C using a Zetasizer Nano-ZS (Malvern
132 Instruments Inc., UK). Each sample was mixed with a sonicator (Branson 450 Sonifier,
133 USA) for 5 min with a microtip before analysis. The measurements were performed in
134 triplicate.

135

136 2.3.2. Rheological study

137

138 The apparent viscosity and flow behavior of the FFS (pure Gel and Gel/CNCs
139 with 0.5 and 5 wt% CNCs at pH 3, 6 and 8) were evaluated on a Physica MCR101

140 rheometer (Anton Paar, Austria) using the concentric cylinder geometry at 40 °C. The
141 shear rate was varied from 0 to 150 s⁻¹ in 10 min. Oscillatory shear deformation tests
142 were also carried out to determine the storage modulus (G') and loss modulus (G'') of
143 the FFS at a constant strain of 1% and frequency of 1 Hz in the temperature range of 60-
144 20 °C using heating/cooling rate of 0.02 °C s⁻¹. Strain values were chosen to be within
145 the linear viscoelastic region based on preliminary measurements. The flow behavior
146 was described by fitting the experimental data to the Power Law model (Eq. 1).

147

$$\sigma = k\dot{\gamma}^n \quad (1)$$

150

151 where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s⁻¹), k is the consistency coefficient
152 (Pa sⁿ) and n is the flow behavior index.

153

154 2.3.3. Polarized light optical microscopy

155

156 Optical images of Gel/CNCs films were taken between crossed polarizers to
157 evaluate the dispersion of the CNCs within the gelatin matrix. The samples were
158 imaged by polarized optical microscopy taken at 10-fold magnification using a
159 microscope Zeiss Axio Vert.A1 (Carl Zeiss Microscopy).

160

161 2.3.4. Scanning electron microscopy (SEM)

162

163 Film surface and the cross-sectional surface were investigated using a JEOL
164 Scanning Electron Microscope (SEM, model Quanta200, FEI, the Netherlands). The
165 samples were first cryo-fractured in liquid N₂ and then fixed onto 90° specimen mounts
166 to be coated with a ca. 5-nm-thick gold layer in an argon atmosphere. The SEM images
167 were taken at accelerating voltages below 10 kV using the secondary electron mode.

168

169 2.3.5. X-ray diffraction (XRD) analysis

170

171 X-ray diffraction patterns were acquired from 2 ° to 60 ° (2 θ) using a Panalytical
172 diffractometer (X'Pert Pro MPD-Ray) with Ni-filtered Cu K α radiation ($\lambda = 1.54 \text{ \AA}$)
173 generated at a voltage of 45 kV and current of 40 mA. The amount of triple helix

174 configuration (X_c in %) in the Gel/CNCs films was calculated in duplicate for each
175 sample using Eq. 2.

176

$$177 \quad X_c = [A_c / (A_c + A_a)] \times 100$$

178 (2)

179

180 where A_c is the area of the diffraction peak at $\sim 2\theta = 8^\circ$, corresponding to the triple helix
181 configuration of gelatin chains, and A_a is the area under the hump located at $\sim 2\theta = 21^\circ$,
182 corresponding to the fraction of amorphous domains in the gelatin films. (Díaz-
183 Calderón, Flores, Gonz, & Enrione, 2017).

184

185 2.3.7. Tensile tests

186

187 The tensile properties of the films were determined by the ASTM D882-09
188 standard method (2009). The film specimens (17 cm x 1 cm) were prepared and
189 equilibrated in a ventilated climatic chamber at $23 \pm 1^\circ\text{C}$ and $50 \pm 5\%$ RH for 48 h. The
190 tests were carried out in an Instron Universal Testing Machine (model 5569, Instron
191 Corp., USA) equipped with a 100 N load cell. The specimens were stretched using
192 crosshead speed of 10 mm/min with clamps initially separated by 100 mm. Tensile
193 strength, Young's modulus, and elongation at break were calculated from the stress-
194 strain curves. Thicknesses were measured using a digital micrometer (Adamel
195 Lhomargy) to the nearest 0.001 mm with ten random measurements per film. The
196 tensile tests were performed with five replicates per film sample.

197

198 2.3.8. Water vapor permeability (WVP)

199

200 WVP was determined using the ASTM E-96-01. The preconditioned film was
201 sealed onto the opening of an aluminum permeation cup (28 mm internal diameter)
202 containing dried calcium chloride. The cup was kept in a ventilated climatic chamber at
203 $23 \pm 1^\circ\text{C}$ and $50 \pm 5\%$ RH. The cup was weighed at least four times for at least 7 days
204 under this controlled environment. For each film, three replicates were performed. The
205 WVP $\text{g mm m}^{-2} \text{h}^{-1} \text{kPa}^{-1}$ was determined using Eq. 3:

206

207 $WVP = (\Delta m / \Delta t) \times (L / A \Delta P)$

208 (3)

209

210 where $\Delta m / \Delta t$ is the mass gain over time (g h^{-1}), A is the exposed film area (m^2), L is the
211 film thickness (mm), and ΔP is the difference of partial pressure.

212

213 2.3.9. Water solubility (WS) determination

214

215 The gelatin films were cut into rectangular ($1 \text{ cm} \times 1 \text{ cm}$) specimen and pre-
216 dried at $105 \text{ }^\circ\text{C}$ for 24 h. The dried specimens were accurately weighed (initial dry
217 weight, w_0), placed in distilled water (30 mL), and stirred at $25 \pm 1 \text{ }^\circ\text{C}$ for 24 h. Filter
218 paper number 1 was prepared by drying at $105 \text{ }^\circ\text{C}$ for 24 h. Then, the film was poured
219 onto the filter paper and dried at $105 \text{ }^\circ\text{C}$ for 24 h and weighed (final dry weight, w_1).
220 WS was measured in triplicate following calculated using Eq 4.

221

222 $WS (\%) = [(w_0 - w_1) / w_0] \times 100$

223 (4)

224

225 2.3.10. Thermogravimetric analysis (TGA)

226

227 Thermogravimetric analysis was carried out in a Q500 equipment (TA
228 Instruments, USA). About 10 mg of each film sample was taken in a standard Ptcrucible
229 and heated from $25 \text{ }^\circ\text{C}$ to $600 \text{ }^\circ\text{C}$ with heating rate of $10 \text{ }^\circ\text{C min}^{-1}$, under inert
230 atmosphere of nitrogen (60 mL/min). Samples before the TGA tests were conditioned in
231 a ventilated climatic chamber at $23 \pm 1 \text{ }^\circ\text{C}$ and $50 \pm 5\% \text{ RH}$ for 48 h.

232

233 2.3.11. Statistical analysis

234

235 Data were subject to analysis of variance (ANOVA). The mean values were
236 compared using the Tukey's test at a confidence level of 95 % ($p < 0.05$) using the Origin
237 software (OriginLab, Northampton, USA).

238

239 2. Results and discussion

240

241 3.1 Physical and rheological properties of Gel/CNCs film-forming solutions

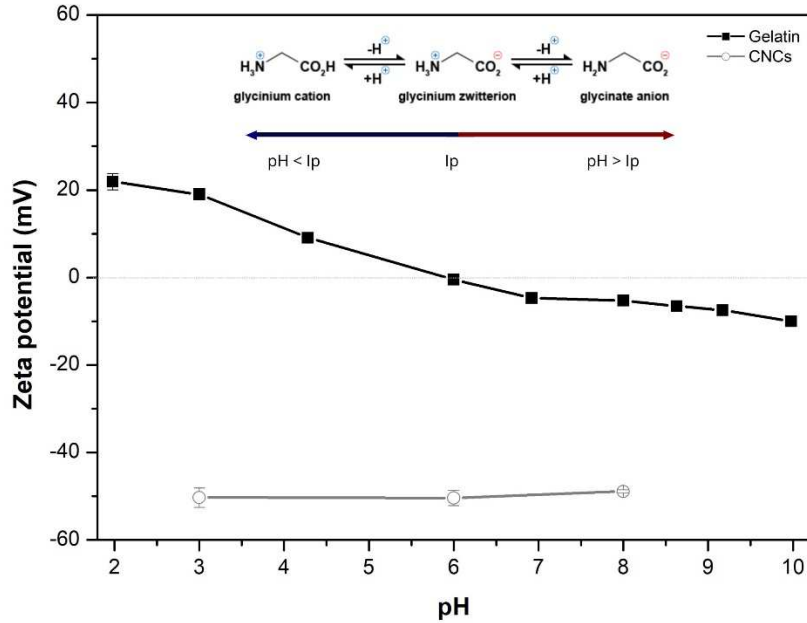
242

243 ζ -potentials provide evidence of the net charges on particle surfaces as a function
244 of pH. Fig. 1 illustrates the evolution of the ζ -potential of gelatin solution and CNCs
245 suspension upon pH variation. The isoelectric point (pI) of gelatin solution occurred at
246 around pH 6, at which a null ζ -potential was observed. This is in agreement with
247 previously reported data for type B gelatins (pH 4.5-6.0) (Ahmed, 2017). Gelatin has
248 the ability to present different charges depending on the pH (Gioffrè et al. 2012). At pH
249 $<$ pI, the gelatin solution showed a net positive charge, which was attributed to the
250 protonation of the amino groups ($-\text{NH}_3^+$), while at pH $>$ pI the solution showed a net
251 negative charge, due to ionization of the carboxyl groups ($-\text{COO}^-$). For the CNCs
252 suspension, the ζ -potential value was found to be negative regardless of the pH. The ζ -
253 potential value of CNCs suspension was around -50 mV indicating a stable suspension
254 in water. According to Lin, Gu, & Cui (2019), ζ -potentials ($<$ -30mV and $>$ + 30 mV)
255 could maintain a stable colloidal system due to the repelling forces between particles.
256 This value can be attributed to the sulfate groups (SO_3^-) grafted on the CNCs surface
257 (Habibi et al. 2010), agreeing with a previous study (Silva et al. 2019).

258

In aqueous solutions, electrostatic charges are one of the main driving forces for
259 interactions between charged biopolymers (Behrouzain, Razavi, & Joyner, 2020). For
260 the Gel/CNCs FFS at pH $<$ pI, the gelatin and CNCs are expected to form complexes
261 stabilized by electrostatic attractive interactions, while at pH $>$ pI the mixture should
262 exhibit electrostatic repulsive interactions between the negatively charged COO^- groups
263 of gelatin and SO_3^- groups of CNCs. Similar behaviors for protein-polysaccharides were
264 recently reported elsewhere (Razzak, Kim, & Chung, 2016; Voron'ko, Derkach,
265 Kuchina, & Sokolan, 2016). As expected, the ζ -potential of the Gel/CNCs FFS at pH 6
266 and pH 8 decreased with increasing the CNCs concentration (Fig. S2). However, at pH
267 3, the addition of CNCs did not change significantly the ζ -potential values of the
268 Gel/CNCs FFS compared to the pure Gel solution at the same pH, confirming that
269 electrostatic attractive interactions occurred between the positively charged gelatin
270 chains and negatively charged CNCs in acidic medium.

271



272

273 Fig.1. ζ -Potential values of neat Gel solution and CNCs suspension (0.25 wt%)
 274 at 25 °C as a function of pH.

275

276

277 The rheological behavior of the Gel/CNCs FFS was evaluated at different pH.
 278 The apparent viscosity and parameters calculated by Ostwald de Waele model are
 279 presented in Table 1. The flow curves are shown in Fig. S3. Experimental shear stress
 280 vs. shear rate curves were fitted to the Ostwald de Waele model ($r^2 > 0.99$), which has
 281 been widely used to describe non-Newtonian fluids over the most important shear rate
 282 ranges (Ahmed, 2017). All the FFS exhibited a shear-thinning behavior ($k > 0$; $0 < n <$
 283 1) and their apparent viscosities (η_{ap}) decreased as the shear rate increased. This
 284 pseudoplastic behavior of Gel/CNCs FFS became progressively more pronounced at
 285 high CNCs contents and with attractive (pH 3) and repulsive (pH 8) electrostatic
 286 conditions. As expected, the addition of CNCs increased the apparent viscosity of the
 287 Gel/CNCs FFS. The electrostatic attraction between the CNCs and gelatin molecules at
 288 pH 3 leads to the formation of gel networks through ionic cross-linking (Kwak, Lee,
 289 Park, Lee, & Jin, 2020). By contrast, the electrostatic repulsive forces between gelatin
 290 and CNCs at pH 8 can stretch the gelatin molecules and favor mutual orientation of
 291 macromolecules and creation of non-covalent interactions (Voron'ko et al. 2016). This
 292 could decrease the mobility of protein molecules, thus increasing the resistance between
 293 gelatin FFS layers. The minimum viscosity was found at the Gel isoelectric point (pH
 294 6),

294

295 Table 1. Apparent viscosity (η_{ap}) parameters of Ostwald de Waele model
 296 (consistency index (K), and flow index (n)) and transition temperatures of Gel/CNCs
 297 film-forming solution at pH 3, 6, and 8.

Gel/CNCs film-forming solution									
	Electrostatic attractive forces pH 3			Isoelectric point pH 6			Electrostatic repulsive forces pH 8		
	0	0.5	5.0	0	0.5	5.0	0	0.5	5.0
CNCs (wt%)	0	0.5	5.0	0	0.5	5.0	0	0.5	5.0
Parameters of Ostwald de Waele model									
k (mPa s ⁿ)	0.01	0.03	1.03	0.01	0.03	0.01	0.01	0.02	2.25
n	0.92	0.83	0.32	0.97	0.97	0.90	0.90	0.96	0.24
r ²	0.993	0.998	0.997	0.999	0.999	0.992	0.999	0.999	0.990
η_{ap} (mPa) at 1 s ⁻¹	10.1	25.0	887.3	10.9	17.5	12.7	7.79	14.0	1788
Transition temperature (°C)									
Gel-sol	32.1	32.0	Nd	34.6	35.1	35.0	35.6	35.1	37.8
Sol-gel	19.4	22.7	Nd	22.7	22.8	22.6	22.4	22.6	Nd

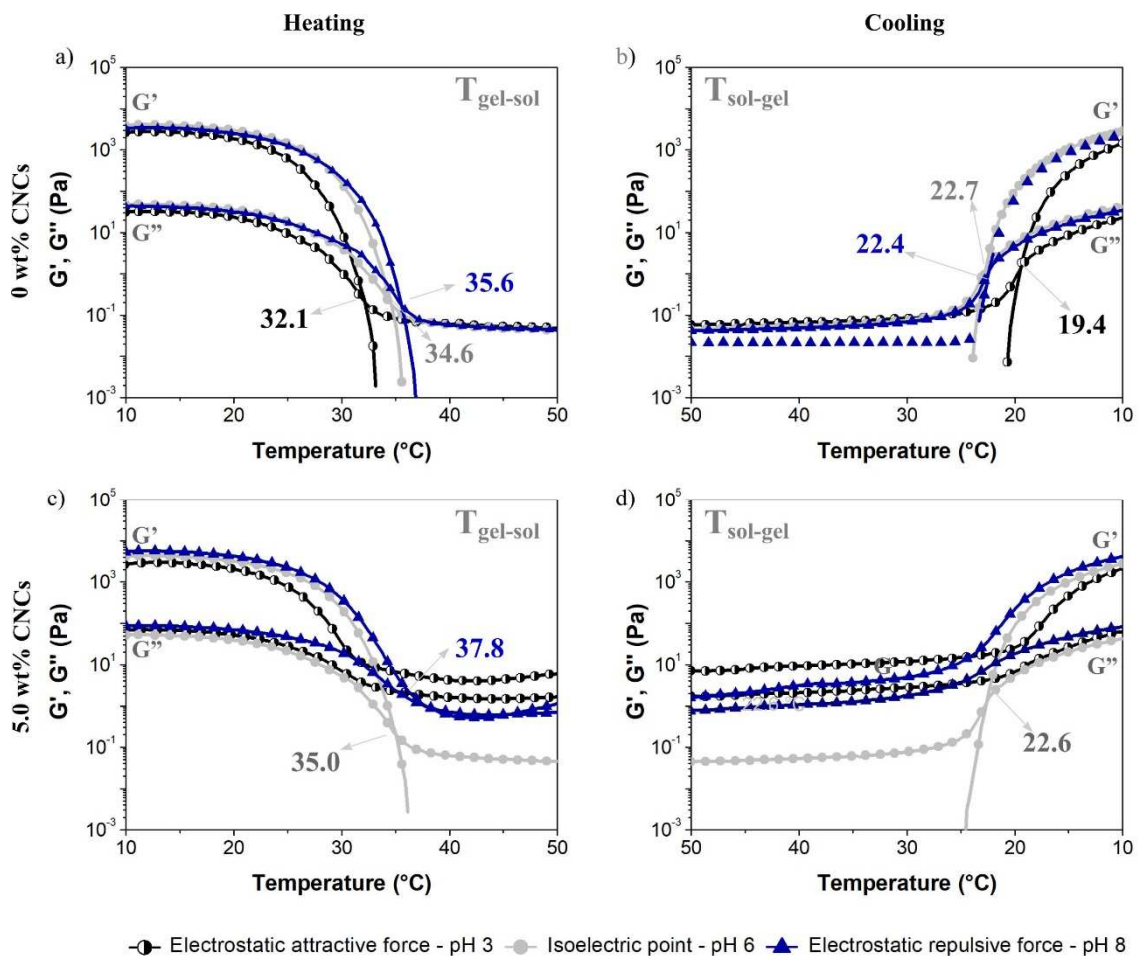
298 Nd: not detected.

299

300 Dynamic rheological measurements can be used to monitor structures in weak
 301 gels or viscoelastic fluids (Behrouzain & Razavi, 2018). These measurements provide
 302 key information for gel processability in continuous solvent casting or 3D printing. Fig.
 303 2 shows the storage modulus (G') and loss modulus (G'') of the Gel/CNCs FFS with 0
 304 and 5 wt% CNCs at different pH obtained from heating and cooling scans. All the
 305 samples presented a similar rheological behavior, regardless of the pH or CNCs content.
 306 The viscoelastic modulus (G' or G'') were higher at lower temperatures (gel-like
 307 domain) than at higher temperatures (sol-like domain), with a well visible inflection
 308 between both domains ($G'=G''$). This inflection is typical of physical gels, and is
 309 defined as the gel-sol transition (Fig. 2a,c) and sol-gel transition (Fig. 2b,d) on the
 310 heating and cooling curves, respectively. Similar profiles have been observed in gelatin-
 311 based systems by several authors (Alexandre, Lourenço, Bittante, Moraes, & Sobral,
 312 2016; Fernando et al. 2014). The gel-sol temperatures (heating curves) of the Gel/CNCs

313 FFS was more affected by the Gel/CNCs electrostatic interactions (Table 1). A
 314 remarkable hysteresis, of about 12 °C, was also noticeable (Fig. S3). Furthermore, the
 315 addition of 5.0 wt% CNCs to the gelatin solution at pH 3 resulted in an elastic gel-like
 316 behavior ($G' > G''$) even above the gel-sol temperature. According to Qiao et al. (2016),
 317 proteins can form complex coacervates through interactions of oppositely charged
 318 particles, as previously indicated by the ζ -potential values of the Gel/CNCs FFS at pH
 319 3. Complex coacervation can lead to precipitation of polymer complexes through the
 320 formation of a dense media that can be treated as being composed of interconnected gel-
 321 like network structures, consistent with the assumption that the CNCs are
 322 electrostatically "cross-linked" along the gelatin chains. Consequently, the gel-sol and
 323 sol-gel transitions of the Gel/CNCs FFS were not observed due to Gel/CNCs
 324 electrostatic attractive forces. The Gel/CNCs FFS with 5% CNCs at pH 8 also did not
 325 exhibit the sol-gel transition and presented a solid-like behavior, indicating the
 326 existence of physical crosslinks by hydrogen bonding as mentioned before.

327



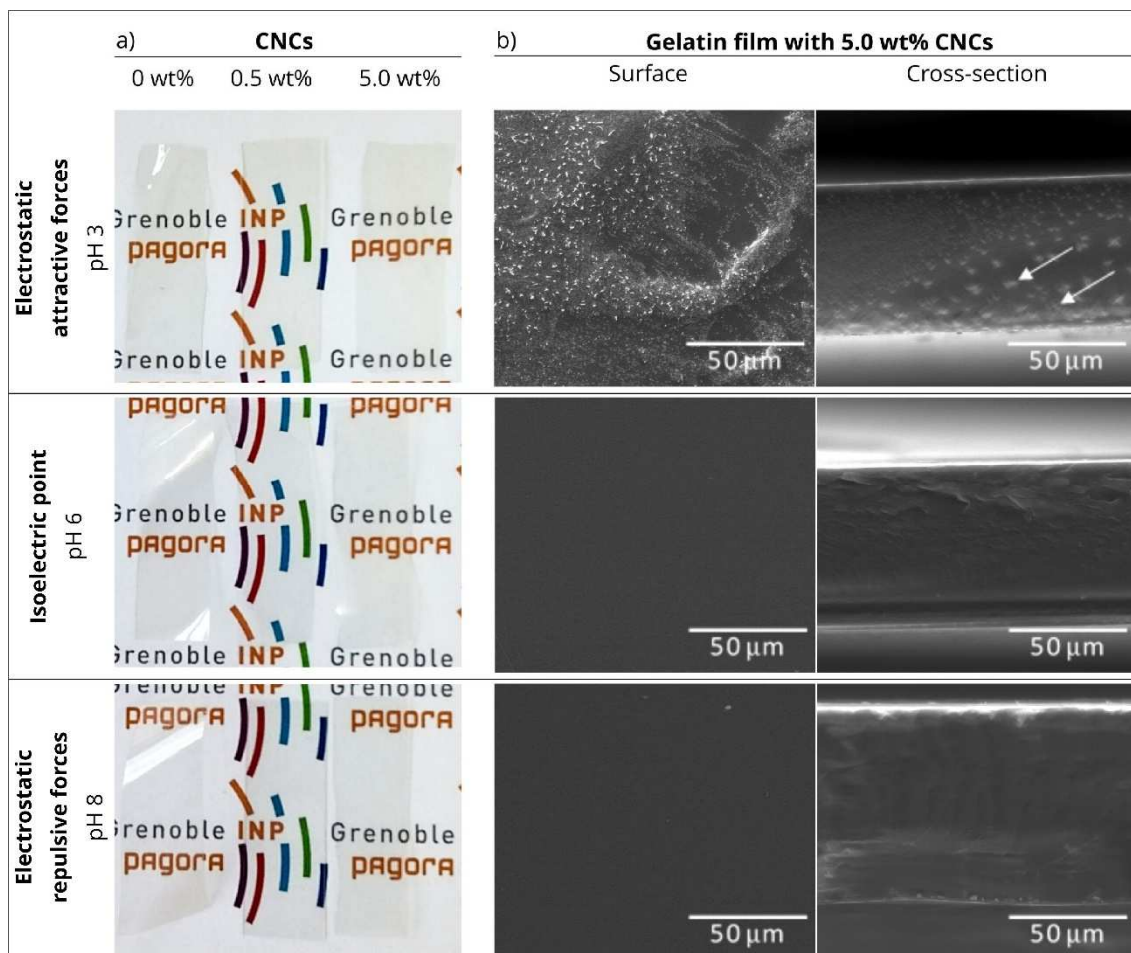
328

329 Fig. 2 Viscoelastic modulus of Gel/CNCs FFS at different pH and CNCs
330 contents. Heating (a and c) and cooling (b and d) at 1 °C min⁻¹.

332 3.2. Characterization of Gel/CNCs nanocomposite films

334 3.2.1 Morphological and structural characterizations

335 The photographs of the Gel/CNCs films are shown in Fig.3a. All the films
336 displayed good macroscopic homogeneity and transparency. Cracks on their surface
337 were also absent. The morphology of the Gel/CNCs films obtained from different pH
338 was investigated by SEM (Fig.3b). The Gel film with 5.0 wt% CNCs at pH 3 showed
339 aggregates (white arrows), on both cross-sectional and air-drying surfaces, suggesting
340 the formation of large CNCs-gelatin coacervates. This was also confirmed by polarized
341 light optical microscopy images (Fig. S4). The same effect was observed by Li et al.
342 (2018) for gelatin/gum arabic mixtures at pH 3.5. They found that complex coacervates
343 were spontaneously formed between gelatin and gum arabic by effective electrostatic
344 interactions at low pH, where gelatin and gum arabic presented opposite surface
345 charges. By contrast, the electrostatic attraction between gelatin and CNCs is weakened
346 by increasing the pH, and the coacervates disappeared at pH 6 and 8 (Fig. 3b). The
347 Gel/CNCs films showed homogeneous and continuous surface with no white dots,
348 which suggest that the CNCs were homogeneously distributed throughout the film
349 structures (Leite, Battirola, Escobar da Silva, & Gonçalves, 2016).



351

352 Fig.3. (a) Photographs of Gel/CNCs films with different CNCs contents, (b) SEM

353 micrographs of air-drying and cross-sectional surfaces of Gel/CNCs films with 5.0 wt%

354 CNCs at pH 3, pH 6 and pH 8. White arrows indicate CNCs-gelatin coacervates at pH

355 3.

356

357 The XRD patterns of the films obtained from different pH and CNCs contents

358 are shown in Figs. 4a-b. The formation of gelatin film is accompanied by the

359 renaturation of triple helical structures of protein molecules (Lin et al. 2019). The XRD

360 diffractograms showed a wide background with a band at $2\theta \sim 21^\circ$, which is typically

361 related to the large amorphous Gel fraction (Bigi, Panzavolta, & Rubini, 2004). The

362 band at $2\theta \sim 8^\circ$ has been previously attributed to the presence of triple helix structure

363 present in collagen and renatured gelatin (Quero et al. 2018). The area of this peak has

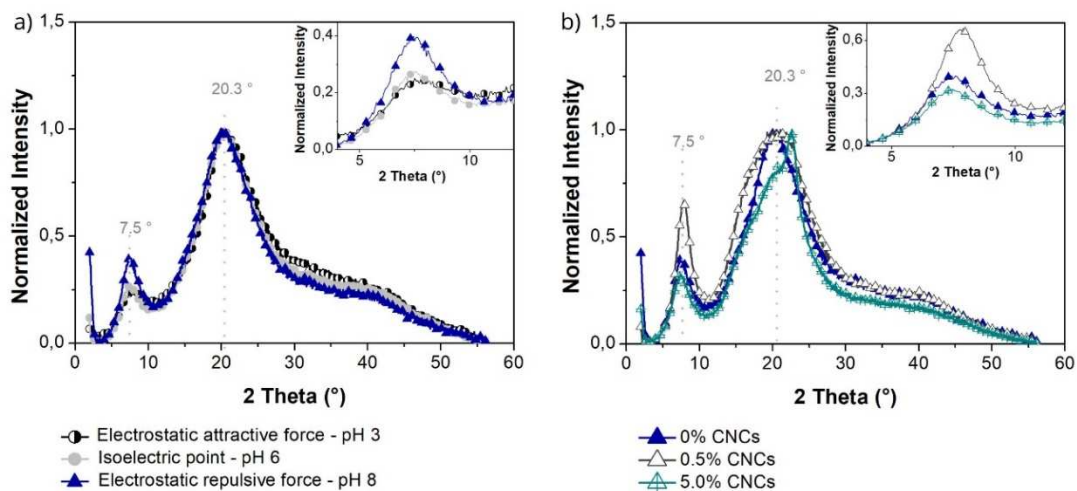
364 been found to be directly related to the triple helix content of gelatin-based composites

365 (Badii et al. 2014; Bigi et al. 2004; Yakimets et al. 2005). As seen in the insert in Figs.

366 4a-b, the band area at $2\theta \sim 8^\circ$ changed with the pH and CNCs content. The triple helix

367 content was 9.4, 6.3, and 6.9% for Gel films with 0 wt% CNCs at pH 8, 6 and 3,

368 respectively (Table S1). According to Ahsan & Rao (2017), the triple helix content of
 369 type B gelatin decreases with decreasing pH, most likely due to the structural changes in
 370 gelatin to mild denaturation, which was complete at around pH 2.5. The highest triple
 371 helix content (13.5%) was observed in the film with 0.5 wt% CNCs at pH 8. This
 372 behavior may result from deprotonation of the gelatin molecules at alkaline pH,
 373 favoring the formation of hydrogen bonds (Ahmed, 2017), which are essential to
 374 stabilize the gelatin triple helices. The formation of hydrogen bonds between CNCs and
 375 gelatin chains (both inside and outside helical fragments), also favors the regeneration
 376 and stabilization of triple helix structures (Bigi et al. 2004; Giofrè et al. 2012).
 377 However, the addition of 5.0 wt% CNCs markedly diminished the triple helix content,
 378 suggesting an increased random coil conformation of gelatin chains. Similar behavior
 379 was observed by Yakimets et al., (2005) who found changes in the structural and
 380 molecular order of gelatin films induced by water content. This result may also suggest
 381 that the folding process from random coil to triple helix is influenced by pH and CNCs
 382 amount.
 383



384 Fig.4. XRD patterns of (a) pure Gel film obtained from FFS with pH 3, 6 and 8
 385 (b) Gel/CNCs films obtained from pH 8 and different CNCs contents. The magnified
 386 view of the 2θ range 3 – 12° is shown as an insert.
 387

388
 389 3.2.2 Mechanical properties

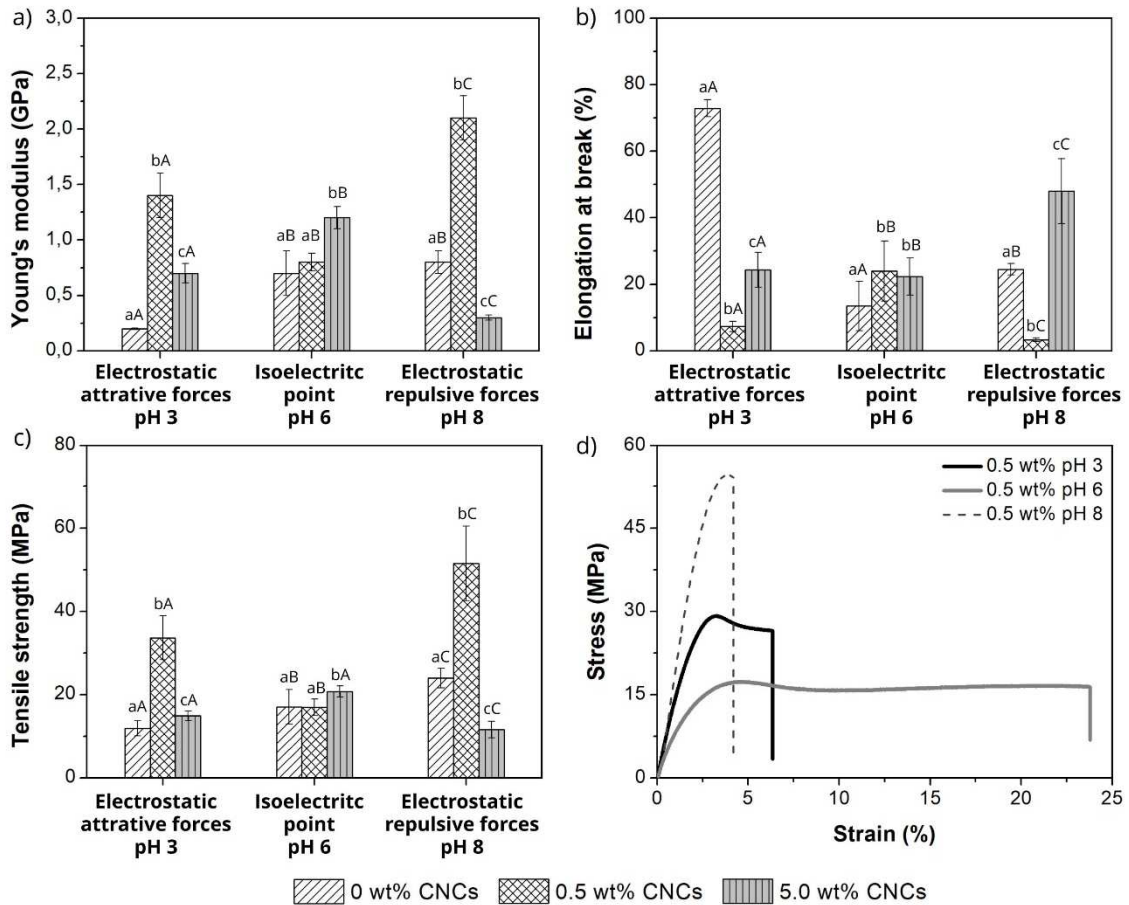
390
 391 The Young's modulus, elongation at break, and tensile strength of Gel/CNCs
 392 films are presented in Fig.5. The nanocomposite films containing 0.5 wt% CNCs at pH

393 3 and 8 presented significantly increased Young's modulus and tensile strength, and
394 decreased elongation at break compared to the pure Gel films ($p < 0.05$). This effect was
395 more pronounced at pH 8, whose Young's modulus and tensile strength increased by
396 152% and 56%, respectively ($p < 0.05$). At pH 3, the increases in Young's modulus and
397 tensile strength for the 0.5 wt% CNCs content may be attributed to the electrostatic
398 attractive interaction between gelatin and CNCs. When pH was increased to 6, no
399 significant changes in the tensile properties of the 0.5 wt% Gel/CNCs film were
400 observed ($p > 0.05$), most likely due to the reduction in the electrostatic interactions
401 above the gelatin pI. At pH 8, the increases in Young's modulus and tensile strength are
402 most likely due to the regeneration of triple helixes, which increases the overall
403 crystallinity of the films (Bigi et al. 2004). Gioffrè et al. (2012) observed that pH higher
404 than 9 and lower than 5 considerably reduced the triple-helix content and, consequently,
405 reduced the Young's modulus and elongation at break of pigskin type A gelatin films.
406 Quero et al. (2018) attributed the improved mechanical performance of 15 wt%
407 microfibrillated cellulose (MFC)-added salmon gelatin films to the high triple-helix
408 content of the nanocomposites compared to similar nanocomposites obtained from
409 bovine gelatin.

410 When the CNCs content was increased to 5.0 wt%, the Young's modulus and
411 tensile strength of the Gel/CNCs films slightly increased at the Gel pI (pH 6), but
412 drastically decreased at pH 3 and 8 ($p < 0.05$). For the acidic condition (pH 3), such
413 decreases could be attributed to the large aggregates formed by the CNCs-gelatin
414 complex coacervates (Celebi & Kurt, 2015). These aggregates provided a heterogeneous
415 distribution of the CNCs within the gelatin matrix, as revealed by SEM (Fig.3b), acting
416 as defects in the film structure, thus reducing the mechanical resistance of the Gel/CNCs
417 nanocomposites (Cao, Zavattieri, Youngblood, Moon, & Weiss, 2016). For the alkaline
418 condition (pH 8), despite the uniform dispersion of the CNCs within the Gel matrix, the
419 triple-helix content decreased, and the electrostatic Gel-CNCs repulsion in the films
420 may have increased the free volume in the gelatin matrix (Lin et al. 2019). These
421 behaviors could explain the reductions in Young's modulus and tensile strength, and the
422 significant increase in elongation at break, respectively, for the 5 wt% CNCs-added Gel
423 nanocomposite film ($p < 0.05$) at pH 8. The moderate but gradual increases in Young's
424 modulus and tensile strength at pH 6 may relate to the lower triple helix content
425 compared to pH 8. These lowered effects also led the Gel/CNCs nanocomposites
426 obtained from the Gel pI to show a more ductile mechanical behavior, as illustrated in

427 Fig. 5d. Overall, these results denote that pH and CNCs content significantly regulate
 428 the mechanical properties of gelatin films.

429



430

431 Fig. 5. (a) Young's modulus, (b) elongation at break, (c) tensile strength, and (d) stress-
 432 strain curves of Gel/CNCs films as a function of pH and CNCs content. Lowercase
 433 letters (a, b, c) - Mean values in the same pH bearing the same letter are not statistically
 434 different according to the Tukey's test ($p > 0.05$). Capital letters (A, B, C) - Mean
 435 values in the same CNCs amount bearing the same letter are not statistically different
 436 according to the Tukey's test ($p > 0.05$).

437

438 3.2.3 Water affinity and barrier properties

439

440 The WVP of the Gel/CNCs films obtained from different pH and CNCs contents
 441 are presented in Table 2. No significant differences ($p > 0.05$) among the WVP for
 442 gelatin films with 0% CNCs were observed concerning the pH variations. These results
 443 agree with previous findings on fish myofibrillar protein films (Shiku et al. 2003), but

444 differ from those previously reported for soy protein isolate films, which showed lower
445 WVP at pH values above the soy protein isoelectric point (Gennadios, Brandenburg,
446 Weller, & Testin, 1993). The WVP at 50% RH of the Gel/CNCs films were lower than
447 those observed for other films based on gelatin at 0% RH (B.-S. Chiou et al. 2008); the
448 results found were close to 1.8 g mm/m² h kPa (25 °C at 0% RH) for bovine and porcine
449 gelatin films. The addition of CNCs to gelatin at pH 3 and pH 6 significantly decreased
450 the WVP by 68% and 39% (p < 0.05), respectively, of the resulting films. According to
451 Qiao et al. (2013), the addition of counterions screens the electrostatic repulsions
452 between charges in the polymer, which, in turn, allows the chains to collapse and
453 assume a more compact conformation. In a previous study, Santos et al. (2014) reported
454 that the WVP of protein films decreased by increasing the CNCs loading. They found
455 that fish gelatin films with 15 wt% CNCs exhibit WVP values of approximately 2 g
456 mm/m² h kPa (25 °C at 85% RH). George & Siddaramaiah (2012) reported that 4 wt%
457 of bacterial cellulose nanocrystals reduced the WVP of gelatin and attributed this to the
458 low hygroscopicity of the highly crystalline cellulose nanocrystals (Fig S3). They found
459 WVP values around 0.175 g mm/m² h kPa (25 °C at 50% RH). By contrast, as expected,
460 the addition of CNCs to the gelatin matrix at pH 8 increased the WVP since the
461 electrostatic repulsion in alkaline Gel/CNCs films can increase the free volume, which
462 resulted in greater water vapor diffusion. Overall, the WVP values of the gelatin/CNCs
463 films in the present study were lower than those of previous gelatin films reinforced
464 with CNCs.

465 The water solubility results are summarized in Table 2. No film fully dissolved
466 or broke apart after 24 h at 25 °C, suggesting that they were formed by stable network.
467 The solubility of the Gel films decreased by increasing the pH since higher pH favors
468 the increase of protein-protein interactions and the exposition of hydrophobic groups
469 that increase surface hydrophobicity gelatin and decrease the water solubility (Díaz,
470 Candia, & Cobos, 2017) The addition of CNCs at pH 6 and pH 8 did not significantly (p
471 < 0.05) change the Gel film solubility. However, a drastic decrease in water solubility
472 was observed at 0.5 wt% CNCs and pH 3. This improvement in the water resistance can
473 be attributed to the formation of the Gel-CNCs complex coacervates, as shown in the
474 polarized light optical microscopy images (Fig. S4). These results are in agreement with
475 Esteghlal et al. (2018), who suggested that the decrease in solubility is due to charge
476 reduction after complexation and formation of crosslinked gelatin-CMC films. Yin et al.
477 (2005) also found a similar behavior for chitosan/gelatin polyelectrolyte complexes.

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481
482

Table 2. Water vapor permeability at 50% RH and 25 °C and water solubility of the gelatin films with different pH and CNCs content.

CNCs content (%)	WVP (g mm/ m ² h KPa)		
	Electrostatic attractive force	Isoelectric point (pI)	Electrostatic repulsive force
	pH 3	pH 6	pH 8
0	0.204 ± 0.085 ^{aA}	0.180 ± 0.027 ^{aA}	0.131 ± 0.020 ^{aA}
0.5	0.065 ± 0.013 ^{bA}	0.111 ± 0.022 ^{bB}	0.158 ± 0.022 ^{aB}
5.0	0.189 ± 0.009 ^{aA}	0.109 ± 0.019 ^{bB}	0.262 ± 0.034 ^{bC}
Water solubility (%)			
0	65.3 ± 2.55 ^{aA}	31.7 ± 4.81 ^{aB}	15.7 ± 3.30 ^{aC}
0.5	14.6 ± 1.24 ^{bA}	21.5 ± 6.55 ^{aA}	16.9 ± 4.97 ^{aA}
5.0	42.1 ± 2.52 ^{cA}	32.4 ± 1.30 ^{aB}	17.1 ± 9.46 ^{aB}

483 Mean ± standard deviation. Lowercase letters (a, b, c) - Mean values in the same
484 column bearing the same letter are not statistically different according to the Tukey's
485 test (p > 0.05). Capital letters (A, B, C) - Mean values in the same row bearing the same
486 letter are not statistically different according to the Tukey's test (p > 0.05).

487

488 3.2.4 Thermo stability

489

490 The Gel/CNCs films were further characterized by TGA and the degradation
491 temperatures are reported in Table S2. All TGA curves are presented in Fig. S5. All
492 samples followed a similar decomposition profile. The first mass loss was due to
493 moisture and volatiles evaporation (Noorbakhsh-Soltani et al. 2018). Pure gelatin films
494 at pH 3 showed the lowest moisture content (3%) and the addition of 5.0 wt% CNCs
495 increased the weight loss at this stage, with exception of the Gel/CNCs film at pH 6
496 (Table S2), which may be due to less availability of free hydroxyl groups of the gelatin
497 matrix due to hydrogen bonding with CNCs at pH 6. This result is in line with the
498 increase in Young's Modulus and reduction in the WVP of the Gel/CNCs film with 5.0
499 wt% at pH 6. A significant mass loss occurred at temperature (T_{onset}) above 200 °C,
500 mainly associated with the decomposition of gelatin and glycerol molecules. The
501 thermal stability of Gel films increased near the gelatin isoelectric point, which is the
502 result of a more compact form of gelatin (Alberto & Gabriela, 2012). This result is in
503 agreement with Gioffrè et al. (2012), who reported a reduction in the thermal stability of

504 gelatin films prepared at relatively high and low pH. The higher thermal stability was
505 found for the Gel/CNCs films with 0.5% CNCs at pH 3 and pH 8, which showed
506 increases of 14.4 and 15.3 °C in the T_{onset} , respectively, when compared to pure gelatin
507 films at the same pH. This improvement might be attributed to the formation of more
508 rigid structures, like the Gel-CNCs coacervates formed at pH 3. This may also be
509 related to the high concentration of triple helix structures present in Gel/CNCs films at
510 pH 8, as shown by XRD (Fig.4). In addition, the formation of hydrogen bonds between
511 CNCs and gelatin can also influence the protein denaturation process and, as a
512 consequence, lead to an increased film thermal stability, as justified by George &
513 Siddaramaiah, (2012). Previous studies have shown that the addition of CNCs increased
514 9 °C T_{onset} for gelatin films with 10 wt% CNCs (Mondragon et al. 2015), 10.6 °C for
515 gelatin films with 2.5 wt% CNCs (Leite, Ferreira, Corrêa, Moreira, & Mattoso, 2020)
516 and a decrease of around 6 °C for gelatin films with 1.5 wt% CNCs, when compared to
517 pure gelatin films (Alves, dos Reis, Menezes, Pereira, & Pereira, 2015).

518 The gelatin film with 0.5 wt% CNCs obtained from pH 8, which had the highest
519 triple helix content, was chosen to evaluate the effect of the drying casting temperature
520 on the mechanical, thermal and barrier properties of Gel/CNCs nanocomposites. The
521 obtained results for the film samples dried at 25 °C (“cold-dried”), 35 °C (gelation
522 temperature) and 80 °C (“hot-dried”) are listed in Table 3. The gelatin films dried at 25
523 °C showed the highest Young’s modulus and tensile strength. This was likely due to the
524 fact that cold-dried films had a longer drying time (24 h), i.e. sufficient time for the
525 reorganization of gelatin structures as triple helices than the hot-dried films (6 h). Chiou
526 et al. (2009) observed that gelatin films dried below their gelation temperature
527 contained crystallinity triple-helix structures, whereas films dried above their gelation
528 temperature were virtually amorphous. Films with helical structures exhibit higher
529 tensile strength and Young’s modulus than amorphous films (Badii, MacNaughtan,
530 Mitchell, & Farhat, 2014).

531 Furthermore, the thermal stability of gelatin films increased when the drying
532 temperature was decreased (Fig. S5). These results might be explained by the larger
533 amount of energy needed to break the hydrogen bonds in the triple helix structures
534 formed in the cold-dried gelatin films (Fig. S6). The WVP results showed no statistical
535 difference concerning the drying temperature ($p > 0.05$). Bor Sen Chiou et al. (2008)
536 showed that mammalian gelatin films had higher levels of renaturation than fish gelatin
537 films. However, the authors observed that this greater amount of helical structures did

538 not lead to better water barrier properties, proving that WVP is not proportional to the
 539 renaturation level in gelatin films. The mechanical, thermal and barrier results suggest
 540 that the drying temperature used in the casting process and the addition of CNCs are
 541 significant factors that affect the structure and physical properties of Gel/CNCs films.

542

543 Table 3: Tensile, thermal properties, water vapor permeability, and triple-helix content
 544 of gelatin films with 0.5% CNCs at pH 8 as a function of different drying temperatures.

Drying temperature (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)	T _{onset} (°C)	WVP (g mm/ m ² h KPa)	Triple helix content (%)
25	2.1 ± 0,2 ^a	51.5 ± 8,95 ^a	3.31 ± 0.53 ^a	266.5	0.16 ± 0.02 ^a	13.5
35	1.0 ± 0,2 ^b	20.9 ± 5,01 ^b	3.57 ± 0.71 ^a	252.6	0.23 ± 0.08 ^a	8.7
80	1.2 ± 0,1 ^b	19.1 ± 0,45 ^b	2.98 ± 0.29 ^a	251.4	0.24 ± 0.16 ^a	0.3

545 Mean ± standard deviation. Mean values in the same column bearing the same letter are
 546 not statistically different according to the Tukey's test (p > 0.05).

547

548 Conclusion

549

550 Gelatin films reinforced with CNCs were successfully developed over a broad
 551 pH range. A comprehensive understanding of the pH role on the properties of
 552 Gel/CNCs films obtained by solution casting was provided by examining the possible
 553 electrostatic interactions and film structure involved. The different electric charges of
 554 gelatin chains attained at different pH led to different gelatin-gelatin and CNCs-gelatin
 555 interactions. Under electrostatic attractive forces (pH 3), the increase in the CNCs
 556 content resulted in the formation of complex coacervates. This behavior was not
 557 observed in the Gel/CNCs films above the Gel isoelectric point pH 6)I, The physical
 558 properties of gelatin films were significantly enhanced with the use of CNCs at 0.5 wt%
 559 CNCs and pH 8 due to the high triple helix content in the films. Furthermore, the drying
 560 temperature significantly hindered the gelatin triple helix formation, and consequently,
 561 had an inverse effect on the mechanical, thermal, and water barrier properties of the
 562 Gel/CNCs nanocomposites. In summary, modulating pH, CNCs amount, and drying
 563 temperature is a suitable strategy for tailoring the properties of nanocellulose-reinforced
 564 gelatin films to cover a broader range of requirements for food packaging applications.
 565 This strategy also illustrates a route for physicochemical control of the properties of
 566 polymer nanocomposites based on ionizable nanoparticles and polyelectrolytes.

567

568 **Credit Author Statement**

569 **Liliane Samara Ferreira** Leite: Conceptualization, Methodology, Formal
570 analysis, Investigation, Writing – original Draft. **Francys Kley Vieira Moreira:**
571 Supervision, Conceptualization, Writing – review & Editing. **Luiz Henrique**
572 **Capparelli Mattoso:** Supervision, Conceptualization, Writing – review & Editing.
573 **Julien Bras:** Supervision, Conceptualization, Writing – review & Editing.

574

575 **Declaration of competing interest**

576 The authors declare that they have no known competing financial interests or
577 personal relationships that could have appeared to influence the work reported in this
578 paper.

579

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593

594 **Reference**

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