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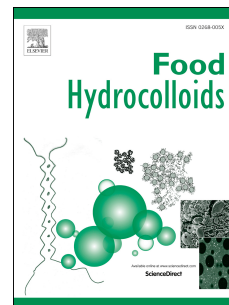


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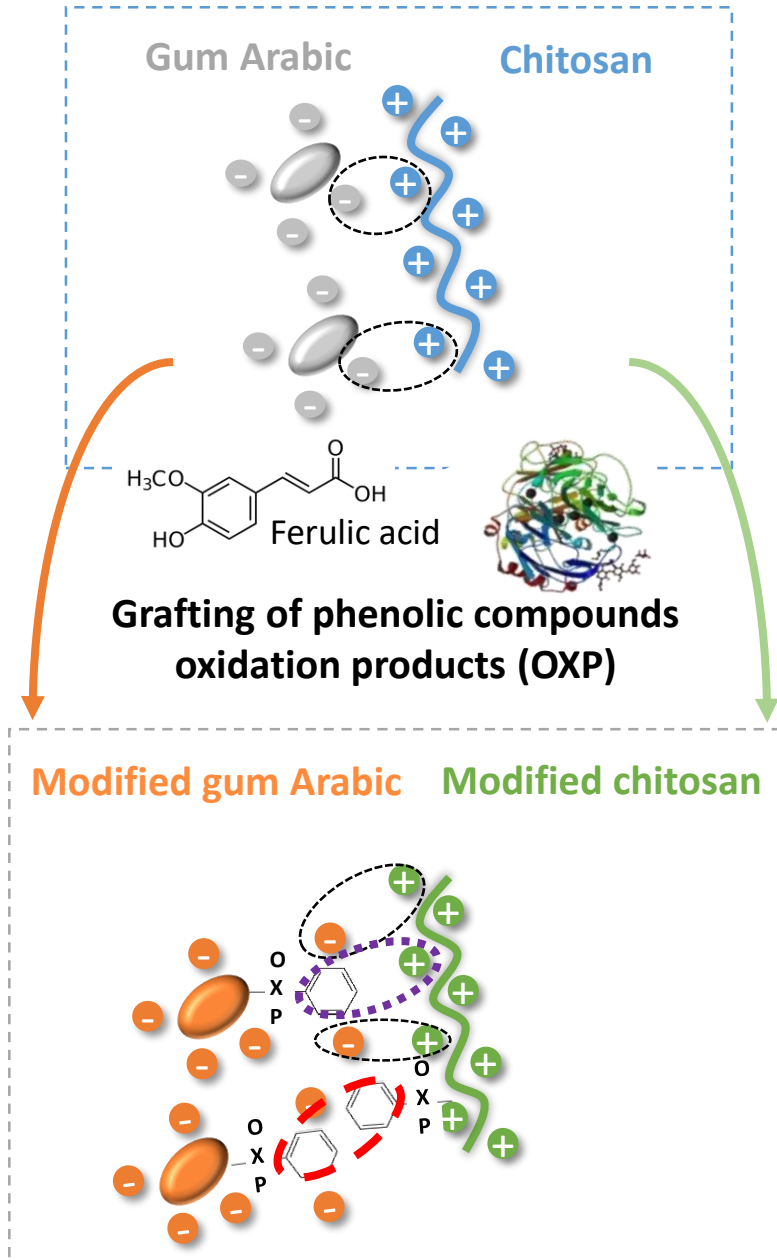
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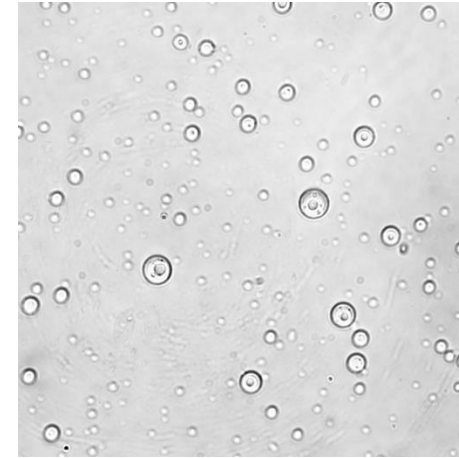
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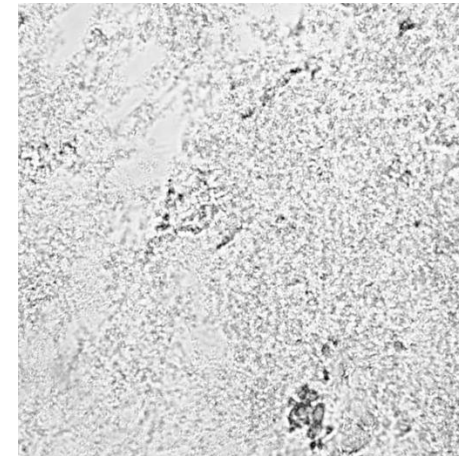
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**Coacervation**

Electrostatic interactions



New interactions: Aggregation

 Electrostatic interactions
 π -cation interaction
 π - π stacking


1 **Polysaccharides enzymatic modification to control the coacervation or the**
2 **aggregation behavior: a thermodynamic study**

3

4 Marie E. Vuillemin, Florentin Michaux, Aurélie Seiler, Michel Linder, Lionel Muniglia and

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8 **Abstract**

9 The effect of enzymatic modification of polysaccharides on the formed supramolecular
10 structures was studied. Gum Arabic and chitosan were modified by covalent grafting of ferulic
11 acid oxidation products so that they could establish new interactions such as π interactions. The
12 stoichiometry of the interaction was studied by ITC measurements, as well as its strength and
13 related thermodynamic parameters. These experiments were supported by Zeta potential and
14 turbidity measurements and optical microscopy observations. Grafting of phenolic compounds
15 onto the polymers increased their ability to exhibit π - π stacking and π -cation interactions and
16 led to the formation of various supramolecular structures. For native gum Arabic and chitosan
17 assemblies, the stoichiometry value determined by ITC was consistent with optical microscopy,
18 macroscopic observations and Zeta potential (around a molar ratio gum Arabic:chitosan of 3).
19 The structures formed were coacervates. For the modified polymers, the optimal molar ratio
20 determined by Zeta potential measurements (3.27) differed from the one determined by ITC
21 (6.3). This meant the formation aggregates was not only due to electrostatic interactions but
22 also to other type of interactions (new π - π stacking and π -cation interactions). It was concluded
23 that by modifying the polymers, it was possible to control the supramolecular structures formed.

24 **Keywords:** Complex coacervation; supramolecular structures; Isothermal titration calorimetry;
25 gum Arabic; chitosan; interactions.

26 **1. Introduction**

27 The balance of interactions between molecules or between molecules and solvent
28 represents the driving force for the formation of self-assembled colloidal systems. In recent
29 years, the compartmentalized environments of cells have been mimicked in bottom- up systems
30 through the use of condensed polymeric phases (Aumiller et al., 2016; Crowe & Keating, 2018;
31 Jia et al., 2014; Poudyal et al., 2019). In these phases, oppositely charged polyelectrolytes
32 condense into a polymer- rich phase due to their mutual electrostatic interactions. This process
33 is called complex coacervation (Yewdall et al., 2019). Coacervates result from spontaneous
34 mechanisms coming from interactions between building blocks as surfactants, proteins,
35 polysaccharides (Kötz et al., 2001; Meyer et al., 2006). Several types of interactions can exist
36 to produce such complex structures as electrostatic, hydrophobic, Van der Waals (Sahoo et al.,
37 2018; Whitesides et al., 1991). These are described by their strength and their range (Campbell
38 & Reece, 2004; Neel et al., 2017).

39 The understanding of the formation of supramolecular structures represents a key point to
40 consider a possible control of the organization at the colloidal scale. Modifying the interactions
41 in a given system to investigate their influence on the colloidal structure formed is therefore of
42 major interest. There are two ways of modifying these interactions. The first one consists in
43 controlling the physical-chemical properties of the medium like pH and/or ionic strength to
44 modify electrostatic interactions for example. This strategy is well known and widely reported
45 in the literature (Butstraen & Salaün, 2014; Coelho et al., 2011; Espinosa-Andrews et al., 2007;
46 Kizilay et al., 2011; Vuillemin et al., 2019). To modify interactions between two molecules, a

47 second possible strategy is to chemically modify the building blocks. This can lead to decrease,
48 suppress or strengthen some potential interactions. Assemblies formed from biopolymers
49 (protein, polysaccharides) appear to be good candidates for this purpose since the structures
50 formed depend on the type and strength of the interaction. Since these molecules are often
51 polyelectrolytes, electrostatic interactions frequently direct the formation of complexes,
52 coacervates or aggregates. It is recognized that strong interactions between the building blocks
53 mainly lead to the formation of aggregates while weaker interactions promote the formation of
54 coacervates (Comert and Dubin, 2017). Modifying the chemical structures of the molecules can
55 change their interactions and lead to controlling the resulting colloidal structure. In this work,
56 the influence of chemical modifications of polysaccharides on the supramolecular structure they
57 spontaneously form in an aqueous phase was investigated. Therefore, Gum Arabic (GA) and
58 chitosan (CN) were chosen for their ability to interact *via* electrostatic interactions in a given
59 pH range and spontaneously form coacervates in water (Vuillemin et al., 2019).

60 CN is a polymer of glucosamine and N-acetyl glucosamine linked by β (1–4) glycosidic
61 bonds (Soni et al., 2018). It is derived from the deacetylation of chitin from crustacean shells.
62 The free amino groups of chitosan are protonated in solution, when the pH is below its pKa (i.e.
63 between 6.3 and 7.0 (Beppu & Santana, 2002)). In other words, CN is positively charged when
64 protonated, which remains a property that few polysaccharides exhibit and it is the primary
65 reason that led to the choice of CN in several studies on complex coacervation (Avadi et al.,
66 2010; Butstraen & Salauen, 2014; Espinosa-Andrews et al., 2007, 2010; Maqbool et al., 2010;
67 Roldan-Cruz et al., 2016; Sakloetsakun et al., 2015; Tan et al., 2016). Moreover, it has an
68 extended conformation in acetic acid solution, so its charges are accessible (Espinosa-Andrews
69 et al., 2007; Schatz et al., 2004). Furthermore, it possesses attractive properties as this
70 biopolymer is film-forming, biodegradable, biocompatible and exhibits antimicrobial and
71 antioxidant activities (Fathi et al., 2014; Soni et al., 2018; Sonia & Sharma, 2012).

72 CN was chosen as the polycation for this study and GA was selected as the polyanion.
73 GA is a sticky exudate of air-solidified tree sap from the stems or branches of Acacia trees
74 (Avadi et al., 2010). There are several species of Acacia. However, the two most exploited
75 species are *Acacia senegal* and *Acacia seyal*, which essentially produce brittle gum (McNamee
76 et al., 1998). GA contains three peculiar fractions: 85–90 wt. % of arabinogalactan, 10 wt. %
77 of arabinogalactan-protein complex and 2 wt. % of one or two glycoproteins (Lopez-Torrez et
78 al., 2015). These fractions confer several different chemical groups to gum Arabic. It possesses
79 amino groups due to its protein content. In addition, it contains carboxyl groups present on its
80 glucuronic acid residues (Padala et al., 2009), which are responsible for the charge of GA in
81 aqueous solution above its pKa of 3.6. GA is a hydrophilic polysaccharide that is highly soluble
82 in water. It has a globular structure in aqueous solution (Moschakis et al., 2010; Renard et al.,
83 2012). Furthermore, it is used in various industrial applications as an additive in food
84 formulation (E414) or for encapsulation (Mosquera et al., 2012), for vectorization in the
85 pharmaceutical industry (Avadi et al., 2010; Bosnea et al., 2014, 2017), as an adhesive in the
86 cosmetic industry and for various other applications (Verbeken et al., 2003).

87 Several authors have already studied the complex coacervation of GA and CN (Butstraen
88 & Salaün, 2014; Coelho et al., 2011; Espinosa-Andrews et al., 2007, 2013; Tan et al., 2016).
89 The influence of several parameters such as pH, ionic strength, temperature, or polyelectrolyte
90 concentration on GA and CN coacervation has been well characterized but there is still a need
91 to deepen the knowledge on the thermodynamic pathways that govern this phenomenon. In
92 recent studies, the mechanism of coacervate formation and the thermodynamic parameters
93 driving the complex formation have been investigated (Vuillemin et al., 2019). It was
94 demonstrated that pH and temperature affected the thermodynamics of GA-CN interactions
95 (especially at 45 °C and pH 5.5) and that coacervation was driven by enthalpy. The electrical
96 charges arise from the presence of negatively charged carboxylic groups on GA and positively

97 charged amine groups on CN, respectively. These chemical groups can be easily modified to
98 induce (i) a decrease in electrical charge by replacing the chemical function with another
99 chemical group and (ii) an addition of new functional groups that can introduce new possible
100 interactions, thus obtaining different supramolecular structures.

101 Some authors have previously attempted to modify chitosan by chemical methods to shed
102 light on the impact of modification on coacervate formation with gum Arabic (Huang et al.,
103 2015, 2016, 2017). They showed that modification of chitosan by N, O-carboxymethylation or
104 O-carboxymethylation reduced electrostatic interactions while maintaining high coacervation
105 yields. Other driving forces such as hydrophobic interaction could be considered for chitosan
106 and gum Arabic to form coacervates, especially at high temperatures. This could initially lead
107 to a deeper understanding of the mechanisms and forces driving complex formation. The
108 modification of polymers could lead to different conditions of complex formation than the
109 optimal ones obtained with native polymers and to the formation of different supramolecular
110 structures (particles, aggregates, coacervates...). However, the modification route used in
111 existing studies on the subject is chemical modification. To make this strategy green and to
112 modify chitosan and gum Arabic using the same methodology, a sustainable way consists in an
113 approach using the laccase-catalyzed oxidation reaction of ferulic acid. This approach has the
114 advantage of being eco-acceptable, as it is implemented under mild, aqueous conditions and at
115 room temperature. Besides, it is not extremely specific and can be implemented on a
116 considerable number of substrates. However, this method presents some disadvantages, such as
117 the fact that the reaction is difficult to control, the products can be grafted at various locations,
118 and the structure of the products formed remains poorly understood. However, the carboxylic
119 and amino groups present on polysaccharides have already been modified by this strategy.
120 Indeed, in a study by Aljawish (Aljawish et al., 2012), CN was functionalized by grafting ferulic
121 acid (AF) oxidation products using *Myceliophthora thermophila* laccase. The authors showed

122 that the functionalization of CN was achieved on C2 by forming a Schiff base (the formation
123 of a C=N bond was proven by FTIR analyses). Grafting of these phenolic oxidation products
124 onto the carboxyl group was also studied on GA (Vuillemin et al., 2021). It was demonstrated
125 that gum Arabic was grafted with oxidation products, close to the C6 of galactose units or close
126 to the C5 of glucuronic acid units of gum Arabic. Furthermore, it was shown that the grafted
127 products were mainly ferulic acid dimers, i.e. compounds with two aromatic rings, which could
128 lead the modified polymers to establish new interactions such as π - π stacking or π -ion
129 interactions. This strategy was implemented here to modify GA and CN. This paper aims to use
130 these structure-modified polymers to contribute to the knowledge the formation of self-
131 assembled colloidal systems. Thermodynamic parameters associated with the interactions were
132 determined by Isothermal Titration Calorimetry (ITC). Measurements of Zeta potential and
133 turbidity were compared to ITC results. These results were supported by microscopic
134 observations to understand the mechanism of formation of the supramolecular structures.

135 **2. Materials and Methods**

136 **2.1. Materials**

137 Chitosan (CN) MMW (medium molecular weight, Mw 310 kDa verified by SEC-MALS
138 experiments (Aljawish et al., 2014), deacetylation degree of 75%) was purchased from Sigma-
139 Aldrich (France). Gum Arabic (GA) Instantgum AA from *Acacia senegal* (Mw 333 kDa
140 determined by SEC-MALS experiments (Vuillemin et al., 2021)) was a gift from Nexira
141 (France). Ferulic acid >99% (FA) was purchased from Sigma-Aldrich (France). Acetone,
142 methanol and ethanol were purchased from Carlo Erba (France). Acetic acid, NaOH and
143 ultrapure water were of analytical grade. The enzyme used for the modification of the polymers
144 was commercialized as "Novozym® 51003" from Novozymes (Bagsvaerd, Denmark). It is a

145 fungal laccase from *Myceliophthora thermophila*, a polyphenol oxidase. The laccase stock
146 activity was $32\,038 \pm 1\,322$ LAMU.g⁻¹.

147

148 **2.2. Methods**

149 **2.2.1. Polymers modification**

150 The functionalization of gum Arabic (GA) and chitosan (CN) was performed according
151 to the standard procedure of our laboratory (Aljawish et al., 2012; Vuillemin et al., 2020, 2021).
152 For chitosan (CN), the method was adapted from Aljawish et al. (Aljawish et al., 2012). For
153 gum Arabic (GA), the method was the same as that used by Vuillemin et al. (Vuillemin et al.,
154 2020, 2021). Briefly, 1 g of polysaccharide was dispersed in 45 mL of phosphate buffer
155 (50 mM, pH 7.5) and stirred at 4 °C overnight. Since the polysaccharides were not soluble in
156 this buffer, functionalization was performed under heterogeneous conditions. The dispersion
157 was stirred at 450 rpm for 1 h at 30 °C. 5 mL of ferulic acid dissolved in methanol (50 mM)
158 was added to the batch. Once the temperature was stable, 13.5 UI.mL⁻¹ of Novozym® 51003
159 was added to trigger the reaction. The batch was then stirred at 30 °C for 50 min.

160 For chitosan (CN), the reaction was stopped by filtration of the reaction medium under
161 vacuum with Ministar-RC membranes (Sartorius, porosity 0.2 µm). The recovered
162 functionalized chitosan (FCN) was washed with phosphate buffer pH 7.5, ultrapure water,
163 methanol, ethanol and then acetone to remove all traces of substrates and enzyme on the
164 chitosan derivative.

165 For gum Arabic (GA), the reaction was stopped by adding 150 mL of cold ethanol. The
166 unfunctionalized GA precipitated. The resulting mixture of functionalized GA (FGA) and

167 ungrafted FA oxidation products (OXP) soluble in the phosphate buffer/ethanol mixture and
168 insoluble unfunctionalized GA was then centrifuged for 20 min at 12000 g with a Beckmann
169 centrifuge (Beckman Coulter Inc., Villepinte, France). The precipitate contained
170 unfunctionalized GA (verified by FTIR measurements) and some of the phosphate buffer salts,
171 whereas the supernatant contained the functionalized GA and the non-GA-grafted ferulic acid
172 oxidation products. Ethanol was then removed using a BUCHI R144 rotary evaporator (BUCHI
173 SARL, Rungis, France) at a boiling temperature of 40 °C under 175 mbar. Some water in the
174 buffer solution was also evaporated by decreasing the pressure to 72 mbar.

175 The functionalized polysaccharides were frozen and then lyophilized for 72 h. The
176 resulting powders were stored in a desiccator until use.

177 **2.2.2. Polymers purification**

178 Native chitosan (NCN), functionalized chitosan (FCN), native gum Arabic (NGA) and
179 functionalized gum Arabic (FGA) were dialyzed against acetic acid (1% v/v) and water,
180 respectively. A high-grade quality regenerated membrane (MWCO 10 000 Da from Membrane
181 Filtration Products Inc.) was used to remove excess salts and possibly ungrafted FA oxidation
182 products (OXP). Dialysis was stopped when the conductivity outside the membrane was equal
183 to the one of acetic acid (1% v/v) or ultrapure water. The samples were then centrifuged for
184 30 min at 12,000 g to remove insoluble material and air bubbles and then freeze-dried and
185 stored at 4 °C. The molecular weights of the two polysaccharides were confirmed by SEC-
186 MALS measurement after dialysis. Grafting of the oxidation products onto chitosan did not
187 significantly alter its molecular weight (310 kDa). However, the functionalization of gum
188 Arabic changed its molecular weight (333 kDa for NGA and 213 kDa for FGA) (Vuillemin et
189 al., 2021).

190 **2.2.3. Polymers stock solutions preparation**

191 Stock solutions of 0.02% w/v NCN and FCN (6.45×10^{-4} mM) and 0.50% w/v NGA and
192 FGA (1.49×10^{-2} mM and 2.35×10^{-2} mM, respectively) were prepared by dissolving purified
193 NCN and FCN or NGA and FGA in acetic acid (1% v/v in ultrapure water). The solutions were
194 stirred on a magnetic plate at 400 rpm overnight at 4 °C to ensure that the polymers were
195 completely dissolved. The NCN and FCN solutions were prepared at 0.02% w/v (6.45×10^{-4}
196 mM), a concentration for which its viscosity was low and suitable for ITC experiments. The
197 concentration of NGA and FGA was adjusted to 0.50% w/v (1.49×10^{-2} mM and 2.35×10^{-2} mM,
198 respectively) based on preliminary measurements to optimize the concentrations for complete
199 titration without signal saturation.

200 **2.2.4. Polymers mixtures preparation**

201 The polymer mixtures were prepared at 45 °C, pH 5.5 by adding acetic acid (1 N) or
202 NaOH (5 N) as required, and different molar ratio of polymers. The required amount of polymer
203 solutions was placed 15 min in a thermostatic degassing chamber (TA instruments, Waters
204 SAS, France) to reach the target temperature and remove bubbles. The NGA or FGA solutions
205 were then added to the NCN or FCN solutions.

206 **2.2.5. Isothermal titration calorimetry (ITC)**

207 ITC measurements were performed with an Affinity ITC equipped with a standard
208 volume (965 μ L) gold cell (TA instruments, Waters SAS, France) to measure the dissociation
209 constant (K_d) and the enthalpic change due to the interaction between CN and GA. The entropy
210 and the Gibbs free energy were obtained from the following equations:

$$211 \quad \Delta G = - R.T.\ln(1/K_d) \quad (\text{Eq. 1})$$

212 $T \cdot \Delta S = \Delta H - \Delta G$ (Eq. 2)

213 With ΔG : Gibbs free energy variation ($\text{kJ} \cdot \text{mol}^{-1}$), ΔH : enthalpy variation ($\text{kJ} \cdot \text{mol}^{-1}$), ΔS :
214 entropy variation ($\text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), T : temperature (K) and R is the specific gas constant
215 ($8.3144598 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$).

216 Ultrapure water was used in the reference cell. The injector microsyringe ($320 \mu\text{L}$), was
217 loaded with NGA or FGA stock solution (0.50% w/v NGA or FGA ($1.49 \times 10^{-2} \text{ mM}$ and 2.35×10^{-2}
218 mM , respectively) in 1.00% v/v acetic acid solution). $5 \mu\text{L}$ was sequentially injected 50 times
219 every 300 s into the titration cell filled with NCN or FCN solution (0.02% w/v NCN or FCN
220 ($6.45 \times 10^{-4} \text{ mM}$) in 1.00% v/v acetic acid solution). At the end of the experiment, the molar ratio
221 of the mixture in the cell was 8.7:1 for the mixture with NGA and 13.6:1 for the mixture with
222 FGA. The polymer concentrations were chosen based on preliminary measurements to optimize
223 the concentrations for complete titration without signal saturation. For each measurement, the
224 pH, temperature and acetic acid solvent were the same in the cell and in the syringe to limit the
225 dilution effect. The heat of dilution from the blank titration was subtracted from the raw data in
226 all experiments. Data acquisition was performed using ITC Run software and data were
227 analyzed with the NanoAnalyse software (TA instruments, Waters SAS, France). The
228 “Independent” model was selected as the most robust model from the plot residuals to fit the
229 obtained isotherms. All measurements were performed in triplicate.

230 **2.2.6. Approximate Zeta potential**

231 The approximate Zeta potential of the polymers alone (0.02 w/v for NCN and FCN
232 ($6.45 \times 10^{-4} \text{ mM}$) and 0.50% w/v for NGA and FGA ($1.49 \times 10^{-2} \text{ mM}$ and $2.35 \times 10^{-2} \text{ mM}$,
233 respectively)) and mixtures was calculated from electrophoretic mobility measurements at
234 controlled temperature ($45 \text{ }^\circ\text{C}$) using a Zetasizer Nano-ZS instrument (Malvern Panalytical,

235 UK) equipped with a He/Ne ion laser ($\lambda = 532$ nm). The measurements were collected on a
236 detector at 173° . The instrument determined the Zeta potential with the Smoluchowski equation
237 (Jayme et al., 1999). All measurements were performed in triplicate.

238 **2.2.7. Turbidity measurements**

239 Turbidity of the mixtures was measured with a UV-visible spectrophotometer (Shimadzu
240 1280, Japan) at 660 nm. The transmittance (T) of the spectrophotometer was calibrated with
241 0.1 N acetic acid to 100% and the turbidity of the samples was reported as $100 - T\%$. The relative
242 turbidity (τ) was calculated from the equation 3:

$$243 \quad \tau = -\frac{1}{L} \ln \frac{I_t}{I_0} \quad (\text{Eq. 3})$$

244 Where L was the optical path length (1 cm) and I_t and I_0 represented the transmitted light
245 intensity and the incident light intensity, respectively. All measurements were made in
246 triplicate.

247 **2.2.8. Optical microscopy**

248 The mixtures were observed by optical microscopy (Olympus BH2, Japan) at x40
249 magnification. They were observed as prepared, i.e. without dilution of the samples. The images
250 were recorded with a 5 MPixel CMOS camera (Olympus) under the control of ToupView
251 software. The micrographs presented are representative of the samples.

252 **2.2.9. Statistical analysis**

253 Values were expressed as mean \pm standard deviation. Differences between mean values
254 were evaluated using one-way analysis of variance (ANOVA), which was performed with

255 Excel for Statistical Analysis and XLSTAT. Differences were considered statistically
256 significant when the p_{value} was less than 0.05.

257 **3. Results and discussion**

258 **3.1. Influence of polymer modification over their approximate Zeta** 259 **potentials**

260 Since coacervation is assumed to be primarily driven by electrostatic interactions, the
261 influence of pH on polymer behavior was first investigated.

262 According to Table 1, native and modified polysaccharides were charged at pH 5.5 and
263 45 °C. Grafting of the oxidation products did not change the approximate Zeta potential of CN
264 in acetic acid solution (17.4 ± 0.7 mV for NCN and 18.1 ± 0.2 mV for FCN). The grafted
265 oxidation products were mainly ferulic acid dimers grafted onto the amine groups of chitosan
266 (Aljawish et al., 2012, 2014). With positive charges of the amine groups replaced by oxidation
267 products, the charge of the polymer would be expected to decrease. The approximate Zeta
268 potential of CN at pH 3.0 was different between NCN and FCN (71.3 ± 2.8 mV and $62.5 \pm$
269 0.4 mV at 25 °C, respectively) confirming the grafting. These results are consistent with those
270 obtained by Aljawish et al. (Aljawish et al., 2012). Nevertheless, at pH 3.0, neither NGA nor
271 FGA were charged. Therefore, it was preferred to work at pH 5.5 so that each polymer was
272 charged. The difference in Zeta potential observed between NCN and FCN at pH 3.0 was
273 already small (12 %). Since at pH 5.5 the charge of NCN and FCN was lower, due to a pH close
274 to their pK_a (6.3), the influence of the grafting on the charge of the polymer was less marked
275 and may enter into instrumental errors, explaining why no significant difference was observed.
276 Furthermore, at the selected temperature and pH (45 °C, pH 5.5), the hydrodynamic radius of
277 chitosan in solution was higher than under standard conditions (room temperature) and the

278 flexibility of the chains increased (Chen & Tsaih, 1998; Fan et al., 2012). Thus, under the
 279 conditions studied (pH close to the pKa of chitosan and temperature of 45 °C), the lack of
 280 charge difference between NCN and FCN could also be attributed to chain flexibility.

281 Table 1: Approximate Zeta potential of native chitosan (NCN, 0.02% w/v), functionalized
 282 chitosan (FCN, 0.02% w/v), native gum Arabic (NGA, 0.50% w/v) and functionalized gum
 283 Arabic (FGA, 0.50% w/v) solutions in acetic acid 1.00% v/v at pH 5.5, 45 °C. “a, b, and c”
 284 mean that the statistical analysis showed no significant differences between the results.

285

Approximate Zeta Potential (mV)			
NCN 0.02% w/v in acetic acid	FCN 0.02% w/v in acetic acid	NGA 0.50% w/v in acetic acid	FGA 0.50% w/v in acetic acid
17.4 ± 0.7^a	18.1 ± 0.2^a	-9.7 ± 0.7^b	-21.7 ± 1.1^c

286

287 According to Table 1, the charge of FGA was twice as high as that of NGA. Indeed, the
 288 oxidation products were mainly composed of ferulic acid dimers and trimers that were
 289 potentially negatively charged causing an increase in the negative charge of the polysaccharide
 290 after grafting (Vuillemin et al., 2021).

291 Many authors have previously reported that charge stoichiometry between NGA and
 292 NCN (when all NCN charges were neutralized by all NGA charges) was achieved for a
 293 composition of five times more NGA than NCN (Butstraen & Salaün, 2014; Espinosa-Andrews
 294 et al., 2010, 2013; Vuillemin et al., 2019). The results in Table 1 suggested that since the
 295 modification did not change the surface charge of chitosan, the introduction of FCN instead of
 296 NCN into the mixture should not change the stoichiometry. However, since the surface charge
 297 of FGA was twice that of NGA, it appeared that twice the amount of FGA compared to NGA
 298 would be required to neutralize chitosan charges and achieve stoichiometry.

299 To confirm that more FGA than NGA was needed to achieve stoichiometry and to shed
300 light on the thermodynamic pathways of complex formation, whether the polymer was modified
301 or not, ITC measurements were performed.

302 **3.2. Influence of the polymers modification on the thermodynamic** 303 **parameters involved in the complexation process**

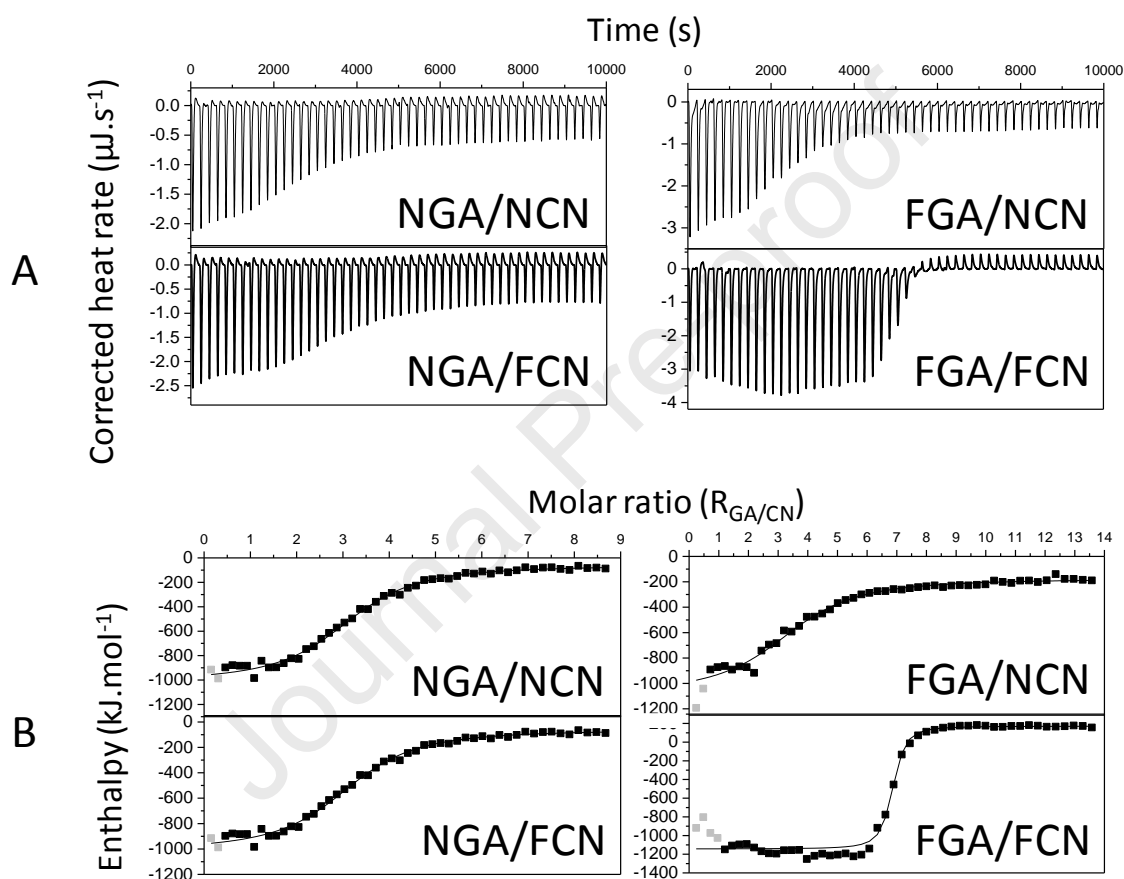
304 The influence of the modification of gum Arabic and chitosan structures over the
305 thermodynamic parameters involved in the interaction process was studied using ITC at pH 5.5
306 and 45 °C.

307 The corrected heat rates recorded after each gum Arabic injection were predominantly
308 negative in all cases (Figure 1A). This means that the major contribution of the interaction was
309 mainly exothermic and that the interactions were spontaneous. Each peak corresponded to the
310 energy released by the interaction between chitosan and gum Arabic after each injection.
311 Initially, typical titration curves were observed. The intensities of the exothermic peaks
312 decreased as a function of molar ratios until they stabilized. This stabilized sequence, related to
313 a dilution phenomenon, corresponded to exothermic peaks. Furthermore, endothermic peaks
314 were also observed at the end of the titration just after each exothermic peak. These phenomena
315 were observed for all mixtures except the FGA/FCN mixture, for which no exothermic peaks
316 were observed at the end of titration, only relatively high endothermic peaks. It is clear that the
317 exothermic peaks are mainly related to the electrostatic interaction between the polymers
318 (Priftis et al., 2012; Duhoranimana et al., 2018; Vuillemin et al., 2019). Solvent reorganization
319 also accounts for a significant part of the exothermic signal, with extensive hydrogen bonding
320 networks at the complex interface making the enthalpy change more favorable, even if

321 counterbalanced by an entropy penalty (endothermic peaks) (Turgeon et al., 2007). The
 322 complexes were formed until the charges were neutralized.

323

324



325

326 Figure 1: (A) Corrected heat rate upon time of mixtures between native gum Arabic (NGA) or
 327 functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN)
 328 at pH 5.5 at 45 °C. (B) Enthalpy and fit upon gum Arabic/chitosan molar ratio of mixtures
 329 between native gum Arabic or functionalized gum Arabic and native chitosan or functionalized
 330 chitosan at pH 5.5 at 45 °C. Grey points recorded at the beginning of the titration were excluded
 331 from the fit.

332 The endothermic peaks at the end of the titration could be related either to aggregation,
 333 reorganization within the system, or hydrophobic interactions (Kayitmazer, 2017; Weinbreck
 334 et al., 2004). Indeed, the endothermic peaks with the highest intensities were observed when

335 FCN was present in the mixtures. Both NGA and FGA had hydrophobic moieties from the
336 protein fraction or grafted moieties, whereas NCN did not. Grafting of phenolic compounds
337 onto chitosan provided hydrophobic moieties capable of exhibiting novel interactions with
338 NGA and FGA.

339 Finally, the successive appearance of exothermic and endothermic signals is typical of
340 ITC measurements on complex formation for many systems (Turgeon et al., 2007). It has been
341 shown to be induced either by other energetic contributions such as the release of water
342 molecules and ions, by conformational changes of polysaccharides, by the aggregation of
343 polymer complexes or to the different steps involved in coacervation (Gonçalves et al., 2005;
344 Harnsilawat et al., 2006; Ziegler & Seelig, 2004; Hadian et al., 2016). The absence of
345 exothermic peaks at the end of the FGA/FCN titration could mean that these phenomena did
346 not occur in this system when gum Arabic was in excess, or be related to a reorganization
347 phenomenon of the system.

348 The binding isotherms were fitted using an “Independent” model (Figure 1B). This model
349 was used to determine the thermodynamic parameters involved in the interactions such as
350 dissociation constant, interaction enthalpy and stoichiometry. The enthalpy upon molar ratio
351 followed a typical titration curve. The interaction stoichiometry (n) corresponds to the inflection
352 point of the sigmoid curve. As it changed upon mixture, the stoichiometry is clearly not the
353 same. The enthalpy was also different upon mixture, which means that the thermodynamic
354 parameters were also different for the conditions studied. The free energy and thermo-entropic
355 contribution were calculated from Eq. 1 and Eq. 2. The results obtained are presented in Table
356 2.

357 Table 2: Thermodynamic parameters (dissociation constant (K_d), stoichiometry (n), enthalpic
358 contribution (ΔH), thermo-entropic contribution ($T\Delta S$) and Gibbs free energy (ΔG)) of binding
359 between native gum Arabic (NGA)/functionalized gum Arabic (FGA) and native chitosan

360 (NCN)/functionalized chitosan (FCN). “a, b and c” mean that the statistical analysis showed no
 361 significant differences between the results, for each column. Results with the same letter means
 362 there was no significant difference between them.

363

Mixtures	$K_d \cdot 10^{-08}$	n	ΔH (kJ.mol ⁻¹)	T ΔS (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)
NGA NCN	14.5 ± 0.6^a	2.7 ± 0.2^a	-965.2 ± 40.7^a	-923.6 ± 40.9^a	-41.6 ± 0.2^a
NGA FCN	13.6 ± 5.3^a	3.1 ± 0.2^{ab}	-916.3 ± 48.8^a	-874.3 ± 47.7^a	-42.0 ± 1.1^a
FGA NCN	17.7 ± 6.9^a	3.4 ± 0.3^b	-913.3 ± 14.7^a	-872.2 ± 13.7^a	-41.1 ± 1.0^a
FGA FCN	0.4 ± 0.2^b	6.3 ± 0.6^c	-1331.5 ± 23.3^b	-1280.4 ± 24.5^b	-51.1 ± 1.2^b

364

365 For all conditions, the ΔG was always negative, meaning that self-assembly was always
 366 spontaneous whether the polysaccharides were functionalized or not. The K_d values were
 367 always low (from 0.4×10^{-8} to 17.7×10^{-8}) corresponding to strong interactions (an interaction
 368 is considered strong when the K_d value is less than 10^{-6} (Qin & Gronenborn, 2014)). The K_d
 369 value was lower for the FGA/FCN mixture. This could mean that the strength of the interaction
 370 was greater when both polymers were functionalized. This could potentially be related to the
 371 increased charge of FGA in comparison to NGA and to the grafting of the oxidation product
 372 onto gum Arabic and chitosan, which could promote further interactions between them.

373 The ΔH values were negative in all cases, as were the T ΔS values. The ΔH values, mainly
 374 related to electrostatic interactions (Aberkane et al., 2010; Kayitmazer, 2017; Vuillemin et al.,
 375 2019), are considered favorable to interactions when they are negative (negative contribution
 376 to ΔG , see Eq.1). On the contrary, the values of T ΔS , related to hydrophobic interactions
 377 (Kayitmazer, 2017; Weinbreck et al., 2004), are favorable to interactions when they are
 378 positive. In the given cases, ΔH values were always favorable to interactions (from $-913.3 \pm$

379 $14.7 \text{ kJ}\cdot\text{mol}^{-1}$ to $-1331.5 \pm 23.3 \text{ kJ}\cdot\text{mol}^{-1}$) and $T\Delta S$ values were always unfavorable to
380 interactions (from $-872.2 \pm 13.7 \text{ kJ}\cdot\text{mol}^{-1}$ to $-1280.4 \pm 24.5 \text{ kJ}\cdot\text{mol}^{-1}$).

381 Regarding the stoichiometry values (Table 2), the involvement of FGA may have resulted in a
382 change in stoichiometry. For mixtures containing NGA, the stoichiometry was the same ($2.7 \pm$
383 0.2 for NGA/NCN mixtures and 3.1 ± 0.2 for NGA/FCN mixtures). However, for mixtures
384 containing FGA, the stoichiometry was different (3.4 ± 0.3 for FGA/NCN mixtures and $6.3 \pm$
385 0.6 for FGA/FCN mixtures). The difference between mixtures containing NGA and FGA was
386 expected from the change in the approximate Zeta potential of gum Arabic after
387 functionalization (Table 1). However, the difference in stoichiometry observed between the
388 FGA/NCN and FGA/FCN mixtures (3.4 ± 0.3 and 6.3 ± 0.6 , respectively) was surprising,
389 because the approximate Zeta potential of chitosan was the same at pH 5.5 after
390 functionalization. This result suggests that electrostatic interactions are not the only types of
391 interactions between the two modified polymers. This could indicate that when both polymers
392 were modified, potential hydrophobic interactions, π - π stacking, cation- π and anion- π induced
393 by the grafting of phenolic compounds could lead to a change in complexation stoichiometry
394 and an increase in polymer content. However, hydrophobic interactions would mainly lead to
395 aggregation, as already studied by Kaibara (2000) on the elastin/water system (Kaibara et al.,
396 2000). While the ΔG was almost the same for all conditions examined, it was significantly more
397 negative for FGA/FCN mixture ($-51.1 \pm 1.2 \text{ kJ}\cdot\text{mol}^{-1}$). Furthermore, this mixture had the lowest
398 K_d ($0.4 \times 10^{-8} \pm 0.2 \times 10^{-8}$), which means that under these conditions the interaction between
399 modified gum Arabic and modified chitosan was the most favorable.

400 The enthalpic and entropic parameters did not change whether chitosan was functionalized or
401 not (Table 2). However, the functionalization of both polymers had an impact on them. ΔH
402 varied from $-965.2 \pm 40.7 \text{ kJ}\cdot\text{mol}^{-1}$ for NGA/NCN mixtures to $-1331.5 \pm 23.3 \text{ kJ}\cdot\text{mol}^{-1}$ for

403 FGA/FCN mixture while $T\Delta S$ varied from $-923.6 \pm 40.9 \text{ kJ.mol}^{-1}$ to $-1280.4 \pm 24.5 \text{ kJ.mol}^{-1}$,
404 respectively. This means that the enthalpy contribution became more favorable to the
405 interaction while the entropy contribution became less favorable when both polymers were
406 modified. This makes sense, as the functionalization of gum Arabic, while increasing its
407 hydrophobicity and/or the possibility for it to undergo π - π stacking, cation- π and anion- π
408 interactions, increased its negative charges, likely leading to more electrostatic interaction.

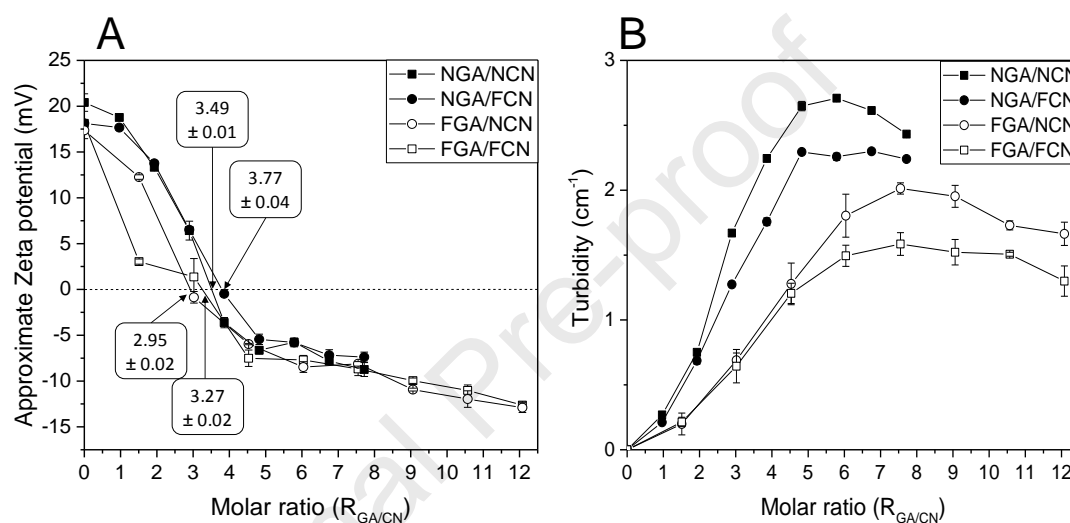
409 A surprising result was obtained from the comparison of the enthalpic and entropic
410 contributions of FGA/NCN and FGA/FCN. Since the charges of chitosan were the same
411 whether it was functionalized or not, the observed difference could not be explained by an
412 increase in electrostatic interaction. However, although the thermodynamic parameters did not
413 indicate a significant role of hydrophobic interactions in the complexation process, the
414 isotherms (Figure 1A) showed an endothermic contribution at the end of the titration. This could
415 mean that in the case of FGA/FCN mixtures, there could be a competition between electrostatic
416 interactions, which are strong at the beginning of the titration until stoichiometry is reached,
417 and hydrophobic interactions, which are predominant at the end of the titration.

418 By measuring the approximate Zeta potential upon polymers molar ratio, the turbidity of the
419 samples after mixing and with the support of optical microscopy, a more thorough
420 understanding of the type of interactions that were occurring should be gained in order to
421 elucidate the complexation mechanism.

422

423 3.3. Characteristics of polymer mixtures upon molar ratio

424 The influence of the molar ratio on the approximate Zeta potential and turbidity of the
 425 samples was studied for each potential mixture (Figure 2). The experiments were performed at
 426 pH 5.5, 45 °C. Measurements and observations were performed 5 min after mixing the
 427 polymers solutions.



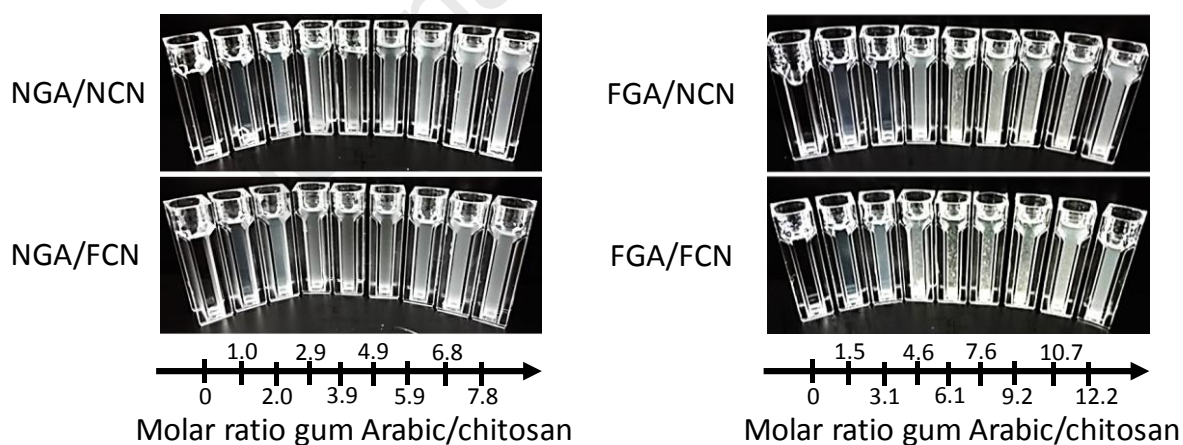
428
 429 Figure 2: (A) Approximate Zeta potential (mV) and (B) turbidity (cm^{-1}), of mixtures between
 430 native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or
 431 functionalized chitosan (FCN) upon gum Arabic/chitosan molar ratio at pH 5.5 and 45 °C,
 432 5 min after mixing.

433
 434 When mixing gum Arabic and chitosan, the approximate Zeta potential decreased upon
 435 addition of gum Arabic (Figure 2A). This is because the charges of chitosan (NCN or FCN)
 436 were progressively neutralized upon addition of FGA or NGA to the mixtures. The molar ratio
 437 for which the approximate Zeta potential of the mixture was zero was not exactly the same as
 438 the stoichiometry obtained by ITC measurements (Table 2), but similar trends were reported
 439 for all mixtures except for the FGA/FCN mixture. The optimal molar ratio (for which charges

440 were neutralized) was lower by Zeta potential measurements than by ITC measurements for
 441 FGA/FCN mixtures (3.27 ± 0.02 versus 6.3 ± 0.6 , respectively). This means that the optimal
 442 molar ratio obtained by ITC for FGA/FCN interaction was not simply due to electrostatic
 443 interactions. This confirmed that other types of interactions such as π - π stacking, cation- π and
 444 anion- π were at work for this mixture at 45 °C.

445 Upon molar ratio, the turbidity of the mixtures increased up to a molar ratio of 4.9:1 for
 446 the mixtures containing NGA and 7.6 for mixtures containing FGA, then decreased slightly
 447 (Figure 2B). For molar ratio lower than these, the turbidity was different for the different
 448 mixtures, indicating the formation of various supramolecular structures (coacervates or
 449 aggregates of different size and shape). These differences may be related to the shape of the
 450 formed samples, as can be seen in Figure 3.

451

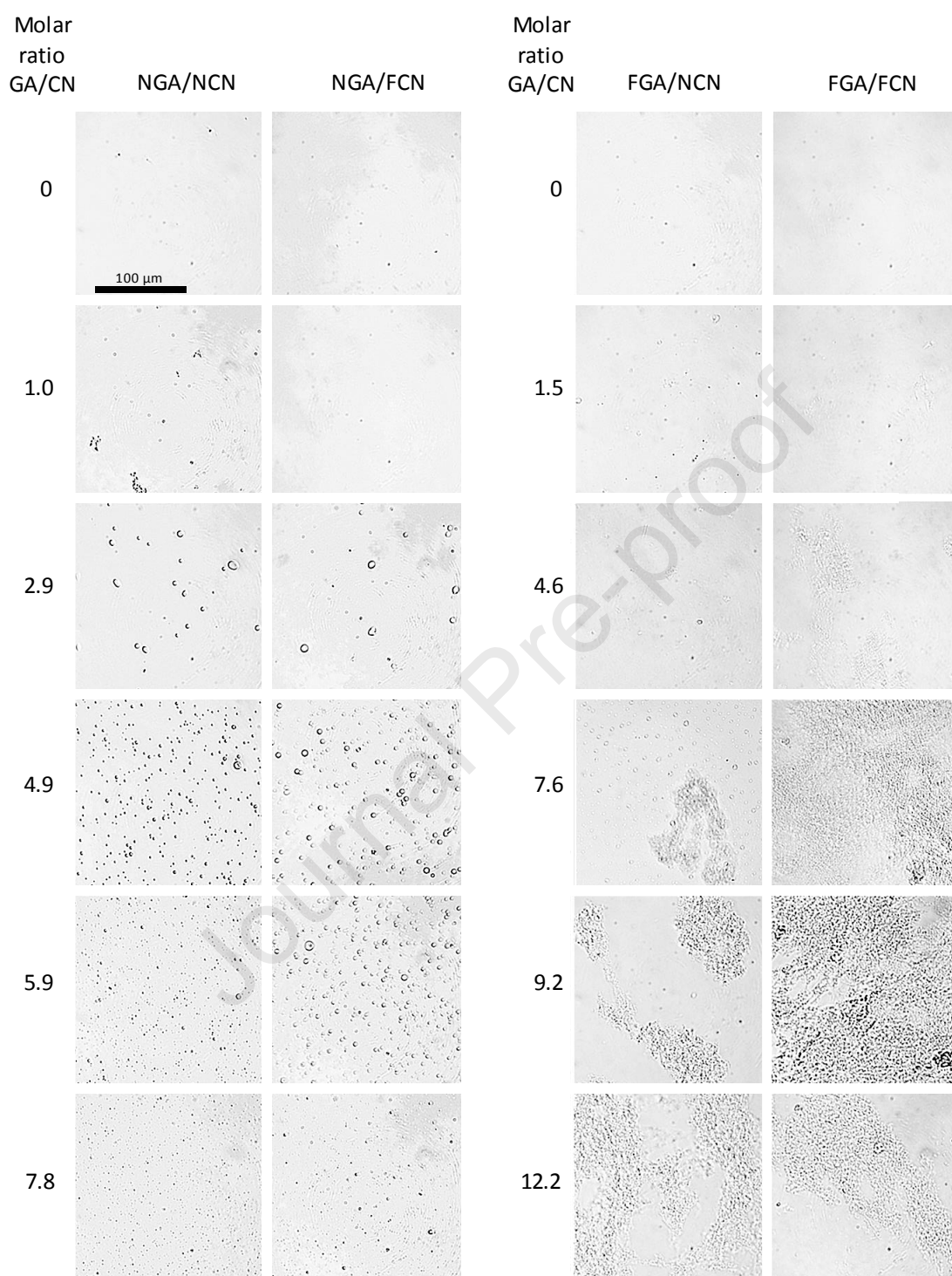


453 Figure 3: Macroscopic observations of mixtures between native gum Arabic (NGA) or
 454 functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN)
 455 mixtures upon molar ratio at 45 °C, 5 min after mixing.

456 From the macroscopic observation in Figure 3, it can be observed that the mixtures
 457 exhibited different behavior depending on the polymers involved and the molar ratio. Some

458 samples appeared “homogeneously” turbid while aggregates were observed on others. Mixtures
459 containing NGA were turbid while the presence of FGA seemed to lead to the formation of
460 aggregates at certain molar ratios. In the photographs of the mixtures (Figure 3), it would appear
461 that the mixtures become more turbid for NGA/NCN and NGA/FCN mixtures upon molar
462 ratios. However, for mixtures containing FGA, the mixtures became turbid upon FGA addition
463 until a molar ratio was reached when aggregation occurred (for ratio 3.1 for FGA/FCN mixtures
464 and for the 6.1 ratio for FGA/NCN mixtures). To confirm the presence of coacervates and
465 aggregates in the samples, the mixtures were observed by optical microscopy (Figure 4).

466



467

468 Figure 4: Optical micrographs of mixture between native gum Arabic (NGA) or functionalized
469 gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN) mixtures upon
470 different GA/CN molar ratios 5 min after mixing at 45 °C, pH 5.5.

471 For molar ratio below 1.0, no supramolecular structures were observed for all mixtures.
472 When NGA was added to NCN or FCN up to a molar ratio of 2.9, a few coacervates appeared.
473 They became more numerous for a molar ratio of 4.9. Upon addition of NGA, coacervates
474 tended to disappear (molar ratio 7.8).

475 For FGA/NCN mixtures (Figure 4), no supramolecular structures were observed for a
476 molar ratio of 7.6. For a molar ratio of 7.6, a coexistence of two types of supramolecular
477 structures was observed: there were both coacervates and aggregates. Above a molar ratio of
478 9.2, no more coacervates were observed, but the aggregates became larger and more numerous.
479 For FGA/FCN mixtures, no coacervates were observed at any molar ratio. However, aggregates
480 started to appear for a molar ratio of 4.6, and they became larger until the molar ratio of 7.6 was
481 reached. For a molar ratio of 9.2, it appears that the density of aggregates decreased. The latter
482 result was positively correlated with the turbidity and macroscopic aspect of the mixtures
483 (Figure 2 and Figure 3). This could be due to a reorganization within the different systems that
484 would correlate with the endothermic events observed on ITC measurements above a molar
485 ratio of 8 (Figure 1A). By linking the ITC measurements, the thermodynamic parameters
486 obtained from fitting the raw data, to macroscopic observations, Zeta potential measurements,
487 turbidity measurements and optical micrographs, a mechanism was proposed to explain what
488 happened within the different systems.

489 **3.4. Possible mechanism of the interaction between gum Arabic and** 490 **chitosan**

491 The interactions between NGA and NCN were electrostatic interactions between the
492 carboxyl groups of gum Arabic and the amine groups of chitosan (Butstraen & Salaün, 2014;
493 Espinosa-Andrews et al., 2007, 2010; Vuillemin et al., 2019). The two polymers attracted each

494 other due to their opposite charges, followed by the release of water and counterions. Then,
495 upon addition of gum Arabic, the charges of the two polymers were balanced. Upon addition
496 of gum Arabic beyond stoichiometry, reorganization of the complexes occurred until the
497 complexes disintegrated to reach equilibrium in the systems (observed in previous studies on
498 NCN and NGA (Vuillemin et al., 2019)).

499 Studies often agree that there is an initial step of soluble complexes formation prior to the
500 actual coacervation step. Recent studies have focused on these complexes formed between
501 oppositely charged polyelectrolytes as precursors to coacervates and multilayers (Comert et al.,
502 2018). The presence of these small precursors has been reported to increase the turbidity of
503 samples, and it has been suggested that this is related to an increase in the size or number of
504 complexes or soluble aggregates. In the optical micrographs, for the first few molar ratios, even
505 though no supramolecular structures were visible, it could be assumed that soluble complexes
506 or soluble aggregates were formed, due to the increased turbidity of the samples (Figure 2 and
507 Figure 3).

508 For the NGA/NCN system, up to a molar ratio of 1.0, no complexes were visible (Figure
509 4). However, it was possible that supramolecular structures had formed, but they were too small
510 to be seen by optical microscopy. This was confirmed by the turbidity of the mixtures (Figure
511 2) rising before the first coacervates could be observed by optical microscopy (Figure 4). When
512 mixed beyond a ratio of 2.9, these two oppositely charged polysaccharides began to form
513 coacervates (Figure 4). When more NGA was added to NCN, more charges of the polymers
514 interacted. The stoichiometry value determined by ITC (2.7) was confirmed by optical
515 microscopy (first supramolecular structures at 2.9), macroscopic observations (first turbid
516 samples at 2.0) and Zeta potential (optimal ZP at 3.49). At ratio 7.8, when NGA was in excess,
517 the complexes became smaller. To reach an equilibrium in the system, the GA could desorb

518 and the coacervates could change. This assumption was consistent with the small endothermic
519 event at the end of the titration (Figure 1). This could be explained by this reorganization within
520 the system.

521

522 For the NGA/FCN system, the same phenomena were observed (Figure 4). The ITC
523 results, macroscopic and microscopic observations as well as Zeta potential and turbidity
524 indicated no change when replacing NCN with FCN. This means that the coacervation process
525 was probably the same. Since the charges of FCN and NCN were the same at the chosen pH
526 and temperature, the interactions between FCN and NGA were likely mainly electrostatic
527 interactions. If another phenomenon occurred, such as hydrophobic interactions or π -anion
528 interactions between NGA and the grafted oxidation products of FCN, the experiments did not
529 reveal them.

530

531 For the FGA/FCN system, no coacervation phenomena were clearly observed in
532 photographs of the sample or in micrographs (Figure 3 and Figure 4). Up to a molar ratio of
533 3.1, the aspect of the sample became turbid but no supramolecular structures were visible by
534 optical microscopy. This means that before this ratio, small complexes were formed. After a
535 ratio of 4.6, aggregates appeared (Figure 4). The optimal molar ratio determined by Zeta
536 potential measurements was consistent with the appearance of the turbidity (3.27) and the
537 appearance of aggregates was consistent with the optimal molar ratio determined by ITC (6.3).
538 This means that the formation of soluble complexes was primarily driven by electrostatic
539 interactions but the formation of aggregates would be driven by other forces. ITC parameters
540 confirmed that the presence of FGA and FCN increased the contribution of the enthalpic

541 parameter. This could be due to the fact that both polymers are grafted with oxidation products,
542 which leads to more π - π stacking, π -cation and π -anion interactions increasing the enthalpic
543 contribution. The presence of exothermic hydrophobic interactions could not be excluded, but
544 their effect on the titration isotherms would probably be concealed by all the other interactions
545 involved in the aggregation phenomenon. However, at the end of titration, hydrophobic
546 interactions between FGA and FCN would be predominant (Figure 1).

547 The increased charge of FGA in comparison to NGA could increase the strength of its
548 interactions with chitosan. It is widely accepted that strong attractive properties promote
549 aggregation (Comert & Dubin, 2017). The polyelectrolyte structure also modulates the type of
550 phase separation observed. Indeed, the structure of the modified polymers changed their steric
551 hindrance, consequently modifying the accessibility of polysaccharides to each another. It was
552 revealed in previous studies that the glass transition temperature of gum Arabic decreased upon
553 functionalization, meaning that the grafting of phenolic compounds would have added to the
554 steric hindrance of FGA and made its chains more flexible, allowing it to undergo more
555 interactions (Vuillemin et al., 2020, 2021). The increase in FGA charge, leading to stronger
556 electrostatic interactions, combined with increase in steric hindrance and the increase in
557 polymer flexibility would be responsible for the aggregation phenomenon observed for the
558 FGA/FCN mixtures.

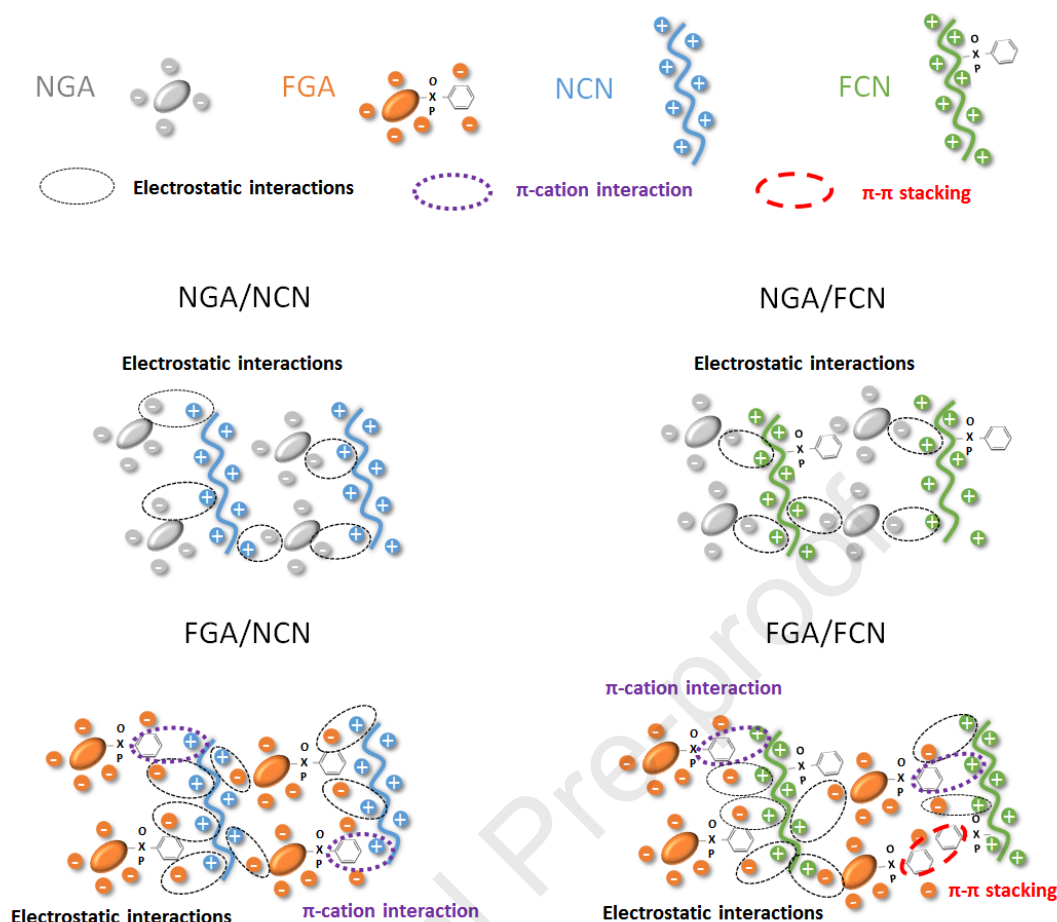
559

560 For the FGA/NCN system, the turbidity of the sample increased after the addition of FGA
561 (Figure 2). A slight difference was observed at a molar ratio of 1.5, but the turbidity was higher
562 at a ratio of 4.6. At a molar ratio of 6.1, aggregates appeared (Figure 3). The optimal molar ratio
563 obtained by Zeta potential measurements (2.95) and ITC measurements (3.4) were almost the
564 same, but no supramolecular structures were visible by optical microscopy until a molar ratio

565 of 7.6. This means that before this ratio, soluble complexes were formed. However, at a ratio
566 of 7.6, two distinct phenomena were observed by macroscopic observations. Coacervates and
567 aggregates were present in the mixture. In the literature, the differentiation between the two
568 mechanisms is often obscured by the common use of turbidity to detect these transitions
569 (Comert & Dubin, 2017). Both mechanisms induce an increase in turbidity, but microscopy is
570 needed to know whether the turbidity arises from small soluble complexes, coacervation or
571 aggregation. By adding more FGA to the mixture, only aggregates were visible in the optical
572 micrographs.

573 A surprising result was the difference between the NGA/NCN mixture and the FGA/NCN
574 mixture. Indeed, the ITC results (Table 2) were not significantly different for these mixtures
575 and the stoichiometries were almost the same. However, turbidity showed the appearance of
576 aggregates for the FGA/NCN mixtures as well as the appearance of coacervates for a molar
577 ratio of 7.6 (Figure 3) which were confirmed by optical microscopy (Figure 4). Furthermore,
578 while the stoichiometry obtained by ITC was almost the same for NGA/NCN and FGA/NCN
579 mixtures, the stoichiometry obtained by approximate Zeta potential measurements was lower
580 for the FGA/NCN mixtures. This could be explained by the fact that a lower amount of FGA
581 was required to neutralize the charge of chitosan since FGA was more charged than NGA (Table
582 1). The fact that the charge stoichiometry and the one obtained by ITC were close (2.95 ± 0.02
583 and 3.4 ± 0.3 , respectively) could explain why the two phenomena coexist for a molar ratio of
584 3.9 in the optical micrographs.

585



586

587 Figure 5: Schematic representation of the possible interactions between native gum Arabic
 588 (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized
 589 chitosan (FCN)

590 At the chosen pH and temperature, due to their charges under these conditions,
 591 NGA/NCN and NGA/FCN exhibited typical « weakly charged polyelectrolytes » coacervation
 592 behavior and steps, mainly driven by electrostatic interactions (negative enthalpy), with the
 593 entropy of counterion release playing only a minor role (Turgeon et al., 2007). The fact that
 594 these mixtures exhibited the same behavior suggests that mainly electrostatic interactions
 595 occurred between NGA and NCN and NGA and FCN (Figure 5).

596 In the FGA/NCN system, several phenomena were observed. First, soluble complexes
 597 were formed. Then, coacervates appeared as well as aggregates (Figure 4). Then, the
 598 coacervates disappeared and only aggregates remained, meaning that stronger interactions were

599 present (Comert & Dubin, 2017). These were probably mainly more electrostatic interactions,
600 due to the higher charge of FGA, but they could also be due to π -cation interaction between the
601 amine groups of NCN and the phenol groups of the oxidation products grafted onto FGA
602 (Figure 5).

603 Based on the fact that coacervation usually occurs when the interactions are weak and on
604 the contrary, aggregation is a consequence of strong interactions, it might be surprising if
605 coacervation occurred before aggregation or at the same time. However, the strength of the
606 interactions should not be regarded as the sole strength of electrostatic and π -cation interactions
607 between one FGA and one NCN but has the number of interactions in the system. Though some
608 interactions are individually weak, they can become collectively important (Biedermann &
609 Schneider, 2016; Neel et al., 2017). One explanation for the coexistence of coacervation and
610 aggregation would be that the “collective importance” of the π -cation interactions between FGA
611 and NCN was low at a given molar ratio, leading to coacervation, and that when the molar ratio
612 increased, this “collective importance” increased with the amount of FGA aromatic rings,
613 leading to the disappearance of coacervates in favor of aggregates. Another explanation could
614 be based on the fact that chitosan opens the structure of FGA when they interact. This would
615 lead to exposure of FGA phenolic groups from the grafted oxidation products to the solvent,
616 which would decrease the affinity of the polymer for the aqueous solvent. This could lead to an
617 aggregation phenomenon.

618 For the FGA/FCN system, soluble complexes appeared to have been formed at the
619 beginning of the titration, but then only aggregates were visible. The strength of the interaction
620 was higher than for the other systems (Table 2) and the enthalpy was the most favorable in this
621 system. These stronger interactions could be due to FGA, as described for the FGA/NCN
622 system, but other interactions were involved since the results for the FGA/FCN system were

623 different from the ones for FGA/NCN systems. These interactions were not involved in the
624 mixtures containing NCN or NGA/NCN systems, meaning that they were due to the
625 modification of both FGA and FCN. These strong and numerous interactions would lead to
626 aggregation instead of coacervation. The aggregation phenomenon could be related to the fact
627 that both polymers were grafted with oxidation products, which leads to more π - π stacking and
628 π -cation interaction, thus increasing the enthalpic contribution (Figure 5). The increase in FGA
629 charge likely also contributed to enhanced electrostatic interactions. Similarly, the increase in
630 steric hindrance and the increase in polymer flexibility induced by grafting of phenolic
631 compounds onto the polymer is known to promote the aggregation phenomenon.

632

633 **4. Conclusions**

634 This study provided a better understanding of the impact of the interaction balance
635 between two polymers on the formation of self-assembled structures. By comparing the
636 interaction stoichiometry (ITC results) and charge stoichiometry (approximate Zeta potential
637 results), it was shown that the interaction of chitosan with modified gum Arabic was not solely
638 due to electrostatic interactions. The strength of the interaction (K_d measured by ITC) was
639 higher for the mixture containing modified chitosan and modified gum Arabic, which meant
640 that other interactions occurred between those two (new π - π stacking and π -cation interactions).
641 The balance between these different interactions (their nature but also their collective strength)
642 led not only to the formation of coacervates but also to the formation of aggregates (confirmed
643 by turbidity measurements, macroscopic observation of the mixtures and optical microscopy).

644 By managing the physicochemical conditions of the medium and by judiciously choosing
645 the polymers (modified or not) it is possible to control the structures formed. Moreover, with

646 modified polymers, it is possible to form coacervates or aggregates under conditions different
647 from the optimal conditions with native polymers. With native polymers, to control the
648 structures formed, the physicochemical conditions of the medium must be controlled. When
649 these structures are used *in vivo* or to mimic the compartmentalized environments of cells for
650 example, the conditions of the medium are already fixed. The use of polymer modification
651 seems to be a promising way to control the structures formed in a given medium for
652 encapsulation purposes, for example.

653

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Table captions

Table 1: Approximate Zeta potential of native chitosan (NCN, 0.02% w/v), functionalized chitosan (FCN, 0.02% w/v), native gum Arabic (NGA, 0.50% w/v) and functionalized gum Arabic (FGA, 0.50% w/v) solutions in acetic acid 1.00% v/v at pH 5.5, 45 °C. “a, b, and c” mean that the statistical analysis showed no significant differences between the results.

Table 2: Thermodynamic parameters (dissociation constant (K_d), stoichiometry (n), enthalpic contribution (ΔH), thermo-entropic contribution ($T\Delta S$) and Gibbs free energy (ΔG)) of binding between native gum Arabic (NGA)/functionalized gum Arabic (FGA) and native chitosan (NCN)/functionalized chitosan (FCN). “a, b and c” mean that the statistical analysis showed no significant differences between the results, for each column. Results with the same letter means there was no significant difference between them.

Table 1:

Approximate Zeta Potential (mV)			
NCN 0.02% w/v in acetic acid	FCN 0.02% w/v in acetic acid	NGA 0.50% w/v in acetic acid	FGA 0.50% w/v in acetic acid
17.4 ± 0.7^a	18.1 ± 0.2^a	-9.7 ± 0.7^b	-21.7 ± 1.1^c

Table 2:

Mixtures	$K_d \cdot 10^{-08}$	n	ΔH (kJ.mol ⁻¹)	$T\Delta S$ (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)
NGA NCN	14.5 ± 0.6^a	2.7 ± 0.2^a	-965.2 ± 40.7^a	-923.6 ± 40.9^a	-41.6 ± 0.2^a
NGA FCN	13.6 ± 5.3^a	3.1 ± 0.2^{ab}	-916.3 ± 48.8^a	-874.3 ± 47.7^a	-42.0 ± 1.1^a
FGA NCN	17.7 ± 6.9^a	3.4 ± 0.3^b	-913.3 ± 14.7^a	-872.2 ± 13.7^a	-41.1 ± 1.0^a
FGA FCN	0.4 ± 0.2^b	6.3 ± 0.6^c	-1331.5 ± 23.3^b	-1280.4 ± 24.5^b	-51.1 ± 1.2^b

Figure captions

Figure 1: (A) Corrected heat rate upon time of mixtures between native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN) at pH 5.5 at 45 °C. (B) Enthalpy and fit upon gum Arabic/chitosan molar ratio of mixtures between native gum Arabic or functionalized gum Arabic and native chitosan or functionalized chitosan at pH 5.5 at 45 °C. Grey points recorded at the beginning of the titration were excluded from the fit.

Figure 2: (A) Approximate Zeta potential (mV) and (B) turbidity (cm^{-1}), of mixtures between native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN) upon gum Arabic/chitosan molar ratio at pH 5.5 and 45 °C, 5 min after mixing.

Figure 3: Macroscopic observations of mixtures between native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN) mixtures upon molar ratio at 45 °C, 5 min after mixing.

Figure 4: Optical micrographs of mixture between native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN) mixtures upon different GA/CN molar ratios 5 min after mixing at 45 °C, pH 5.5.

Figure 5: Schematic representation of the possible interactions between native gum Arabic (NGA) or functionalized gum Arabic (FGA) and native chitosan (NCN) or functionalized chitosan (FCN).

Figure 1:

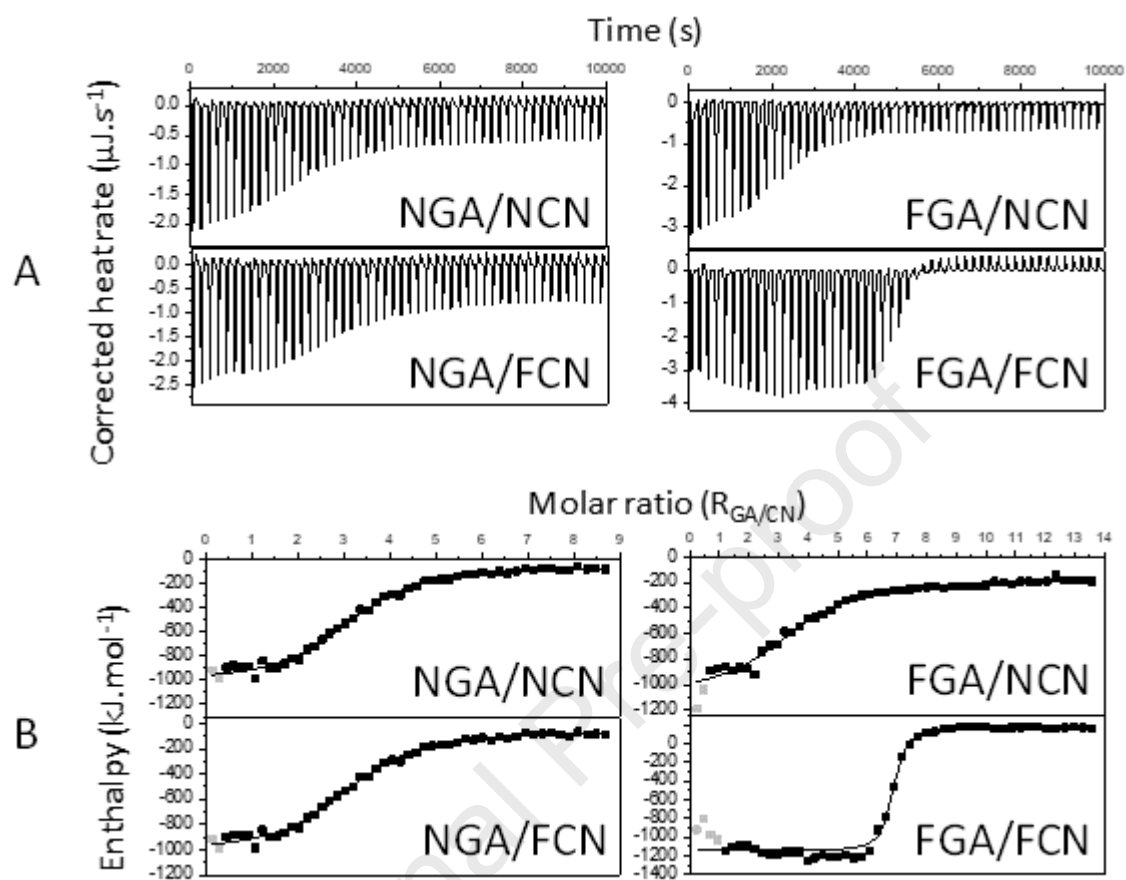


Figure 2:

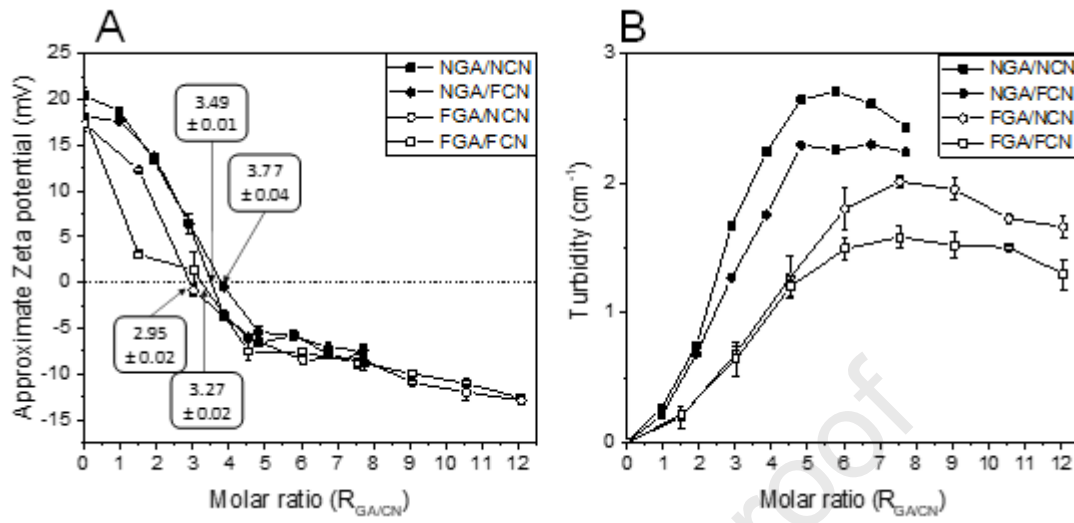


Figure 3:

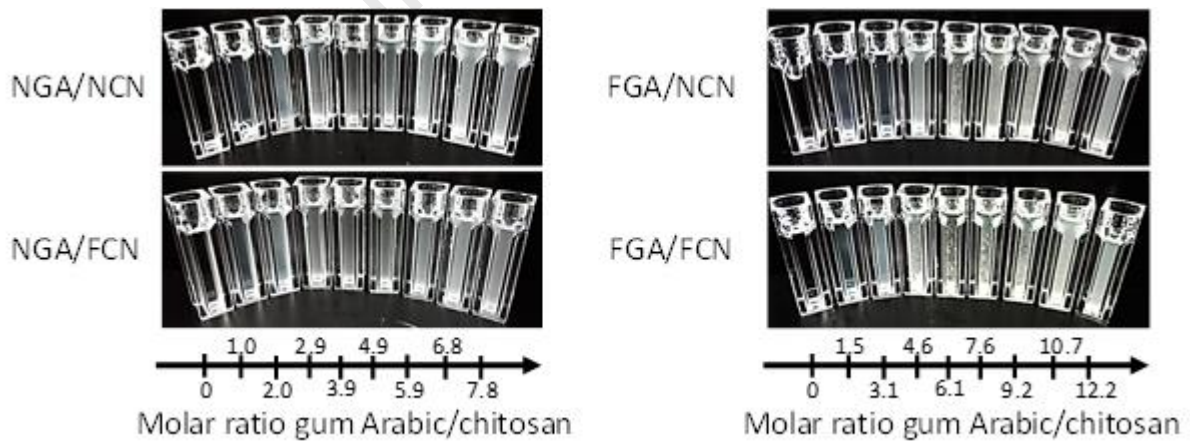


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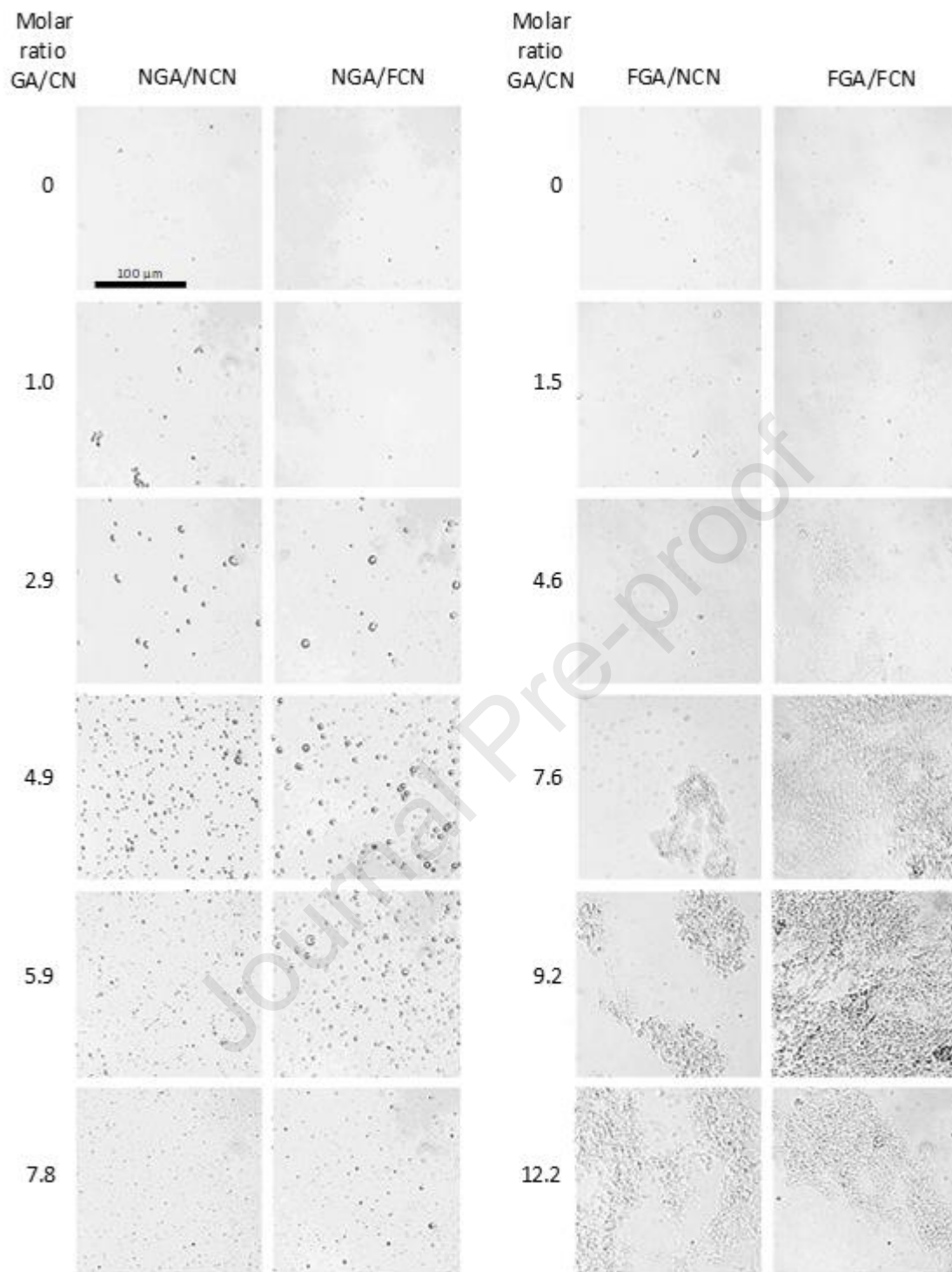
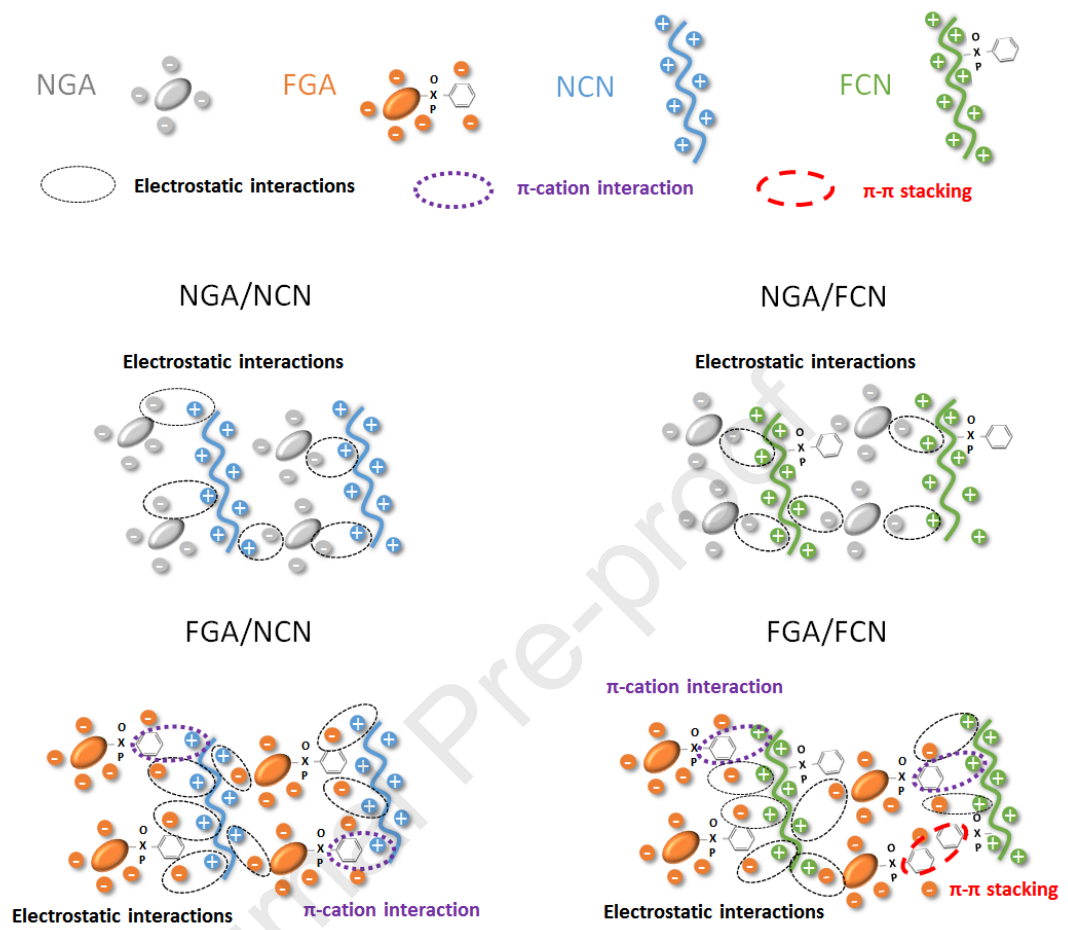


Figure 5:



Highlights:

- Grafting phenolic compounds onto polymers changed interactions and objects.
- Polymer functionalization increased their ability to exhibit π interactions.
- New interactions type, led to the formation of different objects.
- In some conditions, coacervates and aggregates coexisted.

Journal Pre-proof

Declaration of interests

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

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