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1 **Interactions between infernan and calcium: from the molecular level to the**
2 **mechanical properties of microgels**

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15

16 **Abstract**

17 With the increasing need for hydrogels with tunable properties for specific biomedical
18 applications, a complete understanding of the structure-function relationship of polymers used
19 for hydrogel development remains crucial for their optimal use. In the present study, by
20 combining experimental and theoretical approaches, the structure-function relationship of a
21 bacterial exopolysaccharide, infernan, displaying both glycosaminoglycan-mimetic and gelling
22 properties, was investigated at molecular and microscopic levels. Atomic force microscopy
23 (AFM) experiments and molecular dynamics simulations were applied to determine the
24 persistence length of individual infernan chains before studying their association induced by
25 calcium. Infernan-based microgels were then produced using microfluidics and their

26 mechanical properties were characterized by AFM methods. The mechanical properties of
27 EPS/calcium microgels were finely tuned by varying the crosslinking density of their network,
28 either by calcium or EPS concentrations. The obtained set of viscoelastic microgels with
29 different elastic modulus values opens several possibilities for their applications in tissue
30 engineering.

31 **Keywords**

32 **Atomic force microscopy, molecular dynamics simulations, persistence length, viscoelastic**
33 **properties, microfluidics.**

34

35 **1. Introduction**

36 Polysaccharides constitute a highly diverse family of macromolecules in primary and
37 secondary structures, which further determine their valuable functional properties.
38 Polysaccharides occur in plant (e.g. pectin, cellulose) and algal (e.g. alginate, carrageenan,
39 fucan) cell walls, and in animal tissues (glycosaminoglycans, GAG). They are also secreted by
40 microorganisms, including bacteria (e.g. xanthan, gellan, welan, hyaluronic acid). These
41 ubiquitous macromolecules are essential for cells, tissues and the whole organism, in which
42 they fulfil crucial structural and biological functions. Both structure and molecular weight of
43 polysaccharides determine their functionalities, which are largely exploited in several domains.
44 For instance, physicochemical, in particularly gelling, properties of some anionic
45 heteropolysaccharides, such as alginate, pectin, carrageenan and xanthan, have been explored
46 for many decades in food, cosmetics and more recently in biomedical applications (Augst,
47 Kong, & Mooney, 2006; Freitas, Coimbra, Souza, & Sousa, 2021; Kumar, Rao, & Han, 2018;
48 Pacheco-Quito, Ruiz-Caro, & Veiga, 2020). They contribute largely to the economy of
49 glycoscience (Corolleur, Level, Matt, & Perez, 2020). The common feature of these
50 polysaccharides is their ability to form a physical gel in the presence of divalent cations, e.g.
51 calcium (Ca^{2+}). In the case of alginate, gel formation results from the association of single
52 helical chains rich in guluronic acid (GulpA) domains into junction zones through the Ca^{2+} ions
53 coordinated between them, as described in the “egg-box model” (Grant, Morris, Rees, Smith,
54 & Thom, 1973). For pectin gelation, a modified model called the “shifted egg-box model” was
55 proposed to explain the association of pectin domains rich in galacturonic acid (GalpA) into
56 junction zones through Ca^{2+} (Braccini, & Pérez, 2001). In contrast to alginate and pectin, where
57 carboxylic oxygen atoms and water oxygen coordinate the divalent ion, iota-carrageenan
58 gelation results from inter-helix bridges stabilized by Ca^{2+} coordinated by negatively charged
59 sulfate groups and water molecules (Janaswamy, & Chandrasekaran, 2008). Xanthan is an

60 example of a bacterial exopolysaccharide (EPS), having, in its repeating unit, a negatively
61 charged trisaccharide side chain attached to a cellulose-like backbone (Jansson, Kenne, &
62 Lindberg, 1975). In the helical conformation adopted by xanthan chains, the side chains remain
63 aligned with the backbone and stabilize the overall structure by non-covalent interactions,
64 mainly hydrogen bonding (Moorhouse, Walkinshaw, & Arnott, 1977). The presence of Ca^{2+}
65 stabilizes xanthan in its ordered helical conformation, where the main binding sites are likely
66 constituted between the terminal pyruvate groups of the side chains, while the main chains play
67 a minor role in the cation complexation (Bergmann, Furth, & Mayer, 2008; Lambert, Milas, &
68 Rinaudo, 1985). Recently, we have shown that a bacterial EPS secreted by the deep-sea
69 hydrothermal vent bacterium *Alteromonas infernus*, called infernan (GY785 EPS), was also
70 able to gel in the presence of Ca^{2+} (Zykwinska *et al.*, 2019; Gélébart *et al.*, 2022). Similarly to
71 xanthan, infernan is also a highly branched heteropolysaccharide. However, its repeating unit
72 is slightly more complex (Roger, Kervarec, Ratiskol, Collic-Jouault, & Chevolot, 2004). The
73 trisaccharide main chain, composed of glucose (Glc), GalpA and galactose (Galp), is
74 covalently linked in the sequence: $\rightarrow 4\text{-}\beta\text{-D-Glc p-(1}\rightarrow 4\text{)-}\alpha\text{-D-Galp A-(1}\rightarrow 4\text{)-}\alpha\text{-D-Galp-(1}\rightarrow$, is
75 branched by a hexasaccharide side chain. Indeed, GalpA residue of the main chain is substituted
76 at O-3 by two glucuronic acids (Glc pA), one Gal and one Glc giving the following sequence:
77 $\beta\text{-D-Glc p-(1}\rightarrow 6\text{)-}\alpha\text{-D-Galp-(1}\rightarrow 4\text{)-}\beta\text{-D-Glc pA-(1}\rightarrow 4\text{)-}\beta\text{-D-Glc pA-(1}\rightarrow$. In addition, the two
78 Glc pA of the side chain are each substituted by a terminal Glc. The interesting feature of this
79 EPS is its anionic nature resulting from the presence of uronic acid residues and sulfate groups.
80 Indeed, GalA of the main chain is substituted at O-2 by one sulfate group. In contrast to xanthan,
81 native infernan of high molecular weight (HMW), 2,000,000 g/mol, cannot gel. However, a
82 physical Ca^{2+} gel was formed by a medium molecular weight (MMW) derivative, EPS DR
83 (240,000 g/mol), obtained by radical depolymerization of the native HMW EPS (Zykwinska *et*
84 *al.*, 2019; Gélébart *et al.*, 2022). Besides gelling properties with divalent cations, the negatively

85 charged EPS is also able to bind to positively charged proteins, such as basic growth factors,
86 and this property has a huge potential in tissue engineering (Merceron *et al.*, 2012; Rederstorff
87 *et al.*, 2017; Zykwinska *et al.*, 2018; Zykwinska *et al.*, 2019; Gélébart *et al.*, 2022). Indeed,
88 growth factors constitute a key element in tissue engineering strategies since they stimulate
89 essential cellular processes (proliferation, migration, differentiation). Growth factor delivery
90 systems, such as microgels, have intensely been explored to enhance growth factor bioactivity
91 and sustain their local release to the surrounding microenvironment (Caballero Aguilar, Silva,
92 & Moulton, 2019; Subbiah & Guldberg, 2019). By associating both physicochemical (gelling)
93 and biological (GAG-mimetic) properties of infernan derivatives, we have recently developed
94 growth factor-loaded microgels for cartilage and bone engineering purposes (Zykwinska *et al.*,
95 2019; Gélébart *et al.*, 2022).

96 The objective of the present study was to gain further insight into infernan (GY785 EPS)
97 gelling properties induced by the presence of Ca^{2+} . The knowledge of the structure-function
98 relationship remains crucial for the future development of infernan-based microgels for
99 particular applications, especially in tissue engineering. For this purpose, polysaccharide- Ca^{2+}
100 interactions were firstly studied at the molecular level by combining an experimental approach
101 based on atomic force microscopy (AFM) with a theoretical one, exploiting molecular
102 dynamics simulations. AFM constitutes a powerful tool allowing to image biological structures
103 with nanometric resolution, to measure the forces between single molecules with piconewton
104 sensitivity and to probe the mechanical properties (Cuenot, Bouvrée, & Bouchara, 2017; Florin,
105 Moy, & Gaub, 1994; Hinterdorfer & Dufrêne, 2006; Zykwinska *et al.*, 2018). Molecular
106 dynamics simulations allowed to establish the molecular conformations of the polysaccharide
107 chains upon Ca^{2+} binding. The impact of both EPS and Ca^{2+} concentrations on the mechanical
108 properties of infernan-based microgels, formulated using capillary microfluidics, was further
109 assessed by AFM measurements. The main hypothesis of the present study was how the

110 structure-function properties on the molecular level influence the mechanical properties of
111 infernan-based microgels at the microscale.

112 **2. Materials and Methods**

113 **2.1. Production of native infernan and medium molecular weight (MMW) EPS derivative** 114 **(EPS DR_{MMW}), and their characterization**

115 Native EPS was produced through fermentation (Raguénès *et al.*, 1997). Briefly,
116 *Alteromonas infernus* was cultured at 25°C pH 7.4 in a 30 L fermenter (Techfors 30 L INFORS,
117 Switzerland) containing 20 L of Zobell medium, composed of 5 g/L of tryptone, 1g/L of yeast
118 extract and 33.3 g/L of aquarium salts. For EPS biosynthesis, 30 g/L of glucose was added at
119 the beginning of the batch. Native EPS excreted in its soluble form and remaining in the culture
120 medium was recovered after 48h by a centrifugation step (9000 g, 45 min). The supernatant
121 was then ultrafiltrated on a 100 kDa cut-off membrane and freeze-dried.

122 An EPS DR_{MMW} of 260,000 g/mol was prepared from native EPS using a free-radical
123 depolymerization process (Gélébart *et al.*, 2022). Native EPS and EPS DR_{MMW} were
124 characterized for their monosaccharide composition, weight-average molecular weight and
125 sulfur content (Gélébart *et al.*, 2022).

126 **2.2. Microgel formation by capillary microfluidics**

127 EPS DR_{MMW} was structured into microgels using a home-made capillary microfluidic co-
128 flow device (Figure S1). The dispersed phase containing EPS DR_{MMW} aqueous solution at 2, 3
129 or 4% (w/w) was delivered through a silica tube with an interior diameter of 75 µm inserted
130 into chromatography tee fitting. The continuous phase, a sunflower seed oil (Fluka), was
131 delivered through a Fluorinated Ethylene Propylene (FEP) 1/16" tube (ID 750 µm, OD 1.57
132 mm) (Cluzeau Info Labo, France) perpendicularly to the tee fitting. The dispersed phase co-
133 flowed in a FEP 1/16" tube (ID 750 µm, OD 1.57 mm) containing the continuous oil phase.

134 The dispersed phase was applied at 2 $\mu\text{L}/\text{min}$ and the continuous phase at 120 $\mu\text{L}/\text{min}$, to
135 produce microdroplets. The flow rates were controlled using a Microfluidic Flow Control
136 System MFCSTM-EZ with a flow-rate platform (Fluigent). The device was run for 45 min per
137 sample. Microdroplets were recovered in a collecting bath containing 10 mL of 360 mM (for
138 EPS DR_{MMW} at 2, 3 and 4% (w/w)) or 1 000 mM CaCl₂ (for EPS DR_{MMW} at 4% (w/w)) under
139 magnetic stirring to avoid their coalescence. Microgels formed directly upon the microdroplet
140 contact with CaCl₂ solution were stored overnight at 4°C in either 360 mM or 1 000 mM CaCl₂.
141 Microgel suspensions were washed three times with the same CaCl₂ solutions before AFM
142 experiments. Microgels remaining in 360 mM CaCl₂ were also equilibrated in 2 mM CaCl₂ for
143 24h at 4°C.

144 **2.3. Microscopy**

145 Microgels recovered in collecting baths were observed using an optical microscope (Optika,
146 Italy). The size of microgels (N = 30) was determined using Optika Vision Lite software.
147 Scanning electron microscopy SEM observations on freeze-dried microgels were performed
148 using JEOL JSM 7600F instrument (JEOL, Japan) working at an accelerating voltage of 5KV.
149 Images were collected by the Everhart-Thornley secondary electron detector of the chamber,
150 far enough of the lens to ensure good depth of field.

151 **2.4. AFM**

152 *2.4.1. AFM imaging*

153 For AFM imaging, EPS DR_{MMW} aqueous solution at 1 mg/mL was diluted at 5 $\mu\text{g}/\text{mL}$ in
154 water. For EPS DR_{MMW}/CaCl₂ samples, CaCl₂ aqueous solution at 10 mg/mL was mixed (1:1,
155 v/v) with EPS DR_{MMW} aqueous solution at 1 mg/mL. The remaining mixture was then diluted
156 in water at either 5 $\mu\text{g}/\text{mL}$ or 50 $\mu\text{g}/\text{mL}$. Five μL of each diluted solution were deposited on
157 freshly cleaved mica surface and left overnight to allow slow evaporation of water under

158 ambient conditions. These conditions favor a slow transition from the hydrated state to the dried
159 state, contrary to a quick-drying with pressurized air.

160 The surface of samples was systematically imaged on four zones using a NanoWizard®
161 Atomic Force Microscope (JPK, Germany) operating in air in intermittent contact mode. A
162 standard rectangular cantilever (Nanosensors NCL-W) with a free resonance frequency of 165
163 kHz and a spring constant of ~40 N/m was used. Before AFM observations, an AFM tip with a
164 radius curvature of ~10 nm was cleaned by UV-ozone treatment. High-resolution images (2 μm
165 x 2 μm, 2048 x 2048 pixels) were acquired for EPS DR_{MMW} samples, while lower resolution
166 (10 μm x 10 μm, 512 x 512 pixels) was applied for EPS DR_{MMW}/CaCl₂ samples. All images
167 were only flattened to remove the background curvature. The height measurements of
168 polysaccharide chains were performed using JPK Data Processing software (JPK, Germany).
169 A home-made Matlab program performed both histograms of measured heights and the
170 statistical analysis of adsorbed chains. After digitizing the EPS DR_{MMW} chain contours from
171 high-resolution AFM images, the mean square end-to-end distance $\langle R^2 \rangle$ was calculated by
172 varying the internal contour length (s) from the pixel size to the total length. Only nonlinear
173 single chains without overlapping or coiling were selected for these statistical measurements.
174 In addition, their contour lengths have to be higher than 145 nm (as the infernan chain length
175 was estimated at ~250 nm from the mean value of molecular weight) to avoid an overestimation
176 of the persistence length with shorter chains. The persistence length, L_p , was then determined
177 by fitting the experimental data ($\langle R^2 \rangle - s$ curve) by the 2D worm-like chain model using the
178 least-squares method (Bettini, Pozzan, Valdevit, & Frontali, 1980; Rivetti, Guthold, &
179 Bustamante, 1996). Only L_p values corresponding to the best fits (with a coefficient of
180 determination > 0.95) were used to calculate the mean value.

181

182

183 2.4.2. *Mechanical measurements on microgels*

184 Before mechanical measurements by AFM, microgels were immobilized on a Petri dish
185 glass surface functionalized with poly-L-lysine (PLL, Sigma). The glass surface was firstly
186 washed with ethanol before being exposed to UV-ozone treatment for 10 minutes. Then 200 μL
187 of PLL at 0.01% was deposited for 30 minutes. After washing with MilliQ water, the surface
188 was dried, and 20 μL of microgel suspension were deposited and incubated for 30 minutes. All
189 AFM experiments were performed in CaCl_2 at room temperature at different concentrations
190 (2mM, 360 mM and 1 000 mM). Indentation and relaxation experiments were carried out with
191 cantilevers (SQube) having a colloidal glass sphere of 5 μm in diameter. The cantilever spring
192 constant, calibrated using the thermal noise method implemented in the AFM setting (JPK
193 software), was comprised between 0.14 and 0.17 $\text{N}\cdot\text{m}^{-1}$. Before indentation experiments,
194 several approach-retract force curves were performed on different microgels (for the chosen
195 CaCl_2 concentration) to accurately determine the force to be applied corresponding to an
196 indentation depth of ~ 500 nm. This depth corresponds to a good compromise to avoid the long-
197 range interactions and to be within the validity domain of the Hertz contact model. Then,
198 conventional force curves were recorded at a constant speed (1 $\mu\text{m}/\text{s}$) on microgels with a
199 maximum indentation depth of ~ 500 nm. This low cantilever velocity was chosen to neglect
200 the hydrodynamic drag forces (proportional to the cantilever velocity) exerted on the cantilever
201 when experiments are carried out in a liquid medium. These forces were then converted into
202 force-indentation curves, and the approach part was fitted by the Hertz model to determine the
203 apparent elastic modulus of microgels. Modified force curves were performed for micro-
204 rheological measurements by adding a holding phase of 3 s between the approach and retraction
205 parts (recorded at high speed, 20 $\mu\text{m}/\text{s}$). During the holding phase, the force relaxation was
206 measured over time while the indentation depth was kept constant at 500 nm within microgels.

207 The time-dependent shear relaxation modulus was then directly determined with the
208 experimental parameters using the Lee-Radok equation (Lee & Radok, 1960).

209 **2.5. Molecular dynamics simulations**

210 The molecular dynamics simulations were performed using program package AMBER12
211 (Case *et al.*, 2012) in the isotherm isobar thermodynamic ensemble at 300K, using the force-
212 field parameters for GLYCAM06 for saccharides (Kirschner *et al.*, 2008). The AMBER force
213 field was used for ions and the TIP3 model for water molecules, following the protocol
214 described previously (Makshakova, Zykwiniska, Cuenot, Collic-Jouault, & Perez, 2022) with
215 trajectories in the range of 100 ns and output frequency of 10 ps. From the succession of
216 glycosidic torsion angles extracted from the trajectory, chain samples having up to 1 000
217 monosaccharide units were constructed and processed.

218 **3. Results and Discussion**

219 **3.1. Infernan-calcium interactions study at the molecular level by AFM imaging and** 220 **molecular dynamics simulations**

221 Native EPS characterized by its very high molecular weight (2,000,000 g/mol) could not
222 gel in the presence of Ca^{2+} (Zykwiniska *et al.*, 2019). Long thick fibres previously observed by
223 AFM prevented most likely from efficient chain-chain associations and formation of stable
224 junction zones necessary for gelling. In contrast, EPS derivative of medium molecular weight
225 (EPS DR_{MMW}), 240,000 g/mol, prepared by free-radical depolymerization of the native EPS,
226 formed a gel with Ca^{2+} . An increase in fibre diameter was observed after Ca^{2+} addition,
227 emphasizing that the formation of stable junction zones was favored by shorter polysaccharide
228 chains (Zykwiniska *et al.*, 2019). In the present study, to gain further insight into the ability of
229 infernan derivatives to form a physical gel, EPS/ Ca^{2+} interactions were firstly investigated at
230 the molecular level using dual, experimental (AFM) and simulation (molecular dynamics)

231 approach. For the experimental part, EPS DR_{MMW} of 260,000 g/mol was prepared by free-
232 radical depolymerization of native EPS. The depolymerization reaction had no significant
233 impact on the polysaccharide composition (Gélébart *et al.*, 2022). From the consideration of
234 the infernan derivative molecular weight and the molar mass of the nonasaccharide repeating
235 unit (1 567 g/mol), it can be estimated that infernan chains contain around 166 repeating units
236 of ~250 nm in length.

237 *3.1.1. Persistence length of infernan derivative*

238 AFM and molecular dynamics simulations were first applied to investigate the
239 conformational behavior of individual EPS chains before calcium addition. High-resolution
240 AFM imaging in the air was largely used to visualize the conformations of various flexible
241 (bio)polymers and filaments such as DNA, amyloid fibrils and polysaccharide chains (Morris
242 *et al.*, 2001; Rivetti *et al.*, 1996; Relini *et al.*, 2010; Schefer, Usov, & Mezzenga, 2015;
243 Zykwinska *et al.*, 2018; Zykwinska *et al.*, 2019). The usually applied method consists in
244 deposition of diluted solution on a flat substrate to favor isolated chains before imaging in a dry
245 state. Figure 1A presents dried, highly-diluted EPS DR_{MMW} solution (5 µg/mL), showing the
246 worm-like behavior of EPS chains in 2D. The distribution of measured heights revealed that
247 mainly individual monomolecular chains of 0.54 ± 0.07 nm in height were observed (Figure
248 1B). Similar results have been reported for other anionic polysaccharides, such as alginate,
249 pectin, xanthan or gellan (Decho, 1999; Morris *et al.*, 2001; Schefer *et al.*, 2015; Zdunek,
250 Pieczywek, & Cybulska, 2021; Zykwinska, Gaillard, Boiffard, Thibault, & Bonnin, 2009).

251 Before characterizing the 2D conformations adopted by infernan chains, it seems essential
252 to determine how the adsorption affects the chain conformation. During the deposition process,
253 the conformational changes induced by the transition from free three-dimensional (3D) chains
254 remaining in solution to chains adsorbed on the surface depend on the polysaccharide-substrate
255 interaction strength. Indeed, in the case of weak interactions, chains can freely relax and are

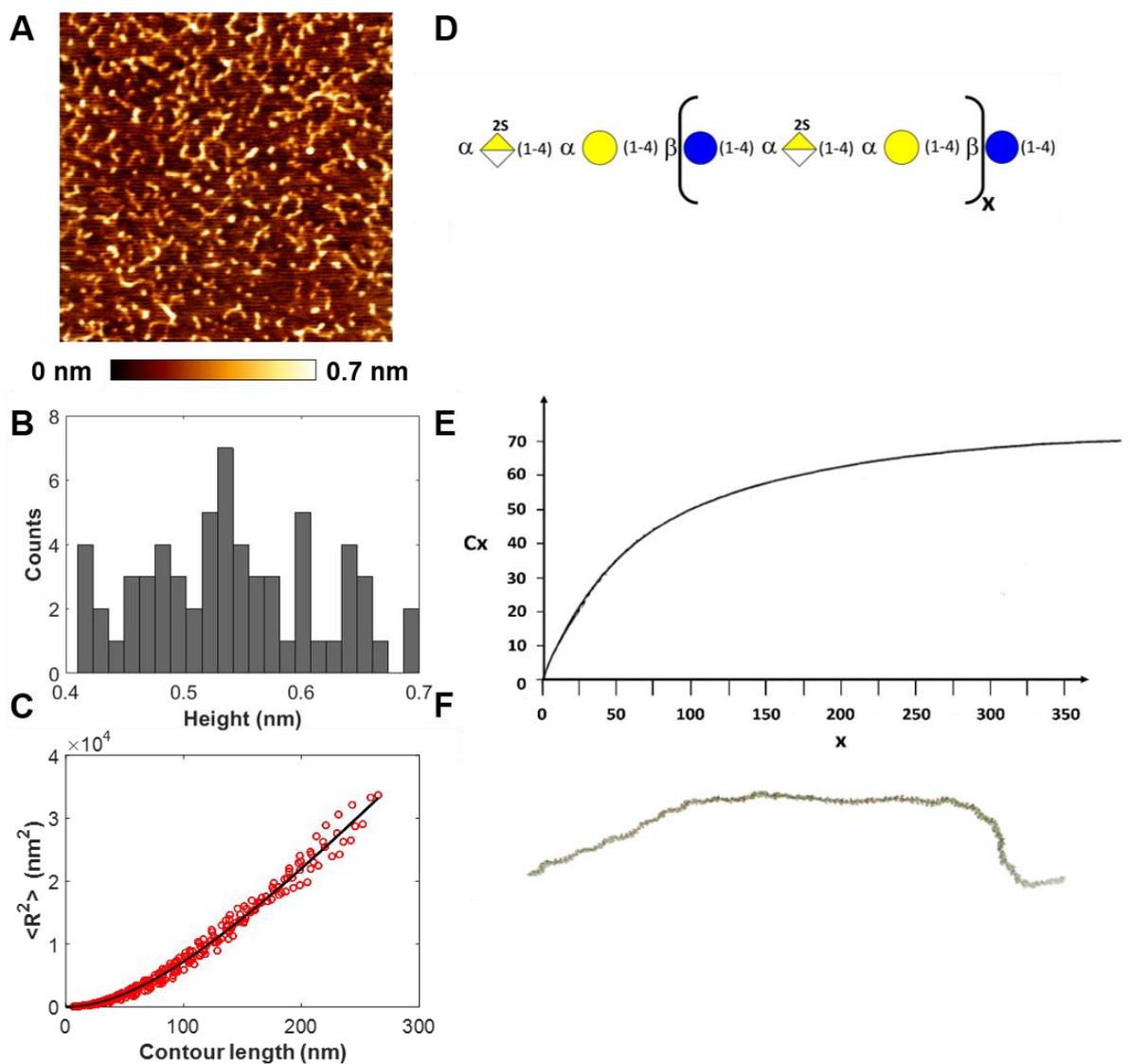
256 fully equilibrated on the surface. The resulting 2D equilibrated conformations can be treated
 257 with the same formalism of free chains in solution (Rivetti *et al.*, 1996). Conversely, chains are
 258 kinetically trapped on the surface without equilibrating when strong interactions occur and the
 259 adopted 2D conformations reflect the history of chain-surface contact points. These non-
 260 equilibrated chain conformations are expected to correspond to the projection of 3D chain
 261 conformations to the surface (Bettini *et al.*, 1980; Rivetti *et al.*, 1996). In our case, weak
 262 interactions between infernan chains and hydrophilic mica surface involving hydrogen bonds
 263 and van der Waals interactions will favor 2D equilibrated chain conformations on the substrate.
 264 Such an equilibrated behavior was observed for another anionic polysaccharide, carrageenan,
 265 adsorbed on a positively charged mica surface. This mica functionalization led to an increase
 266 of electrostatic interaction contribution, which favored chain immobilization on the surface
 267 without preventing carrageenan chains from adopting 2D equilibrated conformations (Schefer
 268 *et al.*, 2015).

269 A statistical analysis of geometrical shapes of individual infernan chains was then
 270 performed by measuring the 2D mean-square end-to-end distance ($\langle R^2 \rangle$) from AFM images.
 271 In Figure 1C, the plot of $\langle R^2 \rangle$ as a function of the internal contour length, s , (*i.e.* the distance
 272 between two contour segments describing the chain) is reported for s varying between the pixel
 273 size (~ 0.97 nm) and the total contour length. This $\langle R^2 \rangle - s$ relationship can be theoretically
 274 expressed for semi-flexible polymer chains by the 2D worm-like chain (WLC) model with the
 275 following equation (Bettini *et al.*, 1980; Rivetti *et al.*, 1996):

$$\langle R^2 \rangle = 4L_p \left(s - 2L_p \left(1 - \exp \left(-\frac{s}{2L_p} \right) \right) \right) \quad (1)$$

277 where L_p is the polymer persistence length. This physical parameter is defined as the
 278 characteristic length scale over which the tangent vectors along the chain contour correlate,
 279 usually attributed to the local resistance of the chain to bending (Bettini *et al.*, 1980). The

280 persistence length was then determined as the only adjustable parameter by fitting the $\langle R^2 \rangle$
281 experimental data by equation (1) using the least-squares method. The excellent agreement
282 between experimental and theoretical $\langle R^2 \rangle - s$ dependence indicates a good description of 2D
283 infernan chains by the 2D WLC model (Figure 1C). An average L_p value of 24.8 ± 7.6 nm was
284 determined from these fits performed on 22 single infernan chains with contour lengths ranging
285 from 145 nm to 275 nm, in accordance to the chain length of ~ 250 nm estimated from infernan
286 molecular weight. The obtained L_p value is in good agreement with published values using the
287 same AFM method, such as L_p values for carrageenan chains without calcium comprised
288 between 17 nm (lambda carrageenan) and 32 nm (iota and kappa carrageenans) (Schefer *et al.*,
289 2015). Alkali-soluble pear pectin displayed the persistence length of 129 ± 92 nm in water,
290 assessed by AFM (Cieřla *et al.*, 2021).



291
 292 Figure 1. (A) AFM height image (2 μm x 2 μm) of EPS DR_{MMW} single chains. EPS DR_{MMW} at 5 $\mu\text{g}/\text{mL}$
 293 in water deposited on a mica surface and imaged in intermittent contact mode in air after drying. (B)
 294 Height distribution of imaged EPS DR_{MMW} chains and (C) mean-square end-to-end distance ($\langle R^2 \rangle$)
 295 measured on a single chain from AFM image as a function of the contour length. The dark line
 296 corresponds to the theoretical relation of the 2D worm-like chain model. (D) Schematic representation
 297 of the backbone structure of infernan, where the monosaccharides are depicted following the SNFG
 298 representation. (E) Variation of the characteristic ratio, C_x as a function of x , being the number of
 299 infernan repeating units. (F) Snapshot of a typical infernan chain containing 300 repeating units.

300 The persistence length, L_p of a polysaccharide can be calculated from molecular modelling
 301 results. Figure 1D shows a schematic representation of the backbone structure of infernan using

302 the SNFG representation for the monosaccharides by specifying the repeating unit, x . Because
303 of the spatial separation afforded by the rigid sugar residues which are interpolated between the
304 flexible linkages, the independence of the sets of glycosidic torsional angles from neighboring
305 sets within the polysaccharide chain is a valid assumption, at least as a first approximation, for
306 almost all glycosidic linkage types except a (1→2) linked monosaccharides. Therefore, the
307 configurational energy for the macromolecular chains is decomposed into additive
308 contributions from the conformational energy of adequately chosen segments such as dimeric
309 fragments. Spatial representative polysaccharide chains are described by specifying values for
310 all torsion angles (Φ , Ψ) of the set (Brant & Goebel, 1975). Assuming that interactions between
311 monosaccharides further away than the first neighbors are not significant, a chain sample may
312 be generated by confining examination to the conformations occurring at the glycosidic
313 linkages. One way to perform such a task is to extract the values of the consecutive glycosidic
314 linkages from the molecular dynamics simulation trajectories and assess these values to a
315 continuously growing polysaccharide chain (Kroon-Batenburg, Kruiskamp, Vliegenthart, &
316 Kroon, 1997). In the generation, no effort is made to eliminate long-range excluded-volume
317 interactions. Each set of torsion angles is assumed to occur independently from all the rest.
318 Therefore, a chain of p residues can be considered as consisting of $(p-x)$ independent chains of
319 a degree of polymerization, x , for which the properties may also be evaluated. The persistence
320 length, L_p was calculated from the characteristic ratio C_∞ , a dimensionless descriptor valid to
321 compare unperturbed polymers chain dimensions (*i.e.* dimensions of a macromolecule
322 subjected to only short-range interactions) of different polysaccharides, corresponding to the
323 asymptotic behavior of C_x (*i.e.* obtained for high values of x). The smaller the C_∞ value, the
324 more flexible a polymer chain. The characteristic ratio, C_x , defined in function of x , is evaluated
325 using the following equation:

$$326 \quad C_x = \frac{1}{Nxl_0^2} \sum_{k=1}^N r^2 k \quad (2)$$

327 where N is the number of chains, k is the number of samples, and l_o is the average virtual bond
328 length connecting glycosidic oxygens. The virtual bond lengths were derived from the
329 conformation analysis. The characteristic features of the main backbone of infernan chain in its
330 random conformation were simulated for chain samples having up to 1 000 monosaccharides.
331 In Figure 1E, C_x first increases as a function of x and becomes independent of x when the
332 asymptotic behavior is reached for about 300 repeating units. The corresponding infernan chain
333 containing 300 repeating units is presented in Figure 1F. The specific value C_∞ determined from
334 Figure 1E is about 65 indicating a significant extension of the infernan chain. This behavior
335 closely resembles that calculated for homogalacturonan and some other (1→4)-linked uronic
336 acid-rich polysaccharides (Braccini, Grasso, & Perez, 1999), since it arises from the similarity
337 of the sequence of linkages between the main backbone of infernan and uronic acids. The
338 persistence length, L_p can then be calculated from $L_p = l_o C_\infty/2$ (Kroon-Batenburg *et al.*, 1997).
339 A value of 140 Å was found for the persistence length of the infernan chain. Similar simulated
340 values were reported for homogalacturonan (140 Å, 110 Å), cellulose (145 Å) or more flexible
341 hyaluronan (75 Å) (Cros, Garnier, Axelos, Imbert, & Perez, 1996; Noto, Martorana, Bulone,
342 & San Biagio, 2005; Kroon-Batenburg *et al.*, 1997; Haxaire, Braccini, Milas, Rinaudo, & Perez,
343 2000). The persistence length of infernan chains calculated from the molecular dynamics
344 simulations on free chains in solution, with the L_p value of 14 nm, agreed with the value
345 obtained from AFM imaging of 24.8 ± 7.6 nm. This confirmed the correct assumption that
346 imaged chains were fully equilibrated on the substrate before AFM imaging.

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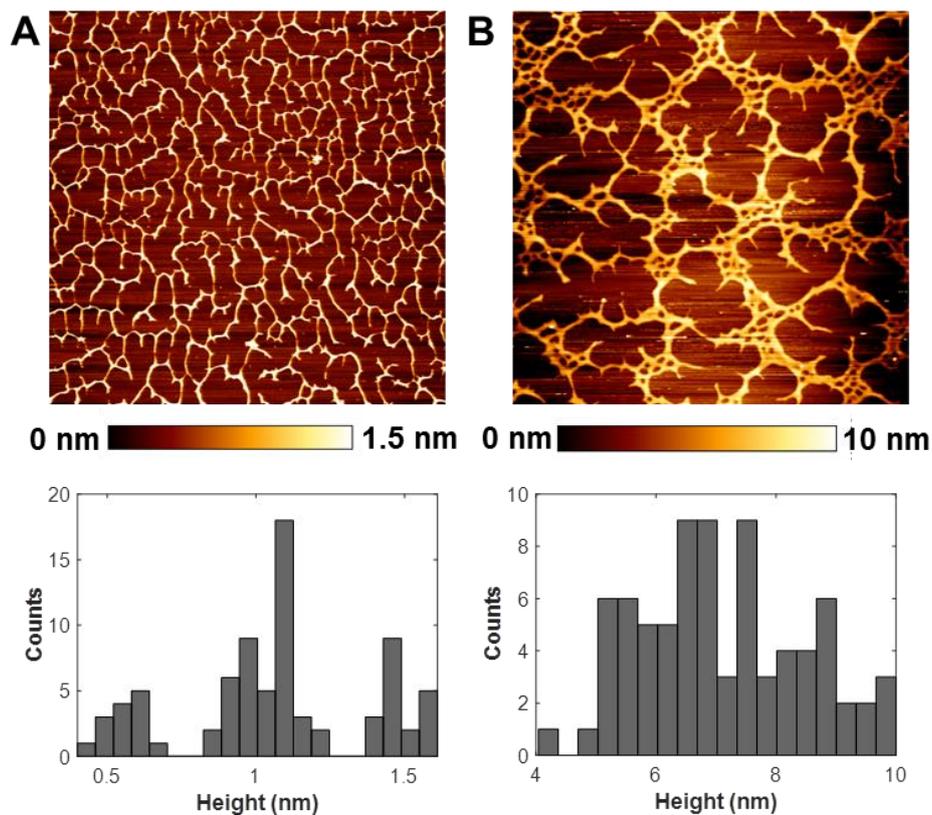
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352 3.1.2. Chain-chain associations through calcium ions

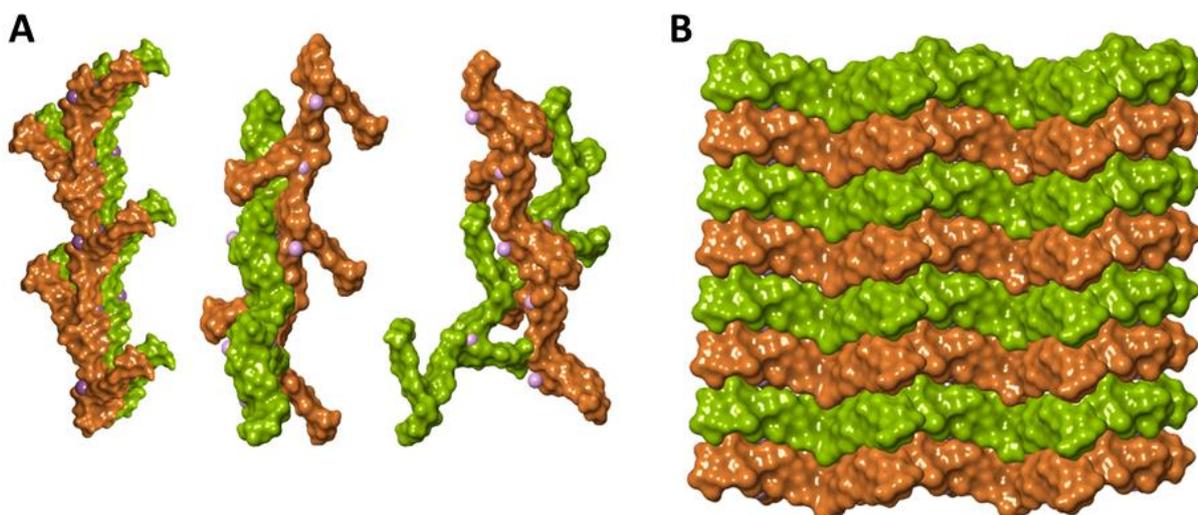
353 AFM imaging was firstly performed on highly-diluted EPS DR_{MMW}/Ca²⁺ solution (5
354 $\mu\text{g/mL}$) dried on a mica surface to investigate infernan chain-chain associations induced by
355 Ca²⁺ (Figure 2A). Histogram of measured heights from AFM images clearly revealed three
356 distinct peaks at 0.55 ± 0.07 nm, 1.04 ± 0.09 nm and 1.49 ± 0.07 nm. The imaged network of
357 highly branched fibers suggested that despite monomolecular chains of ~ 0.5 nm in height,
358 several chains associated with Ca²⁺ were also present. Less-diluted EPS DR_{MMW}/Ca²⁺ solution
359 ($50 \mu\text{g/mL}$) offered an opportunity to observe a dense network setting (Figure 2B). Height
360 measurement on these fibers indicated a further increase in their diameters from ~ 5 nm to ~ 10
361 nm, highlighting additional chain-chain associations. The branching points displayed the same
362 height as fibers in their linear parts.



363 Figure 2. AFM height images ($10 \mu\text{m} \times 10 \mu\text{m}$) and height distribution: (A) EPS DR_{MMW}/CaCl₂ solution
364 diluted at $5 \mu\text{g/mL}$ and (B) EPS DR_{MMW}/CaCl₂ diluted at $50 \mu\text{g/mL}$. All sample solutions in water were
365 dried on a mica surface at 20°C before imaging in intermittent contact mode in air.

366 To better understand chain-chain association induced by Ca^{2+} leading to the formation of a
367 stable and homogeneous network observed by AFM, molecular dynamics simulations were
368 performed. We have recently reported that the disordered state of the infernan macromolecule
369 displays a continuous series of pseudo helical segments exhibiting either around a two-fold or
370 a five-fold structure (Makshakova *et al.*, 2022). It was shown that the side chains of infernan
371 are essential for creating the Ca^{2+} chelating sites in the region of the branching point involving
372 three adjacent residues: βGlcP and $\alpha\text{GalpA-2S}$ from the backbone and $\beta\text{GlcP A}$ from the side
373 chain. Our previous computations showed that only the two-fold helical shape and its
374 subsequent ribbon-like structure allows bringing neighboring chains in proximity without steric
375 conflicts. The two-fold helical conformation of the backbone, presenting the appropriate Ca^{2+}
376 cavity, may induce the formation of the polysaccharide chain pairing, providing the chains in
377 their favorable orientation. The coordination of Ca^{2+} within one chain reaches completion with
378 the oxygen atoms of a sulfate group belonging to the neighboring chain. The resulting “junction
379 zone” is based on chain-chain interactions that lead to strong dimer associations with essential
380 contributions from the van der Waals and hydrogen bonding interactions. Calcium ions, sulfate
381 groups and uronic acids have specific positions in well-adapted cavities (Makshakova *et al.*,
382 2022). The feature of the interaction is the heterotypic mode of binding, which contrasts with a
383 monotypic type of interactions characteristic of the binding mode of anionic polysaccharides,
384 such as alginate and pectins described by the “egg-box” model (Braccini & Perez, 2001). Such
385 a chain-chain association can propagate and result in the formation of rigid elements.
386 The models of chain-chain pairing induced by Ca^{2+} involving two adjacent polysaccharide
387 chains are presented in Figure 3A. Both lateral and twisted chain stacking conformations are
388 possible. Chain-chain associations can further continue, leading to several chains stacking
389 together (Figure 3B). The height of the two stacking chains is around 1.6 nm, while eight
390 stacking chains measure 6.5 nm in height. The obtained values are close to those measured from

391 AFM images of highly-diluted (5 $\mu\text{g/mL}$) and less-diluted (50 $\mu\text{g/mL}$) EPS DR_{MMW}/Ca²⁺
392 solutions (Figure 2). In highly-diluted solutions, the fiber height corresponds to a pairing of at
393 least two chains, while in less-diluted solutions, the fiber height suggests stacking of more than
394 six polysaccharide chains. Twisted helical conformations that may adopt two adjacent chains
395 explain the formation of the complex network of connected fibers observed in Figure 2.



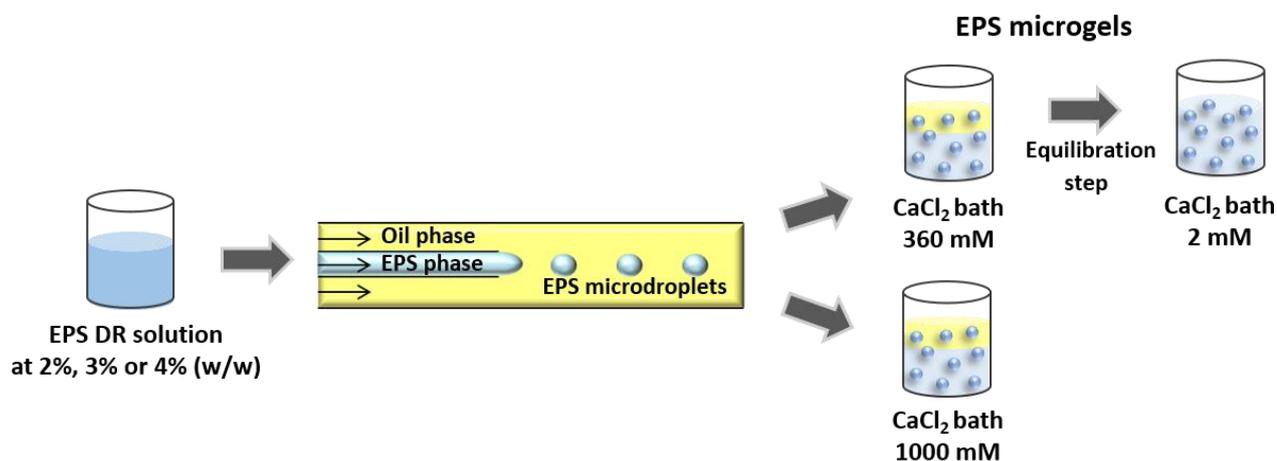
396 Figure 3. The models of infernan chain-chain stacking through Ca²⁺ ions. (A) Lateral and twisted
397 pairings of two polysaccharide chains. (B) The lateral pairing of eight polysaccharide chains. The
398 adjacent polysaccharide chains are displayed as surfaces in green and brown color; the Ca²⁺ ions are
399 given as van der Waals spheres in violet.

400 3.2. Impact of infernan-calcium interactions on the mechanical properties of microgels

401 3.2.1. Microgel formation by capillary microfluidics

402 In the following step, the impact of infernan chain-chain associations mediated by Ca²⁺ on
403 the mechanical properties of EPS-based microgels was studied. Such microgels were previously
404 designed for growth factor encapsulation and release for tissue engineering applications
405 (Zykwinska *et al.*, 2019; Gélébart *et al.*, 2022). Indeed, the mechanical properties of hydrogels
406 deeply affect the release properties of encapsulated bioactive molecules and regulate essential
407 cellular processes, including migration, proliferation and differentiation (Vinning, & Mooney,
408 2017). These properties are related to the hydrogel network nature determined by chemical

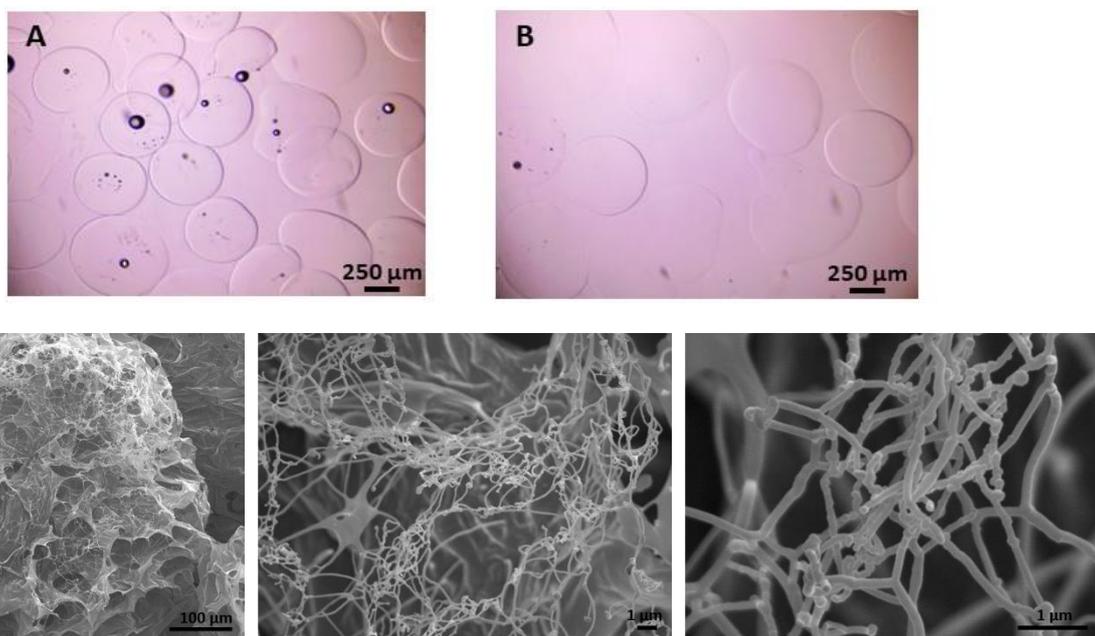
409 (covalent) or physical (non-covalent) crosslinks and their density. Herein, the impact of both
410 EPS and CaCl_2 concentrations on mechanical properties of physical microgels was investigated
411 using AFM. EPS DR_{MMW} aqueous solutions at three concentrations (2, 3 and 4% (w/w)) were
412 formulated into microdroplets using a homemade capillary microfluidic co-flow device (Figure
413 S1 and 4). Microdroplets formed inside a microfluidic device were recovered in the collecting
414 bath containing either 360 mM CaCl_2 (for EPS DR_{MMW} at 2, 3 and 4% (w/w)) or 1 000 mM
415 CaCl_2 (for EPS DR_{MMW} at 4% (w/w) only) under rapid magnetic stirring to avoid their
416 coalescence. Gelation of the EPS was initiated immediately after microdroplet contact with
417 CaCl_2 solution, which additionally decreased the coalescence phenomenon.



418 Figure. 4. Schematic representation of EPS DR_{MMW} based microgel formulation using capillary
419 microfluidics. EPS DR_{MMW} aqueous solution was used for microgel formulation at three different
420 concentrations: 2, 3 or 4% (w/w). The EPS microdroplets formed inside a microfluidic device gelled
421 immediately after their recovery in either 360 mM or 1 000 mM CaCl_2 collecting bath. In addition, an
422 equilibration step in 2 mM CaCl_2 was applied to microgels formed in 360 mM CaCl_2 to reach a
423 physiological calcium concentration.

424 Monodisperse microgels of $450 \pm 61 \mu\text{m}$, $463 \pm 63 \mu\text{m}$ and $485 \pm 54 \mu\text{m}$ in diameter were
425 obtained for EPS DR_{MMW} solutions at 2, 3 and 4% (w/w) in 360 mM CaCl_2 , respectively (Figure
426 5A). Microgels crosslinked in 360 mM CaCl_2 were also equilibrated in 2 mM CaCl_2 for a further
427 24h to reach a physiological calcium concentration within the microgels (Figure 5B). During

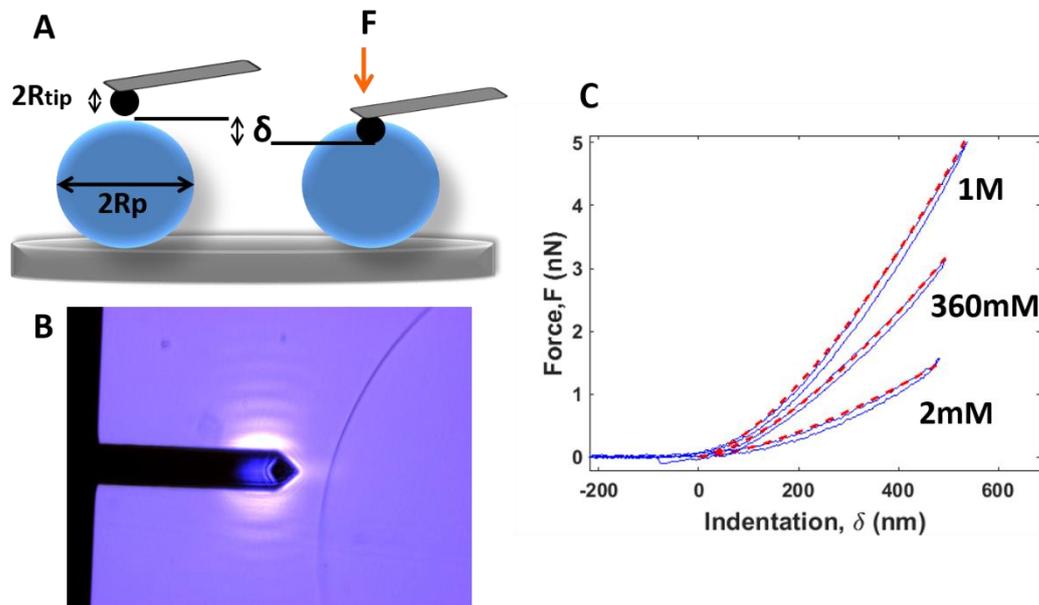
428 this ionic concentration change, the density of cross-links between polysaccharide chains
429 mediated by Ca^{2+} ions decreased, leading to a looser network compared to the initial network
430 formed in 360 mM CaCl_2 . This equilibration step led to a weak microgel swelling since an
431 increase in microgel diameter was observed: $537 \pm 89 \mu\text{m}$, $535 \pm 53 \mu\text{m}$ and $593 \pm 77 \mu\text{m}$ for
432 EPS DR_{MMW} solutions at 2, 3 and 4% (w/w), respectively (Figure 5B).
433 Microgels prepared from an EPS DR_{MMW} solution at 4% (w/w) and recovered in the calcium
434 bath of the highest concentration (1 000 mM CaCl_2) were of $557 \pm 58 \mu\text{m}$ in diameter. No
435 significant differences in morphologies were noticed between microgels recovered in different
436 calcium baths, as revealed by SEM (Figure 5C). In all cases, a porous structure was clearly
437 observed with the presence of large fibers of ~ 100 to ~ 250 nm in width. These thick fibers result
438 from further chain-chain associations mediated by Ca^{2+} ions taking place in microgels prepared
439 at higher EPS and CaCl_2 concentrations, when compared to diluted samples prepared for AFM
440 imaging.



441 Figure 5. Optical images of EPS DR_{MMW} microgels at 4% (w/w) recovered in 360 mM CaCl_2 (A) and
442 equilibrated in 2 mM CaCl_2 for 24h (B). Scanning Electron Microscopy (SEM) images of freeze-dried
443 EPS DR_{MMW} microgels at 4% (w/w) recovered in 360 mM CaCl_2 (C).

444 3.2.2. Mechanical properties of microgels by AFM

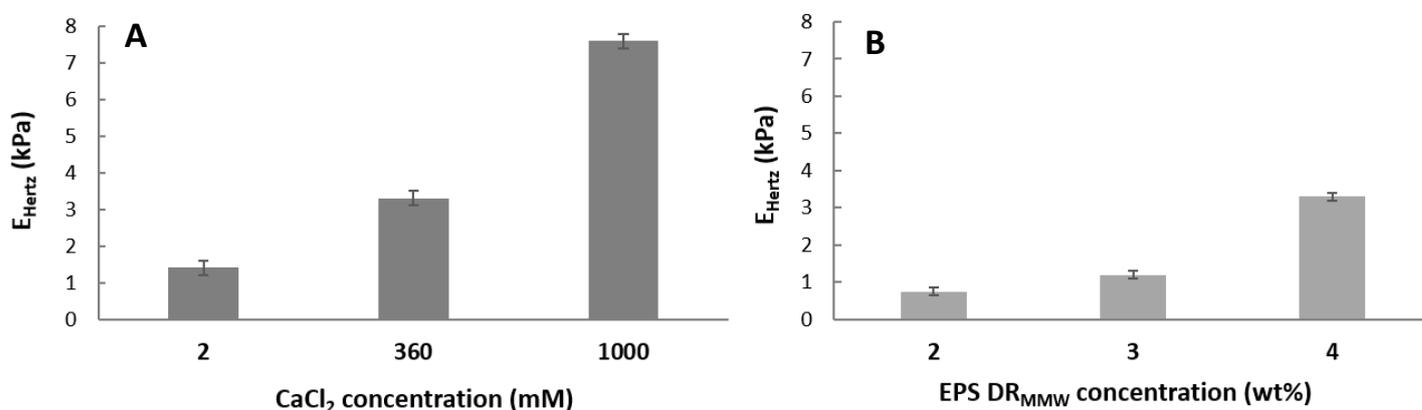
445 Unlike macroscopic hydrogels for which rheological measurements give a direct access to
446 their mechanical properties, determining these properties at microscale is more challenging.
447 Over the last twenty years, the development of AFM-based methods has opened different ways
448 to investigate the local mechanical properties of soft nano- and micrometer- sized samples
449 (Berry, Biviano, & Dagastine, 2020; Cuenot, Alem, Louarn, Demoustier-Champagne, & Jonas,
450 2008; Cuenot, Radji, Alem, Demoustier-Champagne, & Jonas, 2012; Moreno-Guerra *et al.*,
451 2019). Indeed, mechanical properties can be measured by operating either in the time or
452 frequency domains through conventional force curves, force/indentation modulation, creep or
453 force relaxation (Moreno-Guerra *et al.*, 2019; Efremov, Okajima, & Raman, 2020). In the
454 present work, combined AFM methods were used to characterize the mechanical properties of
455 microgels. Firstly, microgels were indented by a glass sphere of 5 μm in diameter at ~ 500 nm
456 to obtain conventional force (F)-indentation (δ) curves (Figure 6). Figure 6C shows the
457 approach-retract F - δ curves obtained for three microgels formulated at 4% (w/w) and recorded
458 in 2 mM, 360 mM or 1 000 mM CaCl_2 . The Hertz model was then used to fit the approach
459 curves to extract the apparent elastic modulus, E_{Hertz} (Hertz, 1881), which characterizes the
460 ionic crosslinking density of microgels, *i.e.* the number of junction zones formed between
461 infernan chains, involving sulfate and carboxylic groups, and calcium ions, as previously
462 described (Makshakova *et al.*, 2022). A good agreement between fitted and experimental curves
463 was obtained for the different experimental conditions.



464 Figure 6. (A) Schematic representation of the experimental setup where a microgel of radius R_p is
 465 indented by a depth δ by a spherical probe of radius R_{tip} attached to an AFM cantilever. (B) Optical
 466 image showing the curvature of a probed microgel compared with the length of $225\ \mu\text{m}$ (width $48\ \mu\text{m}$)
 467 of a used cantilever. (C) Experimental approach-retract force-indentation curves (in blue) were
 468 performed in different CaCl_2 solutions (2 mM, 360 mM and 1 000 mM) with Hertz's model fit (in red)
 469 of the approach curve.

470 The influence of the calcium concentration on the resulting apparent elastic modulus, E_{Hertz} ,
 471 measured on 5 to 7 microgels at fixed EPS DR_{MMW} concentration (4% (w/w)) was presented in
 472 Figure 7A. E_{Hertz} increased with increasing calcium concentration from $1.4 \pm 0.2\ \text{kPa}$ at 2 mM
 473 to over $3.3 \pm 0.1\ \text{kPa}$ at 360 mM, and then to $7.6 \pm 0.4\ \text{kPa}$ at 1 000 mM CaCl_2 , indicating that
 474 the crosslinking density increased with the ionic strength. A five-fold increase in the elastic
 475 modulus of microgels was observed between 2 mM and 1 000 mM CaCl_2 . It appeared further
 476 that the equilibration step applied to the microgels to reach a physiological calcium
 477 concentration (from 360 mM to 2 mM CaCl_2) led to about a two-fold decrease in the elastic
 478 modulus. Such a decrease results most likely from the loss of calcium initially present within
 479 microgels, directly impacting the crosslink density of the network.

480 It was further observed that increasing EPS DR_{MMW} concentration at a fixed calcium
 481 concentration (360 mM CaCl₂) led to an increase in E_{Hertz} of microgels from 0.75 ± 0.15 kPa at
 482 2% (w/w) to over 1.2 ± 0.1 kPa at 3% (w/w), and then to 3.3 ± 0.2 kPa at 4% (w/w) (Figure
 483 7B). Therefore, the mechanical properties of microgels can also be tuned by the EPS
 484 concentration, as a two-fold increase in EPS concentration, from 2 to 4% (w/w), led to a four-
 485 fold increase in apparent elastic modulus.



486 Figure 7. (A) The impact of $CaCl_2$ concentration (2, 360, 1 000 mM) on the apparent elastic modulus
 487 (E_{Hertz}) at constant EPS DR_{MMW} concentration (4% (w/w)). (B) The impact of EPS DR_{MMW}
 488 concentration (2, 3 and 4% (w/w)) on the apparent elastic modulus (E_{Hertz}) at constant $CaCl_2$
 489 concentration (360 mM).

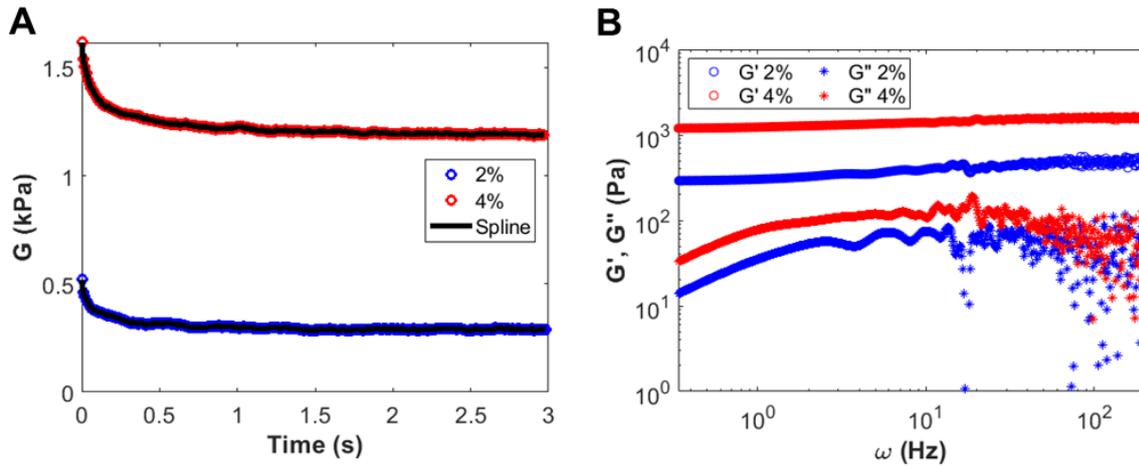
490 A hysteresis occurring between the approach and retraction F - δ curves observed in Figure
 491 6C indicates that microgels exhibit time-dependent mechanical properties instead of purely
 492 elastic behaviour. Indeed, hydrogels possess both solid- and liquid-like features indicating that
 493 the mechanical energy is stored and dissipated, respectively. Thus, their behavior is somewhere
 494 between a purely elastic and strictly viscous material. For macroscopic hydrogels, the
 495 straightforward approach is to characterize these viscoelastic properties by determining the
 496 complex shear modulus, $G^*(\omega)$, by rheological oscillatory measurements over a broad range of
 497 discrete frequencies. AFM-based experiments have been conducted for micrometer-sized

498 hydrogels to determine their frequency-dependent viscoelastic properties using
 499 force/indentation modulation methods (Mahaffy, Park, Gerde, Kas & Shih, 2004; Xu,
 500 Koslowski & Raman, 2012; Nalam, Gosvami, Caporizzo, Composto & Carpick, 2015). An
 501 alternative to these frequency-dependent measurements is to perform modified force curves by
 502 adding a holding phase between the approach and retraction parts and recording the force
 503 relaxation over time while the indentation depth is kept constant (Darling, Zauscher, Block, &
 504 Guilak, 2007). In the present work, we investigated the frequency-dependent behavior of the
 505 elastic and viscous components for microgels with two extreme elastic modulus values, *i.e.* for
 506 the EPS DR_{MMW} concentration of 2 and 4% (w/w) (Figure 7B). The time-dependent shear
 507 relaxation modulus of the sample, $G(t)$, can be directly obtained from the force measured over
 508 time, $F(t)$, using the relation introduced by Lee and Radok for a spherical rigid indenter
 509 penetrating a viscoelastic sample (Lee & Radok, 1960):

$$510 \quad F(t) = \frac{8\delta_0^{3/2}}{3(1-\nu)} \left[\left(\frac{1}{R_{tip}} \right)^{1/3} + \left(\frac{1}{R} \right)^{1/3} \right]^{-3/2} G(t) \quad (3)$$

511 where δ_0 is the constant indentation depth, ν is the Poisson's ratio of the microgel, R_{tip} and R
 512 are the tip radius and the microgel radius, respectively. In our experiments, the following
 513 parameters were used: $R_{tip} = 2.5 \mu\text{m}$, $R = 230 \mu\text{m}$, $\delta_0 = 500 \text{ nm}$ and $\nu = 0.5$ as the widely
 514 accepted value for hydrogels (Moreno-Guerra *et al.*, 2019, Efremov *et al.*, 2020). Interestingly,
 515 $G(t)$ directly corresponds to the evolution of $F(t)$ as the constant of proportionality in equation
 516 (3) is fully determined by the experimental conditions (Moreno-Guerra *et al.*, 2019). Figure 8A
 517 shows the raw data obtained for the shear relaxation modulus of microgels at 2 and 4% (w/w).
 518 An attractive approach to determine the viscoelastic properties of microgels, as an alternative
 519 of modulation methods, consists of performing the Fourier transform of the time derivative of
 520 $G(t)$ leading to $G^*(w)$, where w denotes the angular frequency (Evans, Tassieri, Auhl, & Waigh,
 521 2009; Moreno-Guerra *et al.*, 2019). Contrary to the direct analysis of force relaxation curves,

522 this transformation into the frequency regime avoids the use of viscoelastic constitutive models.
523 The real, $G'(w)$, and imaginary, $G''(w)$, components of the complex shear modulus, describe
524 the frequency-dependent elastic (storage modulus) and viscous (loss modulus) characters of the
525 sample respectively. To calculate $G'(w)$ and $G''(w)$ from experimental data, the analytical
526 procedure of Fourier transform, developed by Evans *et al.*, was applied to equation (3) (Evans
527 *et al.*, 2009; Moreno-Guerra *et al.*, 2019). This approach offers the advantage to control the
528 range of accessible, continuous frequencies by the experimental temporal window. Indeed, the
529 lowest frequency is related to the relaxation duration (*i.e.* 3 s), and the highest frequency is
530 determined from the inverse of the sampling rate (*i.e.* the time-step). To increase this highest
531 frequency, cubic spline interpolation of experimental $G(t)$ was performed before the Fourier
532 transform to reduce the time-step from 0.01 s to 0.005 s (Figure 8A). Finally, the viscoelastic
533 properties of microgels are available over several decades of continuous angular frequencies
534 between 0.33 Hz and 200 Hz. No significant dependence on the frequency in the examined
535 frequency range was observed for the storage modulus and the loss modulus obtained for
536 microgels at 2 and 4% (w/w) (Figure 8B). $G'(w)$ and $G''(w)$ values were both higher for
537 microgel at 4% (w/w) ($G'(w) \sim 1\,000$ Pa) compared to 2% (w/w) ($G'(w) \sim 250$ Pa), in agreement
538 with the elastic modulus values measured from force-indentation curves (Figures 6 and 7).
539 Indeed, a factor of about 3 exists between the storage modulus and the elastic modulus in the
540 case of hydrogels ($G' = E/2(1 + \nu)$ with $\nu \sim 0.5$), (Efremov *et al.*, 2020). G' and E_{Hertz} were about
541 250 Pa and 750 Pa for microgels at 2% (w/w) while these values were respectively of 1 kPa and
542 3.3 kPa for microgels at 4% (w/w). In addition, no crossover occurred between these two
543 moduli, with $G'(w)$ always higher than $G''(w)$, indicating that the solid-like behaviour of
544 microgels was more pronounced than the liquid-like character. The small hysteresis appearing
545 in the force-indentation curves (Figure 6C) tends also to confirm this result.



546

547 Figure 8. (A) Shear relaxation modulus, $G(t)$, measured over time for EPS DR_{MMW} microgels at 2 and
 548 4% (w/w). Experimental data $G(t)$ was interpolated using the cubic spline method. (B) Storage $G'(w)$
 549 and loss $G''(w)$ moduli obtained by Fourier transforming of $G(t)$.

550 The mechanical properties of infernan-based microgels measured by AFM showed the
 551 elastic moduli between 0.8 kPa and 8 kPa, depending on calcium and EPS concentrations. They
 552 are similar to the values reported for other microgels based on synthetic and natural polymers
 553 (Oevreeide *et al.*, 2021). $G'(w)$ and $G''(w)$ moduli of these microgels correspond to those
 554 usually measured for engineered matrices and soft tissues, such as skin, brain and lung
 555 (Chaundhuri, Cooper-White, Janmey, Mooney, & Shenoy, 2020). In general, the elastic
 556 modulus of microgels varies between 0.1 and 500 kPa depending on the nature of network
 557 crosslinks (e.g. covalent for chemical microgels or non-covalent for physical microgels) and
 558 the AFM method used (Oevreeide *et al.*, 2021). In the context of polysaccharide based-
 559 microgels crosslinked with Ca^{2+} , alginate represents the most explored natural polymer.
 560 Alginate microgels developed for single-cell encapsulation by Mao *et al.* (2016) showed elastic
 561 moduli measured by AFM between 0.3 kPa and 16 kPa, depending on alginate molecular weight
 562 and calcium concentration used for microgel formulation using microfluidics. In another study,
 563 alginate/ Ca^{2+} microgels displaying elastic modulus of ~ 13 kPa (compression tests) were
 564 reported (Ahn, Rath, Tsao, Bentley, & Raghavan, 2021). Alginate/ Ca^{2+} microparticles obtained

565 after shrinkage of alginate/Na⁺ microdroplets produced using microfluidics showed elastic
566 modulus of ~ 47 kPa (AFM measurements) (Zhang *et al.*, 2020). For macroscopic alginate
567 hydrogels crosslinked with calcium and elaborated to study cellular responses in three-
568 dimensional matrices, elastic moduli of ~9 kPa and ~17 kPa (rheological measurements) were
569 reported (Chaundhuri *et al.*, 2016). It was shown that a hydrogel with an elastic modulus of 9
570 kPa induced adipogenesis, while for a higher elastic modulus of 17 kPa, osteogenesis was
571 observed. Alginate/Ca²⁺ hydrogels prepared to assess the effect of mechanical properties
572 (elastic modulus, stress relaxation) on cell spreading and proliferation displayed elastic moduli
573 from 3 kPa to 49 kPa (compressive tests) (Bauer *et al.*, 2017). The elastic modulus of hydrogels
574 increased with calcium concentration for the same alginate concentration.

575 **4. Conclusion**

576 In the present study, an experimental approach using a set of AFM-based methods was
577 combined with a theoretical one, exploiting molecular dynamics simulations to investigate the
578 effects of interactions between infernan and Ca²⁺ on the interchain associations and the
579 mechanical properties at two different levels. At the molecular level, AFM imaging was firstly
580 applied to assess the conformational behavior of infernan chains before Ca²⁺ addition.
581 Statistical analysis performed on individual chains allowed to determine their average height of
582 0.54 ± 0.07 nm and persistence length of 24.8 ± 7.6 nm. The measured structural features of
583 infernan chains agreed well with those obtained from molecular dynamics simulations.
584 Secondly, AFM imaging of infernan chains after Ca²⁺ addition revealed the stacking of several
585 polysaccharide chains. Generated models of chain-chain pairing induced by Ca²⁺, showing both
586 lateral and twisted chain stacking, perfectly agreed with AFM observations of highly-diluted
587 and less-diluted samples. The ability of infernan chains to associate through Ca²⁺ and form
588 physical gel was further exploited to prepare EPS/Ca²⁺ microgels using microfluidics. In these
589 microgels, further chain-chain association was noticed, as assessed by increasing fiber diameter

590 observed on SEM images. The impact of chain-chain associations, modulated by different EPS
591 and CaCl₂ concentrations, on the mechanical properties of microgels formed was then assessed
592 by AFM. The apparent elastic modulus of microgels measured from force-indentation curves
593 varied from ~1 kPa to ~8 kPa, depending on EPS and CaCl₂ concentrations. It appeared that the
594 modulus can be tuned by five-fold, with increasing CaCl₂ concentration from 2 mM to 1 000
595 mM, and by four-fold, with increasing EPS concentration from 2 to 4% (w/w). In addition, the
596 rheological properties of microgels were determined by AFM from the shear relaxation
597 modulus measured over time. The frequency-dependent elastic character of microgels was in
598 good agreement with the elastic modulus values obtained from force-indentation curves. The
599 viscous component was always lower than the elastic one indicating that the microgels did not
600 exhibit a pronounced liquid-like character in the accessible frequency range, between 0.33 Hz
601 and 200 Hz. Finally, by modulating the crosslinking density of the physical network, the
602 resulting mechanical properties of microgels can be tuned to design hydrogels with appropriate
603 properties for specific applications in tissue engineering.

604

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614 **Makshakova:** Conceptualization, Methodology, Investigation, Formal analysis, Writing –
615 Original Draft, Writing – Review & Editing; **Perrine Gélébart:** Investigation, Formal analysis,
616 Writing – Review & Editing; **Corinne Siquin:** Investigation, Formal analysis, Writing –
617 Review & Editing; **Nicolas Stephant:** Investigation, Formal analysis, Writing – Review &
618 Editing; **Sylvia Collic-Jouault:** Writing – Review & Editing; **Serge Perez:** Conceptualization,
619 Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review &
620 Editing; **Stéphane Cuenot:** Conceptualization, Methodology; Investigation, Formal analysis,
621 Validation, Writing – Original Draft, Writing – Review & Editing.

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