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1 **INTRAGASTRIC STRUCTURING OF ANIONIC POLYSACCHARIDE KAPPA-**
2 **CARRAGEENAN FILLED GELS UNDER PHYSIOLOGICAL *IN VITRO* DIGESTION**
3 **CONDITIONS**

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26 **ABSTRACT**

27 In the present work, sodium alginate (SA), low methoxyl pectin (PEC) and κ -carrageenan (κ -
28 CAR) were evaluated for their intragastric structuring ability by means of light and dynamic
29 oscillatory rheology. SA and PEC solutions, their Ca^{2+} complexed gel analogues as well as
30 their binary blends with ionically or thermally set sheared κ -CAR gels, were subjected to *in*
31 *vitro* orogastric conditions. SA and PEC – Ca^{2+} complexed sheared gels exerted the highest
32 vulnerability to digestive fluid exposure due to the dialysis of egg-box dimer structures via
33 proton-calcium exchange. Incorporation of SA and PEC systems to κ -CAR gels prevented the
34 loss of mechanical strength of the gastric gels due to the ability of κ -CAR to undergo
35 spontaneous gelation in the presence of Na^+ and K^+ ions. Binary blends of SA and PEC – Ca^{2+}
36 complexed sheared gels with κ -CAR- Ca^{2+} gels exerted a significantly lower mechanical
37 strength loss sensitivity against pH and counterion composition of the gastric fluids.

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49 **Keywords:** alginates; pectins; intragastric structuring; pre-absorptive digestion conditions; acid
50 gelation; ionotropic gelation.

51 **1. INTRODUCTION**

52 Over the last decades the prevalence of obesogenic lifestyle associated with the consumption
53 of highly calorific food products, limited uptake of essential micronutrients and dietary fibre,
54 as well as restricted physical activity have led to an alarming increase of obesity and obesity
55 related health complications (Lake and Townshend, 2006). As result, health disease such as
56 type II diabetes, metabolic syndrome, hypertension, coronary artery disease, stroke,
57 osteoarthritis, liver and gall bladder disease, and obstructive sleep apnoea have been evidenced
58 (Kopelman, 2007). In addition, the association of obesity to several forms of cancer such as
59 endometrial, kidney, postmenopausal breast and colocteral adenoma has been reported
60 (Kopelman, 2007; Vigneri et al., 2006). Modulation of eating behaviour via suppressing
61 appetite and controlling the dietary and calorific value of food are widely recognised as
62 effective strategies to counteract obesity. As for appetite suppression, it has been demonstrated
63 that satiety is a responsive construct of the combination of environmental, physiological and
64 neurobiological signalling which involves food choice and intake based on the cross-modal
65 orosensory perception response, as well as pre-absorptive (gastric stretching and emptying,
66 suppression of digestive enzymes activity) and post absorptive (macro- and micro-nutrients
67 absorption, regulation of the gut microbiota) parameters (Bellisle, 2008; Chambers et al., 2015;
68 Fiszman and Varela, 2013a, 2013b; Llewellyn and Wardle, 2013).

69 Food macromolecules including proteins, dietary fibre and lipids are known as having a pivotal
70 impact on satiety signalling; however, this is generally attained via different mechanistic
71 physiological and neurobiological pathways (Fiszman and Varela, 2013b). As concerns to
72 dietary fibre, their satiety suppression effectiveness stems from their chemical and functional
73 aspects such as thickening and gelling ability, water and oil holding capacity,
74 fermentability/digestibility, absorption and mucoadhesivity (Brownlee, 2011; Fiszman and
75 Varela, 2013a, 2013b; Kristensen and Jensen, 2011). In a pre-absorptive digestion context,

76 dietary fibre can induce a plausible suppression of appetite via their ability to prolong
77 orosensory exposure (oral processing/mastication, secretion of saliva and gastric juice) and to
78 modulate the gastric response to food ingestion, e.g. activation of stomach mechanoreceptors
79 triggering stomach distension due to intragastric structuring, reduction of gastric enzymes
80 activity and delay of gastric emptying. Therefore, soluble dietary fibre exerting a fair
81 thickening ability and/or self- or co-structuring (in the presence of other macronutrients) ability
82 under acidic conditions, such as pectins, seaweed extracts (alginates and carrageenans), root
83 extracts (konjac gum), microbial synthesised gums (curdlan and gellan) and cellulose
84 derivatives (HPMC, CMC), have been scrutinised as potential intragastric structuring materials
85 (Borreani et al., 2016; Bradbeer et al., 2014; Fiszman and Varela, 2013a; Garrec et al., 2013;
86 Logan et al., 2015; Morell et al., 2014; Soukoulis et al., 2016; Spyropoulos et al., 2011; Zhang
87 et al., 2014). In addition, biopolymer assisted structural and interfacial engineering methods
88 have also been developed to reduce the energy density and promote satiety response of staple
89 processed food (Norton et al., 2006 & 2015). For example, crosslinked gel networks
90 (hydrogels) and ionotropically gelled microparticulates (fluid gels), protein-polysaccharide
91 assembled structures, as well as highly viscified aqueous systems (w/w) or o/w emulsions
92 are only some of the structurally bespoke food models promoting satiation response via their
93 intragastric structuring ability (Norton et al., 2015). When it comes to scrutinising the
94 intragastric ability of biopolymer structure engineered food models, adopting physiological
95 pre-absorptive digestion conditions is of paramount importance. Soukoulis and co-workers
96 (2016) have demonstrated that the adoption of a harmonised *in vitro* digestion protocol
97 (INFOGEST) was associated with evidently diversified structuring performance of sodium
98 alginate based o/w emulsions throughout gastrointestinal passage. Therefore, parameters such
99 as the pH fluctuation due to human host physiological diversity and stomach fullness state, as
100 well as the counterions complexity of the individual simulating pre-absorptive digestive fluids

101 (including the oral phase) should be considered as validating criteria of the foreseen intragastric
102 structuring performance of food biopolymers.

103 In the present work we aimed to investigate the intragastric structuring ability of gel composites
104 comprising anionic polysaccharides as the responsive construct of intrinsic (ionotropic, random
105 to ordered coil and acid self-induced gelling ability) and extrinsic (pH of the gastric chymes,
106 concentration and ionic strength of the simulating pre-absorptive digestive fluids) parameters.
107 The morphological and mechanical characteristics of the gastric chymes were assessed by
108 means of optical microscopy and dynamic oscillatory rheology respectively.

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110 **2. MATERIALS AND METHODS**

111 **2.1 *Materials***

112 Low viscosity sodium alginate (250 mPa·s, 2% w/w in water at 25°C, M/G ratio = 1.6,
113 mannuronic to guluronic acid content 61-31, $M_w = 1.43 \times 10^5 \text{ g mol}^{-1}$), κ -carrageenan (5-25
114 mPa·s, 0.3% w/w in water at 25°C), anhydrous calcium carbonate, δ -glucono-lactone, and
115 porcine pepsin (ca. 474 U/mg) were purchased from Sigma Aldrich (Leuven, Belgium). All
116 other chemicals, unless otherwise stated, were from the same supplier and of analytical grade
117 quality. Low calcium reactivity apple pectin (45 % degree of esterification, 80% galacturonic
118 acid content, $M_w = 2 \times 10^5 \text{ g mol}^{-1}$) was kindly provided as a gift from Herbstreith and Fox
119 GmbH (Neunbürg, Germany). All biopolymers listed were used without any further
120 purification.

121 **2.2 *Preparation of the biopolymer based solutions and Ca^{2+} mediated gel systems***

122 Two grams of biopolymer (sodium alginate, κ -carrageenan, or pectin) were dispersed into 200
123 mL of deionised 18 M Ω water (Millipore, USA), heated at 80 °C, kept at the same temperature
124 for 1 h to allow complete dissolution and then the obtained aliquots were cooled at 50 °C and
125 left to fully hydrate overnight under constant magnetic stirring. To prevent microbial spoilage,

126 a small amount of sodium azide (0.002% w/w) was added. One hundred mL aliquots of sodium
127 alginate, pectin and κ -carrageenan solutions (2% w/w) were mixed with anhydrous calcium
128 carbonate in order to achieve a final concentration of 40 mM. The biopolymer solutions were
129 successively ultrasonicated (5 min, 90% amplitude, UP200S, Hielscher GmbH, Teltow,
130 Germany) to ensure uniform distribution of CaCO_3 . Finally, the biopolymer solutions were
131 mixed with δ -glucono-lactone (at a 2:1 GDL to CaCO_3 ratio), covered with aluminium foil and
132 kept under magnetic agitation (1000 rpm) at 50 °C for 6 h to allow ionotropic gelation of the
133 biopolymers triggered via the *in situ* release of Ca^{2+} ions for 6 h. Evaporated water was added
134 back and the gels were cooled at ambient temperature under stirring (20±2 °C). A similar
135 approach was also used in the case of thermally set κ -carrageenan systems, i.e. the heated
136 solutions were left to cool down (ca. 2 °C/min) to ambient temperature under constant
137 magnetic agitation as previously described. Binary blends (1:1) of the Ca^{2+} complexed sodium
138 alginate and pectin with either ionically or thermally set κ -carrageenan were also prepared. All
139 biopolymer comprising systems were stored overnight at ambient temperature before carrying
140 out the *in vitro* pre-absorptive digestion experiments.

141 **2.3 *In vitro* pre-absorptive digestion of the biopolymer solutions and Ca^{2+} mediated gels**

142 The gastric structuring ability under simulated physiological conditions was studied adopting
143 the INFOGEST static standardised *in vitro* model as previously described by Minekus et al.
144 (2014). In brief, 5 g of the biopolymer system (solution or gel), preconditioned at 37±1 °C,
145 were transferred into 50 mL plastic centrifuge tubes and mixed with 5 mL of simulated salivary
146 fluid (SSF) (pH = 7, K^+ = 18.8, Na^+ = 13.6, Mg^{2+} = 0.15, Ca^{2+} = 1.5 mM). The obtained oral
147 phase was successively mixed with 10 mL simulated gastric fluid (SGF) (pH = 2, K^+ = 7.8,
148 Na^+ = 72.2, Mg^{2+} = 0.1, Ca^{2+} = 0.15 mM) and incubated at 37 °C for 1 h into a shaking water
149 bath (GFL GmbH, Germany) operated at 100 rpm simulating a physiologically achievable
150 antral shear rate (Vardakou et al., 2011). Simulated gastric chyme systems were cooled down

151 to 25 °C and were successively characterised by means of dynamic oscillatory rheology. *In*
152 *vitro* digestion experiments were carried out in triplicate.

153 In addition, a duplicate batch of model gastric chymes adjusted to a pH ranging from 1 to 4,
154 corresponding to stomach conditions varying from the fasted (starvation) to fed (full stomach)
155 state respectively, was also prepared adopting either physiological (systems diluted with SSF
156 & SGF) or non-physiological (systems diluted exclusively with Millipore water) pre-absorptive
157 digestion conditions.

158 **2.4 Dynamic oscillatory rheological measurements**

159 Dynamic oscillatory rheological measurements of the initial biopolymer aqueous systems as
160 well as of the obtained gastric chymes were carried out in an Anton-Paar rheometer (MCR 302,
161 WESP, Graz, Austria). Initial biopolymer aliquots were measured using a cone plate geometry
162 (CP50-1) whilst the gastric chyme suspensions were analysed by means of double gap
163 concentric cylinder geometry (DG 26.7). All measurements were performed at 25 ± 0.03 °C.
164 Strain-sweep (0.001 to 1000%) measurements of the biopolymer aliquots and gastric chymes
165 were carried out at 1Hz to determine the linear viscoelastic region (LVR). Therefore, the
166 viscoelastic properties of all biopolymer systems were determined by small frequency
167 amplitude sweeps (0.1 to 10 Hz) at a constant strain of 0.1%.

168 Thermo-oscillatory scans at a constant strain of 0.1% and frequency of 1 Hz were also carried
169 out to investigate the melting behaviour of the biopolymers. To prevent water evaporation the
170 cone-plate edge was covered with a small amount of silicon oil. A cooling-heating protocol
171 was applied as follows: a) heating from 10 to 70 at 2 °C/min, b) holding at 70 °C for 5 min and
172 c) cooling from 70 to 10 °C at 2 °C/min. The elastic modulus (G') and storage modulus (G'')
173 curves crossover points, at heating and cooling step were calculated to define melting and
174 gelation temperature (midpoint) of the biopolymer systems, respectively (Suppl. Table 1).

175 **2.5 Light microscopy**

176 The structure conformational changes of the biopolymer fibres during the pre-absorptive
177 digestion conditions (saliva and gastric phases) were qualitatively assessed by means of optical
178 microscopy. A small amount (ca. 1 mL) of the individual biopolymer containing gastric chyme
179 was mixed with either 0.25 mL of toluidine blue solution (0.05% w/w in distilled water) or
180 0.25 mL of saffronin solution and vortexed for 30 s. Toluidine blue was used to stain κ -
181 carrageenan whereas saffronin was used to stain sodium alginate and pectin containing
182 systems. A 1:1 toluidine blue to saffronin mixture was used to stain the binary biopolymer
183 based gastric chymes. Then, ca. 200 μ L of the stained biopolymer aliquot was deposited on a
184 glass slide and covered carefully by a glass cover slip so to avoid the entrapment of air bubbles.
185 Samples were visualised at a magnification of 10 \times using a Zeiss optical microscope (Axio Vert
186 A1, Zeiss GmbH, Germany).

187 ***2.6 Statistical analyses***

188 Normal distribution of data and equality of variance were verified by normal distribution plots
189 and box-plots, respectively. One way ANOVA was performed on complex viscosity data of
190 the simulated gastric chymes in order to evaluate the significance of pH and ionic strength
191 conditions. All analyses were carried out using SPSS v.19 statistical software (IBM Inc.,
192 Chicago, IL, USA).

193 **3. RESULTS AND DISCUSSION**

194 ***3.1 Thermo-oscillatory characterisation of the biopolymer aliquots***

195 The biopolymer aqueous systems were subjected to thermo-oscillatory scanning within the
196 linear viscoelasticity region (frequency 1Hz, strain 0.1%) to assess their mechanical response
197 on their mixing with simulated pre-absorptive digestion fluids (saliva and gastric juice) as well
198 as to determine the physical state transitions, i.e. sol to gel, during the fabrication of the sheared
199 biopolymer gels (Fig. 1). Corroborating the literature data, only κ -carrageenan (CAR) systems
200 exerted a clear sol-gel transition occurring at ca. 39 $^{\circ}$ C as result of its ability to undergo random

201 coil to double helix structure conformation (Tecante and Nez Santiago, 2012). As for sodium
202 alginate (SA) and apple pectin (PEC), systems did not exert any distinct physical state transition
203 retaining their domineering viscous-like character across the 10-70°C temperature range
204 (Suppl. Table 1, Fig. 1). Nevertheless, in the case of PEC, rheological spectra exhibited
205 significant hysteresis which possibly is associated with the conformational reorganisation of
206 pectin aggregates as function of temperature. Indeed, Muhidinov et al. (2011) demonstrated
207 that by heating PEC aqueous systems from 20 to 60°C, Higgins coefficient (K_h) was reduced
208 attaining a minimum value around 40°C which was primarily attributed to the disintegration
209 of pectin aggregates. Further increase of temperature resulted in the increase of K_h indicating
210 the restructuring of protein molecules into small compact structures.

211 Ionotropic (Ca^{2+}) gelation of the biopolymer did not modify significantly the profile of the
212 thermo-rheological spectra in the case of SA and CAR. In the latter case, a more pronounced
213 hysteresis between the melting and cooling curves was observed probably due to the occurring
214 gel-sol-gel transitions, i.e. de-assembly of the aggregated double helices to form random
215 biopolymer coils and vice versa (Kara et al., 2003). In addition, upon heating, CAR- Ca^{2+}
216 sheared gels retained their domineering solid like behaviour ($G' > G''$) in the entire temperature
217 range suggesting a partial de-assembly of the double helices contrarily to pure CAR systems.

218 On the other hand, ionic gelation of PEC resulted in a significant decrease of their
219 responsiveness to temperature e.g. hysteresis loop area.

220 Blending the ionic sheared gels (SA, PEC) with the sheared κ -carrageenan gels (thermoset and
221 ionotropically set) at the ratio of 1:1 (Fig. 1c,d), resulted to hybrid biopolymer gel structures
222 characterised by a domineering solid like behaviour ($G' > G''$) and larger hysteresis loop areas.

223 Although the absence of sol-gel transitions is sufficiently rational for sheared ionic gel
224 mixtures, in the case of SA- Ca^{2+} /CAR and PEC- Ca^{2+} /CAR gels, the dimer structures exerted a
225 governing influence over the structuring ability of κ -carrageenan. On the contrary, in SA/CAR

226 and PEC/CAR gels, sol-gel transitions took place (in the range of ca. 20 to 36 °C), indicating
227 the unobstructed ability of κ -carrageenan to undergo coil to double helix structure
228 conformational changes. Therefore, blending of the former gels with the oral and gastric fluids
229 would expectedly induce a remarkable suppression of the melting points. Indeed, thermo-
230 oscillatory rheological characterisation of κ -carrageenan gels adopting a 4-fold dilution
231 protocol (simulating mixing with oral and gastric fluids) with deionised water revealed a
232 considerable suppression of the melting and gelation temperature points, i.e. ca. 28 and 31 °C,
233 respectively (data not shown).

234 ***3.2 Viscoelastic profiling of the biopolymer aqueous systems***

235 The major physical state aspects of the biopolymer gels were assessed by means of dynamic
236 oscillatory rheology (frequency sweeps) in the LVR (Fig. 2) at ambient (25 °C) temperature.
237 As concerns the individual biopolymer aqueous systems (Fig. 2a), only κ -carrageenan
238 exhibited a domineering solid-like behaviour ($G' > G''$). A predominant liquid-like behaviour
239 was observed for SA ($G' \ll G''$) indicating the sterically hindered interaction of the biopolymer
240 chain segments (Ma et al., 2014), whilst a rather viscous liquid-like behaviour ($G' < G''$, loss
241 factor ($\tan\delta$) < 1.5) was observed in the case of PEC, with both moduli being fairly frequency
242 dependent; the latter is representative of topological interaction (hydrogen bonding and
243 hydrophobic interactions) of pectin side chain segments (Ström et al., 2014). When
244 biopolymers underwent Ca^{2+} complexation (Fig. 2b), an evident increase of the storage moduli
245 was achieved, which can be attributed to the aggregation of the intermolecular junctions of the
246 either egg-box (SA, PEC) or double helix (CAR) dimer structures (Draget et al., 2006; Fraeye
247 et al., 2009; MacArtain et al., 2003). As concerns the biopolymer type, SA- Ca^{2+} sheared gels
248 experienced an almost 6-order increase of G' , far higher than PEC- Ca^{2+} (> 2.5-order), and
249 CAR- Ca^{2+} (<1.5 order). This appears to be rational considering the low Ca^{2+} reactivity of PEC
250 due to its rather high degree and random pattern of methoxylation (Fraeye et al., 2009). For

251 CAR, the presence of Ca^{2+} promotes the aggregation of the junction zone (which are also
252 formed in the case of the sheared thermoset gels) resulting to the formation of a fine network
253 without superstrands and therefore the observed differences in the G' values were rather
254 moderate (Hermansson et al., 1991).

255 As for the binary biopolymer gel systems comprising thermoset κ -carrageenan sheared gels
256 (CAR), a cooperative effect in terms of viscoelasticity was observed. All systems exerted a
257 dominant solid like character ($G' > G''$) of low sensitivity to oscillation frequency indicating
258 the formation of true gel structures (Picout and Ross-Murphy, 2003). As expected, SA based
259 binary systems were the most responsive to κ -CAR presence. It should be also noted that in the
260 case of SA- Ca^{2+} and PEC- Ca^{2+} based blends, the G' values achieved were ca. 4 to 8-fold higher
261 compared to their exclusively SA- Ca^{2+} and PEC- Ca^{2+} sheared gel analogues. It is therefore
262 hypothesised that due to the high chemical affinity of κ -carrageenan to calcium, an electrostatic
263 intermolecular interaction between the Ca^{2+} conveying domains of SA and PEC chain segments
264 with the negatively charged (e.g. sulfate) side groups of κ -carrageenan might also induced.
265 Indeed, when the binary blends composed of exclusively Ca^{2+} mediated sheared gels (Fig. 2d)
266 were scrutinised, the storage moduli were plausibly comparable to the systems containing
267 thermoset κ -carrageenan sheared gels.

268 ***3.3 Intra-gastric structuring ability of the biopolymer gels***

269 ***3.3.1 Morphological aspects of gastric chymes***

270 The gastric chymes obtained following an 1h exposure to simulated gastric fluids were assessed
271 by means of light microscopy to assess the morphological aspects of the gel microparticulates
272 (Figs. 3-5). In order to have a more comprehensive overview of the counterion composition
273 impact on intra-gastric structuring, acidified biopolymer aliquots obtained by mixing the initial
274 gel systems with deionised water were also microscopically analysed (Soukoulis et al., 2016).
275 In the present work the obtained micrographs provide a tangible visualisation of the structural

276 traits of the particulates without offering the possibility to measure the actual volume fraction
277 of the biopolymer microparticulates. This has also been reported previously when sheared
278 anisotropic hydrogel microstructures compressed between the glass slide and coverslip, were
279 visually analysed (Wolf et al., 2000).

280 Adopting a counterion-free digestion protocol (odd numbered micrographs) was associated
281 with more intact biopolymer structures which consecutively results in larger hydrodynamic
282 radii of the polymeric particulates. The prevalence of monovalent cation species (e.g. Na⁺ and
283 K⁺) in the physiological simulated oral and gastric juices was associated with a remarkable
284 modification of microstructural aspects of the gastric chymes (even numbered micrographs).
285 As concerns the individual biopolymer (non-crosslinked) containing gastric chymes, a fair
286 erosive effect leading to the formation of particulates of smaller hydrodynamic radius was
287 observed (Fig. 3). This corroborates our previous findings on SA based o/w emulsions where
288 the prevalence of monovalent cations was associated with modification of the morphological
289 aspects of the acid self-structured biopolymer (Soukoulis et al., 2016). It has been reported that
290 SA and PEC fibres conditioned in physiological saline undergo significant hydrodynamic
291 volume reduction; this is due to the fibre contraction induced by salting out of the biopolymer
292 i.e. the electrostatic repulsion between the negatively chain segments is reduced in the
293 prevalence of the cation species (Jonassen et al., 2013; Qin, 2004). However, in all cases the
294 biopolymer systems maintained their principal structure conformational aspects, e.g. thin
295 layered/sheet-like, cloudy/aggregated or flocculated/fragmented for CAR, SA and PEC
296 respectively. On the ionotropic gelation, CAR underwent the most pronounced structural
297 changes attaining a predominantly smooth yet aggregated microstructure. Contrarily, SA-Ca²⁺
298 and PEC-Ca²⁺ sheared gels did not undergo any plausible microstructural changes, i.e. in both
299 cases systems maintained their aggregated particulate form.

300 Use of different staining protocols allowed us to sufficiently discriminate between CAR (violet
301 coloured) and SA or PEC (dark orange coloured) rich gel microdomains (Figs. 4&5). As for
302 the binary biopolymer gastric chyme systems containing the thermoset sheared κ -carrageenan
303 gels (Fig. 4), a drastic size reduction of the carrageenan rich microparticulates was achieved
304 regardless the presence and the physical state (dissolved or ionically crosslinked) of either
305 sodium alginate or pectin. However, no significant differences in the acid particulates'
306 morphology were detected when the physiological gastric chymes were assessed, i.e. highly
307 fragmented/eroded (SA/CAR and PEC/CAR) or moderately aggregated (SA-Ca²⁺/CAR and
308 PEC-Ca²⁺/CAR) structures were detected. In line to the aforementioned, the exposure of the
309 SA-Ca²⁺/CAR-Ca²⁺ and SA-Ca²⁺/CAR-Ca²⁺ sheared gels (Fig. 5) to highly acidic conditions,
310 the presence of an extensively aggregated network of microparticulates was confirmed.
311 Nevertheless, *in vitro* physiological gastric chymes exerted a finely aggregated structure
312 compared to their counterion-free analogues which is in agreement to our previous findings.
313 Therefore it can be postulated that the prevalence of sodium in the pre-absorptive digestion
314 fluids has an ion-exchanging role on the both Ca²⁺ triggered dimer structures, e.g. egg-box or
315 double helices, suppressing their intragastric structuring performance (Soukoulis et al., 2016).

316 ***3.3.2 Oscillatory rheological characterisation of the gastric chymes***

317 For the rheological characterisation of the gastric chymes a concentric cylinder geometry was
318 used, which has been previously reported as being relevant for the analysis of aqueous
319 suspensions (Picout and Ross-Murphy, 2003). Although the obtained values of viscoelastic
320 moduli of chymes cannot be directly contrasted to those of the initial gel systems (measured
321 using cone-plate geometry), the obtained rheological spectra can still give insight to the
322 structural changes occurring throughout the pre-absorptive digestion passage. According to the
323 gastric chyme rheographs (Fig. 6), the acid structuring ability of the gels was highly diversified
324 on their exposure to gastric juice conditions. As for the individual based systems, CAR exerted

325 the most pronounced acid structuring ability particularly in its non-crosslinked state. On the
326 other hand, mixing of PEC systems (in solution or sheared gel form) with the simulated oral
327 and gastric fluids resulted to a drastic reduction of the storage moduli, exhibiting a domineering
328 viscoelastic behaviour with a crossover point of the G' , G'' moduli at low ($<1\text{Hz}$) frequencies.
329 Therefore, the latter systems should behave as predominantly low viscosity liquids under the
330 hereby simulating antral forces (ca. 1.7 Hz). It is well established that low methoxylated pectin
331 may undergo gelation in the presence of both divalent and monovalent cation species (Thakur
332 et al., 1997; Yoo et al., 2003). In the latter case, parameters such as the esterification and
333 blockiness degree, the pH and the concentration and type of cation species control the sol-gel
334 transition (Ström et al., 2014). As refers to monovalent cations, the presence of 0.2 M of Na^+
335 is necessary to trigger a significant gelation (true) effect via later aggregation of pectin chains
336 via hydrophobic and van der Waals interactions (Ström et al., 2014). In our case, the low pH
337 of gastric chymes ($\text{pH} < \text{pK}_{\text{a,pectin}}$) together with the relatively low Na^+ (ca. 0.05 M) and the low
338 blockiness and methoxylation degree of PEC obstruct its self-aggregation leading to a loosely
339 structured biopolymer network which conveys the characteristics of rather weak gel only at
340 very low frequencies that fall out the range of expected antral forces (Ström et al., 2014). On
341 the contrary, SA dilute systems attained a clear true gel-like behaviour ($G' > G''$) as result of
342 the formation of intermolecular junctions via the hydrogen bonding of the protonated carboxyl
343 groups of the GG blocks in a cooperative manner with polymannuronic block segments (Draget
344 et al., 2006). When the Ca^{2+} complexed sheared gels were exposed to digestive fluids (Fig. 6b),
345 a noticeable reduction of the mechanical strength of the gels was detected. As proposed to our
346 previous work, the prevalence of monovalent cations triggers the ion exchange (dialysis) of
347 Ca^{2+} by H^+ hampering the intermolecular junctions between the dimer (egg-box) or double
348 helix structures (Draget et al., 2006, 1998; Soukoulis et al., 2016). As expected, the rheographs
349 revealed a higher impact of the digestive fluids on the PEC- Ca^{2+} than the SA gel analogues,

350 which may be attributed to the lower chemical affinity of PEC to sodium or potassium and the
351 constrained ability of the dialysed binding sites to interact via other forms of intermolecular
352 bonding, e.g. hydrogen bonding or hydrophobic interactions (Fang et al., 2008). In the case of
353 CAR-Ca²⁺ the ca. 1.5-order reduction of the G' modulus can be primarily attributed to digestive
354 fluids diluting effect (MacArtain et al., 2003) and at a lesser extent to the antagonistic calcium
355 exchanging activity of Na⁺ under acidic conditions reducing the aggregative bridging of the
356 double helices.

357 Gastric chymes obtained by the digestion of the binary blends of either SA/SA-Ca²⁺ or
358 PEC/PEC-Ca²⁺ with κ-CAR exhibited a persistent plateau of G' which indicates the existence
359 of a highly entangled network comprising the anisotropic gel microparticulates (Norton et al.,
360 2006). Although storage moduli were not significantly different among samples, the loss
361 moduli were generally higher in the case of the SA based systems suggesting a higher level of
362 gel particles entanglement. In addition, the presence of CAR had a dual assisting role in terms
363 of intragastric structuring: first, it counteracted the structural losses due to the 4-fold digestion
364 fluids diluting factor, and second, it prevented the gel mechanical strength loss due to the
365 dialysis of the egg-box Ca²⁺ dimers. It is therefore postulated that the high chemical affinity of
366 CAR with K⁺ and Na⁺ present in the digestive fluids impedes fibres contraction due to H⁺
367 induced ion exchange, facilitating chain segments interaction via hydrogen bonding (Tecante
368 and Nez Santiago, 2012). Complexation of CAR with Ca²⁺ (in the case of SA-Ca²⁺/CAR-
369 Ca²⁺ and PEC-Ca²⁺/CAR-Ca²⁺ sheared gels) was accompanied by a steep reduction of G'
370 moduli (ca. 2-order compared to the initial gel systems), yet the solid like behaviour remained
371 strongly evident. On this occasion, it is assumed that the reduction of the elasticity of the acid
372 gel microparticulates is primarily associated with the dialysis of egg-box dimer structures, and
373 secondarily with the constraining of the aggregative bridging of the CAR double helices.
374 Indeed, the gel particulates elasticity reduction in the case of the PEC-Ca²⁺ containing systems

375 was pronouncedly higher than the SA analogues, due to their lower calcium chemical affinity
376 leading to a sterically favoured ion exchanging effect.

377 ***3.3.3 Impact of counterions composition and pH conditions on the complex viscosity of the*** 378 ***gastric chymes***

379 Evaluating the impact of counterion composition of the simulating pre-absorptive digestion
380 fluids, gastric chymes obtained by blending the initial biopolymer systems with either
381 physiological *in vitro* saliva and gastric fluids or deionised (MilliQ) water were rheologically
382 characterised at 1 Hz and 0.1 % strain (Fig. 7). As clearly depicted, the prevalence of Na⁺ and
383 K⁺ ions was associated with a diversified intragastric structuring performance which was
384 biopolymer dependent. As a general trend, SA based systems were better performing in terms
385 of acid self-structuring in the absence of counterions. In this case, the formation of
386 intermolecular junctions between both across GG blocks and at lesser extent MM block
387 segments is sterically favoured (Draget et al., 2006). On the contrary, the intragastric
388 structuring ability of both individual and binary CAR based biopolymer systems was reduced
389 on their exposure to counterion-free digestive fluids supporting our postulation that
390 electrostatic bridging of monovalent cation species with the sulfate binding sites takes place.
391 As for PEC, solely in the case of the ionotropically structured systems a significant reduction
392 of the intragastric structuring performance was observed in a similar manner to SA-Ca²⁺
393 sheared gels. However, it should be noted, that the binary blends of the ionotropically
394 complexed biopolymers (SA-Ca²⁺/CAR and PEC-Ca²⁺/CAR) were not significantly affected
395 by the ionic composition of the digestive fluids and therefore, their intragastric performance
396 exerts the lowest sensitivity to the implemented digestion protocol.

397 In a consecutive case study, we attempted to assess the magnitude of the impact of simulating
398 pH gastric conditions, ranging from the fasted to the fed stomach state (Dressman et al., 1990),
399 on the acid structuring performance of biopolymer aqueous systems (Fig. 8). Interestingly, the

400 compositional profile of the biopolymer gels was found to crucially affect the responsiveness
401 of intragastric structuring to pH fluctuations. Specifically, the acid self-structuring performance
402 of SA exerted a steep increase (> 3-order) in the nearly fasted stomach pH range e.g. 2-3
403 followed by a gradual decrease of the complex viscosity under harsh pH conditions (pH<2).
404 This is in line with the findings of Andriamanantoanina and Rinaudo (2010) who observed an
405 increase of the storage modulus of SA alginate solutions (in their saline form) at pH<3 reaching
406 a maximum degree of swelling of the gel fraction at pH=2.5; then, at lower pH values a gradual
407 decrease of the suspended gel particulates was reported. It should also be noted that at highly
408 acidic conditions (pH<3) strong cooperative interactions with the participation of
409 polyguluronic and polymannuronic blocks are expected to take place (Draget et al., 2006).
410 Contrarily to SA, PEC systems exhibited an up to 2-order increase of the complex viscosity
411 (depending on their physical state i.e. ionically complexed or free) under moderate acidic
412 environment conditions, e.g. pH = 3. Corroborating the literature data, the acid self-structuring
413 ability of LM PEC is maximised when pH<pK_a (ca. 2.9) and in the presence of fairly sufficient
414 concentration of monovalent (0.2 M NaCl) cation species (Ström et al., 2014). When the
415 responsiveness of the individual κ-CAR systems was studied, a highly diversified acid
416 structuring performance was attained depending on their physical state; complex viscosity of
417 CAR-Ca²⁺ gastric chymes was not significantly affected by pH changes, whilst in the case of
418 their ionically non-complexed analogues a upper structuring plateau was reached in the 2 to 3
419 pH range. Reduction of the pH, resulted in a significant increase of the absolute charge of the
420 CAR molecules facilitating the electrostatic bridging of sulfate groups with the prevalently
421 present monovalent cation species (Tecante and Nez Santiago, 2012). When the cation binding
422 sites of the carrageenan chains are occupied by Ca²⁺ the effect of the pH on the net charge of
423 CAR molecules is reduced leading to lower sensitivity to pH change. On mixing κ-CAR with
424 SA or SA-Ca²⁺ (Fig. 8b), a structuring synergy was observed, with complex viscosity of the

425 gastric chymes remaining at high levels in the complete fasted to moderately fed state pH
426 region i.e. 1-3. An adverse behaviour was also attained in the case of PEC/ κ -CAR systems
427 though their structuring performance was higher in the intermediate to high pH region, e.g. 2.5-
428 4. Finally, gel systems comprising exclusively ionotropically crosslinked biopolymers exerted
429 a rather constrained sensitivity to pH fluctuation, a behaviour that could be primarily attributed
430 to the modulating effect of the κ -CAR- Ca^{2+} gel component.

431

432 **4. CONCLUSIONS**

433 In summary, anionic biopolymers such as sodium alginate, κ -carrageenan and low
434 methoxylpectin can promote *in vitro* intragastric structuring via different mechanistic pathways
435 i.e. ionotropic gelation, acid-self structuring and thickening. As for individual biopolymer
436 systems, SA or CAR exerted the highest structuring performance induced via cooperative
437 junction zone formation of the homopolymeric block segments and electrostatic bridging of
438 gastric juice monovalent ions with the sulfate binding sites respectively. Notwithstanding
439 ionically mediated sheared gels exerted a fairly true gel character, their ability to maintain their
440 structural aspects was diminished on their exposure to simulated digestive fluids. On the other
441 hand, blending of SA or PEC with CAR led to a synergistic gastric structuring effect. This was
442 primarily attributed to the ability of CAR to interact with both monovalent and divalent ions
443 present in the gastric chymes preventing their adverse impact on acid self-structuring of SA
444 and PEC. Finally, binary semi-solid composites comprising Ca^{2+} complexed anionic
445 polysaccharides exhibited the most limited responsiveness to the pH and ionic composition of
446 simulated digestive fluids.

447 **Conflict of interest:** Authors declare no conflict of interest.

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554

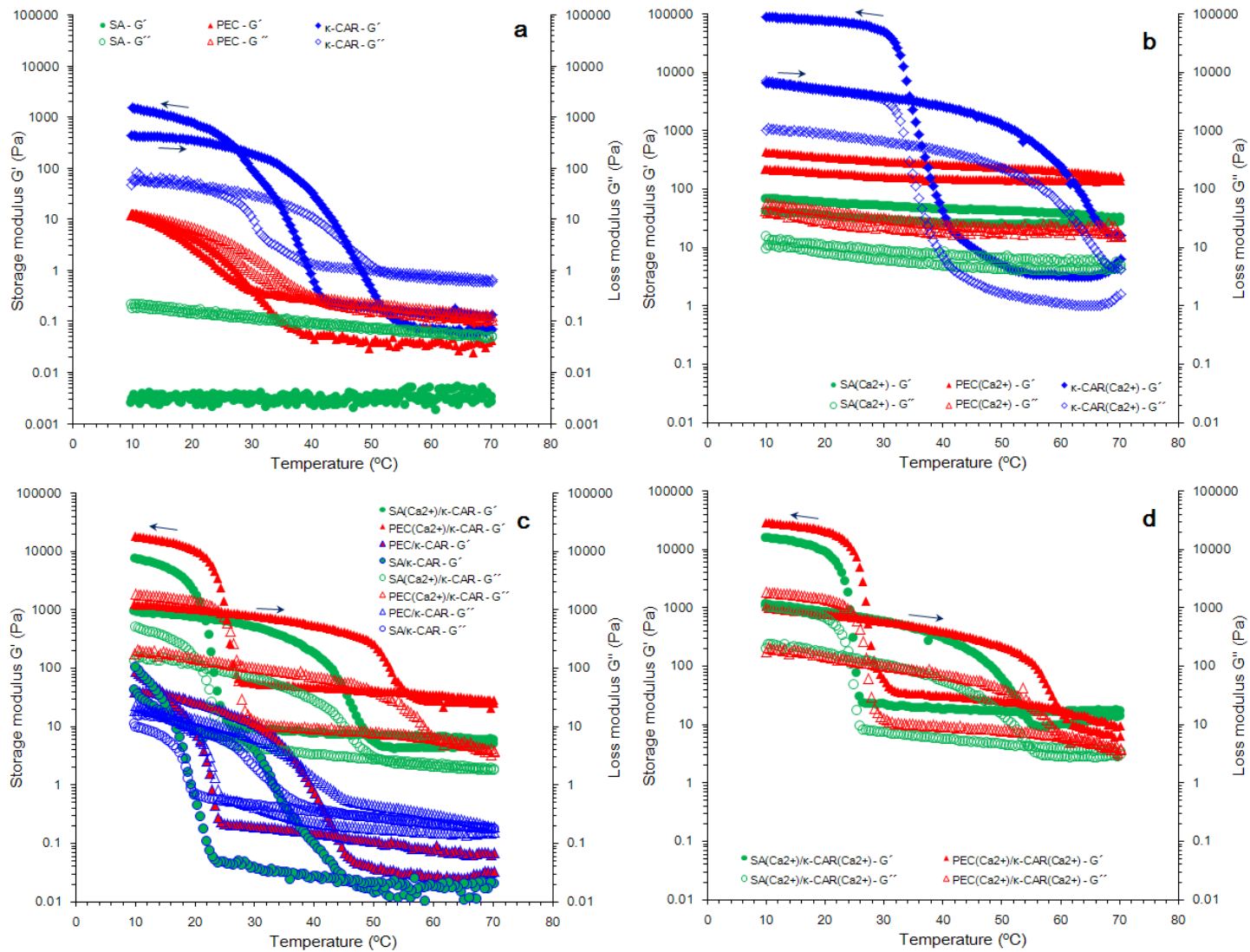


FIGURE 1

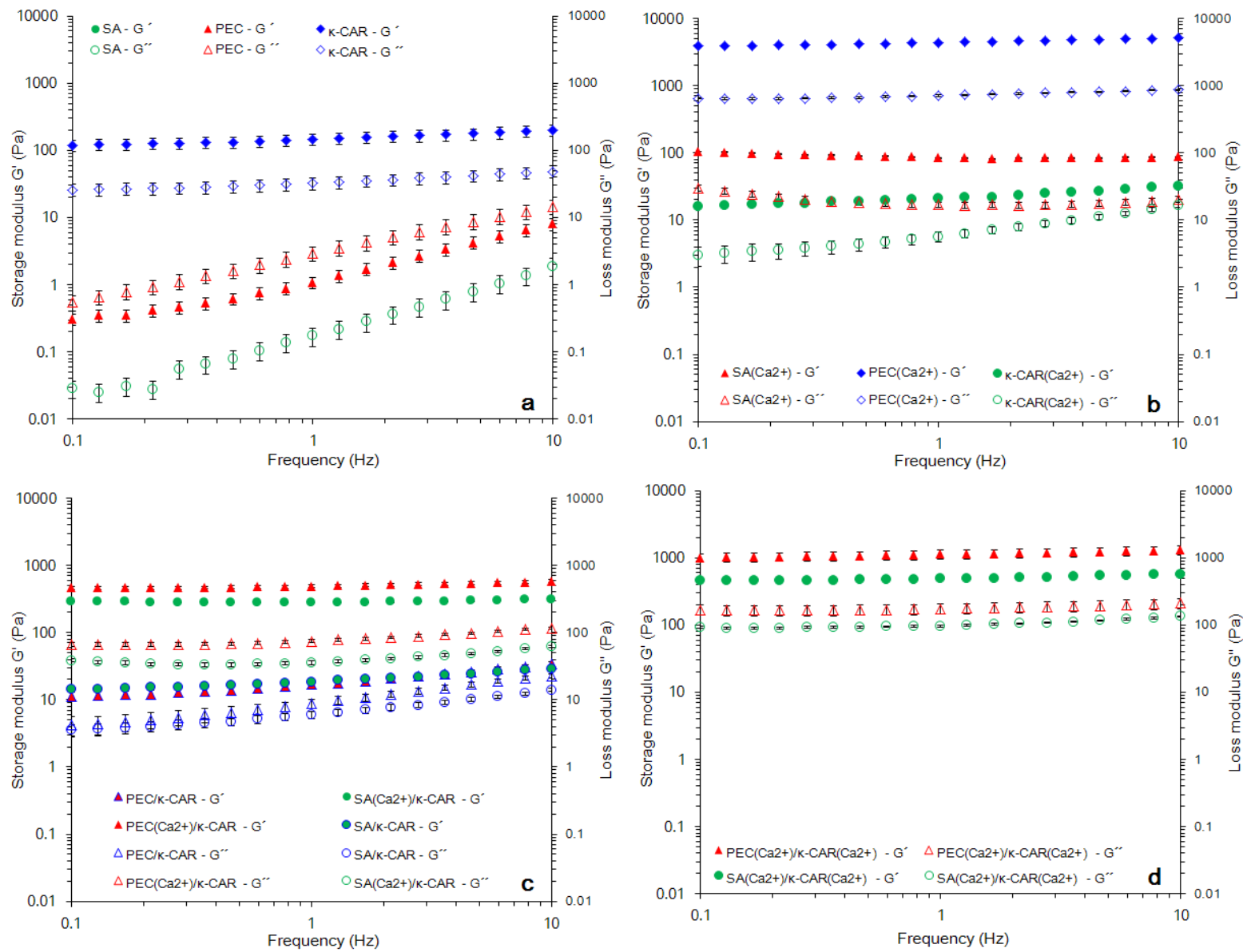


FIGURE 2

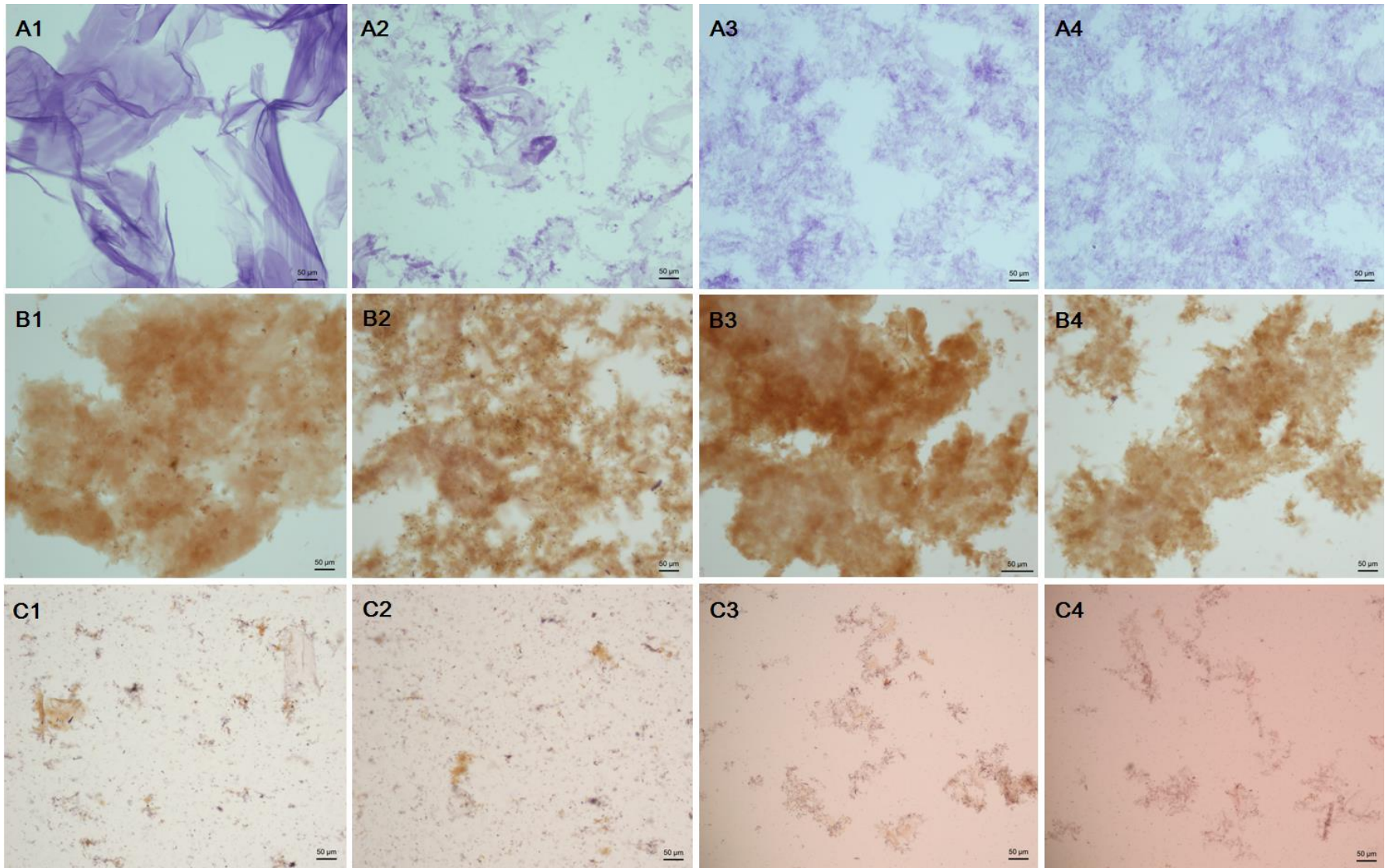


FIGURE 3

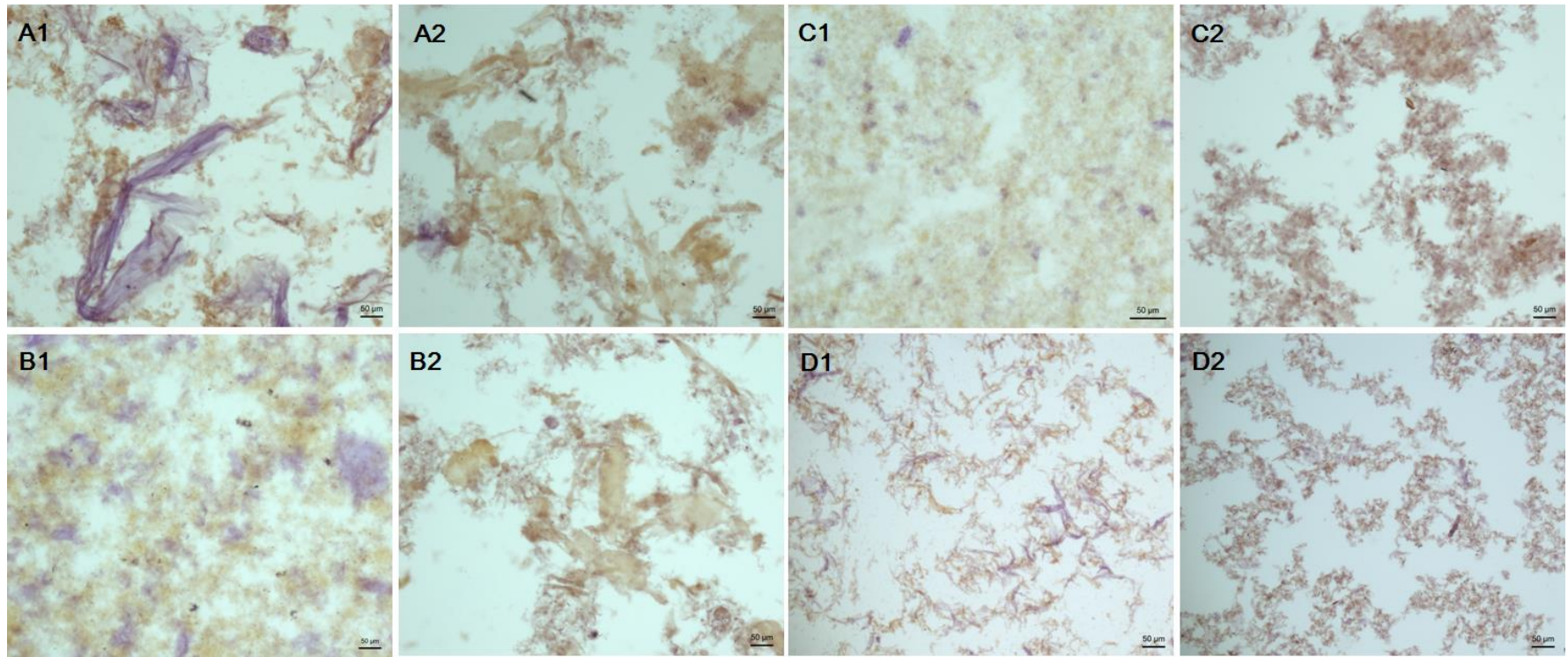


FIGURE 4

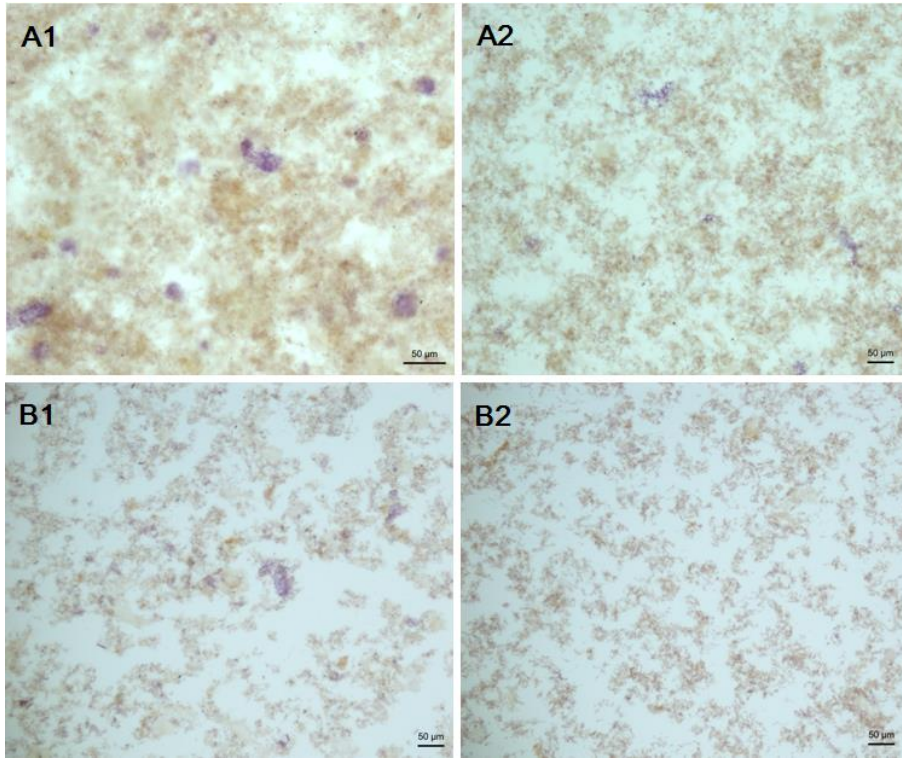


FIGURE 5

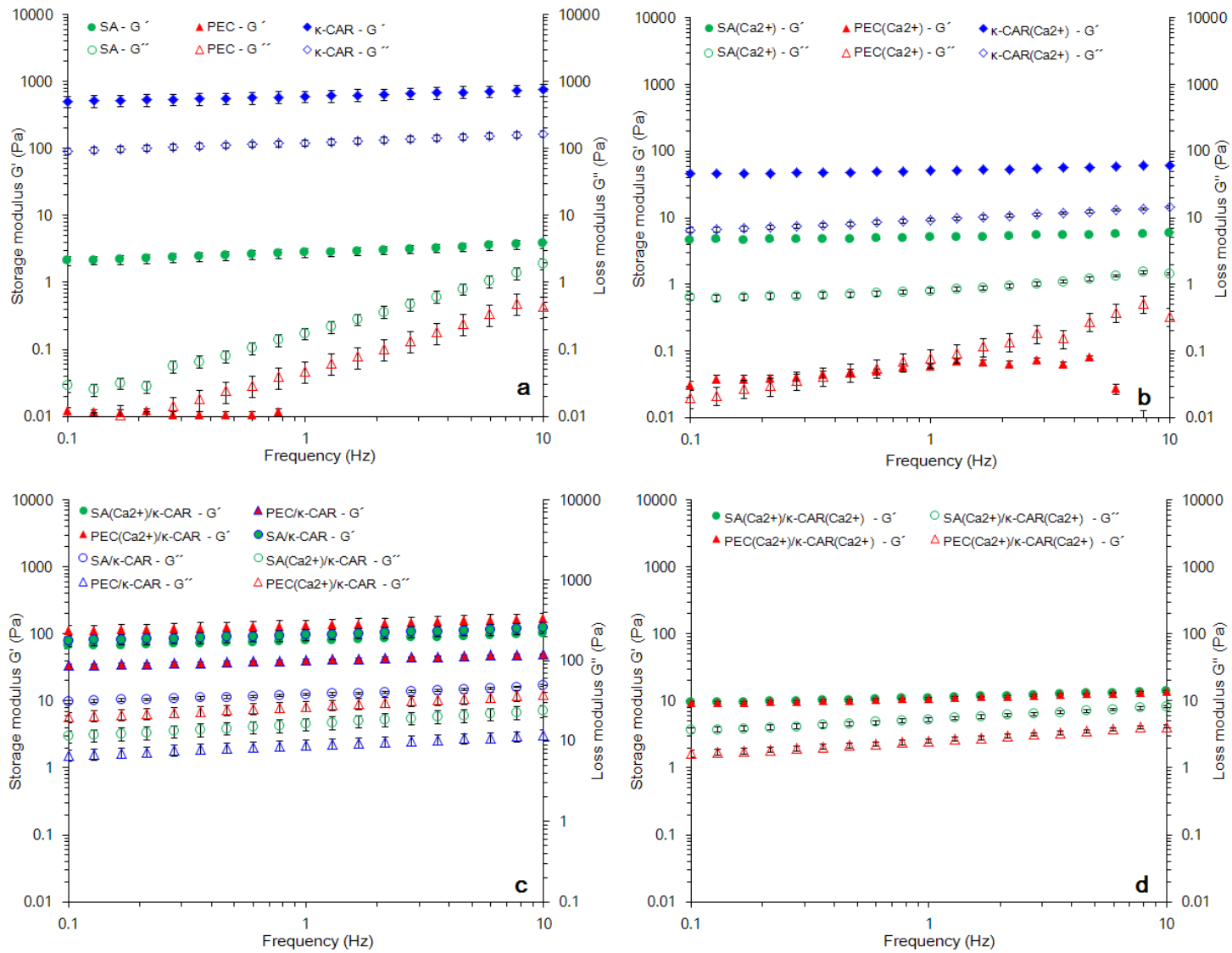


FIGURE 6

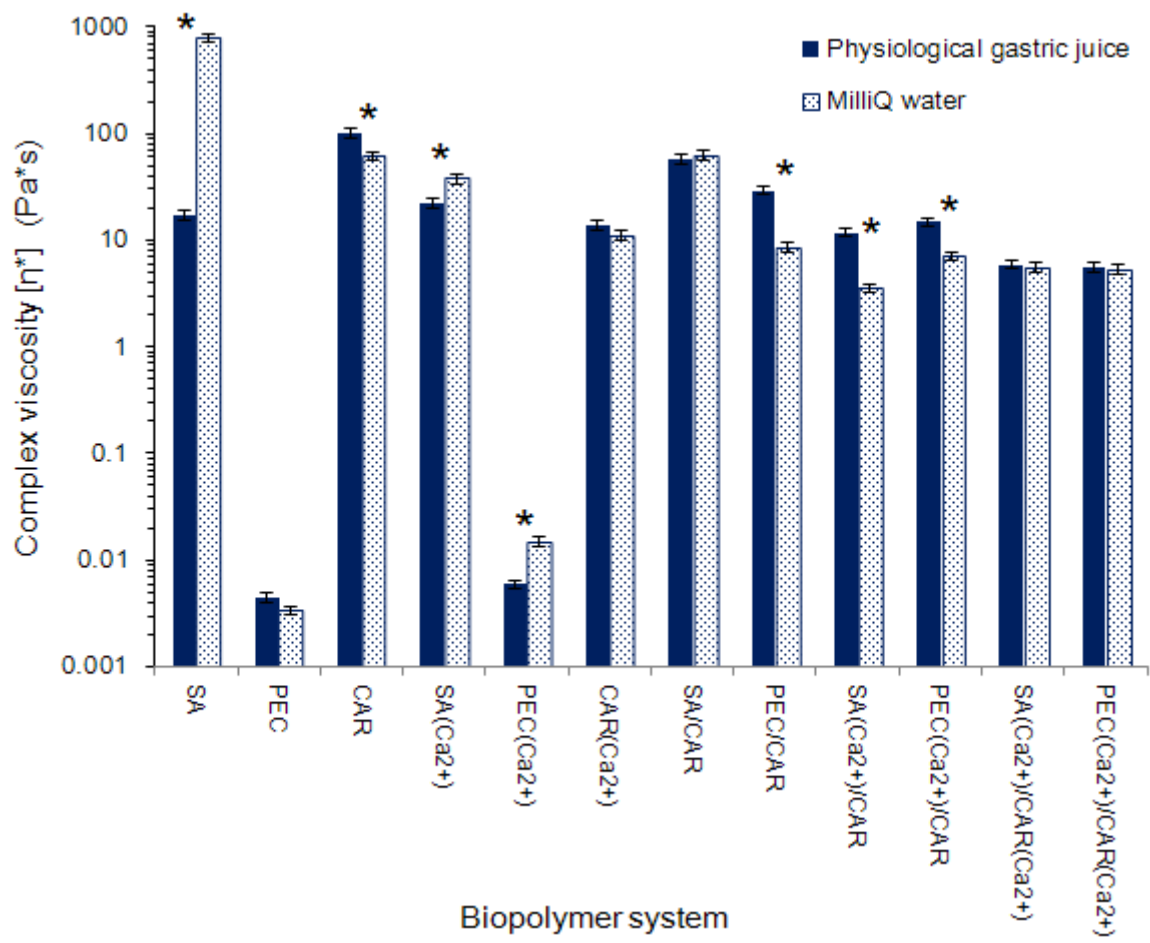


FIGURE 7

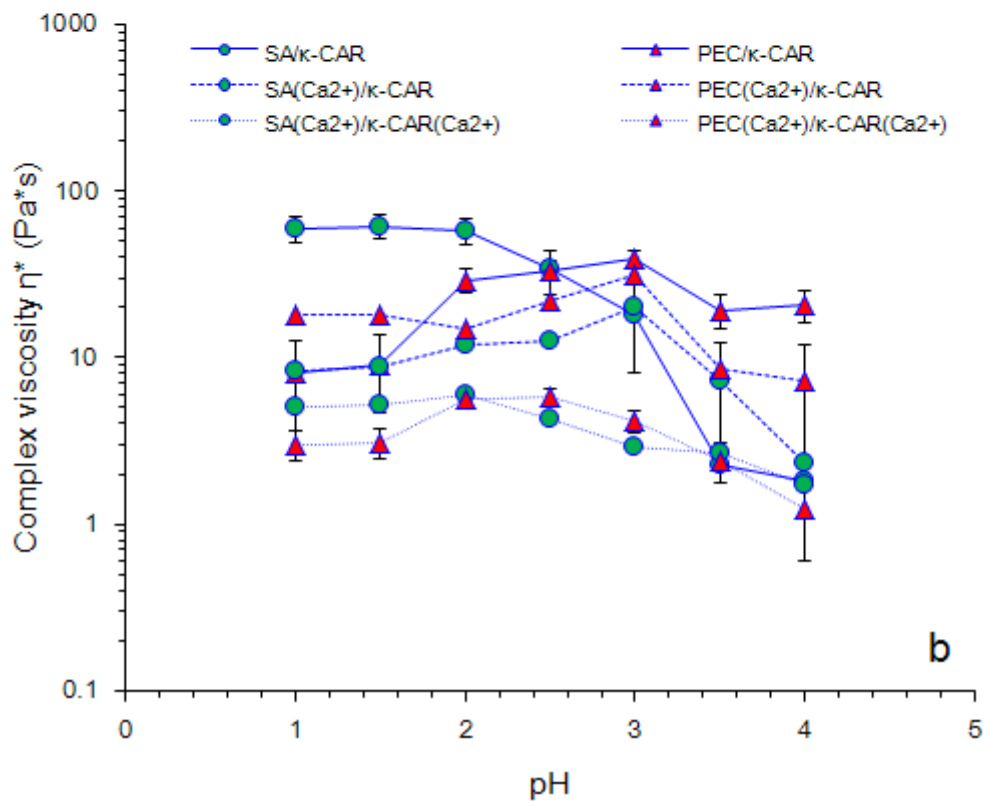
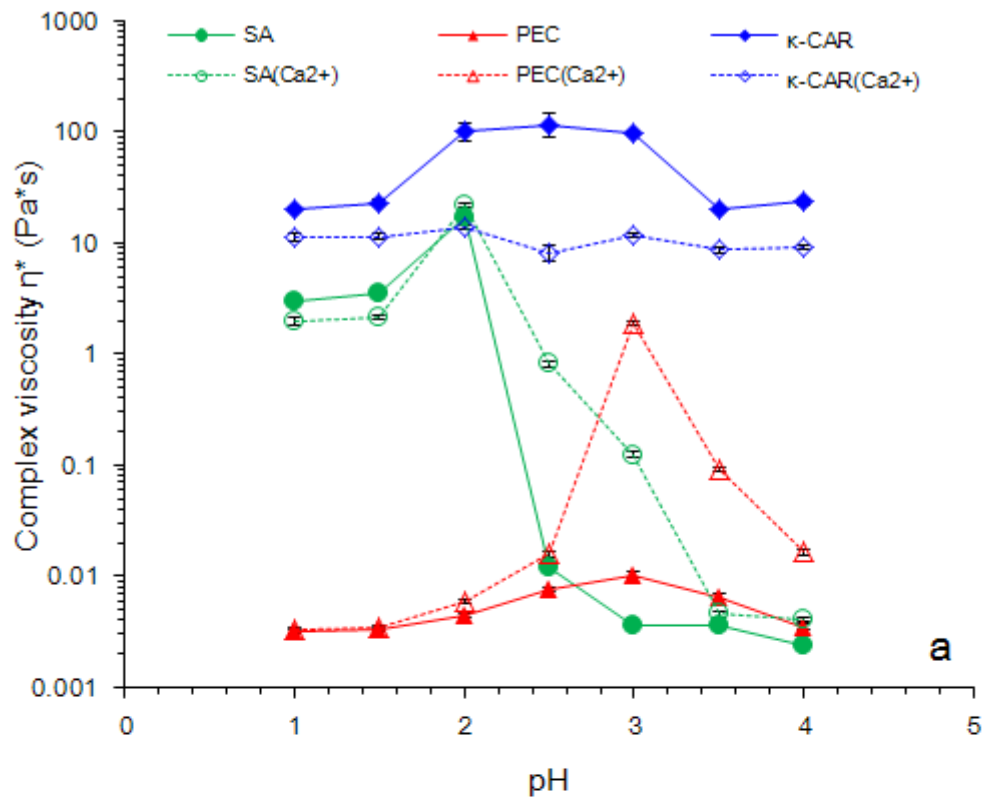


FIGURE 8

SUPPLEMENTARY TABLE 1

Sample	Gelation gel temperature	Melting point temperature
	T _{gel} (°C)	T _{gel} (°C)
SA	nd†	nd
PEC	nd	nd
κ-CAR	38.9 ± 0.5 ^b	46.2 ± 0.7 ^c
SA/κ-CAR	21.0 ± 0.4 ^a	19.5 ± 0.2 ^a
PEC/ κ-CAR	20.5 ± 0.9 ^a	36.0 ± 0.4 ^b
SA-Ca ²⁺	nd	nd
PEC-Ca ²⁺	nd	nd
κ-CAR-Ca ²⁺	nd	nd
SA-Ca ²⁺ /CAR	nd	nd
PEC-Ca ²⁺ /CAR	nd	nd
SA-Ca ²⁺ /CAR-Ca ²⁺	nd	nd
PEC-Ca ²⁺ /CAR-Ca ²⁺	nd	nd