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1 **Biomimetic hydrogel by enzymatic crosslinking of pullulan grafted with ferulic acid**

2

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7

8

9

10 **Abstract**

11 A novel eco-friendly two-step synthesis process of neutral pullulan (PUL)-ferulic acid (FA)
12 conjugates was reported in this work. Ferulic acid was first transformed to activated ferulate-
13 imidazolide using N,N'-carbonyldiimidazole (CDI), a green activated reagent. Issued product
14 was then reacted with pullulan. PUL-FA derivatives were characterized by FTIR and ¹H
15 NMR leading to substitution degrees (DS) between 0.02 and 0.1 (mol FA per mol PUL repeat
16 unit). The study in dilute regime indicated an associative behavior with the presence of
17 aggregate structures in solution due to the hydrophobic interactions between the grafted FA
18 onto polysaccharide backbones. Laccase from *Trametes versicolor* was then used to crosslink
19 polysaccharide chains to obtain biomimetic PUL-FA hydrogels. Gelling's kinetics were
20 analyzed with rheology in dynamic mode showing the impact of laccase amount, DS and
21 concentration. Mechanical and swelling properties appear related only to DS and
22 concentration of PUL-FA products.

23

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25

26 Keywords: hydrogels, polysaccharide, enzyme, antioxidant property

27

28 **1. Introduction**

29 Hydrogels represent a peculiar class of materials. consisting in 3D structures usually based on
30 cross-linked polymer networks. They are characterized by high capacity to absorb aqueous
31 solution and by specific mechanical properties highly dependent on the degree of crosslinking
32 or content interactions (Wong, Ashton & Dodou, 2015). Hydrogels have known a very
33 substantial expansion due to their unique properties and value which aroused manufacturers'
34 interest in different fields specially biomedical (Hoffman, 2002), pharmaceutical (Kashyap,
35 Kumar & Kumar, 2005) and cosmetic (Patravale & Mandawgade, 2008). Beyond their
36 current uses, hydrogels are more and more envisaged in many technological and innovative
37 applications such as scaffolds for tissue engineering (Moreira Teixeira, Feijen, van
38 Blitterswijk, Dijkstra & Karperien, 2012), entrapping of drugs or enzymes (Gasmı, Rihouey,
39 Picton, & Le Cerf, 2014), functional contact lenses (Hu, Tan & Hao, 2016), 3D bio-printing
40 (Tasoglu & Demirci, 2013), anti-aging masks (An et al., 2014) or sunscreen cream or mascara
41 (Yahia et al., 2015). For sustainable development considerations and in particular the
42 limitation of the fossil source of polymers, hydrogels based on natural polymers or their
43 derivatives constitute an interesting alternative. In this way, polysaccharides are primary
44 candidates and research around their chemical crosslinking pathways is dynamic and active.
45 However, the conventional reagents used in such chemical crosslinks are generally dangerous
46 or toxic such as epichlorohydrin (O'Connor et al., 2015) and glutaraldehyde (Choi, Khan &
47 Jung, 2019). Other more safe crosslinkers like sodium trimetaphosphate (STMP) can also be
48 used but the crosslinking pathway requires working at high pH which limits the applications
49 of such systems (Lack et al., 2004).

50

51 Therefore, it is common to use a new generation of hydrogels based on natural derivatives
52 with biomimetic ways of crosslinking as in the vegetal reign and more precisely in structure
53 of some cereal as wheat (Naczka & Shahidi, 2006). It was widely described that ferulic acid
54 (FA) is the most abundant phenolic compound in such structures linked by ester bond to cell
55 wall polysaccharides chiefly arabinoxylans ones (Bento-Silva, Vaz Patto, & do Rosário
56 Bronze, 2018). Number of studies has shown very interesting properties correlated to the role
57 of FA as antioxidant, antiallergic, antimicrobial, anti-inflammatory, antiviral, antithrombotic,
58 antidiabetic, anticancer, antihepatotoxic (Kumar & Pruthi, 2014). In addition to these
59 properties, and as the main idea of our concept, FA presents the capacity to dimerize by
60 oxidation in the presence of enzyme such as laccase (Adelakun et al., 2012), widely
61 distributed in plants, fungi, bacteria and insects (Rivera-Hoyos et al., 2013) and forming with

62 its homologues, the protein superfamily of multicopper oxidases (Sirim, Wagner, Wang,
63 Schmid & Pleiss, 2011). Thus, it was demonstrated that grain and cell wall contain dimers of
64 FA, which have leading to the crosslinking of arabinoxylans chains alone or together with
65 lignin. Such natural enzymatic crosslinking may be useful for plant in order to strengthen
66 their structure (Naczka & Shahidi, 2006) or to elaborate hydrogels around the seeds to fix
67 water needed for growing (Vismeh et al., 2013). Using such arabinoxylans naturally grafted
68 FA with its characteristics for new application fields appears difficult, unsuitable or not
69 versatile for many reasons: delicate extraction with low yield, few global amounts for
70 envisaging large field of applications, very few amount of grafted FA, impossibility to
71 modulate the FA content (i.e. to control both swelling and mechanical properties) and finally
72 impossibility to decline such process on other type of polysaccharides which may be more
73 suitable for specific applications.

74 On the basis of these considerations, the chemical grafting of FA with well-defined and
75 controlled amounts on any polysaccharides represents a great challenge. It could open the way
76 to very large possibilities of modulate hydrogels crosslinked with enzymatic eco-friendly
77 pathway. In this context, our approach is to create natural and versatile hydrogels after
78 chemical grafting of FA whatever the polysaccharide, following by a controlled cross-linking
79 between polysaccharide grafted chains performed by dimerization of FA under laccase action.

80 Polysaccharides can be neutral (arabinoxylan, pullulan, cellulose...), anionic (alginate,
81 hyaluronic acid...) and more rarely cationic for artificial polysaccharides (chitosan,
82 diethylaminoethyl-dextran...). To cover practically all polysaccharides, we decided to work on
83 two model polysaccharides, a neutral one as pullulan (PUL) and an anionic one as
84 carboxymethylpullulan (CMP). This choice is motivated since the chemical pathways for
85 grafting the ferulic acid are different if the polysaccharide evidence carboxylic acid functions
86 or only hydroxyl ones. Our first approach has been oriented toward anionic polysaccharides
87 and achieved outcomes using CMP were already reported (Dulong, Hadrich, Picton & Le
88 Cerf, 2016) showing very nice results. In this previous study, CMP-FA were obtained by
89 activation of the carboxylate functions of CMP with the well-known EDC/NHS activators.

90 Here, the purpose is to covalently graft FA with controlled amounts on pullulan, a water-
91 soluble neutral polysaccharide issued from fermentation of black yeast like *Aureobasidium*
92 *pullulans*. It consists of linear and flexible macromolecular chains composed of maltotriose
93 units linked by α -1,6 (Kato, Okamoto, Tokuya, & Takahashi, 1982). Pullulan is being
94 explored for various applications especially in biomedical due to its biocompatibility (Singh,

95 Kaur, Rana, & Kennedy, 2017). The grafting reaction is provided by carbonyldiimidazole
96 (CDI, considered as an eco-friendly reagent (Métro, Martinez & Lamaty, 2017) in two steps
97 by creating an ester bond between FA and PUL (Wen, Ye, Zhu & Zhao 2016). The efficiency
98 of the reaction was qualitatively and quantitatively verified by characterizing PUL-FA
99 products using several techniques (FTIR and ¹H NMR). The behavior in aqueous solution was
100 studied with size exclusion chromatography (SEC) coupling on-line with several detectors.
101 Then laccase from *Trametes versicolor* was used to crosslink PUL chains to obtain hydrogels.
102 Kinetics and mechanical properties were analyzed with rheology. Swelling, antioxidant and
103 cytotoxicity properties were also investigated.

104 **2. Experimental**

105 *2.1. Materials*

106 Pullulan (PUL) was provided by Hayashibara Biochemical Laboratory (Japan) and ferulic
107 acid was purchased from Acros Organics (France). N,N'-carbonyldiimidazole (CDI) and
108 Laccase [EC 1.10.3.2] from *Trametes versicolor* were supplied by Sigma-Aldrich (France).
109 All other materials are listed in SI file.

110 *2.2. Grafting of FA on PUL*

111 As mentioned previously, a two-step reaction was conducted to get PUL-FA. The first step is
112 to activate FA by CDI. For example, in a two-necked round-bottomed flask equipped with a
113 magnetic stirrer and connected to the N₂ line, equivalent moles of FA and CDI (1.5 mmol)
114 were dissolved in 20 mL of anhydrous DMSO. The reaction was kept under stirring (375 rpm)
115 at 60°C for 16 hours. In the second step, 1.6 g of dried pullulan was dissolved in anhydrous
116 DMSO (60 mL) and added slowly to the previous solution to form PUL-FA through an
117 esterification reaction. Then, the mixture was let at 60°C with continuous stirring for 6 hours.
118 At the end of reaction, the PUL-FA derivative was precipitated in five volumes of acetone and
119 after filtration it was stirred in the same volume of acetone for 24 hours to remove unreacted
120 FA, CDI and imidazole. After that, a second filtration was performed and the obtained
121 product was dried under vacuum at 50°C for 3 days. To eliminate residual traces of solvent,
122 the yellowish powder obtained was dissolved in milli-Q water and dialyzed against milli-Q
123 water until low conductivity of dialysis water (equivalent to milli-Q water one). Finally, PUL-
124 FA derivatives were lyophilized and stored at 4°C.

125 *2.3. Fourier transform infrared spectroscopy*

126 FTIR spectra were carried out using a Nicolet IS50 FT-IR spectrometer (Thermo Scientific,
127 USA) over a wavenumber range of 600–4000 cm⁻¹ by accumulating 128 scans at a resolution
128 of 4 cm⁻¹.

129

130 2.4. Nuclear magnetic resonance spectroscopy

131 ¹H NMR spectra were recorded on a Bruker Avance AC-P 300 MHz spectrometer (USA)
132 using D₂O/NaOD (85/15, v/v) as solvent. The substitution degree of FA onto pullulan (DS_{FA})
133 was determined based on the integral ratio of aromatic and methine protons of FA to α-1,4
134 anomeric protons of pullulan's anhydroglucose unit (AGU) via Eq. 1:

$$135 \quad DS_{FA} = \frac{\frac{I_{\text{aromatic and methine protons of FA}}}{5}}{\frac{I_{\alpha 1,4 \text{ anomeric protons of AGU}}}{2}} \quad \text{Eq. 1}$$

136 The amount of FA can be also expressed as FA equivalent (FA_{eq}) in mg per g of PUL-FA by
137 Eq. 2.

$$138 \quad FA_{eq} = \frac{M_{FA} \times DS_{FA}}{M_{PUL-FA}} \quad \text{Eq. 2}$$

139 with M_{FA}, the molar mass of FA.

140 2.5. Macromolecular characteristics

141 The absolute average number and weight molar masses (respectively Mn and Mw) and molar
142 masses distribution of PUL-FA derivatives were determined using on-line coupling a size
143 exclusion chromatograph (SEC), a multi-angle light scattering system (MALS), a viscosity
144 detector (VD), an ultraviolet spectrophotometer (UV) and a differential refractive index
145 detector (DRI). The details were provided in Supplementary Information.

146

147 2.6. Laccase from *Trametes versicolor* activity

148 Laccase activity was determined using the oxidation method of syringaldazine (Figueroa-
149 Espinoza & Rouau, 1998). The protocol is described in the SI file.

150

151 2.7. Preparation of PUL-FA hydrogels

152 A series of PUL-FA hydrogels was prepared by biomimetic enzymatic crosslinking with
153 following procedures. Different amounts of PUL-FA samples were dissolved, for 24h at room
154 temperature, in 2 mL of 0.1 M citrate-phosphate buffer (pH= 5.5) leading to three different

155 concentrations (40, 70 and 100 g/L). Then, laccase solution at 0.5, 1 and 2 nkat was added.
156 For rheological analysis, the solution is mixed manually (during a precise time added to the
157 analysis time) and immediately poured into the geometry of the rheometer to investigate the
158 gelling kinetics *in situ*.

159 For other analysis, the crosslinking reaction was kept at room temperature during 24h. The
160 obtained hydrogels were thoroughly washed with milli-Q water, frozen and freeze-dried for
161 one week.

162

163 2.8. Rheological measurements

164 Rheological experiments were carried out using a Discovery Hybrid Rheometer HR2 (TA
165 Instrument, U.K.) with standard-size double concentric cylinder geometry in dynamic mode.
166 Crosslinking reactions were performed by recording both elastic modulus (G') and viscous
167 modulus (G''), time dependent, at 1 Hz with 2% strain (in the linear viscoelastic domain).
168 Temperature (25°C) was kept constant using a Peltier and experiments were reproduced twice
169 with good accuracy. Obtained data were analyzed with TRIOS V 4.1.1.33003® software.

170 2.9. Swelling properties

171 For the kinetics of swelling, pre-weighted dried hydrogel (m_0) was immersed in 20 mL of
172 milli-Q water at room temperature. For each kinetics time point, the swollen hydrogel was
173 taken out of the solution and weighted (m_t) after the elimination of water's excess by Kimtech
174 delicate task wipes until swelling equilibrium was reached. The water content Q_t (g/g) was
175 calculated with Eq. 3:

$$176 \quad Q_t = \frac{m_t - m_0}{m_0} \quad \text{Eq. 3}$$

177 The equilibrium water content Q_e is obtained when Q_t stayed constant. Each experiment was
178 carried out in triplicate and results agreed within ± 0.04 g/g standard error.

179

180 2.10. Antioxidant activity and cytotoxicity

181 In order to evaluate the properties of FA, PUL and PUL-FAs, antioxidant and cytotoxicity
182 assays were realized. Furthermore, FA present inside hydrogels has the form of dimers
183 (DiFA). For this reason, we have enzymatically developed these dimers (see SI file) to also
184 estimate their activities.

185

186 2.10.1. Antioxidant properties: DPPH free radical scavenging assay

187 FA, DiFA, PUL and PUL-FAs were measured in terms of radical scavenging ability using the
188 stable radical DPPH• (Parejo et al., 2002). The procedure is detailed in the SI file.

189

190 2.10.2. Cytotoxicity assays

191 The cytotoxicity effect of FA, DiFA, and PUL-FAs were established on mouse fibroblasts
192 cells (L929- ATCC® CCL-1) according to the protocol described in the SI file.

193

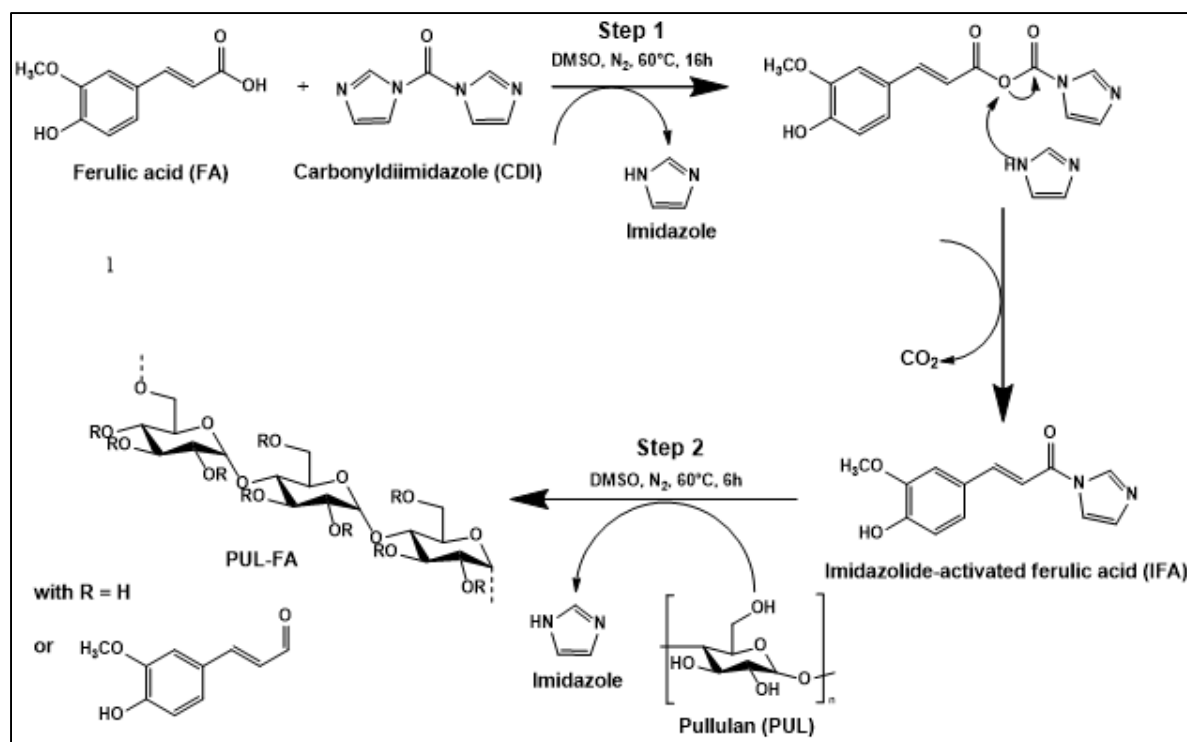
194 3. Results and discussion

195

196 3.1. Synthesis of PUL-FA

197 To get PUL-FA, several reaction protocols were tested according to experiments inspired
198 from literature with an appropriate adjustment on our reagents. Carbodiimide mediated
199 coupling reaction using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride and
200 N-hydroxysuccinimide was used in different solvents and co-solvents (Woranuch, Yoksan, &
201 Akashi, 2015). The Mitsunobu reaction using the coupling reagents triphenylphosphine and
202 diethylazodicarboxylate was also tested in DMSO and DMF (But & Toy, 2007). Furthermore
203 enzymatic esterification applying lipase from *Rhizomucor miehei* was also performed (Schär
204 & Nyström, 2015). Results achieved from all those reactions were unfortunately insignificant
205 and the grafting reaction was invalid or non-reproducible. This could be explained by the
206 solubility difference between hydrophilic PUL and hydrophobic FA and could be due also to
207 the low reactivity of some coupling reagent in used solvents.

208 However, the solution was found using an imidazole-mediated coupling reaction in anhydrous
209 DMSO applying CDI, as illustrated in Fig. 1. Obtained PUL-FAs (with good yield) are
210 yellowish that get darker as DS values increase. Their water solubility is highly dependent on
211 DS; the more their DS increase, the more their water solubility decrease and vice versa. To
212 obtain water-soluble PUL-FA, DS of 0.1 should not be exceeded. The productive parameters
213 used to synthesize PUL-FA with different DS are summarized in Table 1.



214

215 **Fig. 1.** Synthesis of PUL-FA conjugates. Chemical generation of imidazolide-activated FA by
 216 activation with CDI (Step 1). Esterification reaction of pullulan with FA (Step 2).

217

218 Table 1: Synthesis conditions and structural characteristics of PUL-FA according to reaction
 219 shown in Fig. 1

Product	n_{FA}/n_{AGU}^a	DS_{FA}^{th}	$DS_{FA}^{exp}^b$	FA content ^c	Yield
PUL-FA-2%	0.15	0.15	0.02	23.5	85%
PUL-FA-6%	0.30	0.30	0.06	67.5	87%
PUL-FA-10%	0.60	0.60	0.10	108.1	84%

220 ^a Molar ratio of FA over PUL AGU.

221 ^b Degree of substitution of FA determined by NMR (Eq. 1) (standard deviation 5%).

222 ^c FA content expressed in mg of FA/g of PUL-FA derivatives (Eq. 2).

223

224 3.2. Fourier transform infrared spectroscopy

225 FTIR spectral analyses were carried out to confirm the grafting of FA onto PUL (see the SI
 226 file, Fig. SI-3).

227

228 3.3. *Nuclear magnetic resonance spectroscopy*

229 ¹H NMR was used to figure out the DS_{FA} onto pullulan. The spectra and the peak attribution
230 of PUL, and PUL-FA-10% are given in SI (Fig. SI-4). Obtained values (Table 1) were lower
231 than the theoretical ones due to low reactivity and steric hindrance on PUL

232

233 3.4. *Discussion*

234

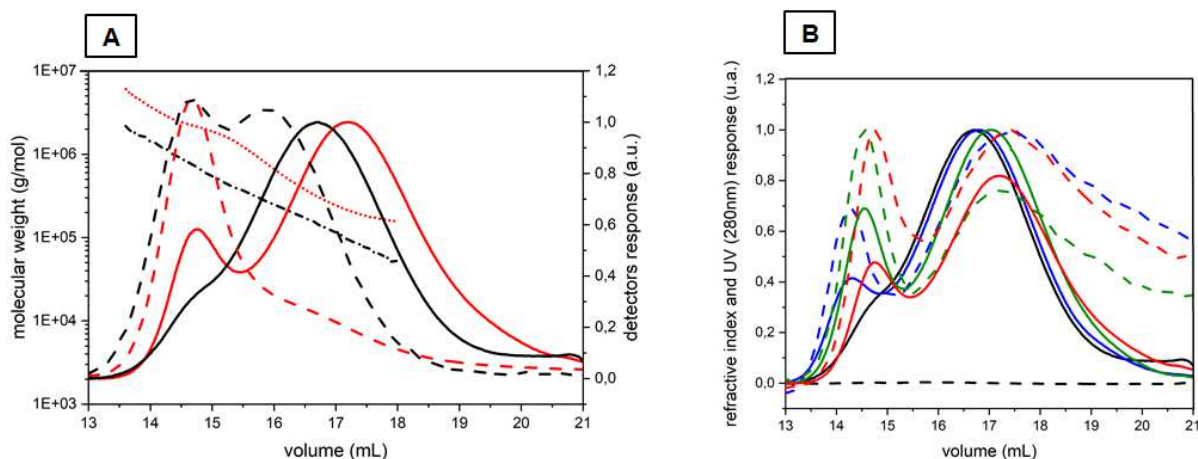
235 The amount of FA contained in PUL-FA shows a maximum value of 108.1 mg of FA/g of
236 PUL-FA (Table 1). Besides, if we compare our values to those obtained on CMP (between
237 8.53 and 83 mg/g) (Dulong, Hadrich, Picton & Le Cerf, 2016), we notice that our FA contents
238 are quietly higher which confirm the good efficiency of the carbodiimidazole chemistry in
239 esterification reaction. In addition, our values are much higher than those naturally observed
240 on arabinoxylans (between 1.6 and 2.3 mg/g) extracted from endosperm of wheat kernel
241 (Carvajal-Millan, Guilbert, Morel & Micard, 2005). This result proves that, we succeeded to
242 graft more FA moieties than in nature and then the possibilities of crosslinking will be more
243 important leading improved higher mechanical properties. However, PUL-FAs contain much
244 lower amounts of FA moieties than those obtained on starch using the same coupling method
245 (Wen, Ye, Zhu & Zhao, 2016). But in this case, starch derivatives were no water-soluble.
246 Higher values of FA content are reported on chitosan based on carbodiimide chemistry
247 (Woranuch & Yoksan, 2013). The water-solubility was ensured by the ionic character of the
248 chitosan, but with limit to a maximum concentration of 1.3 g/L.

249

250 3.5. *Physico-chemical behavior of PUL-FA in dilute regime*

251

252 Fig. 3A illustrates the molar masses distributions of PUL and PUL-FA-10% as a function of
253 elution volume with both light scattering (LS at 90°) and DRI profiles. Chromatograms of
254 PUL-FA-2% and PUL-FA-6% are shown in the SI (Fig. SI-1). Average characteristic
255 variables obtained in LiNO₃ 0.1mol/L at 25°C with polymer concentration at 1 g/L are
256 collected in Table 2. The mass recovery of analyzed samples (ratio between recovered sample
257 from integrated DRI peak and injected quantity) was about 80-90% which indicates that every
258 sample is almost totally eluted through the columns.



259
 260 **Fig. 2.** Elution profiles in LiNO_3 0.1 mol/L at 25°C : **A)** from refractive index (full lines) and
 261 light scattering at 90° (dashed lines) of PUL (black) and PUL-FA-10% (red) together with
 262 molar mass distribution (dotted lines). **B)** from refractive index (full lines) and UV at 280nm
 263 (dashed lines) of PUL (black), PUL-FA-2% (blue), PUL-FA-6% (green) and PUL-FA-10%
 264 (red).

265 In comparison with PUL, chromatograms of PUL-FAs (Fig. 2A and SI 1) are noticeably
 266 different. On one hand, PUL-FAs evidence a new population at low elution volume between
 267 14 and 15.5 mL (i.e. larger hydrodynamic volumes of polymer) together with higher apparent
 268 molar masses. This could be related to the presence of aggregated structures due to
 269 intermolecular hydrophobic interactions between grafted FA expected in aqueous solution,
 270 traducing the amphiphilic character of PUL-FA. On the other hand, one can notice that the
 271 major fraction of PUL-FA appears shifted toward higher elution volumes (i.e. smaller
 272 hydrodynamic volumes of polymer) compared to PUL. This clearly evidences the presence of
 273 a diminishing of PUL-FA size compared to PUL. That could be due either to a degradation of
 274 PUL backbone during the chemical modification or to the evidence of intramolecular
 275 associations between FA hydrophobic grafted moieties leading to strongly compacted
 276 structures. The hypothesis of a degradation can be push aside since the apparent molar masses
 277 distributions of PUL-FA fractions appears always parallel and higher than that of PUL.
 278 Moreover, this observation highlights the hypothesis of intramolecular associations since at a
 279 same elution volume (i.e. at a same hydrodynamic volume of polymer), PUL-FA always
 280 presents higher apparent molar mass than PUL. This is clearly the confirmation of more
 281 compact structure for PUL-FA due to FA intramolecular hydrophobic associations.
 282 Furthermore, the absorbance at 280 nm (according to FA phenolic structure) during the
 283 elution (Fig. 2B) indicates that the elution profile is comparable to the DRI response showing
 284 the homogenous distribution of FA moieties on all the molar masses distribution. This is also

285 observed for the first fraction of PUL-FA at low elution volume corresponding to the
 286 aggregates evidencing a considerable amount of associated grafted FA in such structures.

287
 288 This behavior, classic for amphiphilic polymers, has already been reported with pullulan
 289 grafted with guaiacol derivatives (Kouassi et al., 2017).

290
 291 All of the above qualitative conclusions are fully confirmed looking at data in Table 2. No
 292 diminishing in average apparent molar masses (M_n and M_w) of PUL-FA is noticeable,
 293 evidencing the absence of chemical degradation of pullulan backbone. On the contrary, and
 294 compared to PUL, apparent molar masses of PUL-FA become larger as DS_{FA} increases. This
 295 is largely expected considering intermolecular associations of grafted FA and regarding the
 296 first eluted fraction of PUL-FA. Aggregated structures are also confirmed when regarding the
 297 apparent DP of PUL-FAs which are much larger than real DP according to the increase of
 298 repeating units taking into accounts the DS (i.e. M_0). Moreover, the decrease of the $[\eta]$ when
 299 DS_{FA} increases confirms the densification of the polymer coils because of intramolecular
 300 associations (Dulong, Hadrich, Picton & Le Cerf, 2016). In our case, this result seems to
 301 indicate that intramolecular associations (main fraction) are more pronounced in dilute
 302 medium. The Mark-Houwink-Sakurada (MHS) exponent (a) based on the scaling relationship
 303 between $[\eta]$ and M , confirms the compact conformation of PUL-FA samples. Thus, we notice
 304 a clear decrease of (a) values when DS_{FA} increases, with values lower than 0.5 evidencing the
 305 very compact and dense structures of single coil or aggregates due to hydrophobic
 306 associations between FA. This result fully reinforced our previous conclusions.
 307 This associative behavior was less present with CMP-FA samples due to their anionic nature
 308 (Dulong, Hadrich, Picton & Le Cerf, 2016).

309
 310 **Table 2. Results obtained with SEC/MALS/DRI/VD analyses of PUL and PUL-FA**
 311 **derivatives (at 25°C, in LiNO₃ at 0.1 M and at 1 g/L)**

Sample	M_n (g/mol)	M_w (g/mol)	M_0^a (g/mol)	DP_n apparent ^b	$[\eta]_w$ (mL/g)	a^c	Mass recovery (%)
PUL	144,000	265,000	162	889	76	0.60	90
PUL-FA-2%	231,000	670,000	165.5	1,400	70	0.43	87
PUL-FA-6%	257,000	804,000	172.6	1,489	67	0.42	80
PUL-FA-10%	334,000	815,000	179.6	1,859	57	0.39	70

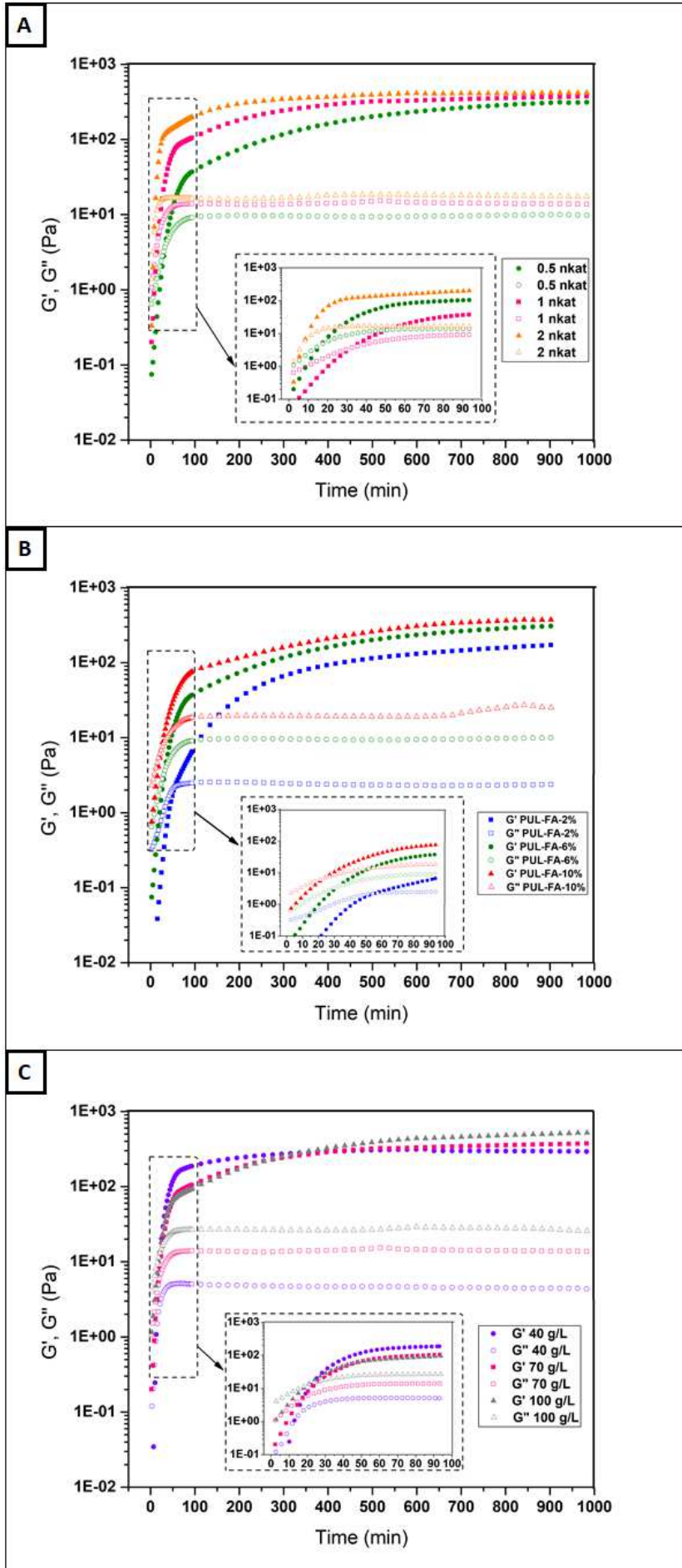
312 ^a M_0 is the molar mass of the repetition unit $M_0 = 162 + (176.2 \times DS_{FA})$

313 ^b $DP_{n \text{ apparent}}$ is the apparent degree of polymerization $DP_{n \text{ apparent}} = M_n / M_0$

314 ^c Integration zone : 15.5 to 18mL

315 *3.6. Rheological measurements*

316 The formation of PUL-FA hydrogels was rheologically investigated during the biomimetic
317 crosslinking reaction of PUL-FA by laccase in citrate-phosphate buffer (0.1M, pH=5.5) .



319 **Fig. 3.** Storage (G' : full symbol) and loss (G'' : empty symbol) moduli versus time during the
320 crosslinking reaction: **A) Influence of enzymatic activity for PUL-FA-6% at 70 g/L** **B)**
321 **Influence of DS_{FA} for PUL-FAs at 70 g/L with laccase at 0.5 nkat.** **C) Influence of PUL-FA**
322 **concentration for PUL-FA-6% using laccase at 1 nkat.**

323

324 All rheological analyses evidence similar profile (Fig. 3). At the beginning, both elastic (G')
325 and viscous (G'') moduli are very low with G' lower than G'' corresponding to the behavior of
326 viscous solution. Then, both moduli increase suddenly and G' exceeds rapidly G'' as gelation
327 proceeds, to become largely higher than G'' . At the end, both moduli reach a plateau or a
328 pseudo plateau indicating the end of gelation. These types of behavior are fully characteristic
329 of a chemical crosslinking leading to the formation of a 3D elastic hydrogel. This clearly
330 evidences the efficiency of the FA grafted moieties biomimetic dimerization induced by
331 laccase via radical reaction (Adelakun et al., 2012; Carvajal-Millan et al., 2005).

332 This gelation profile was also similar to other hydrogels formed through other physical or
333 chemical crosslinks (Chambon, Petrovic, Mac Knight, & Winter, 1986). The crossing point at
334 $G'=G''$ ($\tan \delta = 1$) has been subject of interesting scientific debate. Some researchers consider
335 this crossover as the gel point while others define it as a characteristic point of gelation
336 indicating the beginning of gel's formation by a transition from the purely liquid phase to the
337 solid one dominated by a viscoelastic behavior. Our systems were not in accordance with the
338 Winter-Chambon criterion (Winter & Mours, 1994), following the loss tangent at different
339 oscillation frequencies (Fig SI 5.). Consequently, we will use this point as a qualitative,
340 noticeable indication correlated to the notion of percolation. At least these characteristic
341 points (called gelation point) will permit to obtain a characteristic time (t_{gel}) allowing us to
342 compare the kinetics of various systems according to laccase activity, DS_{FA} or PUL-FA
343 concentration. Another characteristic parameter is the value of G' at the plateau which
344 indicates the mechanical properties of hydrogels also correlated to the number of crosslinks.

345 Several crosslinking reactions with changing enzymatic activity (EA), concentration and
346 DS_{FA} , allowed us to obtain varieties of hydrogels with different kinetics and mechanical
347 characteristics (Table 3). The values are quite close to those obtained with CMP-FA hydrogels
348 (Dulong, Hadrach, Picton & Le Cerf, 2016).

349 The kinetics is largely accelerated when EA increases, with quite similar G' at the plateau
350 (Fig. 4a). Laccase has impact only on the crosslinking kinetics and has no impact on the

351 mechanical properties of final gels (same number of crosslinking points created), agreeing
 352 with the catalytic role of this enzyme in the crosslinking reaction (Dulong, Hadrich, Picton &
 353 Le Cerf, 2016).

354 The amount of FA grafted moieties onto pullulan affects both kinetics and final mechanical
 355 characteristics. The 3D network is formed more rapidly with stronger mechanical properties
 356 as DS_{FA} increases (Fig. 4b). Laccase find easily and more quickly the grafted FA when their
 357 amount increases leading to a tighter network.

358 The increase of PUL-FA concentration during the laccase action induces a denser
 359 entanglement of polymer chains which improves the probability of FA grafted moieties
 360 meeting and reactivity, and consequently increases the crosslinking density (Fig. 4c).
 361 Additionally, this phenomenon could be supported by the presence of intermolecular
 362 hydrophobic associations previously proved with SEC which could lead to a better reactivity
 363 between FA grafts. This slowdown of kinetics could be interpreted by this larger
 364 entanglement achieved with important concentrations which may affect the enzyme mobility.

365 The $\tan \delta$ values ($\tan \delta = G''/G'$) calculated for all final gels were from 0.01 to 0.04 (<1)
 366 confirm the presence of an elastic covalent system (Lack et al., 2004). The weakest hydrogel
 367 reached an elastic modulus of 170 Pa while the strongest one had value of 800 Pa. All of the
 368 hydrogels demonstrate stronger mechanical properties than those obtained by Carvajal-
 369 Millan, Guilbert, Morel & Micard (2005) from the water extractable arabinoxylans found in
 370 endosperm of wheat kernel (G' ranged from 5 to 41 Pa). This result proves that we have not
 371 only use the biomimetic crosslinking phenomenon found in nature but also, we have
 372 successfully improved the mechanical properties of hydrogels.

373 **Table 3. Rheological data of crosslinking reactions of PUL-FA derivatives in citrate-**
 374 **phosphate buffer (0.1M, pH=5.5) at 25°C**

Sample	[C] (g/L)	EA (nkat)	t_{gel} (min)	G'^a (Pa)	G''^a (Pa)	$\tan \delta^a$
PUL-FA-2%	70	0.5	55	170	2	0.01
	40	1	13	260	4	0.02
	70	0.5	33	340	9	0.03
PUL-FA-6%	70	1	17	380	13	0.03
	70	2	8	410	17	0.04

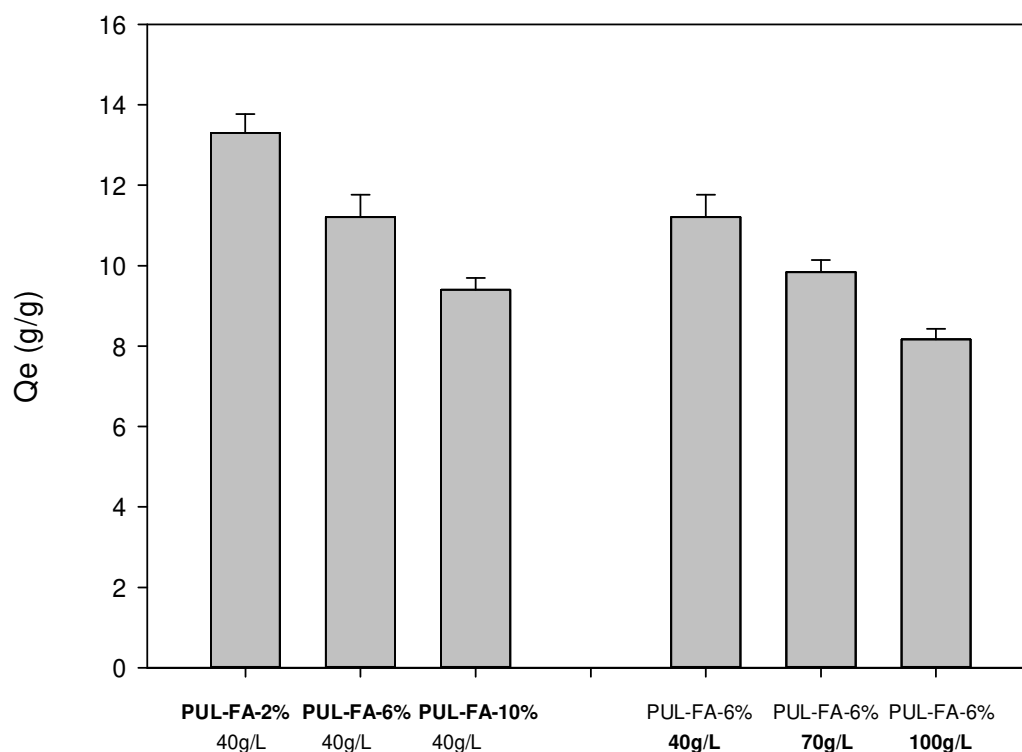
	100	1	20	570	25	0.04
PUL-FA-10%	70	0.5	24	440	19	0.04

375 ^a Values measured at the plateau at the end of the crosslinking reaction

376

377 *3.7. Swelling properties*

378 Usually, the equilibrium swelling capacity of hydrogel is highly dependent on the molecular
379 structure of polymer network, the crosslinking density as well as content interactions of
380 hydrogel. Thus, we have investigated the swelling behavior of elaborated hydrogels as a
381 function of substitution degree and PUL-FA concentration. Unlike the hydrogels obtained
382 from CMP-FA (Dulong, Hadrich, Picton & Le Cerf, 2016) that achieved the maximum
383 swelling degree very fast (in only 20 s), swelling kinetics of PUL-FA hydrogels appears
384 clearly slower reaching a pseudo plateau after 8 days (data not shown). This could be
385 explained by the presence of charges on CMP-FA (anionic polysaccharide) which facilitates
386 the penetration of the swelling solvent as a result of electrostatic repulsion increasing the
387 distance between polymer chains. Moreover, the neutral character of PUL-FA hydrogel and
388 presence of hydrophobic interactions explain the lower swelling degree than CMP-FA
389 hydrogels (factor 7). The same effect was observed on swelling properties of hydrogels based
390 on carboxymethylcellulose and hydroxyethylcellulose (Sannino & Nicolais, 2005).



391

392 **Fig.4** . Effect of DS_{FA} and PUL-FA concentration on the swelling properties of hydrogels in
393 milli Q-water

394 Fig. 4 clearly evidences that the swelling behavior of hydrogels is impacted by the DS_{FA} and
395 the PUL-FA concentration. Logically and in agreement with rheological data, the more is
396 DS_{FA} or the PUL-FA concentration, the less is the swelling. That is due to the increase of the
397 crosslinking density and of hydrophobic interactions which limits the penetration of water
398 inside hydrogel network.

399 Finally, it can be concluded that swelling properties showed very low and slow equilibrium
400 water content due to the non-ionic nature of PUL-FA hydrogels. All results are completely in
401 agreement with the rheological studies with antagonistic tendencies between swelling and
402 mechanical properties.

403

404 *3.8. Antioxidant activity and cytotoxicity*

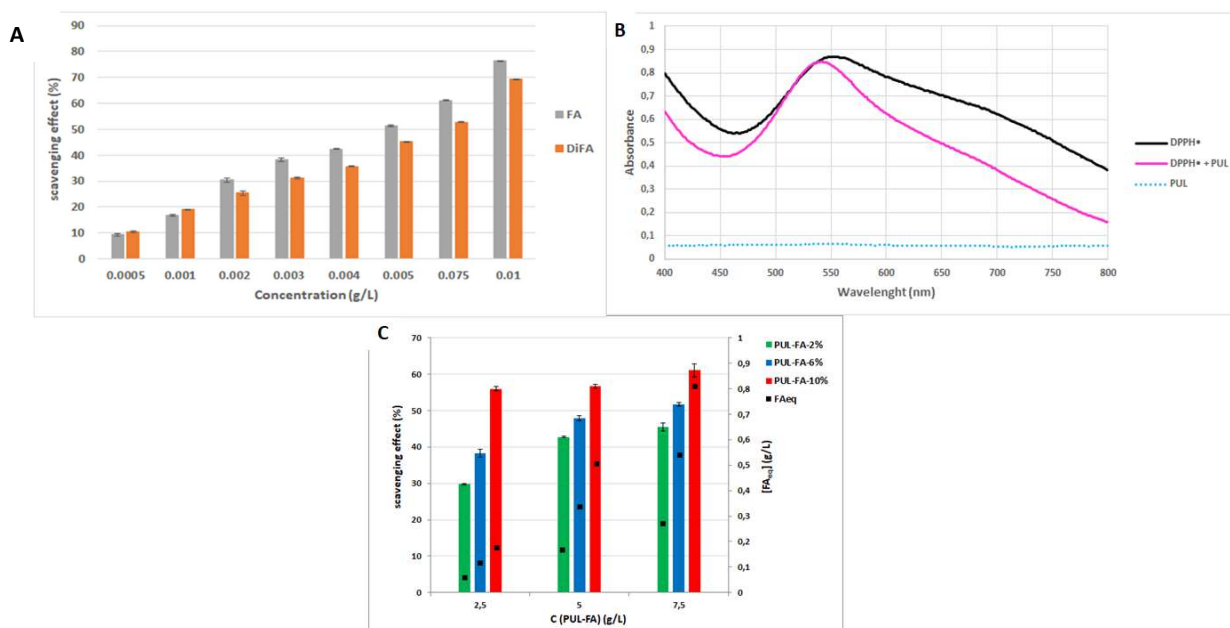
405 *3.8.1. Synthesis of ferulic acid dimers*

406 The synthesis of FA dimers (DiFA) was performed using laccase from *Trametes versicolor*
407 with appropriate amount via radical oxidation phenomenon. The details are given in the SI
408 file.

409

410 *3.8.2. Antioxidant properties: DPPH free radical scavenging assay*

411 The antioxidant properties of FA, DiFA, PUL and PUL-FA derivatives were measured by
412 DPPH free radical method based on calculating the decrease in the absorbance of DPPH•.
413 Obtained results are shown in Fig. 5.



414
 415 **Fig. 5. A)** Free-radical scavenging activity of FA and DiFA after 20 min. **B)** Absorbance of
 416 DPPH (100 μ M) and PUL (5 g/L) without DPPH and in the presence of DPPH. **C)** Free-
 417 radical scavenging activity of PUL-FAs after 20 min. Equivalent concentration of FA are on
 418 the right axis.

419
 420 A kinetics study between 5-30 min was first carried out to determine the time required to
 421 scavenge DPPH• by PUL-FA at different DS_{FA} at the lowest tested concentration (2.5 g/L).
 422 Results had shown that the radical scavenging effect increases slightly by increasing the
 423 analysis time from 5 to 20 min to reach the steady state within 20 min (Fig SI 2). Therefore,
 424 the time to evaluate antioxidant properties was fixed at 20 min.

425
 426 For a better comparison, we have also determined for all tested samples, the half-inhibition
 427 concentration (EC_{50}), a usual way to express the antioxidant activity of a compound which is
 428 defined as the minimum concentration of antioxidant agent able to scavenge 50% of DPPH•.
 429 The higher antioxidant activity, the lower EC_{50} and all acquired data are set out in Table 4.

430
 431 **Table 4. Scavenging effects and half-inhibition concentrations of the DPPH radical**
 432 **(EC_{50}) of FA, DiFA and PUL-FA derivatives**

Product	[C] (g/L)	[FA]eq (g/L)	Scavenging effect (%)	EC_{50} (g/L)
FA	0.01	0.01	76.4 ± 0.2	5.55×10^{-3}

DiFA	0.01	0.01	69.4 ± 0.1	6.53 × 10 ⁻³
	2.5	0.06	29.7 ± 0.1	
PUL-FA-2%	5	0.17	42.8 ± 0.2	9.7
	7.5	0.27	45.5 ± 1.1	
	2.5	0.12	38.3 ± 1.0	
PUL-FA-6%	5	0.34	47.9 ± 0.7	6
	7.5	0.54	51.7 ± 0.4	
	2.5	0.18	55.9 ± 0.6	
PUL-FA-10%	5	0.50	56.6 ± 0.7	0.9
	7.5	0.81	61.0 ± 1.7	

433

434 Both FA and DiFA present antioxidant activity against DPPH• which increases with
435 concentration with a slightly higher activity for FA compared to its dimers (Fig. 5A). This
436 was proved by the EC₅₀ values which was 5.55 µg/mL (28.6 µmol/L) for FA versus 6.53
437 µg/mL (33.63 µmol/L) for DiFA (Table 4). Thus, FA has a slightly higher scavenging effect
438 than its dimers (Dulong, Kouassi, Labat, Le Cerf & Picton, 2018). Indeed, the creation of
439 DiFA requires the condensation of two quinones (radicals) established by the enzymatic
440 oxidation of FA. As a result, some of FA radicals will be involved in the formation of those
441 dimers and then no more available for scavenging DPPH free radical which can explain the
442 decrease in antioxidant activity of DiFA.

443

444 For FA, obtained EC₅₀ was in agreement with literature where values of 22 µmol/L, 25.1
445 µmol/L, 27.8 µmol/L and 28.1 µmol/L were reported (Brand-Williams, Cuvelier & Berset,
446 1995; Cassano, Trombino, Muzzalupo, Tavano & Picci, 2009; Dulong, Kouassi, Labat, Le
447 Cerf & Picton, 2018; Gortzi, Christophoridou & Roussis, 2015). In comparison to its
448 counterparts of non-flavonoid phenolic compounds, FA shown a similar antioxidant activity
449 as for its hydroxycinnamic acid counterpart, caffeic acid (27.75 µmol/L) and lower than two
450 hydroxybenzoic acids, syringic acid (12.2 µmol/L) and gallic acid (6.82 µmol/L) (Gülçin,
451 2006; Salgueiro, Lira, Rumjanek & Castro, 2014). FA possesses also lower antioxidant
452 properties than aminoguaiacol (13.7 µmol/L) but higher than guaiacol (33.5 µmol/L) and
453 eugenol (71.3 µmol/L) (Kouassi et al., 2017; Pérez-Rosés, Risco, Vila, Penalver &
454 Canigual, 2016; Yu, Ren, Nan, & Liu, 2015).

455

456

457 Unlike FA and its dimers, PUL did not present any antioxidant activity (Fig. 5B). We can note
458 that the maximum of PUL precursor with DPPH• curve has an absorbance value like the
459 DPPH• alone. This was also confirmed by the visual aspect of the analyzed samples where no
460 change in color of DPPH• in contact of PUL was observed.

461

462 The antioxidant properties of PUL-FAs were also evaluated (Fig. 5C). Results demonstrated
463 that all grafted PUL present antioxidant properties which increased with concentration and
464 DS_{FA} . As PUL shows no antioxidant activity, antioxidant properties of PUL-FAs are then
465 provided by FA grafted moieties. Calculated values of EC_{50} (Table 4) of PUL-FA-2%, PUL-
466 FA-6% and PUL-FA-10% decrease logically with DS_{FA} increasing and were respectively 9.7
467 g/L (58.6 mmol/L) , 6 g/L (34.7 mmol/L) and 0.9 g/L (5 mmol/L). These values are more
468 important than EC_{50} of FA which confirms a significant decrease in the antioxidant activity of
469 FA when it's grafted onto PUL. This was also confirmed by calculating the grafted FA
470 equivalent concentration $[FA]_{eq}$ in PUL-FA which was added to Fig. 5C on the right vertical
471 axis. In comparison to the tested concentration of FA, the $[FA]_{eq}$ was always higher.

472 Nevertheless, scavenging effect of PUL-FAs was always much lower than those of FA which
473 proves our previous conclusion. This could be attributed to the difficult accessibility of DPPH
474 radicals to FA moieties when they are grafted onto PUL due to the greater difficulty of
475 DPPH• diffusion into polymer solution than a molecular one.

476

477 Obtained EC_{50} value for PUL-FA-10% (6 g/L) can be compared to the EC_{50} of FA grafted
478 starch (0.34 g/L) (Wen, Ye, Zhu & Zhao, 2016). In this case, EC_{50} is lower, reflecting higher
479 antioxidant properties due to the higher FA content for starch-FA derivative (303.9 mg of FA
480 / g of starch-FA derivative). It should be noted that for FA grafted starch, another method was
481 used to determine antioxidant properties due to the poor water solubility of starch-FA
482 derivative. Ferulic acid was also grafted onto chitosan with FA content (66.7 mg of FA / g of
483 chitosan-FA derivative) approximatively similar to our PUL-FA-6% (67.5 mg of FA / g of
484 PUL-FA derivative). The EC_{50} of chitosan-FA derivative (0.75 g/L) is again lower (Liu, Wen,
485 Lu, Kan & Jin, 2014). But this time, this is could be explained by the perfect solubility of this
486 derivative in the medium used for antioxidant activity assays as well as the presence of amine
487 function on the chitosan backbone which brought intrinsic antioxidant activity to this
488 polysaccharide.

489 If we compare the antioxidant character of PUL-FA to CMP-FA, we find that the associative
490 behavior of PUL-FA has a negative effect (Dulong, Kouassi, Labat, Le Cerf & Picton, 2018).

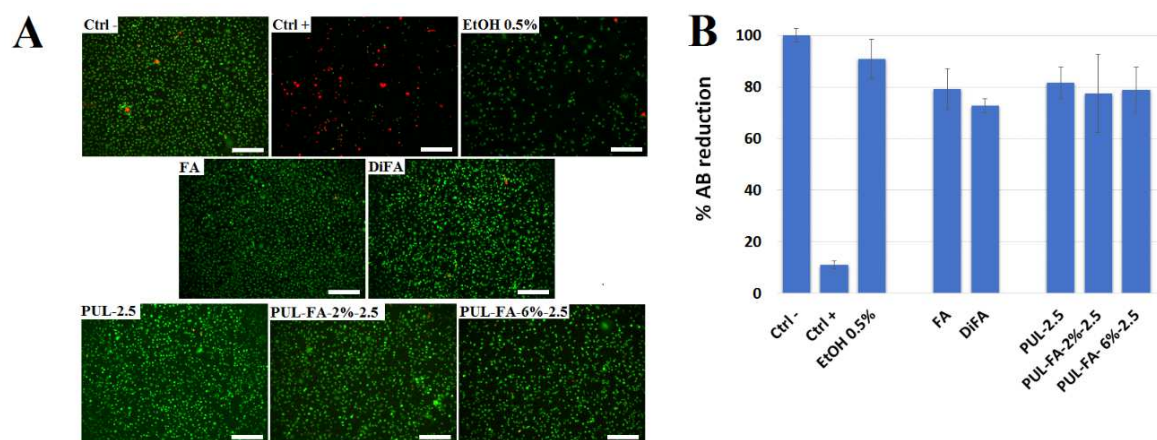
491

492 3.8.3. Cytotoxicity assays

493

494 To check the cytotoxicity of the FA, DiFA, and PUL-FAs, L-929 mouse fibroblast cells were
495 put in contact with all the compounds for 24h and 48h to examine their viability and
496 metabolic activity, respectively. From Fig. 6A, we observe that most of cells appeared in
497 green after fluorescence microscopy inspection, reflecting that cell membranes are not
498 damaged.

499 Next, to validate these qualitative observations, the metabolic activity of cells was assessed
500 using Alamar Blue® assay after 48h of contact. As a reminder, a percentage of AB reduction
501 below 70% of negative control indicates a cytotoxic effect. Figure 6B shows that FA and
502 DiFA, as well as PUL-FAs were above the threshold of 70% AB reduction. Consequently,
503 these results clearly evidence that both FA and DiFA, and PUL- FAs at a concentration of 2.5
504 g/L have no cytotoxic effects towards fibroblasts, whatever the DS_{FA} (2% and 6%).



505

506 **Fig. 6.** Cytotoxicity evaluation of FA, DiFA and PUL-FAs. **A)** Live/Dead® fluorescence
507 microscopy of L-929 cells after 24h contacting; live and dead cells appeared green and red,
508 respectively. **B)** Alamar Blue® (AB) reduction of L-929 cells after 48h in contact. Results are
509 expressed in mean \pm SEM.

510

511 4. Conclusion

512 New advanced biomimetic pullulan hydrogels via an eco-friendly procedure (i.e. enzymatic
513 crosslinking) with improved mechanical and viscoelastic properties were successfully

514 prepared introducing FA moieties onto PUL backbone. Various amount of FA was grafted
515 onto pullulan through a carbodiimidazole mediated coupling reaction via two steps. The
516 reaction efficiencies were very low leading to substitution degrees ranged between 2 and 10%
517 (corresponding to 23.5 and 108.1 mg/g of FA contents in PUL-FAs). All the PUL-FAs are
518 water-soluble, even for the highest grafting amount. In each case, intramolecular hydrophobic
519 associations between FA groups persist and lead to aggregates or isolated dense coils with a
520 compact conformation compared to that of precursor (PUL). Thereafter, hydrogels were
521 obtained by enzymatic crosslinking reaction using laccase from *Trametes versicolor*.
522 Enzymatic activity only impacts the gelation kinetics as a catalyst, while substitution degree
523 and concentration both have effects on the crosslinking kinetics, the rheological and swelling
524 properties of PUL-FA hydrogels. An increase of mechanical properties is logically observed
525 by increasing the DS_{FA} or concentration of PUL-FAs due to the raise of the crosslink density
526 and of hydrophobic interactions. Comparing to CMP-FA hydrogels, PUL-FA hydrogels
527 exhibit slower and lower swellings in water due to the neutral character and high flexibility of
528 pullulan backbone when compared to anionic polysaccharide. Finally, based on the standard
529 ISO 10993-5, the cytotoxicity evaluation of FA, DiFA and PUL-FAs at 2.5 g/L demonstrate
530 that these components are not cytotoxic towards fibroblasts. All realized studies allowed us to
531 better understand this biomimetic phenomenon and then to well control the biomimetic
532 hydrogels for future applications. Further studies are in progress transferring our know-how to
533 a more rigid polysaccharide with more interest in industrial sector.

534

535

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540

541

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543

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