

1 **ADVANCED STRUCTURAL CHARACTERISATION OF AGAR-BASED**
2 **HYDROGELS: RHEOLOGICAL AND SMALL ANGLE SCATTERING STUDIES**

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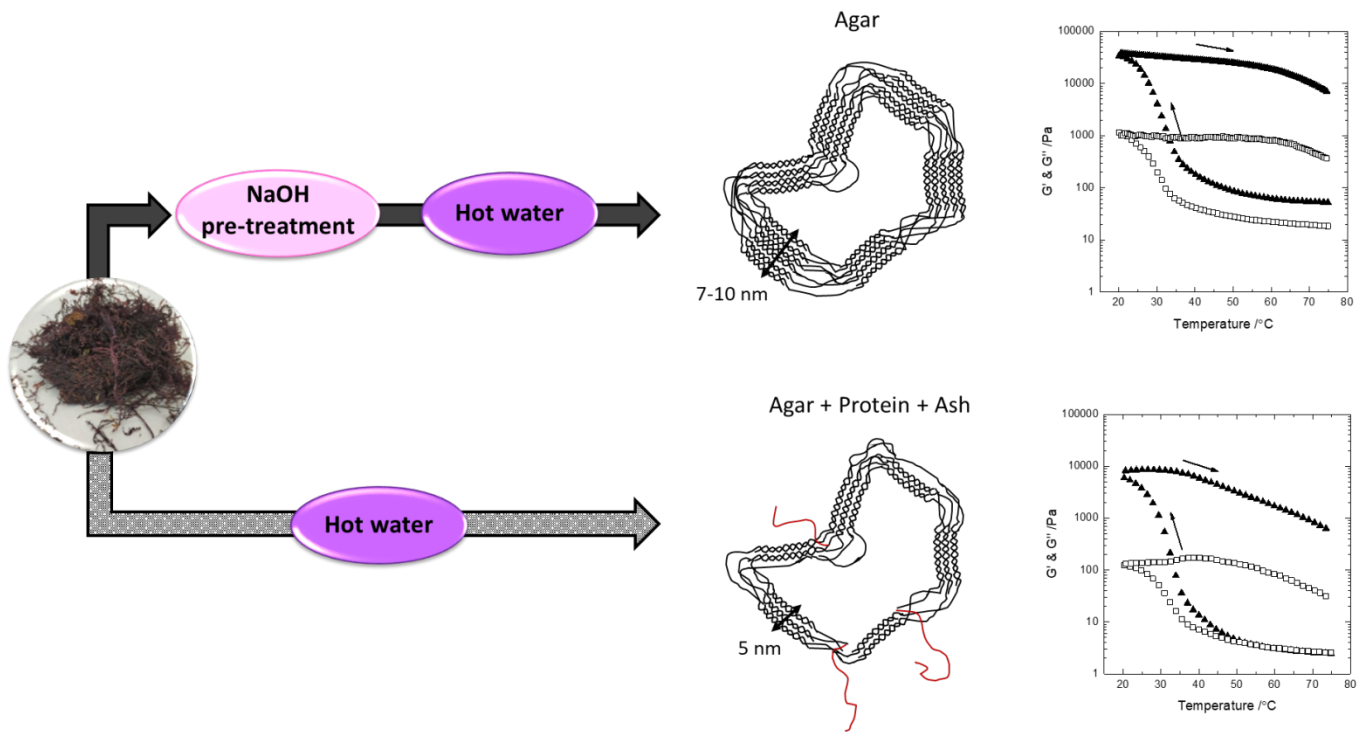
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37 **Abstract**

38 Agar-based extracts from *Gelidium sesquipedale* were generated by heat and combined heat-
39 sonication, with and without the application of alkali pre-treatment. Pre-treatment yielded
40 extracts with greater agar contents; however, it produced partial degradation of the agar,
41 reducing its molecular weight. Sonication produced extracts with lower agar contents and
42 decreased molecular weights. A gelation mechanism is proposed based on the rheological
43 and small angle scattering characterization of the extracts. The formation of strong hydrogels
44 upon cooling was caused by the association of agarose chains into double helices and
45 bundles, the sizes of which depended on the agar purity and molecular weight. These
46 different arrangements at the molecular scale consequently affected the mechanical
47 performance of the obtained hydrogels. Heating of the hydrogels produced a gradual
48 disruption of the bundles; weaker or smaller bundles were formed upon subsequent cooling,
49 suggesting that the process was not completely reversible.

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60 **Keywords:** seaweed; *Gelidium sesquipedale*; rheology; SAXS; SANS; molecular weight

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62 **1. Introduction**

63 Agar is a mixture of polysaccharides which represent the main structural components in the
64 cell walls from several seaweeds (such as *Gelidium* and *Gracilaria*) of the Rhodophyceae
65 (red algae) class. This material is widely used within the food industry and microbiology
66 fields due to its excellent gelling properties, i.e. it forms gels with relatively high thermal
67 stability and gel strength. Agarose is the idealized structure of agar (Araki, 1966), which
68 consists of repeating units of agarobiose or (LA-G)_n (Knutsen, Myslabodski, Larsen, &
69 Usov, 1994); alternating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl
70 groups. However, this backbone is in general masked by substituent groups such as sulphate
71 esters, methyl ethers or pyruvate acid ketals (Duckworth & Yaphe, 1971), which most often
72 reduce the gelling ability and influence gelling temperature and melting behaviour. The
73 native seaweed source and the protocol used for the extraction are determinant factors for
74 the occurrence of the substituent groups in the agar structure and have a strong impact on
75 the physicochemical, mechanical and rheological properties of the extracted agar (Freile-
76 Pelegrín & Robledo, 1997; Sousa, Borges, Silva, & Gonçalves, 2013). Alkaline treatments,
77 which are routinely applied in the agar manufacturing process, convert L-galactose-6-
78 sulphate units into 3,6-anhydro-L-galactose which, in turn, improve the purity and
79 mechanical properties of agar gels (Armisen & Galatas, 1987). Nevertheless, the
80 application of these alkaline pre-treatments may result in partial degradation and loss of the
81 agar during washing and produce significantly lower final extraction yields (Lee et al.,
82 2017; Meena, Prasad, Ganesan, & Siddhanta, 2007). Alternative extraction protocols based
83 on heating treatments, as well as their combination with sonication, have been recently
84 explored for the production of less purified agar-based extracts with the aim of optimizing
85 industrial processes in terms of time and energy consumption (Martínez-Sanz, Gómez-
86 Mascaraque, et al., 2019). These agar-based extracts were reported to form softer gels with

87 antioxidant capacity, which may be interesting for a wide range of alternative applications
88 such as food additives, food packaging structures (Martínez-Sanz, Martínez-Abad, &
89 López-Rubio, 2019) and encapsulation of bioactive components (Alehosseini et al., 2018).

90

91 Despite the great scientific and industrial interests in agar, the mechanism driving the
92 gelation process and the specific role of different components are still not fully understood.

93 The formation of gels has been hypothesized to occur through a sequential two-step process.

94 The first step consists of the transition from a random coil conformation in solution at high
95 temperatures (around 95 °C) to a rigid, ordered structure of double helices upon cooling

96 (Arnott et al., 1974). These helices are thought to provide the junction zones necessary for
97 the gel network formation (Mohammed, Hember, Richardson, & Morris, 1998). Upon

98 further cooling, the helices aggregate to form thick bundles, resulting in the formation of
99 strong gels. Although the formation of double helices seems to be crucial in the gelation

100 process, their aggregation into bundles appears to be principally responsible for the

101 formation of stable and strong gels (Dai & Matsukawa, 2012). In fact, one peculiarity of
102 agar gels is their large thermal hysteresis, i.e. the reported melting temperatures ($T_m \approx 80-$

103 95 °C) are typically much higher than the gelling temperatures ($T_g \approx 28-47$ °C) (Lahrech,

104 Safouane, & Peyrelasse, 2005; Medina-Esquivel, Freile-Pelegrin, Quintana-Owen, Yáñez-

105 Limón, & Alvarado-Gil, 2008). This phenomenon has been attributed to the high stability

106 of the agarose bundles, which are disrupted at temperatures much higher than those at which

107 they are formed upon cooling (Indovina, Tettamanti, Micciancio-Giammarinaro, & Palma,

108 1979). It is not fully understood how the formation of double helices and bundles is affected

109 by the occurrence of agar substituents and much less, by the presence of other components

110 such as proteins, which may interact with agar. Thus, further studies are required to

111 investigate the impact of different factors (such as agar purity, molecular weight and type

112 and amount of substituents) on the gelation mechanism of agar and also to enable the
113 rational design of novel extraction protocols based on the agar requirements that depend on
114 its final intended application. For instance, softer gels could be designed for food related
115 applications where high gel strength is not a requirement (thickening agents), using cheaper
116 and simpler extraction protocols. On the other hand, for microbiology applications, where
117 stronger gels with greater degree of purity are required, a pre-treatment step would be
118 required to remove other components present in the raw seaweed.

119
120 In this context, small angle X-ray and neutron scattering techniques (SAXS and SANS)
121 offer an advantage to investigate the nanoscale structure of agar gels in their native state
122 since, unlike other methods, minimal sample preparation is required thus avoiding
123 structural alterations in the samples. Furthermore, in the case of SANS, the different
124 scattering length of hydrogen and deuterium enables selective scattering length density
125 modification to generate contrast between diverse components in the samples. The
126 possibility of carrying out temperature-resolved experiments represents an additional
127 opportunity to investigate the structural changes taking place during the sol-gel transition
128 in hydrogels. In fact, the combination of SAXS and SANS with complementary techniques
129 has been proven to be extremely powerful to understand the structure of different
130 polysaccharide-based hydrogels (Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz,
131 Fabra, & López-Rubio, 2018; Martínez-Sanz, Mikkelsen, Flanagan, Gidley, & Gilbert,
132 2016; Yu, Yakubov, Martínez-Sanz, Gilbert, & Stokes, 2018). Despite this, the application
133 of SAXS and SANS to investigate the structure and gelation mechanism of agar has been
134 very limited to date and only a few studies, mostly carried out on pure agarose, are available
135 in the literature (Djabourov, Clark, Rowlands, & Ross-Murphy, 1989; Krueger, Andrews,
136 & Nossal, 1994; Rochas, Hecht, & Geissler, 1999). Moreover, the complexity in

137 interpreting the scattering data, and difficulties in identifying a suitable structural model,
138 have prevented the full potential of these techniques in being exploited.

139
140 In this study, we investigated the nanostructure of hydrogels from agar-based extracts using
141 advanced small angle scattering techniques and rheology. The effect of molecular structure
142 and composition of the extracts generated by different extraction methods on the gelation
143 mechanism was evaluated and related to the mechanical properties of the generated gels.

144

145 **2. Materials and methods**

146 **2.1 Materials**

147 Dried *Gelidium sesquipedale* and the commercial grade agar PRONAGAR (batch reference
148 H-3544/19) were kindly donated by Hispanagar (Burgos, Spain). The dry seaweed was
149 ground into a fine powder before further processing.

150

151 **2.2 Agar extraction**

152 Agar was extracted from *Gelidium sesquipedale* seaweed by applying heat or combined
153 heat-sonication extraction procedures, as described in a previous work (Martínez-Sanz,
154 Gómez-Mascaraque, et al., 2019). Briefly, 50 g of dry seaweed powder was dispersed in 500
155 mL of distilled water and heated and held at 90 °C for 2 h with stirring (heat extraction) or
156 subjected to simultaneous heating at 90 °C and sonication using an ultrasound probe
157 operating at 400 W and a constant frequency of 24 kHz for 30 min (combined heat-sonication
158 extraction). The hot agar-based solution was separated from the insoluble material by
159 filtration with muslin cloth. The filtrate was allowed to gel at room temperature and frozen
160 overnight. Subsequently, the material was subjected to two freeze-thaw cycles to remove
161 low molecular weight water-soluble molecules and the purified agar-based gel was freeze-

162 dried. The samples obtained from the heat and the combined heat-sonication extraction
163 protocols were coded as HW and HW-US, respectively. Additionally, the application of a
164 conventional pre-treatment with hot NaOH prior to the extraction process, as described in
165 (Martínez-Sanz, Gómez-Mascaraque, et al., 2019), was also performed for both
166 abovementioned treatments, hence obtaining the samples coded as NaOH+HW and
167 NaOH+HW-US, respectively.

168

169 **2.3 Compositional analysis of the agar-based extracts**

170 *Carbohydrate composition via reductive hydrolysis*

171 A procedure adapted from Quemener and Lahaye (Quemener & Lahaye, 1998) was
172 followed. Typically, 10 mg of sample were dissolved in 5 mL of aqueous myo-inositol (0.5
173 mg/mL) by heating at 95 °C for 45 min. After cooling to 50 °C, 500 µL aliquots were
174 evaporated to dryness at 50 °C in a stream of nitrogen. A pre-hydrolysis was initiated by
175 adding 50 µL of morpholine borane (MMB) (80 mg/ml) and 200 µL of 3 M trifluoroacetic
176 acid (TFA) and heating at 80 °C for 30 min. After cooling, 50 µL of MMB was added and
177 the solution evaporated to dryness again. The main hydrolysis was then performed by adding
178 200 µL of 2 M TFA at incubating at 120 °C for 1 h. Samples were then cooled, 100 µL of
179 MMB added, and evaporated to dryness again. After adding 500 µL of acetonitrile, the
180 samples were evaporated to dryness and acetylation was performed according to Stevenson
181 and Furneaux (Stevenson & Furneaux, 1991). Aqueous solutions of standard sugars
182 (galactose, 3,6 anhydro-L-galactose, glucose and xylose), also containing an internal
183 standard (myo-inositol), were treated in the same way to construct standard curves for
184 quantification. Enzymatically produced di- and tetra-saccharides of 4-sulphated
185 neocarrabiose, DA-G4S and (DA-G4S)₂ respectively from kappa-carrageenan (DA-G4S)_n
186 were included both as a positive control in the GC procedure and for sulphur determination.

187
188 Alditol acetates were separated on an SGE BPX 70 capillary column (25 m x 0.22 mm x
189 0.25 μm) using an Agilent 7890B GC. 1 μL sample was injected and split 40:1 at 250 $^{\circ}\text{C}$.
190 Column helium flow was set to 3 mL/min, the initial temperature was 200 $^{\circ}\text{C}$ and raised to
191 220 $^{\circ}\text{C}$ at a rate of 1 $^{\circ}\text{C}/\text{min}$. Flow was split after the column outlet via a deactivated fused
192 silica column to an Agilent 5977B EI-MSD and FID detector with the FID detector
193 temperature set to 250 $^{\circ}\text{C}$. The MSD transfer line temperature was 300 $^{\circ}\text{C}$, solvent delay 3
194 min and data collection 2.9 scans/s. Mass spectra were collected from 44 to 550 m/z.
195 Identification of eluting peaks was made with EI-MS against literature spectra (Bellion,
196 Brigand, Prome, Welti, & Bociek, 1983; Chizhov, Zolotarev, Usov, Rechter, & Kocchetkov,
197 1971) or NIST mass spectral search program version 2.2. Quantification was made by FID
198 using calibration via the available sugar standards or, where standards were not available,
199 by using relative weight response factors (relative to myo-inositol) computed from published
200 relative molar response (RMRF) factors. For 2-O-Me-3,6 anhydro- galactose (1,4,5-tri-O-
201 acetyl-3,6-anhydro-2-O-methyl galactitol), the RMRF was 0.64 and for 6-O-
202 methylgalactose (1,2,3,4,5-penta-O-acetyl-6-O-methyl galactitol), it was 0.84 (Stevenson &
203 Furneaux, 1991). All data were processed with Agilent Masshunter software. Results are
204 reported as g polysaccharide per 100 g dry weight sample.

205

206 *Sulphur and nitrogen analysis*

207 Sulphur and nitrogen contents were measured by elemental analysis via total combustion
208 using a Vario-EL-cube elemental analyser. Values for sulphur are reported directly as % of
209 dry weight or as degree of sulphate substitution of the idealized neoagarobiose backbone
210 $(\text{LA-G})_n$, using the formula $\text{DS} = 4.5 \times (\text{S}\%/\text{C}\%)$ as proposed by Melo et al. (Melo, Feitosa,
211 Freitas, & De Paula, 2002). The measured sulphur content for $(\text{DA-G4S})_2$ was 7.76 % with

212 a corresponding calculated DS of 1.00 which correlates to one sulphate group per
213 neocarribiose dimer. Crude protein was estimated by the formula %N x 6.25.

214

215 *Ash content*

216 Ash content was determined by the standard method TAPPI T211 om-07. Briefly, dry
217 samples (ca. 1 g) were placed in a muffle furnace for at least 4 h at 525 °C ± 25 °C. Ash
218 content was measured from the ratio of the resulting material divided by the initial dry
219 weight. Determinations were carried out in duplicate.

220

221 **2.4 Weight-average molecular weight and intrinsic viscosity**

222 A similar procedure to that reported by Rochas & Lahaye (Rochas & Lahaye, 1989) was
223 followed, except that a low angle light scattering detector (LALLS) was replaced by a multi-
224 angle light scattering detector (MALLS) and an on-line viscometer rather than a capillary
225 Ubbelohde viscometer was used. To 10-20 mg samples, 0.1 M NaNO₃ containing 0.02 %
226 NaN₃ was added to make a concentration of 1 mg/mL. Tubes were kept in a boiling water
227 bath for 20-30 min, until tube contents visually dissolved. Aliquots of the samples were then
228 centrifuged while still hot at 13300 rpm in a MICROSTATR 17 bench centrifuge (VWR)
229 for 10 min. 1 mL of supernatant was transferred to new tubes and again left in a boiling water
230 bath for 1-2 min. Samples were then transferred to pre-heated vials and the sample tray kept
231 at 95 °C. 100 µL of the unfiltered sample was injected via an autosampler. The
232 chromatography equipment comprised a Shimadzu LC-20 HPLC system delivering 0.1 M
233 sodium nitrate/0.02% azide at 0.5 mL/min to a guard-column (Tosoh PWXL) and then to
234 two serially-connected size-exclusion columns (TosohTSK-gel G6000 PWXL followed by
235 G5000PWXL) thermostatted at 45 °C in a column oven. The detection system comprised a
236 Dawn Helios +8 eight angle MALLS, a Viscostar II viscometer and an Optilab T-Rex RI

237 detector (Wyatt, California, USA). Astra version 6 (Wyatt, California, USA) was used to
238 collect and process raw data. The refractive index increment (dn/dc) was taken to be 0.140
239 mL/g and, assuming low solute concentrations, non-ideality effects were to be assumed
240 negligible; the second virial coefficient (A_2) was therefore set to 0.

241

242 **2.5 Preparation of agar-based dispersions and hydrogels**

243 Dispersions from the agar-based extracts were prepared at a concentration of 1.5% (w/w) in
244 distilled water. To disperse the agar-based extracts, the required amount of freeze-dried
245 sample was added to distilled water and heated to ca. 95 °C for 45 min. Aliquots of the hot
246 solutions were directly transferred to SAXS capillaries or the rheometer plate. For the
247 compression tests and the SANS experiments, the hot solutions were transferred to
248 methacrylate moulds (18 mm diameter) and were cooled to 25 °C overnight to obtain disk-
249 like hydrogel specimens.

250

251 **2.6 Oscillatory rheological measurements**

252 Storage (G') and loss (G'') moduli of the systems were determined using a DHR-3 rheometer
253 from TA Instruments, USA. A cone-and-plate geometry (4 cm diameter, 1° angle and 26 μ m
254 of gap) was used in all measurements. Temperature was controlled using a Peltier plate. To
255 avoid evaporation, the cone was equipped with a solvent trap and an evaporation blocker
256 from TA Instruments and the samples were covered with a layer of paraffin oil. The freshly
257 prepared hot agar solutions, kept at 75 °C until the beginning of the experiments, were added
258 to the rheometer, which was pre-heated at 75 °C. After an equilibration time of 5 min, a
259 cooling step from 75 °C to 20 °C was performed at a constant rate of 1 °C/min and with a
260 constant strain of 1% and frequency of 6.28 rad/s. The samples were then kept at 20 °C
261 during 10 min. Finally, a heating step from 20 °C to 75 °C was carried out at a rate of 1

262 °C/min, 1% strain and a fixed frequency of 6.28 rad/s. Frequency sweeps were also
263 performed at different selected temperatures. The samples, initially equilibrated at 80 °C,
264 were cooled down to the desired temperature at a rate of 1 °C/min and frequency sweeps
265 within the range of 0.05-100 rad/s were carried out, at 1% strain amplitude. All
266 measurements were performed at least in duplicate.

267

268 **2.7 Uniaxial compression**

269 Uniaxial compression tests were performed on an Instron material testing device (model
270 5542, Instron, Norwood, MA, USA). Gel disk specimens were removed from the moulds
271 and placed between flat metal surfaces covered with emery paper to avoid slippage. A 3 cm
272 cylindrical probe was used to compress the gels until fracture, using a 500 N load cell at a
273 crosshead speed of 0.1 mm/s. At least three replicates of each type gel were measured. Force
274 (N) and distance (mm) were converted to true stress (σ_T) and true strain (ε_T) using equations
275 1 and 2 by the instrument software Blue Hill.

$$276 \quad \sigma_T = \frac{F(t)(h_0 - \Delta h(t))}{\pi r_0^2 h_0} \quad (1)$$

$$277 \quad \varepsilon_T = \ln\left(\frac{h_0}{h_0 - \Delta h(t)}\right) \quad (2)$$

278 where h_0 is the initial height of the sample, $\Delta h(t)$ is the change in height during compression
279 and r_0 is the initial radius of the sample. Young moduli (E) were obtained as the slope of the
280 initial linear zone of the true stress vs. true strain plots.

281

282 **2.8 Temperature-resolved small angle X-ray scattering (SAXS) experiments**

283 Small angle X-ray scattering (SAXS) experiments were carried out in the Non Crystalline
284 Diffraction beamline, BL-11, at ALBA synchrotron light source
285 (www.albasynchrotron.es). Aliquots of agar solutions were placed in sealed 2 mm quartz

286 capillaries (Hilgenburg GmbH, Germany) and were left to cool to 25 °C for 24 h to form
287 gels prior to the experiments. The energy of the incident photons was 12.4 keV or
288 equivalently a wavelength, λ , of 1 Å. The SAXS diffraction patterns were collected by
289 means of a Pilatus 1M photon counting detector with an active area of 168.7x 179.4 mm²,
290 an effective pixel size of 172 x 172 μm^2 and a dynamic range of 20 bits. The sample-to-
291 detector distance was set to 6425 mm, resulting in a q range with a maximum value of $q =$
292 0.23 \AA^{-1} , where q is the magnitude of the scattering vector, defined as $q = \frac{4\pi}{\lambda} \sin \theta$, and 2θ
293 is the scattering angle. An exposure time of 0.5 s was selected based on preliminary trials.

294
295 Samples were heated from 25 °C to 95 °C at a heating rate of 1 °C/min, kept at 95 °C for 30
296 min and then cooled down from 95 °C to 10 °C at a cooling rate of 1 °C/min. Data were
297 collected in frames of 30 s, followed by a period of 30 s in which the samples were protected
298 from the beam by a local shutter. Each data frame thus corresponds to a temperature range
299 of 1 °C. The data reduction was treated by pyFAI python code (ESRF) (Kieffer & Wright,
300 2013), modified by ALBA beamline staff, to perform on-line azimuthal integrations from
301 a previously calibrated file. The calibration files were created from a silver behenate
302 standard. The radially averaged intensity profiles were then represented as a function of q
303 using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package
304 (Wavemetrics, Lake Oswego, Oregon). A scattering background corresponding to a quartz
305 capillary filled with water was subtracted from all the samples.

306

307 **2.9 Small angle neutron scattering (SANS)**

308 SANS measurements were performed on the BILBY (Sokolova et al., 2019) instrument at
309 the Australian Centre for Neutron Scattering, ANSTO with a range of neutron wavelengths
310 from 2 to 18 Å. The rear detector was placed at 10 m and the sample-to-detector distance

311 to the top/bottom and left/right curtains were 4.0 m and 3.0 m respectively; the left and right
 312 curtain opening was 350 mm, and that of the top and bottom curtains was 200 mm. This
 313 set-up enables to coverage in q from 0.0023 to 0.98 \AA^{-1} . Pre-formed agar-based hydrogels
 314 were placed in 2 mm path length cells with demountable quartz windows and the cells were
 315 filled with the required solvent (H_2O , D_2O or different $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixtures). To maximize
 316 D/H exchange, prior to the SANS measurements, the hydrogels were initially soaked for 24
 317 h in 50 mL of D_2O or $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixtures and, subsequently, an additional exchange step
 318 with fresh solvent was carried out for at least a further 24 h. Note that this is in vast excess
 319 with respect to the polymer. Data reduction followed BILBY-specific procedures
 320 implemented in the Mantid (Arnold et al., 2014) software suite. The measured intensity was
 321 corrected for scattering contribution from the solvent and empty cells, azimuthally averaged
 322 to $I(q)$ vs. q and placed on an absolute scale.

323

324 **2.10 Data fitting**

325 SAXS and SANS data were simultaneously fitted using the Igor NIST analysis macro suite
 326 (Kline, 2006) and applying a two-level unified model. This model considers that, for each
 327 individual level, the scattering intensity is the sum of a Guinier term and a power-law
 328 function (Beaucage, 1995, 1996):

$$329 \quad I(q) = \sum_{i=1}^N G_i \exp\left(-q^2 \cdot \frac{R_{g,i}^2}{3}\right) + \frac{B_i [\text{erf}(qR_{g,i}/\sqrt{6})]^{3P_i}}{q^{P_i}} + bkg \quad (3)$$

330 where $G_i = c_i V_i \Delta SLD_i^2$ is the exponential prefactor (where V_i is the volume of the particle
 331 and ΔSLD_i is the scattering length density (SLD) contrast existing between the i^{th} structural
 332 feature and the surrounding solvent), $R_{g,i}$ is the radius of gyration describing the average
 333 size of the i^{th} level structural feature, B_i is a q -independent prefactor specific to the type of
 334 power-law scattering with power-law exponent, P_i , and bkg is the background. In this

335 particular case, the largest structural level was modelled only by a power-law (R_{g1} was fixed
336 at a value $\gg q_{\min}^{-1}$ of 5000 Å). The radius of gyration for the second structural level (R_{g2})
337 was linked for all the SAXS and SANS data from the same sample and the background
338 values were fixed for each sample.

339

340 **3. Results and Discussion**

341 **3.1 Composition of the agar-based extracts**

342 Different protocols, based on heat and combined heat-sonication treatments, with (samples
343 coded as NaOH+HW and NaOH+HW-US) and without alkaline pre-treatment (samples
344 coded as HW and HW-US), were applied to generate the agar-based extracts used in this
345 work. As expected, large differences in the composition of the generated extracts existed (cf.
346 Table 1). While the alkaline pre-treatment removed a significant fraction of the non-agar
347 components present in the native seaweed and yielded extracts with higher carbohydrate
348 contents, the extracts generated without applying the pre-treatment step presented a more
349 heterogeneous composition, containing significant amounts of ash and proteins. Similar
350 extracts from the same batch of *Gelidium* have been previously reported to contain minor
351 amounts of polyphenols and lipids (Martínez-Sanz, Gómez-Mascaraque, et al., 2019). It is
352 also interesting to note that, as previously reported (Martínez-Sanz, Gómez-Mascaraque, et
353 al., 2019), the application of the combined heat-sonication treatment seemed to promote the
354 extraction of other non-agar components (such as proteins), while it gave rise to lower
355 carbohydrate contents in the extracts, most likely due to the reduced extraction time used.
356 As expected, the results from the elemental analysis (see Table S1 from the Supplementary
357 Material) showed that the agars produced with the alkali pre-treatment had the lowest
358 sulphur content, comparable to a commercial agar produced from the same *Gelidium* source.
359 It is reasonable to suggest that a major part of the sulphur in the non-alkali treated samples

360 was derived from alkali-labile sulphate; however, the high DS values estimated for these
361 samples would suggest the presence of dominating (L6S-G)_n structures, which are very
362 unlikely to be found. Thus, the high sulphur content in the HW and HW-US extracts must
363 also arise from the presence of other constituents such as co-isolated pigments and proteins.
364 A greater content of sulphur/sulphate and charged polymers will further influence the level
365 of minerals acting as counter ions of these constituents.

366
367 In line with previous works (Martínez-Sanz, Gómez-Mascaraque, et al., 2019; Murano et al.,
368 1992), the alkali-treated samples presented lower weight average molecular weight (Mw)
369 values than the non-purified extracts (cf. Table 1). This has been proposed to arise from the
370 pre-treatment conditions being too harsh for the *Gelidium* seaweed, degrading part of the
371 native agar (Martínez-Sanz, Gómez-Mascaraque, et al., 2019). On the other hand, the use of
372 sonication also led to a decrease in the Mw of the extracts. This is not surprising, since both
373 agar and carrageenan are prone to hydrolysis and depolymerisation at extended heating and
374 energy input, especially due to the lability of 3,6-anhydro (LA or DA) residues (Sousa et al.,
375 2012). However, it should be noted that the extracts produced in the present work had a
376 higher molecular weight than the commercial agar used as a positive control or than values
377 reported in the literature (Rochas & Lahaye, 1989), proving that the applied extraction
378 procedures were not strongly degradative.

379
380 The constituent sugar analysis (cf. Table 2) revealed the common structural elements
381 previously identified in agar from *Gelidium* seaweed, including O-methyl substitutions
382 (Mouradi-Givernaud, A, Givernaud, Morvan, & Cosson, 1992). Alkali-labile precursor
383 elements (L6S) were not positively identified as such, since they cannot be discriminated
384 from 3-linked regular galactose (G-units) in the GC-based analysis. However, the high

385 sulphur content in the HW and HW-US extracts suggests the presence of such elements in
386 these samples. As already anticipated, the agar content in the alkali-treated extracts was
387 much higher than in their non-purified counterparts. The lower agar purity in the alkali-
388 treated extracts, as compared to the commercial sample may be explained by the degradation
389 of the agar during the alkali treatment and the co-extraction of some impurities which were
390 not completely removed during the several washing steps of the extraction protocol. A
391 striking result is the higher content of methylated sugars (LA2M and G6M) in the alkali-
392 treated extracts, which suggests that agar fractions with such structural elements are more
393 strongly embedded in the matrix and became more accessible after alkali treatment. All
394 samples contained a small amount of glucose, which can be attributed to the presence of
395 floridean starch, a storage polysaccharide which is commonly co-extracted with the agar
396 (Carmona, Vergara, Lahaye, & Niell, 1998). Furthermore, as previously reported (Mouradi-
397 Givernaud, Aziza, Hassani, Givernaud, Lemoine, & Benharbet, 1999), some xylose was
398 detected in all samples; however, the same authors also detected the presence of rhamnose,
399 of which we found no evidence in GC-MS analysis of alditol acetates. Compositional
400 differences have been seen to have an impact on the functional properties of hydrogels and
401 films produced from agar-based extracts (Martínez-Sanz, Gómez-Mascaraque, et al., 2019;
402 Martínez-Sanz, Martínez-Abad, et al., 2019), which will ultimately determine the range of
403 applications for which these materials may be suitable. To understand the structural
404 implications, a complete rheological and nanostructural characterization of the agar-based
405 hydrogels was carried out in this work.

406

407 **Table 1.** Basic composition and molecular weight data of the agar-based extracts.

Carbohydrate (%) *	Protein (%)	Ash (%)	Sulphur (%)	DS (%)	Mw (kDa)	PI (Mw/Mn)
-----------------------	-------------	---------	-------------	--------	-------------	---------------

Commercial	83	1.49 ± 0.07 ^a	4.3 ± 0.5 ^a	0.70 ± 0.03 ^a	0.070 ± 0.001 ^a	196	2.0
HW	42	10.55 ± 0.51 ^d	34.7 ± 1.5 ^c	2.22 ± 0.07 ^b	0.32 ± 0.03 ^b	840	1.7
HW-US	30	11.33 ± 0.42 ^d	36.4 ± 0.9 ^c	3.27 ± 0.08 ^c	0.53 ± 0.01 ^c	485	2.8
NaOH+HW	66	4.01 ± 0.08 ^b	11.9 ± 2.1 ^{ab}	0.63 ± 0.01 ^a	0.08 ± 0.01 ^a	487	2.9
NaOH+HW-US	51	7.31 ± 0.07 ^c	13.9 ± 3.9 ^b	0.65 ± 0.02 ^a	0.07 ± 0.02 ^a	265	1.7

408 Values with different letters are significantly different ($p \leq 0.05$). Data were analyzed by ANOVA
 409 followed by a Tukey-test.

410 DS: degree of sulphate substitution; Mw: weight-average molecular weight; PI: polydispersity index.

411 *The total gross carbohydrates were estimated as the sum of the agar, glucose and xylose contents
 412 (cf. Table 2).

413

414 **Table 2.** Carbohydrate composition of the agar-based extracts. The results are expressed as
 415 g polysaccharide per 100 g dry weight sample.

	LA	G	LA2M	G6M	Agar	Glc	Xyl
Commercial	34.0 ± 0.1 ^d	42.9 ± 0.01 ^d	2.8 ± 0.1 ^c	0.4 ± 0.1 ^a	80	1.5 ± 0.1 ^a	0.9 ± 0.1 ^{ab}
HW	15.6 ± 0.5 ^b	23.3 ± 0.3 ^{ab}	1.0 ± 0.2 ^a	0.8 ± 0.03 ^b	40	2.0 ± 0.3 ^a	0.8 ± 0.01 ^a
HW-US	8.5 ± 0.8 ^a	19.1 ± 0.8 ^a	0.5 ± 0.03 ^a	0.1 ± 0.02 ^a	28	1.8 ± 0.2 ^a	0.7 ± 0.03 ^a
NaOH+HW	23.8 ± 1.1 ^c	32.4 ± 1.1 ^c	6.4 ± 0.6 ^b	1.8 ± 0.1 ^c	63	2.2 ± 0.1 ^a	0.9 ± 0.02 ^{ab}
NaOH+HW-US	15.9 ± 1.4 ^b	25.5 ± 2.1 ^b	6.2 ± 0.6 ^b	0.4 ± 0.1 ^a	48	2.1 ± 0.2 ^a	1.1 ± 0.1 ^b

416 Values with different letters are significantly different ($p \leq 0.05$). Data were analyzed by ANOVA
 417 followed by a Tukey-test.

418 LA=3,6-anhydro-L-galactose; G=D-galactose; LA2M=2-O-methyl-3,6-anhydro-L-galactose;
 419 G6M=6-O-methyl-D-galactose; Glc=D-glucose; Xyl=xylose. Nomenclature is according to
 420 (Knutsen et al., 1994).

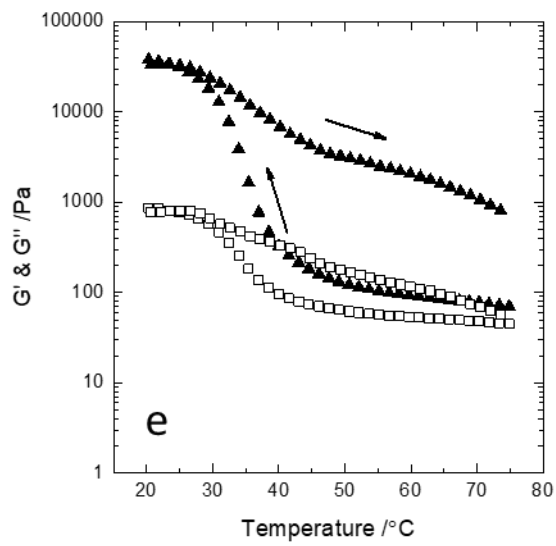
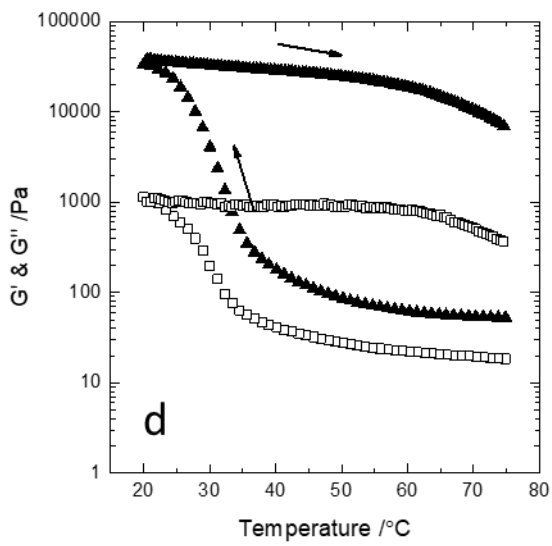
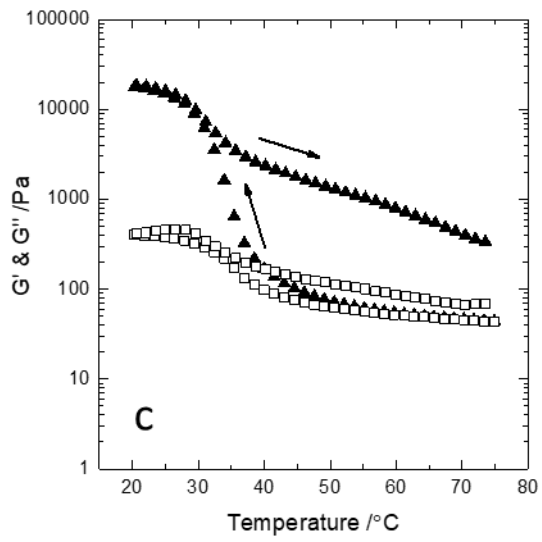
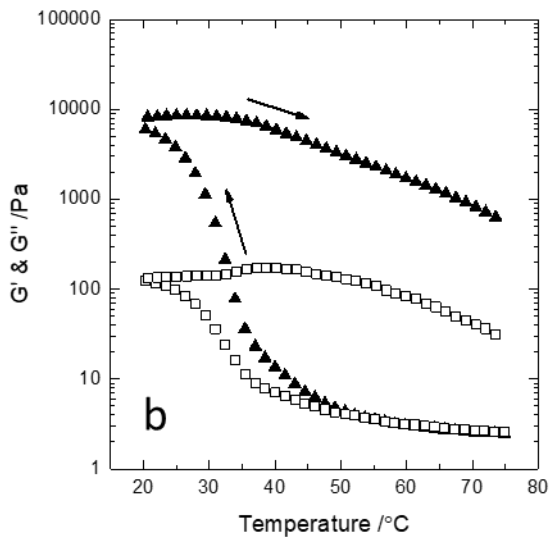
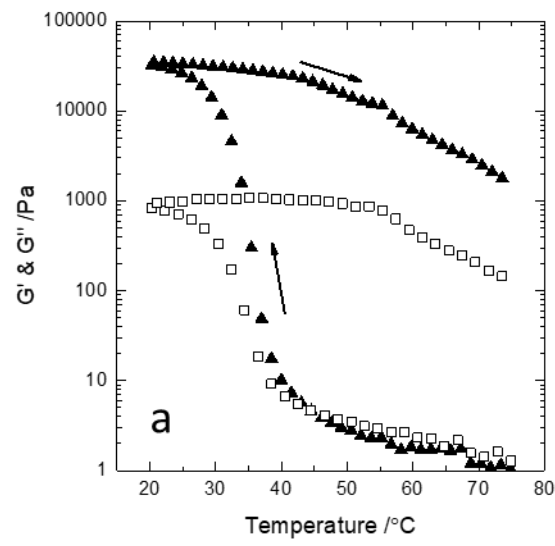
421

422 3.2 Rheological characterization of the agar-based extracts

423 To investigate the effect of the distinct composition on the gelation mechanism of the agar-
424 based extracts, rheological experiments were carried out and representative results are
425 presented in Figure 1. As observed, all the samples showed similar behaviour as a function
426 of cooling. During cooling, an initial stage at which G' and G'' remained almost constant was
427 observed, followed by a sharp increase in both moduli when lowering the temperature below
428 a certain value. The gelling and melting temperatures of agars are often estimated from the
429 crossover point of G' and G'' in cooling and heating ramps (Sousa et al., 2013). However,
430 none of the agar-based samples, except for the commercial sample (for which the crossover
431 point was detected at 45 °C), presented a true solution behaviour ($G'' > G'$); instead they
432 displayed behaviour typical of entangled networks at the initial temperature of 75 °C,
433 especially the purified alkali-treated samples. This behaviour has been previously observed
434 for agarose (Mohammed, Hember, Richardson, & Morris, 1998) and agar (Alehosseini et
435 al., 2018). Although agarose chains are known to associate by forming ordered helical
436 structures, this observation does not necessarily mean that these helices were already formed
437 at 75 °C, but it implies the existence of some kind of molecular association. The greater
438 molecular weights of all the agar-based extracts, as compared to the commercial agar, may
439 explain the occurrence of more extensive molecular interactions in the former. As we did
440 not observe a true cross-over between $G'' > G'$, we defined the apparent gelling temperature
441 by determining the point at which an abrupt rise in G' and G'' took place, in line with other
442 studies (Alehosseini et al., 2018; Russ, Zielbauer, Koynov, & Vilgis, 2013). This transition
443 corresponds to the formation of a well-developed network by aggregation of the agarose
444 helices into larger bundles, which have been previously related to the existence of strong
445 gels (Indovina et al., 1979; Mohammed et al., 1998). As deduced from the values listed in
446 Table 3, this transition took place within the range of 37-42 °C for all the agar-based extracts.
447 This is in agreement with the range of gelling temperatures typically reported in the literature

448 for agar and agarose samples (40-10 °C), depending on the thermal history and cooling
449 conditions (Aymard et al., 2001). Despite the lower agar contents in the HW and HW-US
450 extracts, the temperatures at which gelation occurred were only slightly decreased with
451 respect to the commercial agar and alkali-treated samples. In fact, previous studies showed
452 very little effect of the agarose concentration on the temperature at which aggregation of the
453 double helices took place (Fernández et al., 2008; Millán, Moreno, & Nieto, 2002; Nordqvist
454 & Vilgis, 2011). Moreover, this indicates that the presence of other components such as
455 proteins in the non-purified extracts did not have a strong impact on the temperature at which
456 the agarose helices aggregated to form hydrogels. After equilibration at 20 °C, the samples
457 were heated to 75 °C. Raising the temperature led to a decrease in absolute value of G' and
458 G'' . However, no real melting transition was observed for any of the samples ($G'' > G'$),
459 indicating that the entanglements between the agar molecular chains were not completely
460 disrupted during the heating run. It should be mentioned here that trials were made heating
461 the samples up to 95°C without observing any melting transition; only a reduction in absolute
462 values of moduli (results not shown) were found. A characteristic feature of all the agar-
463 based hydrogels studied here is that they presented large thermal hysteresis (i.e. they showed
464 large differences in their behaviour upon cooling and heating). This hysteresis is typical of
465 agarose hydrogels and has been attributed to the formation of large aggregates of double
466 helices, which remain stable at temperatures much higher than those at which they start
467 associating on cooling (Indovina et al., 1979; Mohammed et al., 1998; Trefna & Ström,
468 2019). The difference between the cooling and heating curves seemed to be greater for the
469 commercial agar and the NaOH+HW agar-based extract compared to the other samples. For
470 instance, a significant difference was observed for these samples between the G' value of the
471 freshly prepared solutions at 75 °C at the beginning of the cooling ramp and G' at 75 °C after
472 gelation and subsequent heating ramp. A plausible explanation for this is that, due to the

473 greater agar content in these extracts (cf. Table 2), more stable aggregates of double helices
474 were formed.
475



477 **Figure 1.** Temperature dependence of G' (filled triangles) and G'' (open squares) moduli of
 478 agar-based extracts during cooling and heating ramps (arrows indicate the direction of the
 479 temperature ramps). (a) Commercial; (b) HW; (c) HW-US; (d) NaOH+HW and (e)
 480 NaOH+HW-US.

481

482 **Table 3.** Rheological and mechanical properties of the different agar-based extracts:
 483 Apparent gelation temperature (T_g), elastic modulus ($G'_{20^\circ\text{C}}$), viscous modulus ($G''_{20^\circ\text{C}}$) and
 484 $\tan \delta$ measured at 20°C , maximum true stress (σ_{max}) and Young's modulus (E).

	T_g ($^\circ\text{C}$)	$G'_{20^\circ\text{C}}$ (kPa)	$G''_{20^\circ\text{C}}$ (kPa)	$\tan \delta$	σ_{max} (kPa)	E (kPa)
Commercial	40	25 ± 1^c	0.6 ± 0.1^{bc}	0.025 ± 0.005^a	29 ± 12^{ab}	21 ± 2^a
HW	39	18.7 ± 0.4^b	0.5 ± 0.1^{ab}	0.028 ± 0.006^a	10 ± 1^a	26 ± 5^a
HW-US	37	7.8 ± 0.4^a	0.20 ± 0.03^a	0.021 ± 0.006^a	5.6 ± 0.5^a	22 ± 4^a
NaOH+HW	42	39 ± 1^d	1.1 ± 0.1^d	0.028 ± 0.003^a	49 ± 6^b	28 ± 1^a
NaOH+HW-US	40	36 ± 2^d	1.01 ± 0.01^d	0.029 ± 0.007^a	39 ± 8^b	33 ± 3^a

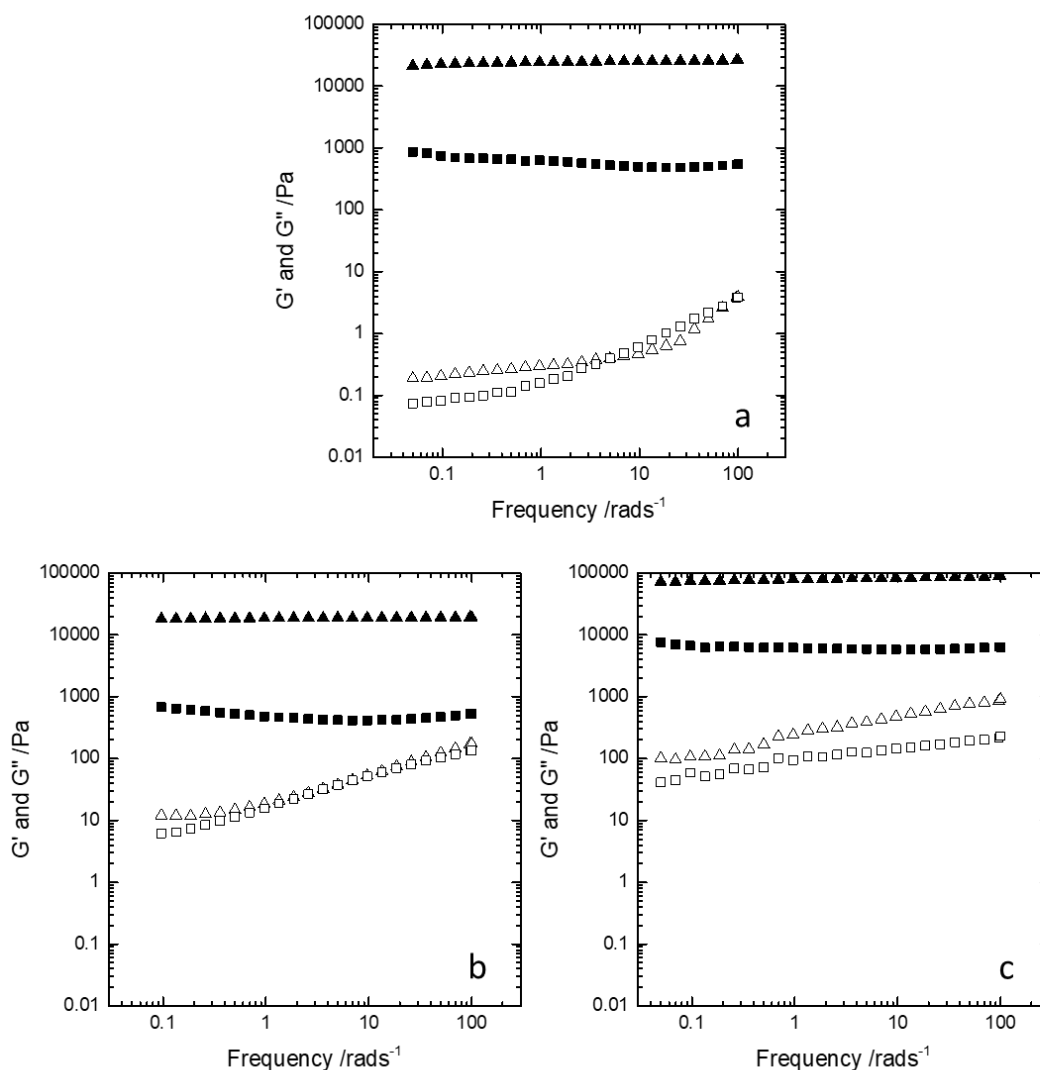
485 Values with different letters are significantly different ($p \leq 0.05$). Data were analyzed by
 486 ANOVA followed by a Tukey-test.

487

488 To gain further insights on the rheological behaviour of the samples, frequency sweeps were
 489 recorded at a higher temperature (80°C) and at a lower temperature at which all the samples
 490 had undergone the transition to strong hydrogels (20°C). Representative plots, shown in
 491 Figure 2, demonstrate distinct behaviour of the samples at the two chosen temperatures. At
 492 80°C , G' and G'' were similar and increased with frequency, which is a behaviour typical of
 493 an entangled or weak gel-like network. In particular, no cross-over point ($G''=G'$) was
 494 detected for the NaOH+HW agar-based extract, which presented a structure characteristic of
 495 an entangled network within the whole frequency range. At 20°C , all the samples showed

496 behaviour typical of strong hydrogels, where G' was at least one order of magnitude higher
497 than G'' and both moduli were independent of the frequency within the studied range (0.1–
498 100 rad/s). The average values for G' and G'' at 20 °C, listed in Table 3, indicate that the HW
499 and HW-US extracts, as expected given the presence of other non-agar components,
500 produced hydrogels with lower absolute values for G' and G'' . On the other hand, the $\tan \delta$
501 ($\tan \delta = G''/G'$) values were similar for all samples (ca. 0.02-0.03), showing comparable
502 amount of elastic response. The most likely explanation is that the presence of other
503 components in the HW and HW-US extracts did not have a dramatic impact on the nature of
504 the interactions established between the agarose chains (i.e. the gelling fraction of the
505 material); however, they did lead to the formation of softer hydrogels mainly due to the lower
506 agar (and agarose) content (i.e. fewer or smaller agarose aggregates, responsible for holding
507 the hydrogel network structure, were formed).

508



509

510 **Figure 2.** G' (triangles) and G'' (squares) as a function of frequency for the agar-based
 511 extracts, measured at temperatures of 80 °C (open symbols) and 20 °C (filled symbols). (a)
 512 Commercial; (b) HW and (c) NaOH+HW.

513

514 In the compression tests (cf. Figure S1) two regions were observed in the plots of all the
 515 hydrogels: (i) a short linear stress-strain region and (ii) a region where stress increased more
 516 markedly until the gel fractured, represented by a peak in the curve. The first linear region
 517 extended up to strains of ca. 30 % for the commercial agar and the NaOH+HW and
 518 NaOH+HW-US extracts, whilst this region was shorter (up to ca. 20 %) for the HW and

519 HW-US extracts. Linearity from strains of 15 % up to the fracture point has been previously
520 reported for agar gels (Nakauma, Ishihara, Funami, Yamamoto, & Higashimori, 2014). As
521 deduced from the parameters listed in Table 3, the hydrogels from the alkali pre-treated
522 extracts were the hardest ones, since they presented the greatest maximum true stress values.
523 On the contrary, and in agreement with the rheological characterization, the presence of other
524 components such as proteins in the less purified extracts gave rise to the formation of softer
525 hydrogels. The highest true stress values for the hydrogels from the alkali pre-treated extracts
526 could be related to (i) the inherent higher agar (and agarose) fraction, (ii) the greater
527 molecular weight of the agar (compared with the commercial grade) and (iii) the lower
528 sulphate content in these extracts. The negative impact of higher sulphate contents in the
529 strength of agar hydrogels (Kumar & Fotedar, 2009; Yousefi, Islami, & Filizadeh, 2013), as
530 well as the ability of alkali pre-treatments to remove sulphate groups from the galactose units
531 (Matsuhiro & Urzúa, 1990) have been widely described in the literature. However, our
532 results demonstrate that the purity and molecular weight of the agar are also critical factors
533 affecting the mechanical performance of agar-based hydrogels. The Young's modulus was
534 estimated from the linear slope of the true stress-strain curves. The modulus of the
535 commercial agar (ca. 21 kPa) was within the range of values previously reported (Ross,
536 Pyrak-Nolte, & Campanella, 2006; Sharma & Bhattacharya, 2014). On the other hand,
537 although minor differences were detected between all the agar-based extracts, the alkali-
538 treated agars seemed to produce the stiffest hydrogels. This might be related to the formation
539 of thicker junction zones in the case of these samples, as later confirmed by the scattering
540 results, particularly in the NaOH+HW hydrogel.

541

542 **3.2 Nano- and micro-structure of agar gels: Small angle X-ray scattering**
543 **characterisation**

544 The different rheological and mechanical behaviour of the agar-based hydrogels were
545 expected to arise from structural differences in the nano- and micrometre scale. Thus, to
546 characterize the native structure of the agar-based hydrogels, SAXS and SANS experiments
547 were carried out. Figure 3A-E shows the experimental data from the SAXS and SANS
548 contrast variation experiments. In general, the shape of the scattering curves was similar to
549 that previously observed for 1-5% w/v agarose hydrogels (Fatin-Rouge, Wilkinson, &
550 Buffle, 2006; Krueger et al., 1994), where three different regions were distinguished: (i)
551 domain I ($q = 0.0023\text{--}0.015 \text{ \AA}^{-1}$) where the scattering intensity for most of the samples
552 showed a power-law behaviour, (ii) domain II ($q = 0.015\text{--}0.1 \text{ \AA}^{-1}$) where the curves showed
553 a shoulder-like feature, and (iii) domain III ($q = 0.1\text{--}0.9 \text{ \AA}^{-1}$) where the SANS curves were
554 mostly dominated by the incoherent scattering arising from the presence of hydrogen atoms
555 in the agar gel structure. The SAXS and SANS scattering curves from the H₂O-soaked gels
556 were not analogous, showing a marked difference within the region $q < 0.005 \text{ \AA}^{-1}$, where the
557 SAXS curves presented a much more marked increase in the scattering intensity. This may
558 be a consequence of the increased interfacial surface scattering existing between the agarose
559 component in the hydrogels and water when using X-rays ($\Delta\text{SLD}_{\text{SAXS}} = 5.85 \text{ cm}^{-2}$) compared
560 to the case for neutrons ($\Delta\text{SLD}_{\text{SANS}} = 2.72 \text{ cm}^{-2}$) as the source of radiation (cf. Table S2 from
561 the Supplementary Material for reference values of the scattering length densities from
562 agarose and water), and noting that the intensity is proportional to the square of the contrast
563 i.e. 4.6 times greater for SAXS.

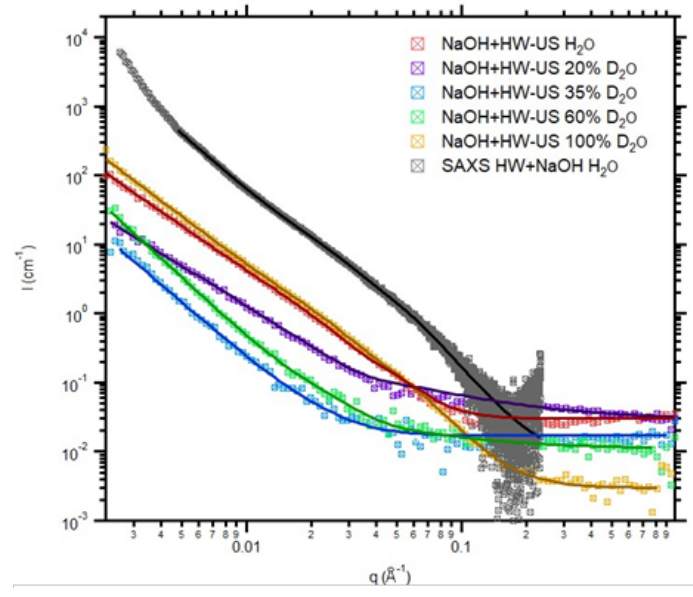
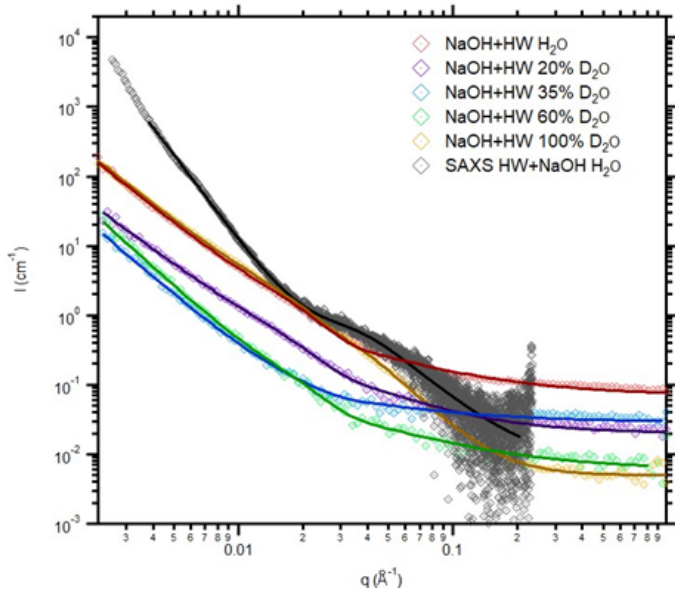
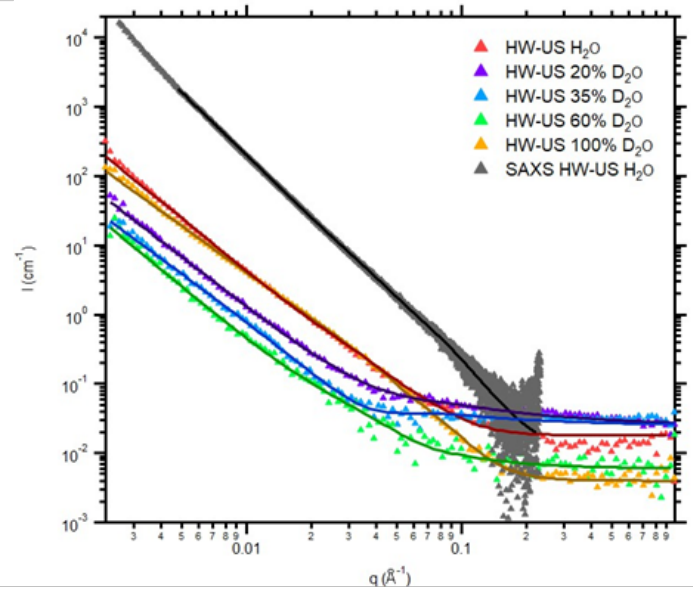
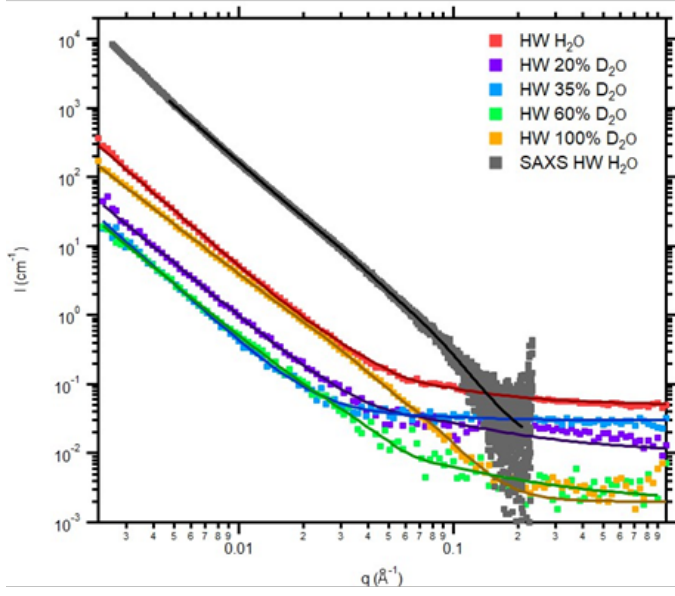
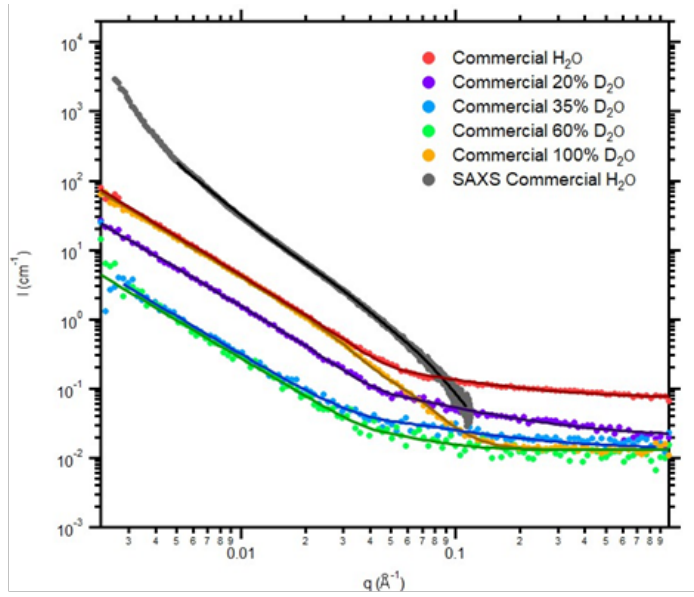
564
565 No specific fitting models have been applied to small angle scattering data from agar and
566 agarose hydrogels in the existing literature and data analysis has been mostly limited to the

567 determination of power-law exponents and cross-sectional radii (Djabourov et al., 1989;
568 Krueger et al., 1994; Rochas et al., 1999; Singh, Aswal, & Bohidar, 2007, 2009).
569 Alternatively, a model based on the combination of two populations of rod-like particles
570 with different diameters was applied to the SAXS data from agarose gels, although the fits
571 were not completely satisfactory (Djabourov et al., 1989). More recently, a correlation length
572 model was successfully applied to describe the SANS data from agarose hydrogels cross-
573 linked with Ca^{2+} (Fatin-Rouge et al., 2006). The correlation length model was also applied
574 to fit the data presented in this work; however, such model was not successful to
575 simultaneously fit the SAXS and SANS contrast variation data from the different agar-based
576 hydrogels. Alternatively, an empirical unified model was used. This model provided
577 satisfactory fits (cf. Figures 3A-E), except for the low q region ($q < 0.005 \text{ \AA}^{-1}$) in the SAXS
578 patterns where the fitting curves deviated from the experimental data (results not shown).
579 The power-law exponents from the domain I (P_1) and the radii of gyration (R_{g2}), estimated
580 from the scattering intensity within the domain II, are summarized in Table 4 (the complete
581 compilation of all the fitting parameters for each sample can be found in Tables S3-S7 of the
582 Supplementary Material). As observed, the power-law exponents for the SAXS patterns
583 were significantly greater than those from the SANS data. This is most likely due to the
584 distinct SLD contrast generated by X-rays and neutrons and to the fact that the low q region
585 in the SAXS data was affected by the presence of large-scale scattering features. Even with
586 this straightforward empirical model, the experimental SAXS data were not well described
587 within the lowest q region. According to the size range corresponding to this low q region
588 ($>130 \text{ nm}$), the occurrence of these scattering features may be associated with the hydrogel
589 network structure in the agar samples. A plausible hypothesis would be the existence of a
590 porous structure with core-shell regions, which would provide different slopes for SAXS
591 and SANS. Such core-shell regions, leading to a reduced slope in the low q region, could

592 arise as a consequence of differences in solvent accessibility between different regions
593 within the sample during solvent exchange treatment when the samples are soaked in
594 D₂O/H₂O, resulting a consequent variations in OH/OD exchange; this is similar to what has
595 been reported previously for cellulose hydrogels (Lopez-Sanchez et al., 2017; Martínez-
596 Sanz, Gidley, & Gilbert, 2016; Martínez-Sanz, Mikkelsen, et al., 2016). Irrespective of its
597 origin, extending the lower q region, i.e. with USAXS/USANS, would be desirable to
598 analyse the data within this region and extract reliable structural information. Supporting the
599 above, the power-law exponents from the SANS patterns varied depending on the sample
600 and on the SLD contrast generated by the different D₂O/H₂O mixtures used. The commercial
601 agar presented exponents of 1.8-1.9 which are similar to the value of 1.7 previously reported
602 for agarose hydrogels (Fatin-Rouge et al., 2006) and consistent with the existence of
603 structures analogous to swollen linear chains in a solvent that is intermediate between a good
604 and theta solvent (Yang et al., 2016). The scattering profile of SANS data for hybrid agar-
605 gelatin hydrogels in the q range of 0.007-0.02Å⁻¹ also showed a power-law behaviour with
606 an exponent of 1.6 for the highest agar ratio (Santinath Singh, Aswal, & Bohidar, 2011).
607 This was attributed to the existence of rod-like structures consisting of agarose double-helix
608 aggregates. The associated radius of gyration was 5.6 nm for the commercial agar (very
609 similar to the correlation length of 5.9 nm previously reported for agarose gels) and was
610 attributed to the thickness of the bundles of agarose double helices (Fatin-Rouge et al., 2006).
611 Interestingly, the power-law exponents for all the agar-based hydrogels were higher than for
612 the commercial agar, with values between ca. 1.9 and 2.9. This is indicative of more
613 branched structures (which is most likely due to a greater degree of chain association or
614 cross-linking) in the case of the produced agar-based hydrogels, which can be correlated
615 with their greater molecular weights. A similar increase in the power-law exponents of
616 agarose hydrogels was observed when increasing the agarose concentration; this was

617 hypothesised to arise from either lower swelling of the junction zones (i.e. agarose bundles)
618 at greater agarose concentrations or from changes in the large-scale structure of the agarose
619 hydrogel network (an decrease in the pore size at higher agarose concentrations) (Krueger et
620 al., 1994). While the radius of gyration slightly decreased for the non-purified agar-based
621 hydrogels (5.0 nm for HW and 4.6 nm for HW-US), larger values were obtained for the
622 purified hydrogels (9.6 nm for NaOH+HW and 6.7 nm for NaOH+HW-US). These results
623 would suggest a greater extent of chain association in the alkali-treated agar-based
624 hydrogels, which may be directly linked to their greater agar content and lower sulphate
625 content. On the contrary, the lower agar content in the HW and HW-US hydrogels led to the
626 formation of smaller aggregates, although the presence of other components did not seem to
627 impair the interconnectivity in the hydrogel network, as suggested by the corresponding
628 power-law exponents. The existence of thicker agarose bundles or aggregates in the alkali-
629 treated agar hydrogels is in agreement with the compression experiments, which showed a
630 stiffer behaviour for these samples as compared to the commercial agar and the HW and
631 HW-US hydrogels.

632



634 **Figure 3.** SAXS and SANS contrast variation data for the agar-based hydrogels. Dots
 635 represent the experimental data and solid lines show the fits obtained by applying the two-
 636 level unified model and simultaneously fitting the six data sets.

637

638 **Table 4.** Fit parameters and contrast values for the SAXS and SANS data from the agar-
 639 based hydrogels. Parameters obtained from the fits of the unified model are power-law
 640 exponents (P_1) and radii of gyration (R_{g2}); contrast match point and scattering length density
 641 (SLD) values have been estimated from the SANS contrast variation experiments.

	Commercial	HW	HW-US	NaOH+HW	NaOH+HW-US
P_1 (SAXS)	2.6	2.7	3.0	3.9	2.7
P_1 (SANS)	1.8-1.9	2.4-2.7	2.3-2.6	2.3-2.8	1.9-2.9
R_{g2} (nm)	5.6	5.0	4.6	9.6	6.7
Contrast match (% D ₂ O)	52 ± 1	54.4 ± 0.8	53.0 ± 0.4	48.0 ± 0.2	44 ± 2
SLD (10 ¹⁰ cm ⁻²)	3.01 ± 0.08	3.19 ± 0.05	3.09 ± 0.02	2.75 ± 0.01	2.5 ± 0.1

642

643 The SLD of the agar-based hydrogels could be estimated, in principle, from the SANS
 644 contrast variation experiments by determining the contrast match point. The scattering
 645 intensity at several points within the q range of 0.0025-0.0055 Å⁻¹ was determined for each
 646 of the five different D₂O/H₂O mixtures used in the experiments and the values were plotted
 647 against the D₂O content. If no deviations from the theoretical behaviour occur (i.e. the
 648 samples are pure and there is no H/D exchange taking place), the so-obtained values should
 649 be properly described by a parabolic function and the intensity should reach a minimum of
 650 zero at the contrast match point for a single phase system. As observed in Figure 4A, for
 651 most of the samples the experimental data deviated from the theoretical parabolic function,
 652 with this effect being more evident in the case of the HW and HW-US hydrogels. Such a

653 deviation from the theoretical behaviour is not surprising, since the agar-based hydrogels are
 654 highly hydrated systems (up to 98% water) in which H/D exchange may have occurred when
 655 the samples were soaked in D₂O/H₂O. In fact, similar behaviour has been reported for highly
 656 hydrated polysaccharide gels such as cellulose-based hydrogels (Martínez-Sanz, Gidley, et
 657 al., 2016; Martínez-Sanz, Mikkelsen, et al., 2016). The contrast match point value for each
 658 agar-based hydrogel, listed in Table 4, was calculated from the minimum of the intensity in
 659 the fitted parabolic functions. Considering that at the contrast match point the solvent SLD
 660 equals that of the sample, the neutron SLD of the agar-based hydrogels could be estimated
 661 by applying the following equation:

$$662 \quad SLD_{Agar} = SLD_{solvent} = (x_{D_2O} \cdot 6.38 \cdot 10^{10}) + ((1 - x_{D_2O}) \cdot (-0.56 \cdot 10^{10})) \quad (4)$$

663 On the other hand, the theoretical SLD value for pure agarose (considering a molecular
 664 formula of C₆H₁₀O₅ (Ramzi, Rochas, & Guenet, 2000)) can be estimated using the following
 665 expression:

$$666 \quad SLD_{Agarose} = N_A \cdot \rho_{Agarose} \cdot \frac{12b_C + 9b_O + 18b_H}{12M_C + 9M_O + 18M_H} = 2.16 \cdot 10^{10} \text{ cm}^{-2} \quad (5)$$

667 where b_i and M_i are the neutron scattering length and mass of the atoms, respectively and
 668 $\rho_{Agarose}$, is the physical density of agarose (1.7 g/cm³ (Rochas et al., 1999)).

669
 670 Considering that (i) the structure of agar differs from the idealized agarose structure and it
 671 contains substituents along the molecular chains (giving rise to more defective regions, with
 672 lower physical density) and (ii) the hydrogels contain a high amount of water, it would be
 673 expected that the agar-based hydrogels should present lower SLD values than that of pure
 674 agarose. Surprisingly, as deduced from Table 4, the SLD from all the samples was greater
 675 than that of pure agarose. This was more evident in the case of the HW and HW-US
 676 hydrogels, which could be originated from the deviation of the contrast variation curves from

677 the theoretical behaviour, induced by a greater H/D exchange taking place in these samples,
 678 as latter commented in the text. In the case of the more purified agars, the NaOH+HW-US
 679 and NaOH+HW extracts presented SLD values closer to that of agarose, as compared to the
 680 commercial agar. This might indicate that the increase in the calculated SLD values arise
 681 from the deviation of the contrast variation curves from their theoretical behaviour due to
 682 H/D exchange. Such an exchange process would be more limited in the alkali-treated
 683 extracts due to their greater molecular weight and crystallinity (Martínez-Sanz, Gómez-
 684 Mascaraque, et al., 2019), hence affecting to a lesser extent the shape of their contrast
 685 variation curves.

686
 687 To estimate the degree of H/D exchange taking place in the agar-based hydrogels, the
 688 theoretical contrast variation curves for pure agarose at different degrees of exchange were
 689 calculated and compared to the experimental data from the pure agar hydrogels. When an
 690 agarose hydrogel is soaked in D₂O, a maximum of four hydrogen atoms, corresponding to
 691 the labile hydroxyl groups, can be exchanged; thus the molecular formula of fully exchanged
 692 agarose would be C₁₂H₁₄D₄O₉. Accordingly, for certain intermediate degree of H/D
 693 exchange ($exc_{H/D}$) the SLD can be estimated as follows:

$$694 \quad SLD_{Agarose \text{ fully exc}} = N_A \cdot \rho_{Agarose} \cdot \frac{12b_C + 9b_O + (18 - (4 \cdot exc_{H/D}))b_H + (4 \cdot exc_{H/D})b_D}{12M_C + 9M_O + (18 - (4 \cdot exc_{H/D}))M_H + (4 \cdot exc_{H/D})M_D} \quad (6)$$

695 Equally so, for a D₂O volume fraction, x_{D_2O} , of 0.5, complete exchange would result in two
 696 labile H atoms being replaced by D. Hence, combining equations (5) and (6), the resultant
 697 theoretical SLD of agarose in each D₂O/H₂O mixture may be calculated by applying the
 698 following equation:

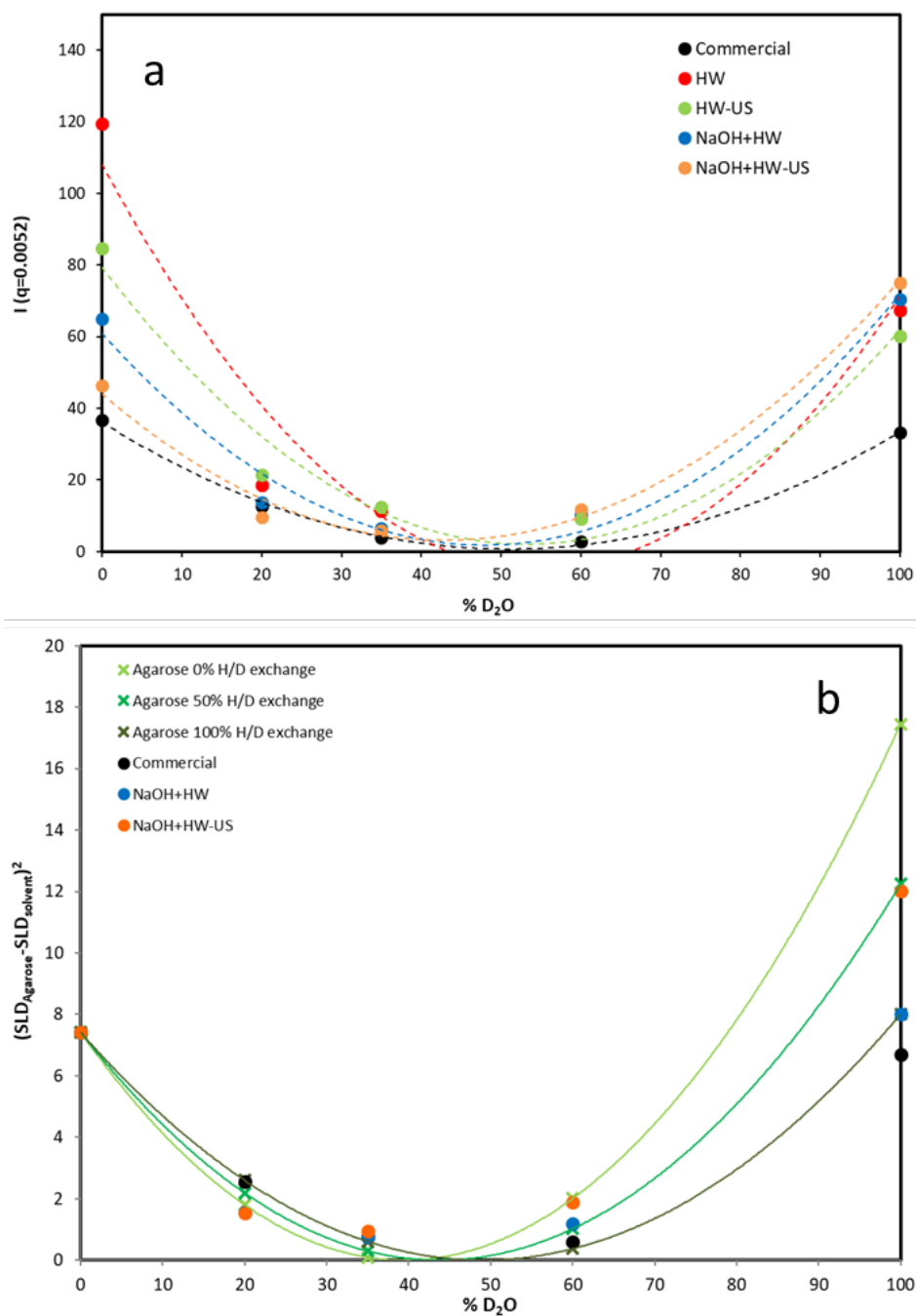
$$699 \quad SLD_{Agarose \text{ exc}} = x_{D_2O} \cdot SLD_{Agarose \text{ fully exc}} + (1 - x_{D_2O}) \cdot SLD_{Agarose} \quad (7)$$

700 for degrees of H/D exchange with the solvent varying between 0 and 1.

701

702 Figure 4B shows the theoretical curves for the term $(SLD_{Agarose} - SLD_{solvent})^2$ plotted
703 against the D₂O volume fraction, considering different degrees of H/D exchange, as well as
704 the experimental data points for the pure agar hydrogels. The first clear observation is that,
705 as already anticipated, the contrast match point is shifted towards greater D₂O volume
706 fractions with greater H/D exchange ratios. This would explain the fact that the shape of the
707 contrast variation curves for the agar hydrogels deviated from the 0% H/D exchange curve,
708 especially for greater D₂O volume fractions, becoming closer to the 100% H/D exchange
709 curve. Moreover, it can be observed how the behaviour of the more crystalline samples
710 (NaOH+HW-US and NaOH+HW) deviate to a lesser extent from the 0% H/D exchange
711 curve; in this case, and as suggested by the rheological characterization, thicker and more
712 stable agarose bundles were formed, consistent with reduced levels of H/D exchange.

713



714

715 **Figure 4.** (a) Contrast variation curves for the agar-based extracts, obtained from the SANS

716 experiments. Solid markers represent the experimental values ($q=0.0052\text{\AA}^{-1}$) and dotted

717 lines correspond to the fitted parabolic functions. (b) Representation of the scattering length

718 density contrast term $(SLD_{Agarose} - SLD_{solvent})^2$ as a function of the D_2O content of the

719 different solvent mixtures used for the contrast variation experiments. Dots represent the

720 experimental values for the agar gels and crosses represent the theoretical values calculated

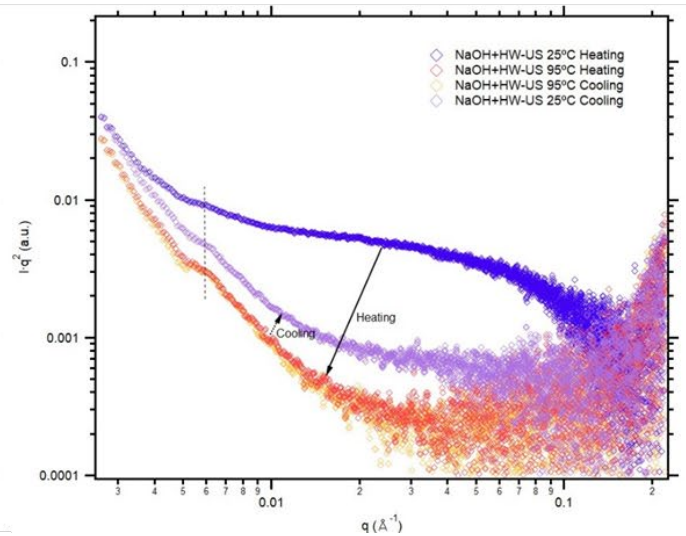
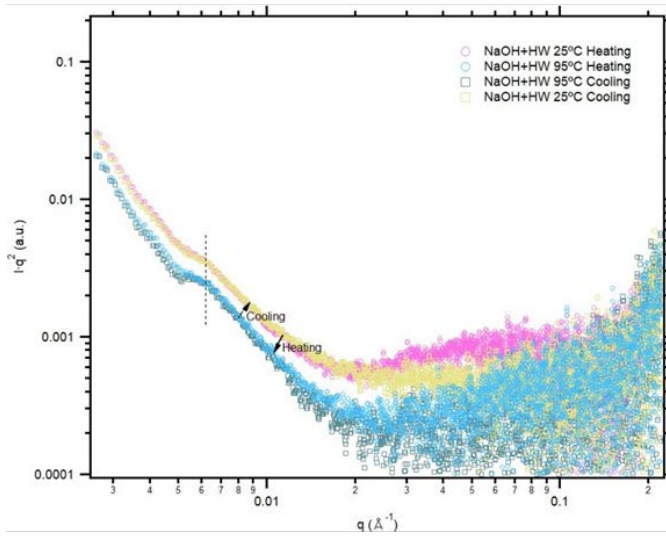
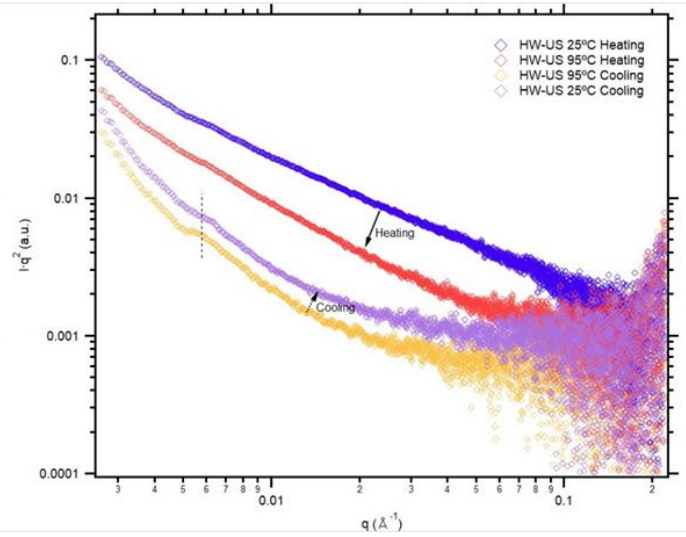
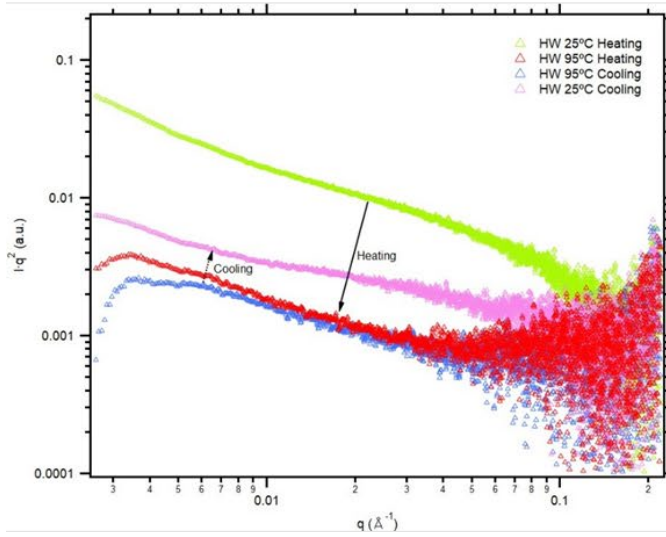
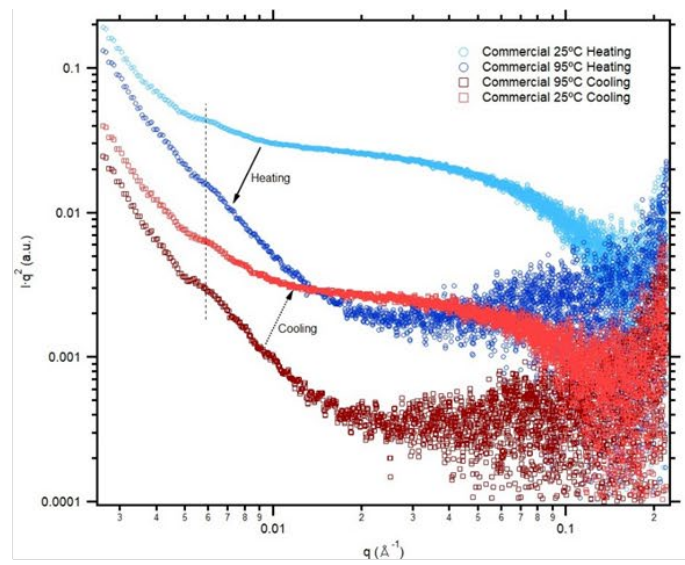
721 by assuming different degrees of H/D exchange; solid lines correspond to the fitted
722 parabolic functions.

723

724 To further investigate the structural changes undergone by the agar-based extracts during the
725 gelation and melting processes, temperature-resolved SAXS experiments were also carried
726 out. The complete set of curves for the heating and cooling ramps can be found in the
727 Supplementary Material section (Figures S2 and S3, respectively). For clarity, only selected
728 curves (those corresponding to the 25 °C and 95 °C curves obtained during the heating and
729 cooling ramps) were used to generate the Kratky plots shown in Figure 5. As observed, the
730 Kratky plots highlighted the appearance of small humps located at ca. 0.006 \AA^{-1} in the SAXS
731 curves from most of the samples. The position and intensity of these features were unaffected
732 by the temperature treatments, although they were less evident in the HW and HW-US
733 samples. The appearance of such features has not been reported before and it is unclear as to
734 whether they are characteristic of form or structure factor effects. A possible origin may be
735 a form factor effect associated with the length of the agarose double helices, whose
736 concentration was greater in the more purified samples and whose structure was not altered
737 during the heating/cooling steps. Again, extending the q range would be desirable to discard
738 any other possible sources such as a structure factor effect derived from the interaction
739 between the agarose bundles or a structure factor effect related to the larger scale porous
740 structure. In general, the scattering intensity is seen to decrease when raising the temperature
741 above a certain point during the heating runs. This may have arisen from a drop in the SLD
742 contrast between the hydrogels and the surrounding solvent due to a decrease in the cross-
743 linking degree of the hydrogels (i.e. disassociation of agarose bundles). This temperature
744 corresponded to ca. 55 °C for the commercial agar, 65 °C for NaOH+HW-US and 85 °C for
745 HW, HW-US and NaOH+HW. The reduction in the scattering intensity was more obvious

746 in the case of the commercial agar; in contrast, the intensity of the NaOH+HW agar-based
747 hydrogel remained almost unaffected. This again could be related to the formation of thicker
748 and more stable aggregates in the latter. Moreover, the shoulder-like feature associated with
749 the presence of agarose double-helix aggregates (located within the range of $q = 0.015\text{--}0.1$
750 \AA^{-1}) was absent in the scattering patterns from the samples at 95 °C, except for the
751 NaOH+HW agar-based hydrogel. Since no true melting behaviour was observed in the
752 rheological experiments (cf. Figure 1), the results from the SAXS heating ramps suggest that
753 although the molecular associations between agarose chains (i.e. agarose double-helix
754 structures) were not destroyed upon heating, the double-helix aggregates, responsible for the
755 formation of strong hydrogel networks, were disrupted in most of the samples. In the
756 particular case of the NaOH+HW agar-based hydrogel, it seems that very strong interactions
757 must have been formed between the agarose double helices, leading to the formation of more
758 stable agarose bundles which remained intact even at 95 °C. This is in agreement with the
759 rheological characterization where, after heating to 70 °C, the G' and G'' moduli still
760 produced much higher values than those from the initial solutions. The characteristic
761 behaviour of the NaOH+HW agar-based extract may be attributed to its higher purity and
762 molecular weight, promoting the formation of a stronger and stiffer network of double-helix
763 aggregates held by hydrogen bonding. The scattering intensity was further reduced when
764 holding the temperature at 95 °C for 30 min only in the case of the commercial and the HW-
765 US agar-based samples, suggesting that structural changes continued taking place. During
766 the cooling ramp, the intensity started to increase when lowering the temperature below 35-
767 25 °C for all the samples. This temperature range is slightly lower than the apparent gelling
768 point determined from the rheological characterization (ca. 40-35 °C, cf. Table 1) which may
769 be due to the distinct thermal history of the samples analysed by each technique or other
770 factors such as the different sample geometry. In the case of the rheology experiments, the

771 samples were gelled directly on the rheometer plate at a cooling rate of 1 °C/min, whereas
772 agar-based dispersions were rapidly cooled down to room temperature inside the SAXS
773 capillaries prior to the temperature ramp experiments. It is known that slow cooling leads to
774 the formation of longer helices than rapid quenching, hence promoting helix-helix
775 aggregation (Mohammed et al., 1998). Interestingly, except for the NaOH+HW sample, the
776 scattering intensity of the hydrogels after the heating and cooling ramps was lower than that
777 of the native hydrogels, suggesting that even though the agarose double-helices could re-
778 aggregate after being disrupted, they do not re-assemble in the same manner. Since the lower
779 scattering intensity after the thermal treatments is indicative of a reduced SLD contrast
780 between the agar-based hydrogel and the surrounding bulk solvent, it may be reasonable to
781 hypothesise that the double-helices re-organize to form less ordered structures, which
782 present lower physical densities or are able to hold greater amounts of water within their
783 structure. This is highly relevant from a technological perspective, since it implies that only
784 very crystalline and pure agars can maintain their structure of double-helix aggregates upon
785 heating, hence preserving their initial mechanical properties and rheological behaviour.
786



789 **Figure 5.** Kratky plots from the SAXS patterns collected at 25°C and 95°C during the heating
790 and cooling ramps. (A) Commercial; (B) HW; (C) HW-US; (D) NaOH+HW and (E)
791 NaOH+HW-US.

792

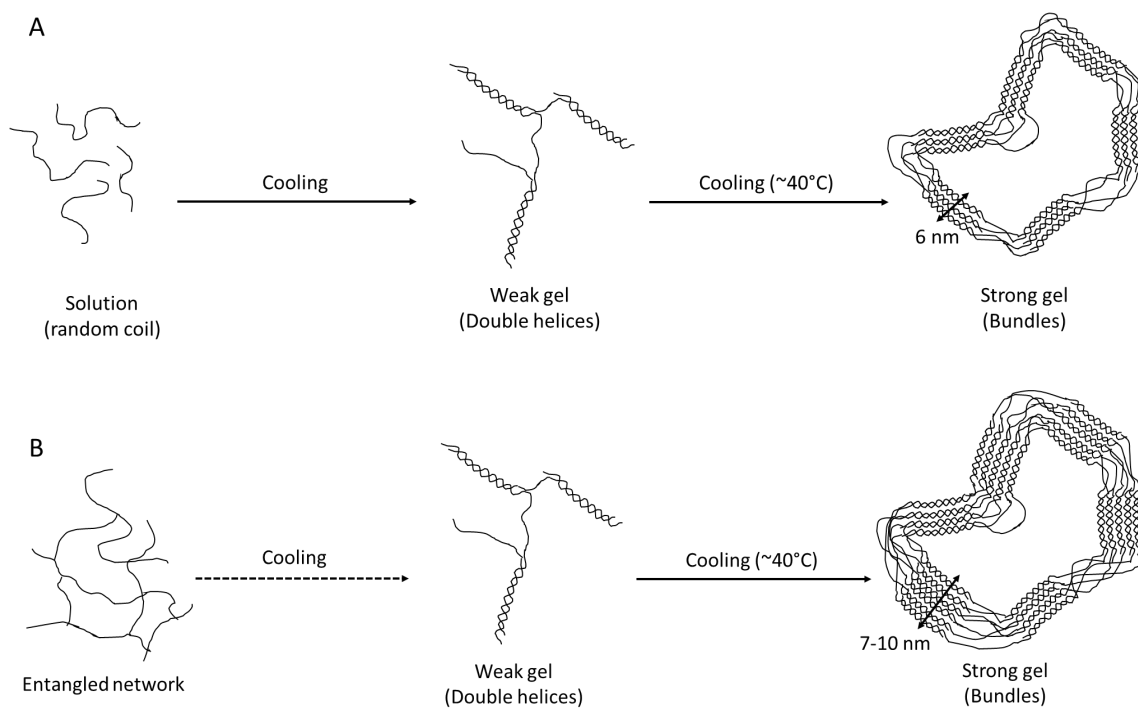
793 Based on the reported results, a mechanism for the structural changes undergone by the
794 different agar-based extracts when subjected to cooling/heating cycles is proposed in Figure
795 6. When the samples were initially heated to prepare aqueous dispersions, only the
796 commercial agar showed true solution behaviour, while the agar-based extracts, due to their
797 higher molecular weights, behaved like entangled networks, as shown by the rheological
798 characterization. Upon cooling, the agar chains (in particular the agarose fraction) started to
799 associate into double helices, which aggregated to form larger structures, referred to as
800 bundles, when decreasing the temperature below 37-42 °C. The formation of these bundles
801 produced strong network structures and, as a result, the obtained hydrogels showed
802 behaviour characteristic of strong gels. Surprisingly, the lower agar content in the non-
803 purified extracts and the presence of proteins and ash did not have a strong impact on the
804 gelation temperature. However, the mechanical performance of the obtained hydrogels was
805 strongly influenced by the agar content, the degree of sulphation and the molecular weight
806 of the samples. As suggested by the scattering results, a greater extent of chain association
807 took place (i.e. thicker agarose bundles were formed) in the alkali-treated extracts, resulting
808 in stronger and stiffer hydrogels. This may be due to the combination of their relatively high
809 agar content, high molecular weight (compared to the commercial agar) and low sulphate
810 content. On the contrary, the lower agarose content and higher degree of sulphation in the
811 non-purified extracts led to the formation of smaller agarose aggregates, producing softer
812 hydrogels. On the other hand, the presence of other components such as proteins did not

813 seem to impair the interconnectivity in the hydrogel network and, thus, the viscoelastic
814 behaviour of the non-purified hydrogels was not strongly affected.

815

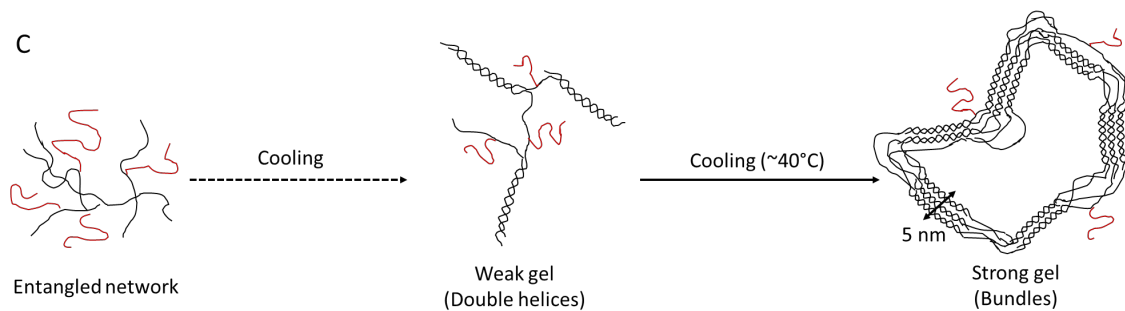
816 The great stability of the agarose bundles was demonstrated by the large thermal hysteresis
817 observed for all samples in the rheology experiments. Although the scattering results show
818 that the structure of bundles was progressively disrupted when increasing the temperature to
819 95 °C, no real melting transition could be detected, suggesting that the entanglements
820 between the agar molecular chains were not completely destroyed. The thicker agarose
821 bundles formed in the particular case of the NaOH+HW extract, were stable even at 95 °C,
822 as shown by the scattering results. Interestingly, although the agarose bundles can re-
823 associate after heating and applying a subsequent cooling cycle, it seems that less ordered
824 structures or slightly weaker inter-chain associations were formed, indicating that unless
825 thicker and more stable bundles are generated (such as in the case of the NaOH+HW extract),
826 thermal treatments have an impact on the structure of the agar-based hydrogels.

827



828

829



830

831 **Figure 6.** Scheme illustrating the proposed nanostructure of different agar-based hydrogels
 832 in their initial “solution” state and after being subjected to cooling ramps. (A) Commercial
 833 agar, (B) HW and HW-US agar-based extracts and (C) NaOH+HW and NaOH+HW-US
 834 agar-based extracts.

835

836 4. Conclusions

837 Agar-based extracts were produced from *Gelidium sesquipedale* using extraction protocols
 838 based on heat and combined heat-sonication treatments, with (NaOH+HW and NaOH+HW-
 839 US) and without (HW and HW-US) the application of an alkali pre-treatment. The
 840 composition of the generated extracts was strongly affected by the preparation method, the
 841 alkaline pre-treatment being critical for the subsequent extraction of more purified extracts,
 842 with higher carbohydrate contents (in particular, agar), lower sulphate contents and lower
 843 amounts of other components such as proteins. On the other hand, the harsh conditions used
 844 for the alkali pre-treatment partially degraded the agar fraction, reducing the molecular
 845 weight with respect to the non-purified extracts. With regards to the agar extraction step, the
 846 application of sonication produced extracts with lower agar contents and molecular weights.

847

848 Aqueous dispersions from the agar-based extracts underwent structural modification upon
 849 cooling due to the association of agarose chains into double helices and bundles. The
 850 temperature at which the bundles were formed was very similar for all the extracts. On the

851 other hand, the mechanical performance of the hydrogels was mostly determined by the agar
852 purity and molecular weight. Higher agar contents and molecular weights promoted a greater
853 extent of intermolecular chain association, hence producing thicker agarose bundles. This in
854 turn gave rise to the formation of stronger and stiffer hydrogels, such as those from the
855 NaOH+HW and NaOH+HW-US extracts, which were also more stable and thermally
856 resistant. The presence of non-agar components in the HW and HW-US extracts did not
857 interfere with their gelation mechanism. The lower agar content in these samples resulted in
858 the formation of softer hydrogels; however, the size of the agarose bundles was not
859 significantly affected as compared to commercial agar. As a result, the stiffness of the
860 hydrogels was similar. Although the formation of bundles is a reversible process, smaller
861 aggregates or slightly weaker structures are typically formed when subjecting the hydrogels
862 to successive heating/cooling cycles.

863

864 These results show that the gelling mechanism is essentially the same for the different agar-
865 based extracts, regardless of their composition. However, importantly, their mechanical
866 performance can be adjusted, depending on the requirements for their final application, by
867 selecting the suitable extraction protocol.

868

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1049

1050 **Supplementary Material**

1051 **Table S1.** Elemental composition of the agar-based extracts (% dry weight).

	N (%)	C (%)	S (%)
Commercial	0.24	43.30	0.70
HW	1.82	27.70	3.27
HW-US	1.69	31.20	2.22
NaOH+HW	0.64	41.90	0.63
NaOH+HW-US	1.17	38.30	0.65

1052

1053 **Table S2.** Neutron and X-ray scattering length densities for native agarose ($C_{12}H_{18}O_9$) and
 1054 after H/D exchange of the labille hydroxyl groups ($C_{12}H_{14}D_4O_9$). The agarose physical
 1055 density was taken as $\rho_{\text{agarose}} = 1.7 \text{ g/cm}^3$ (Rochas et al., 1999).

	Neutron SLD (10^{10} cm^{-2})	X-ray SLD (10^{10} cm^{-2})
Agarose	2.16	15.32
Agarose (D ₂ O exchanged)	3.51	15.13
H ₂ O	-0.56	9.47
D ₂ O	6.38	9.37

1056

1057 **Table S3.** Parameters obtained from fits of the unified model for the commercial agar gel.

1058 Standard deviations on the last digit are shown in parentheses.

	H ₂ O SAXS	SANS				
		H ₂ O	20%D ₂ O	35%D ₂ O	60%D ₂ O	D ₂ O
$B_1 (\text{cm}^{-1} \cdot \text{sr}^{-1} \cdot \text{\AA}^{-P_1})$	0.0018 (3)	0.0006 (3)	0.0004 (4)	0.00005 (2)	0.00005 (2)	0.00067 (2)
P_1	2.611 (4)	1.91 (1)	1.82 (2)	1.9 (1)	1.9 (1)	1.89 (1)
$G_2 (\text{cm}^{-1} \cdot \text{sr}^{-1})$	47.2 (5)	0.46 (1)	0.12 (2)	0.03 (1)	0.03 (1)	0.54 (1)
$R_{g2} (\text{nm})$	5.63 (2)					
$B_2 (\text{cm}^{-1} \cdot \text{sr}^{-1} \cdot \text{\AA}^{-P_2})$	0.0001 (1)	0.0064 (3)	0.003 (1)	0.001 (1)	0.00001 (1)	0.00001 (1)
P_2	3.85 (1)	1.0 (2)	1.0 (1)	1.0 (2)	1.0 (5)	4.0 (1)

1059

1060 **Table S4.** Parameters obtained from fits of the unified model for the HW agar-based gel.

1061 Standard deviations on the last digit are shown in parentheses.

	H ₂ O SAXS	SANS				
		H ₂ O	20%D ₂ O	35%D ₂ O	60%D ₂ O	D ₂ O

B_1 ($\text{cm}^{-1}\cdot\text{sr}^{-1}\cdot\text{\AA}^{-P_1}$)	0.00065 (1)	0.00003 (1)	0.00001 (1)	0.00001 (1)	0.00001 (1)	0.00001 (2)
P_1	2.709 (2)	2.66 (1)	2.517 (1)	2.74 (6)	2.52 (4)	2.39 (1)
G_2 ($\text{cm}^{-1}\cdot\text{sr}^{-1}$)	10.3 (1)	0.39 (1)	0.065 (3)	0.02 (1)	0.05 (1)	0.35 (1)
R_{g2} (nm)	4.95 (2)					
B_2 ($\text{cm}^{-1}\cdot\text{sr}^{-1}\cdot\text{\AA}^{-P_2}$)	0.00003 (1)	0.0018 (1)	0.0018 (1)	0.0001 (4)	0.0004 (1)	0.00001 (1)
P_2	4.0 (1)	1.0 (3)	1.00 (1)	1.6 (5)	1.0 (7)	3.7 (2)

1062

1063 **Table S5.** Parameters obtained from fits of the unified model for the HW-US agar-based

1064 gel. Standard deviations on the last digit are shown in parentheses.

	H ₂ O SAXS	SANS				
		H ₂ O	20%D ₂ O	35%D ₂ O	60%D ₂ O	D ₂ O
B_1 ($\text{cm}^{-1}\cdot\text{sr}^{-1}\cdot\text{\AA}^{-P_1}$)	0.00024 (1)	0.00003 (1)	0.00002 (1)	0.00002 (1)	0.00003 (1)	0.00013 (1)
P_1	2.97 (1)	2.54 (1)	2.39 (3)	2.28 (4)	2.55 (5)	2.25 (1)
G_2 ($\text{cm}^{-1}\cdot\text{sr}^{-1}$)	6.7 (1)	0.35 (1)	0.08 (1)	0.01 (1)	0.05 (1)	0.33 (1)
R_{g2} (nm)	4.59 (3)					
B_2 ($\text{cm}^{-1}\cdot\text{sr}^{-1}\cdot\text{\AA}^{-P_2}$)	0.00002 (1)	0.00001 (4)	0.0025 (1)	0.001 (1)	0.00001 (1)	0.00001 (1)
P_2	3.97 (1)	3.7 (4)	1.0 (2)	1.0 (4)	1.7 (7)	4.0 (2)

1065

1066 **Table S6.** Parameters obtained from fits of the unified model for the NaOH+HW agar-based

1067 gel. Standard deviations on the last digit are shown in parentheses.

	H ₂ O SAXS	SANS				
		H ₂ O	20%D ₂ O	35%D ₂ O	60%D ₂ O	D ₂ O
B_1 ($\text{cm}^{-1}\cdot\text{sr}^{-1}\cdot\text{\AA}^{-P_1}$)	0.0000002 (1)	0.00004 (1)	0.00003 (1)	0.000002 (2)	0.000001 (1)	0.00005 (1)
P_1	3.92 (1)	2.47 (1)	2.29 (6)	2.6 (1)	2.78 (8)	2.45 (2)
G_2 ($\text{cm}^{-1}\cdot\text{sr}^{-1}$)	2.63 (1)	2.46 (2)	0.67 (4)	0.14 (2)	0.22 (1)	3.07 (4)

R_{g2} (nm)	9.58 (3)					
B_2 ($\text{cm}^{-1} \cdot \text{sr}^{-1} \cdot \text{\AA}^{-\text{P}2}$)	0.00018 (1)	0.0066 (1)	0.0009 (3)	0.001 (1)	0.00068 (3)	0.00001 (1)
P_2	2.50 (1)	1.11 (1)	1.4 (1)	1.0 (2)	1.1 (2)	2.93 (2)

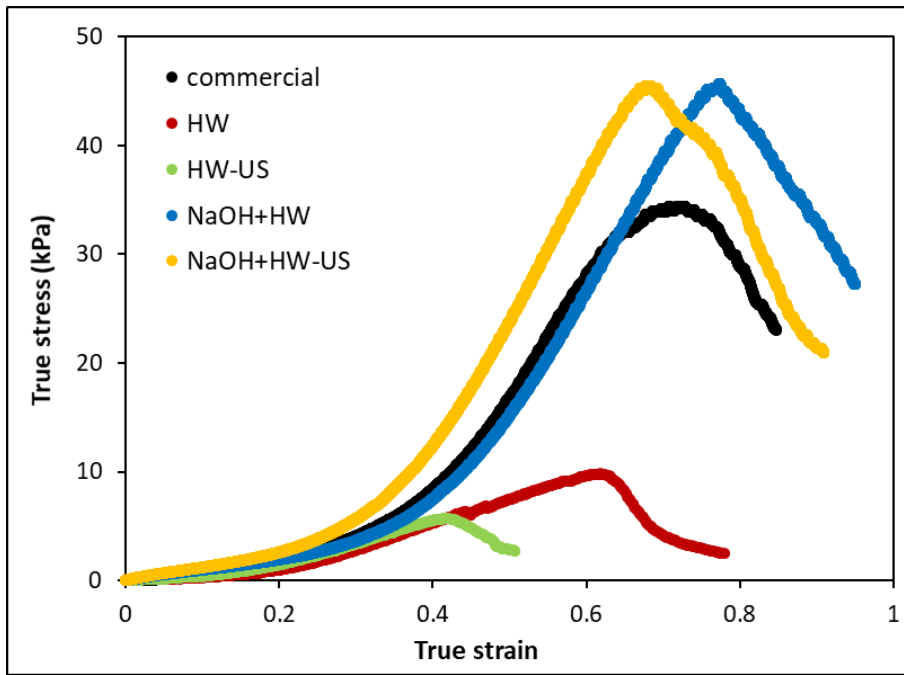
1068

1069 **Table S7.** Parameters obtained from fits of the unified model for the NaOH+HW-US agar-

1070 based gel. Standard deviations on the last digit are shown in parentheses.

	H ₂ O SAXS	SANS				
		H ₂ O	20%D ₂ O	35%D ₂ O	60%D ₂ O	D ₂ O
B_1 ($\text{cm}^{-1} \cdot \text{sr}^{-1} \cdot \text{\AA}^{-\text{P}1}$)	0.0002 (1)	0.00019 (1)	0.00019 (5)	0.000001 (6)	0.000001 (2)	0.00010 (1)
P_1	2.745 (3)	2.16 (1)	1.91 (5)	2.7 (1)	2.87 (5)	2.34 (2)
G_2 ($\text{cm}^{-1} \cdot \text{sr}^{-1}$)	14.1 (1)	0.74 (1)	0.15 (3)	0.03 (1)	0.09 (1)	1.10 (2)
R_{g2} (nm)	6.67 (3)					
B_2 ($\text{cm}^{-1} \cdot \text{sr}^{-1} \cdot \text{\AA}^{-\text{P}2}$)	0.00004 (1)	0.000001 (5)	0.003 (1)	0.001 (1)	0.0003 (1)	0.000001 (1)
P_2	3.56 (1)	4.0 (2)	1.0 (1)	1.0 (2)	1.2 (4)	3.33 (6)

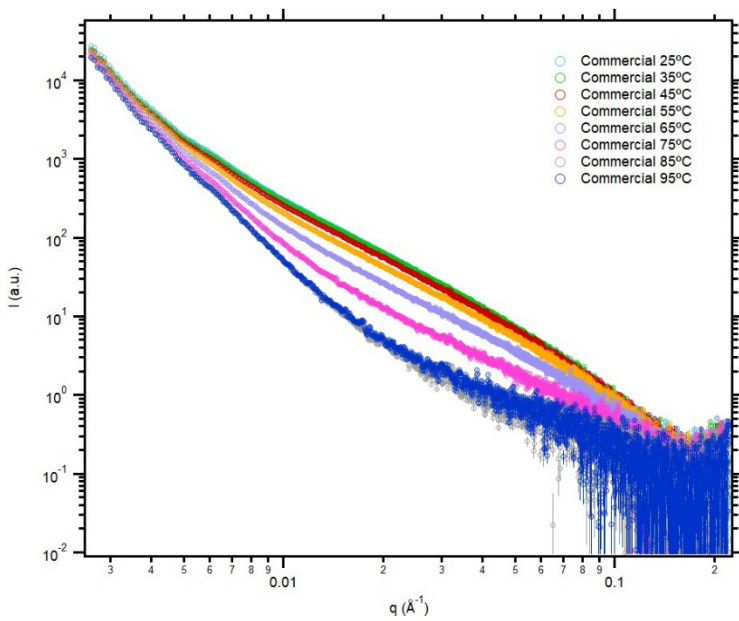
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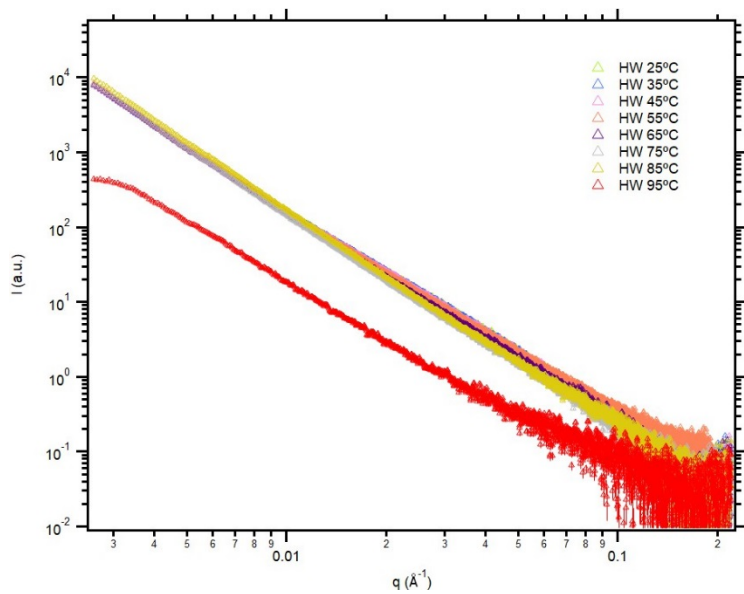
1073 **Figure S1.** Representative compressive true stress-true strain curves for the agar-based
 1074 hydrogels.

1075

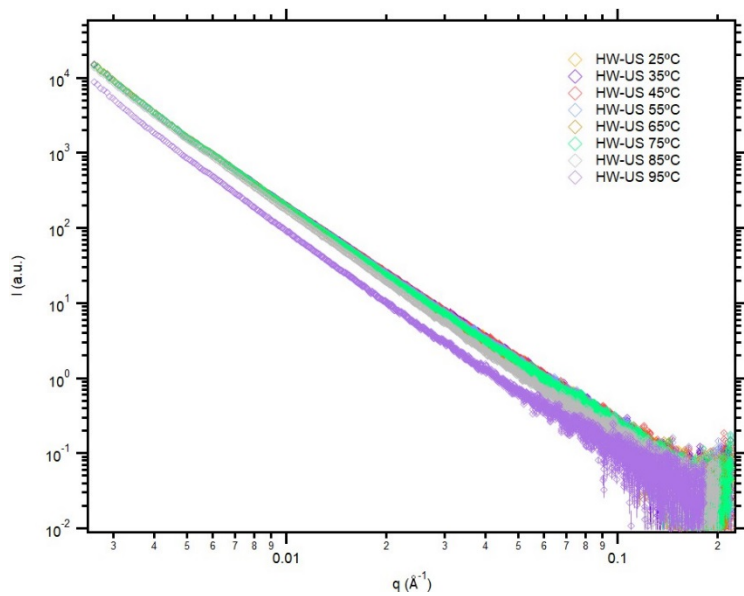


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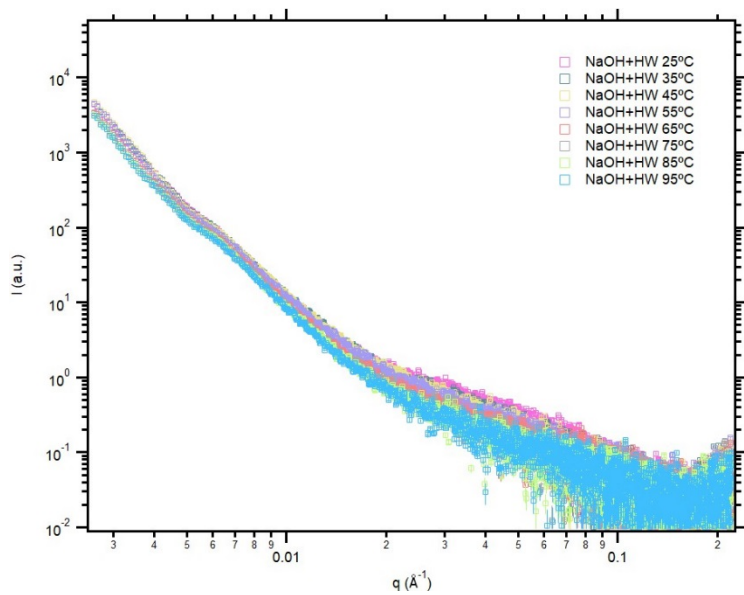
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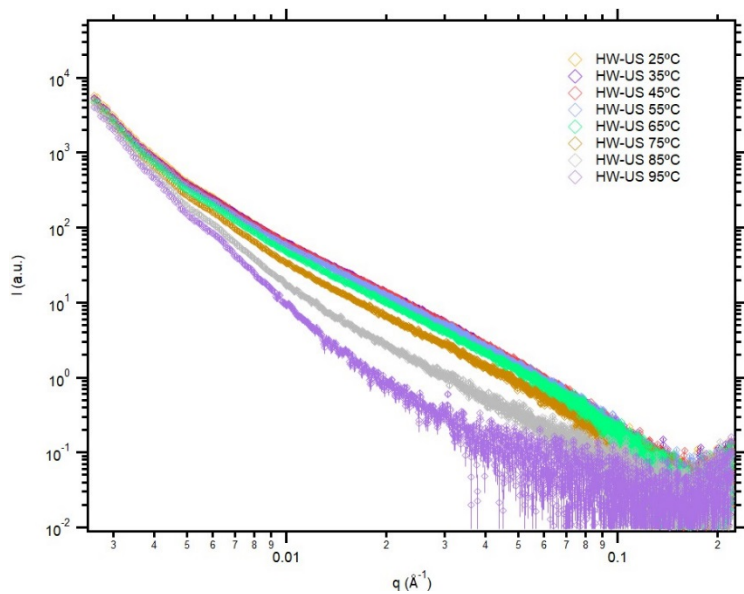


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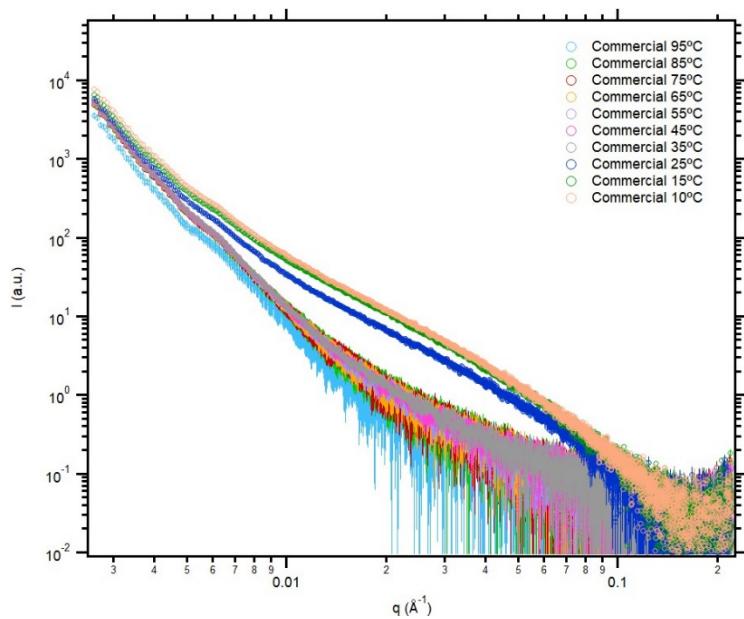


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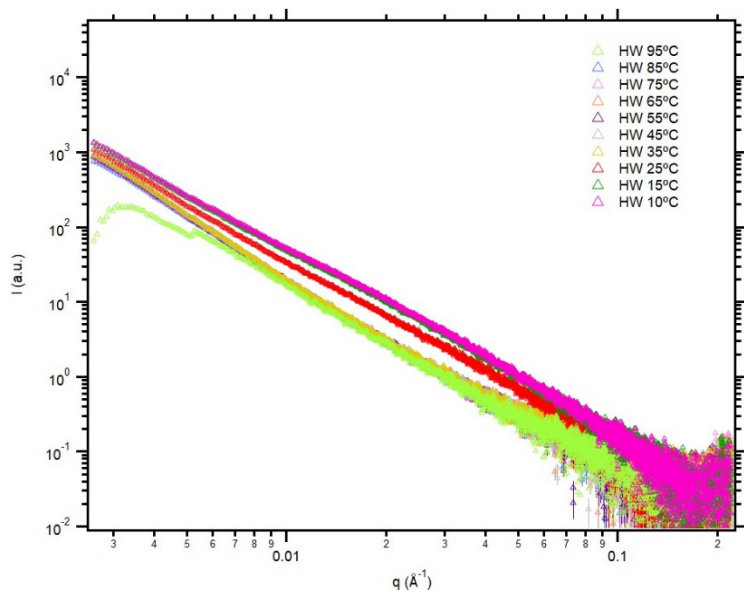
1081 **Figure S2.** SAXS patterns from the heating ramps (25-95°C) of agar-based extracts: (A)

1082 Commercial; (B) HW; (C) HW-US; (D) NaOH+HW and (E) NaOH+HW-US.

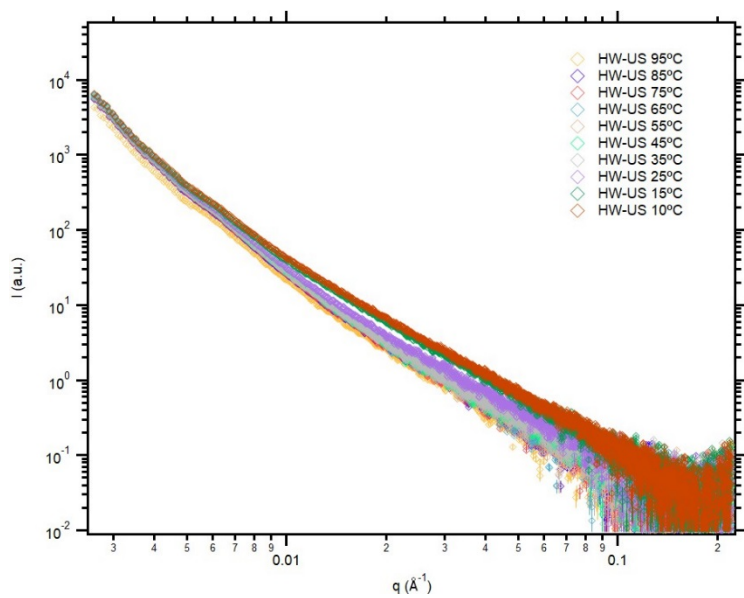
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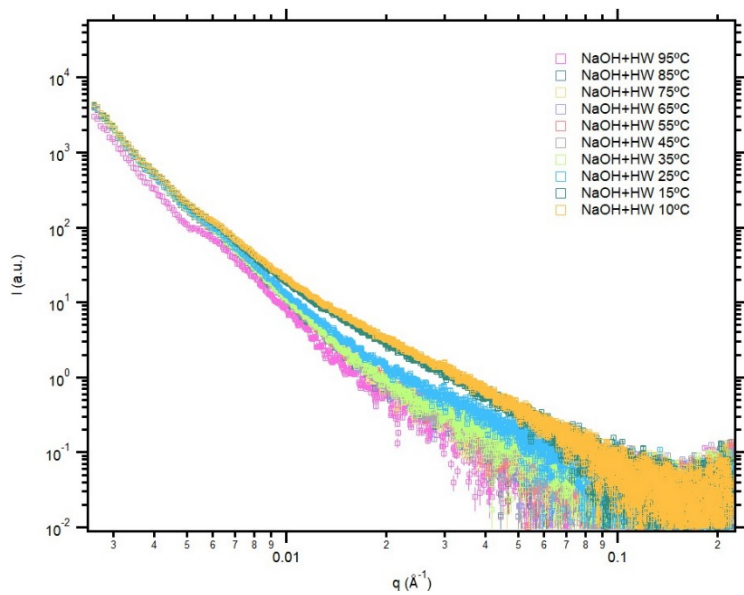
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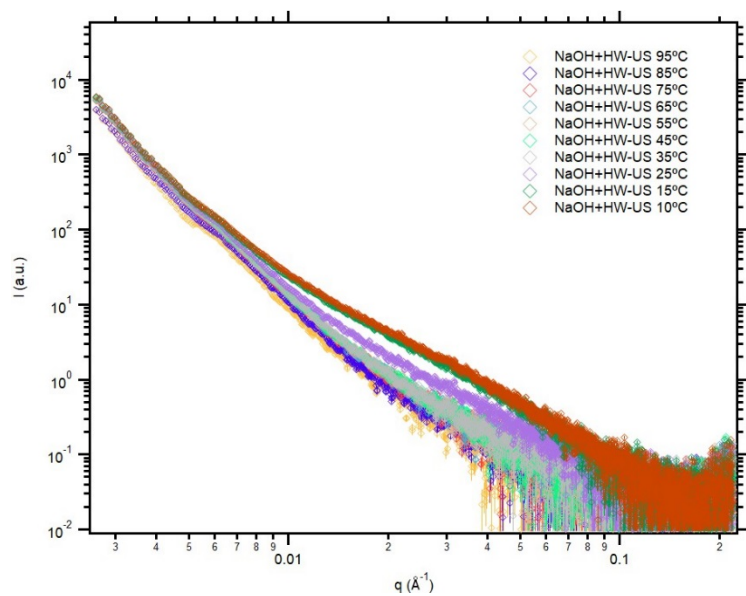
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1089 **Figure S3.** SAXS patterns from the cooling ramps (95-10°C) of agar-based extracts: (A)

1090 Commercial; (B) HW; (C) HW-US; (D) NaOH+HW and (E) NaOH+HW-US.