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DOI:
10.1016/j.carbpol.2021.117758

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Kanyuck, KM, Mills, TB, Norton, IT & Norton-Welch, AB 2021, 'Swelling of high acyl gellan gum hydrogel: characterization of network strengthening and slower release', *Carbohydrate Polymers*, vol. 259, 117758. https://doi.org/10.1016/j.carbpol.2021.117758

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Swelling of High Acyl Gellan Gum Hydrogel: Characterization of Network Strengthening and Slower Release

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Abstract

This study examined the mechanism of swelling for high acyl (HA) gellan gum and the impacts on the hydrogel mechanical properties and the release of a model drug (glucose). Controlling the material properties and the release of entrapped drugs during use in aqueous environments, such as the stomach or bodily fluids, are crucial in designing functional applications. Swelling of HA gellan gum was controlled by varying the osmotic environment with salts and solvents, and effects on the gel network were characterized by uniaxial compression tests, DSC, and rheology. Low ionic strength solutions caused the greatest degree of swelling (up to 400%) and corresponded to a more brittle gel with a greater modulus and greater network enthalpy. Swelling slowed the release of glucose by decreasing the diffusion flux. The osmotic environment was found to produce different functional properties, and it is crucial to consider these changes in the design of formulations.

Keywords: gellan gum; swelling; drug release; gellan hydrogel**;** tissue scaffold; superabsorbent hydrogel

1. Introduction

Hydrocolloid gels are frequently used to create soft-solid structures composed predominately of water in the food, pharmaceutical, and tissue engineering industries. Gellan gum is a carbohydrate hydrocolloid that forms physical gels and is commonly used in each of these industries (Coutinho et al., 2010; Morris, Nishinari, & Rinaudo, 2012; Osmałek, Froelich, Jadach, & Krakowski, 2018; Palumbo, Federico, Pitarresi, Fiorica, & Giammona, 2020; Stevens, Gilmore, & Wallace, 2016). Cytocompatibility, easy processability, mucoadhesion, tuneable mechanical properties, and food-grade status offer many attractive benefits for using gellan gum (Morris, Nishinari, & Rinaudo, 2012; Palumbo, Federico, Pitarresi, Fiorica, & Giammona, 2020; Stevens, Gilmore, & Wallace, 2016). There are two types of gellan gum available: the native or high acyl (HA) and a modified version termed low acyl (LA). The repeating unit of LA gellan gum is →3)-β-D-glucose-(1 → 4)-β-D-glucoronic acid-(1 → 4)-β-D-glucose-(1 → 4)-α-L-rhamnose-(1→ and HA gellan gum has additional substitutions of glyceryl and acetyl units on the 3-linked glucose (Supplementary Figure 1) (Morris, Nishinari, & Rinaudo, 2012; Sworn, 2009).

Both gellan variants form gels by a helix-coil transition occurring upon cooling (after dispersing in hot water 80-90 °C) (Morris, Nishinari, & Rinaudo, 2012). The acetate group on the HA gellan polymer prevents aggregation of double helix chains and follows a fibrous gelation model through end to end associations (Morris, Gothard, Hember, Manning, & Robinson, 1996; Morris, Nishinari, & Rinaudo, 2012). The removal of acyl groups from HA gellan yields a completely different gel texture in LA from modification to the helix structure and aggregation mechanism. HA gellan forms a soft and easily deformable gel, while the gel of LA gellan is firm and brittle (Morris, Nishinari, & Rinaudo, 2012). LA gellan requires monovalent cations to promote double helix formation and divalent cations to allow aggregation of helices, while the HA gellan gelation does not require salt (but it does promote gelation) (Mazen, Milas, & Rinaudo, 1999; Miyoshi & Nishinari, 1999). Recent studies have demonstrated that aggregates of HA gellan, created during the drying process, contribute to the

shear storage modulus (Shinsho et al., 2020). Additional reports suggested a heterogeneous gel structure with crystalline regions and amorphous regions (Yang et al., 2019) presumed to be the helices and aggregates respectively.

The response of gellan gum gels to submersion into aqueous solutions is of interest in both the pharmaceutical and food industries for examining digestion (Lin & Metters, 2006; Norton, Hancocks, & Grover, 2014) and tissue engineering for response to body conditions (De Silva, Poole-Warren, Martens, & in het Panhuis, 2013; Pereira et al., 2011). A common property of charged gel networks, including gellan, is the uptake of water to increase the volume and mass of the gel (defined as swelling). Theory of polymer swelling postulates three driving causes of swelling: polymer-solvent interactions, elasticity, and Donnan potential (Annaka, Ogata, & Nakahira, 2000; Sakai, 2020). Elastic pressure holds the gel network together while the Donnan potential and polymer-solvent interactions drive dissolution. The equation proposes the total osmotic pressure (Π) causing swelling for a charged gel is driven by the summation of polymer-solvent mixing (Π_{mix}), chain elasticity ($\Pi_{elastic}$), and Donnan potential ions (Π_{ion}) (Annaka, Ogata, & Nakahira, 2000; Sakai, 2020):

$$\Pi = \Pi_{mix} + \Pi_{elastic} + \Pi_{ion} \qquad \text{(Eq. 1)}$$

For charged gels with complexed counter ions, the Donnan potential is the greatest influence. Higher concentrations of counter ions inside the gel and low concentration of ions in solutions (in the case of DI water) drives water into the gel (Annaka, Ogata, & Nakahira, 2000; Coutinho et al., 2010). The maximum swelling is limited by the crosslink density (Moe, Elgsaeter, Skjåk-Bræk, & Smidsrød, 1993) and the extent depended on the osmotic gradient (Annaka, Ogata, & Nakahira, 2000; Coutinho et al., 2010).

Large increases in the mass of HA gellan gels have been reported upon submersion in aqueous environments (Cassanelli, Norton, & Mills, 2018b; Chen et al., 2020; de Souza, de Mello Ferreira, I. L., da Silva Costa, M. A., da Costa, M. P. M., & da Silva, 2021; Liu, Wang, Gao, & Bai, 2013). In water, a 2% HA gellan gel increased in mass by 192% (Cassanelli, Norton, & Mills, 2018b). Swelling of freeze

dried HA gellan gum gels have been measured, but it is known the freeze drying process partially destroys the gel structure (Cassanelli, Norton, & Mills, 2018a) so a true comparison cannot be made. Increases in mass ranged from 1,150% to 32,000% for the freeze dried gel (Chen et al., 2020; de Souza, de Mello Ferreira, I. L., da Silva Costa, M. A., da Costa, M. P. M., & da Silva, 2021; Liu, Wang, Gao, & Bai, 2013). In simulated body conditions (typically high salt), both increased (De Silva, Poole-Warren, Martens, & in het Panhuis, 2013; Osmałek, Froelich, Jadach, & Krakowski, 2018) and decreased (Osmałek, Froelich, Jadach, & Krakowski, 2018; Pereira et al., 2011) modulus have been observed but none of these looked at the effect of ion concentration on the swelling.

Swelling of modified LA gellan gum gels was driven by salt concentration (Annaka, Ogata, & Nakahira, 2000; Coutinho et al., 2010). An increased modulus was observed from submersion of LA gellan in salt solutions (De Silva, Poole-Warren, Martens, & in het Panhuis, 2013; Hossain & Nishinari, 2009; Nitta, Ikeda, & Nishinari, 2006; Tanaka & Nishinari, 2007; Yu, Kaonis, & Chen, 2017), acidic solutions (Norton, Hancocks, & Grover, 2014), and even DI water (Hossain & Nishinari, 2009; Nitta, Ikeda, & Nishinari, 2006). It was thought to arise from aggregation of unaggregated helices by the additional counter ions (De Silva, Poole-Warren, Martens, & in het Panhuis, 2013; Hossain & Nishinari, 2009; Nitta, Ikeda, & Nishinari, 2006; Tanaka & Nishinari, 2007; Yu, Kaonis, & Chen, 2017). The hypothesis of ions migrating from an external solution during soaking and causing this further aggregation has been generally accepted (Morris, Nishinari, & Rinaudo, 2012). The behaviour of hardening in DI water has also been observed but cannot be explained by the cation theory (Hossain & Nishinari, 2009; Nitta, Ikeda, & Nishinari, 2006). Hossain and Nishinari (Hossain & Nishinari, 2009) proposed that the swelling caused “stiffening of network chains” which led to the increased modulus but no further analysis or mechanism was given. The behaviour of HA gellan may provide additional understanding because it does not aggregate through counterions, but no comparison has yet been made.

95 Lack of a fundamental understanding of HA gellan swelling and the interesting swelling-hardening of
96 LA gellan necessitate further examination to understand gellan gum behaviour. No studies have
97 comprehensively examined the origin of HA gellan swelling or the structural changes taking place
98 during swelling. Several researches have highlighted the need to examine changes in material
99 properties during usage in aqueous solutions (Stevens, Gilmore, & Wallace, 2016; Yu, Kaonis, &
100 Chen, 2017). This work will investigate the mechanism of swelling of HA gellan gum, the effects of
101 swelling on the network structure, and the impact on release of a small molecule. This work
102 hypothesized that HA gellan swelling is driven by an osmotic imbalance which causes a
103 rearrangement of chains to an extended structure and is accompanied by a physical strengthening to
104 the network. Swelling of HA gellan gum gels was controlled by altering salt concentration gradients
105 and the resulting physical properties and network structure were examined. Additionally, the effects
106 of solvent properties were compared to elucidate mechanisms for the physical change. Lastly,
107 release of glucose from gellan gels under high-swelling and low-swelling environments were
108 compared to determine the impact of swelling.

109 **2. Materials and Methods**

110 **2.1. Materials**

111 HA (LT100) and LA (F) gellan gum were acquired from CP Kelco (Atlanta, USA). The linear polymer is
112 comprised of a repeating sequence of a β-D-glucose, one β -D glucoronate, one β -D-glucose, and
113 one α-L-rhamnose and the chemical structure shown in Supplementary Figure 1 (Morris, Nishinari, &
114 Rinaudo, 2012; Sworn, 2009). HA gellan has acetyl and glyceryl substitutions on the first glucose of
115 the repeating unit at O(2) and O(6) respectively while the acyl groups are removed for LA gellan gum
116 (Morris, Nishinari, & Rinaudo, 2012). The total degree of acylation for LT100 indicated a glycerate
117 group on 90% of those units and an acetyl group on 40% of the units (Kasapis et al., 1999). The
118 molecular weight of HA gellan gum is 1-2 x 10^6 Da and LA gellan gum is 2-3 x 10^5 Da (CP Kelco
119 specifications; Shinsho et al., 2020). Further characterization of this polymer by FTIR and NMR has

120 been published by de Souza et al. (2021). Cations present within the commercial gellan gums were
121 analysed by ICP-OES (Optima 8000 by PerkinElmer, Waltham, USA). Counterions of the HA gellan
122 powder were predominately potassium and contained 19,000 ppm K+, 3,600 ppm Na+, 2,200 ppm
123 Ca^2+ and below LoQ of Mg^2+. Similarly, the LA gellan was also +potassium-type and contained
124 46,000 ppm K+, 6,300 ppm Na+, 1,400 ppm Ca^2+ and 580 ppm Mg^2+. Materials were used as
125 described without any further purification. The DI water was prepared with a reverse osmosis milli-Q
126 water system (Merck, Kenilworth, USA). On the logarithmic plots, DI water is considered as 0.0001
127 mM salt to allow it to be within the bounds of the x-axis. Solutions were prepared from salts (KCl and
128 NaCl, $CaCl_2$) and glucose and purchased from Sigma Aldrich (St. Louis, USA).

129 **2.2. Sample preparation**

130 All gels were prepared by dispersing the hydrocolloid powder at 2% w/w in 90 °C DI water with
131 stirring for two hours to hydrate the polymers. Samples used for glucose released were prepared at
132 4% and mixed with an 80° C glucose solution (at 60%) prior to the cooling and setting of the gel (they
133 were mixed as two solutions). A final concentration of 30% glucose and 2% gellan gum was achieved.
134 Hot solutions were poured into 20mm diameter cylindrical plastic moulds and set at room
135 temperature (20°C ± 1°C) for at least 24 hours before analysis. All samples were prepared in at least
136 triplicate and error bars represent standard deviation.

137 Solvent properties were examined by submerging gels in solutions of DI water with added ethanol,
138 glucose, or glucose with KCl. The 'solvent %' refers to the amount of ethanol or glucose added to the
139 mixture on a total weight ratio. The samples with glucose and KCl were each formulated at a final
140 concentration of 100 mM. All solutions were prepared 24 hours before usage.

141 **2.3. Swelling measurement**

142 Swelling of gellan gum gels was measured by increases in mass after soaking in aqueous solutions.
143 Gels were cut into ~20 mm height pieces from the cylindrical moulds (20 mm diameter) and the

144 mass of 7.5 g ± 1 g weighed. The gel was then placed into 150 mL of solution at room temperature.
145 Salt concentrations of the solutions ranged from 0.0001 mM to 1000 mM as indicated in each figure.
146 After 48 hours the gel was removed using a strainer, pat dry to remove surface water, and weighed.
147 Swelling was quantified using the ratio of initial mass to final mass by the equation where M is the
148 measured sample mass after swelling and M_0 is the initial mass:

149 $$\text{Swelling Ratio } (q) = M/M_0 \quad \text{(Eq. 2)}$$

150 This parameter was proposed by Djabourov (Djabourov, Nishinari, & Ross-Murphy, 2013) and
151 chosen to mimic the values used during release studies. Distinction should be made from another
152 common swelling ratio 'Q' which measures the swelling of a freeze dried gel. As it is known the
153 process of freeze drier partially destroys the gellan network (Cassanelli, Norton, & Mills, 2018a),
154 these gels were not freeze dried prior to measurement. Comparison to values obtained from freeze-
155 dried samples are not equivalent.

156 **2.4. Gel compression and fracture**

157 A compression test was used to measure the physical properties of fresh and swollen gels with a
158 TA.XT.plus Texture Analyser (Stable Micro Systems, Godalming, UK). Analysis was completed
159 immediately after the 48 hour period of submersion. Prior to submersion in the water, all samples
160 had dimensions of 20 mm diameter and 20 mm height. Upon swelling, these dimensions changed.
161 For gels that had swelled, the new radius was measured and accounted for in the calculations and
162 the height was cut to maintain a constant 20 mm. The compression used two parallel plates which
163 were both larger than the dimensions of the sample. Gels were placed on the bottom plate and the
164 upper plate was moved downward at 2 mm/s until fracture occurred. Sample height was recorded
165 during the experiment, and the surface area was calculated by measuring diameter of each
166 individual sample. The Young's modulus was determined by the slope of the initial linear relationship
167 between stress and strain. True stress and true strain were calculated to account for the changing
168 dimensions of the gel during compression.

169

170 **2.5. Differential Scanning Calorimetry (DSC)**

171 Changes in enthalpy and entropy of the gel from submersion in water were analysed with DSC. The
172 instrument was a µDSC3evo by Setaram Instrumentation (Caluire, France) which feature sample cell
173 tubes with ~0.9 mL volume made of Hastelloy and able to be tightly sealed (up to 20 bar). Samples
174 were prepared by cutting cylindrical pieces from the gels to fill the samples cells with 750 mg ± 50
175 mg. Identical mass of DI water was added to the reference cell within ± 10 mg. Thermograph cycles
176 began with a hold at 5 °C for 10 minutes and then increased at 1 °C/min up to 95 °C. After a 10
177 minute hold at 95 °C, the temperature was cooled at 1 °C/min down to 5 °C (to be referred to as the
178 first run). This cycle was repeated again immediately after and termed the second run. Gels were
179 prepared and analysed at least 4 separate times for each sample.

180 **2.6. Rheology**

181 Oscillatory rheology was performed with a Kinexus Rheometer (Malven Panalytical Ltd, Malvern, UK)
182 using a 20 mm parallel plate geometry. Circular slices of a 20 mm diameter were carefully cut to a
183 height of 1.5-2.5 mm and placed directly on the geometry. Differing height between samples was
184 accounted for by loading to a normal force between 0.2 and 0.3 N so the gap ranged from 1.5 mm to
185 2.5 mm. A strain sweep was conducted from 0.01% to 100% at a frequency of 1 Hz and a
186 temperature of 20 °C and all samples had a linear viscoelastic region (LVER) greater than 1%. Prior to
187 conducting the temperatures sweeps the temperature was held constant for 5 minutes at 5° C to
188 allow equilibration and following was raised at 1 °C/min from 5 °C to 90 °C. A frequency of 1 Hz and a
189 strain of 0.1% was used. Three replicates were analysed for each sample and error bars show the
190 standard deviation.

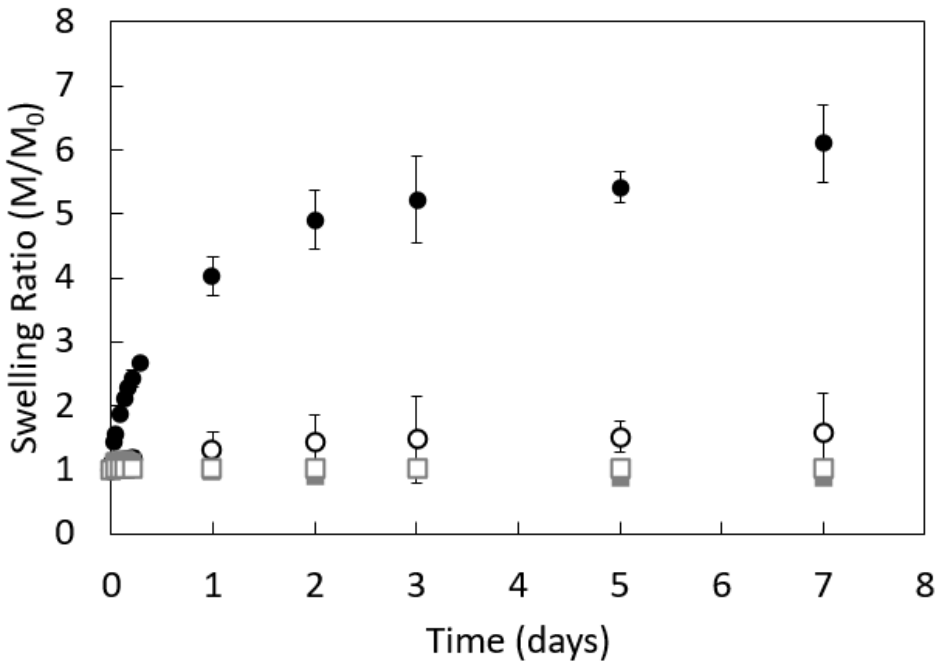
191 **2.7. Release**

192 To compare release of a small molecule (glucose) from gels, a model system was used. Gels were
193 placed into 150 mL of aqueous solution (DI water or 50 mM KCl) at 37 °C to mimic body
194 temperature. A shaker with 200 RPM was used for mixing the bulk solution and the gels were held in
195 place with dialysis tubing. Gels were prepared with 30% glucose and set for 24 hours prior to
196 measurement (section 2.2). Before analysis, gels were cut into 1 mL pieces (1 cm height) and four
197 were utilized in each release experiment (5 g total). The concentration of glucose in the aqueous
198 solution was measured at each time point with a refractometer (Rudolph Research J357 automatic
199 refractometer from Hackettstown, USA). For every sample, the release after 24 hours was 100 ± 5%
200 of the expected glucose concentration and thus values were normalized to the maximum release to
201 minimize the effect of sample variability.

202 **3. Results and Discussion**

203 **3.1. Swelling of gellan gum**

204 Submersion of gellan gels into water caused enlargement of the network and an absorption of water
205 resulting in a higher mass. Swelling of HA gellan and LA gellan are shown over the course of 7 days in
206 Figure 1 and additional data is shown in Table 1. HA gellan had a much greater swelling ratio than for
207 LA gellan and both had greater swelling in DI water than in 50mM KCl. Neither gellan appeared to
208 reach a single equilibrium swelling value, with HA gellan continuing to increase while LA gellan began
209 to decrease after 180 minutes (Table 1). For LA gellan gum, the maximum swelling ratio was reached
210 at 180 minutes while HA gellan continued to increase logarithmically up to 14 days. In agreement
211 with previous work, the maximum swelling of LA gellan occurred between 120 and 180 minutes
212 (Nitta, Ikeda, & Nishinari, 2006). An initial structural rearrangement was followed by a low degree of
213 dissolution of polymer chains for LA gellan in DI water. The absence of HA gellan gum dissolution in
214 DI water was interesting. These differences in apparent equilibrium likely reflect the essential
215 participation of cations in the gelation of LA gellan but not HA gellan.



216

217 **Figure 1**. Changes in swelling ratio of 2% high acyl (HA) gellan (●) and low acyl (LA) gellan (■) during

218 submersion in DI water (filled symbol) and in 50 mM KCl (unfilled symbol ○ and □) for up to 7 days at

219 room temperature.

220 **Table 1**. Swelling ratio of high acyl (HA) gellan and low acyl (LA) gellan gum gels after submersion in

221 150mL of solution the indicated solution. Averages are reported with ± the standard deviation.

	60 mins	**120 mins**	**180 mins**	**24 hours**	**2 days**	**7 days**	**14 days**
2% HA gellan gum in DI water	1.57 ± 0.06	1.87 ± 0.06	2.11 ± 0.09	4.03 ± 0.3	5.03 ± 0.4	6.10 ± 0.6	7.10 ± 0.6
2% HA gellan gum in 50mM KCl	1.09 ± 0.01	1.12 ± 0.02	1.14 ± 0.01	1.31 ± 0.02	1.42 ± 0.01	1.59 ± 0.03	1.66 ± 0.03
2% LA gellan gum in DI water	1.15 ± 0.02	1.15 ± 0.06	1.19 ± 0.02	0.99 ± 0.02	0.91 ± 0.05	0.86 ± 0.04	0.82 ± 0.03
2% LA gellan gum in 50mM KCl	1.03 ± 0.01	1.04 ± 0.004	1.05 ± 0.003	1.04 ± 0.004	1.03 ± 0.003	1.04 ± 0.01	1.04 ± 0.01

222

223 The unique ability for HA gellan to increase in size by 400%, without indication of chain dissolution,
224 was of particular interest. A practical time point of 2 days after submersion in water was chosen for
225 future measurements, when the change appeared to slow for HA gellan, although this does not
226 represent a true equilibrium value. Characterization of this swelling by controlling solution
227 properties and measuring the resulting structural and mechanical properties were the subject of this
228 research.

229 **3.2. Ionic influence on HA gellan gum swelling**

230 Swelling is driven by the total osmotic contribution (Π) and is a combination of polymer-solvent
231 mixing (Π_{mix}), chain elasticity ($\Pi_{elastic}$), and the Donnan potential (Π_{ion}) for charged polymers (Eq. 1).
232 Helix formation is assumed to be unchanged by the swelling and instead causes a tertiary
233 rearrangement of the polymer. A change in the hydrodynamic volume would be reflected in Π_{mix}.
234 The existing gel network resists swelling by $\Pi_{elastic}$ and acts to hold the gel together. Cationic
235 polymers are well known to swell from a high Π_{ion} if the salt concentration outside the gel is lower
236 than inside the gel (Annaka, Ogata, & Nakahira, 2000). The term swelling has sometimes been be
237 used to describe the change from a compact rigid polymer in a glassy state to an extended large
238 pored rubbery network (Lin & Metters, 2006), however in this work the transition is from a soft
239 loose network to a more rigid and extended network. Both cases involve an increased proportion of
240 water per unit of polymer, but with differing effects on the material properties.

241 The following experiments will control for the Donnan contribution (Π_{ion}) to examine the effect of
242 swelling on the HA gellan gum gel network. Effects of external ionic concentration on the swelling of
243 a 2% HA gellan gel are shown in Figure 2. In DI water and low concentrations of chloride ions (0.01
244 mM and below), the swelling ratio of 2% HA gellan was at a maximum of 5.0 (Figure 2 zone 1).
245 Sigmoidal shape of the curve was consistent with Donnan equilibrium (whereby swelling is caused
246 from an imbalance of ions inside and outside the gel) and also supported by the similarity of the ions
247 (Annaka, Ogata, & Nakahira, 2000). The end of the linear portion of the sigmoid and beginning to

248 approach an asymptote (between 10 and 50 mM) is thought to be the equivalence point. Here the
249 internal ionic concentration is likely equal to the outside concentration and would correspond to a
250 Donnan effect of near zero (Figure 2 zone 2). Calculating the internal gel ionic concentration from
251 the ICP data (section 2.1) estimates a 15 mM cationic concentration (10 mM for K+ alone) in a 2%
252 gel. Falling between these two concentrations (of 10 and 50mM), the measured values are
253 consistent with an expected equivalence point (15 mM). High ionic concentrations in the external
254 solution (above 50 mM) and the Donnan effect alone should actually promote deswelling (swelling
255 ratio lower than 1 and a loss of water from the gel). In this region, contributions from Π_{mix} must
256 cause a net positive swelling force. Not until 1,000 mM KCl does the swelling ratio drop below one
257 (Figure 2).

258 Contribution of the Donnan effect to the total swelling was estimated by comparing the maximum
259 swelling ratio (5.0) to the ionic balanced swelling (1.4). A large proportion (90%) of the swelling in DI
260 water was consistent with osmosis-driven salt imbalance. The other 10% of swelling is likely a
261 contribution from Π_{mix} (Eq. 1). For LA gellan, a comparable salt dependant swelling has been
262 previously established (Annaka, Ogata, & Nakahira, 2000; Coutinho et al., 2010; Nitta, Ikeda, &
263 Nishinari, 2006). In addition to the increase in mass of 400% from the Donnan effect, soaking gellan
264 in water also led to a network strengthening that could not be explained by the change in
265 concentration.

266 **3.2.1. Mechanical properties**

267 Mechanical properties of these swollen gels were compared using uniaxial compression testing to
268 measure the Young's Modulus (Figure 3) and the strain to fracture (Figure 4). Submerging HA gellan
269 gels in aqueous solution caused an increase in modulus (Figure 3) but interestingly was not directly
270 related to the swelling (R^2 = 0.11). Whether in DI water or up to 10 mM KCl, the higher modulus was
271 consistent (13 kPa ± 0.8 kPa) while the swelling ratio ranged from 5.0 to 1.9 (Figure 2). At
272 concentrations of salt greater than 50 mM, there was an increasing modulus predicted to be caused

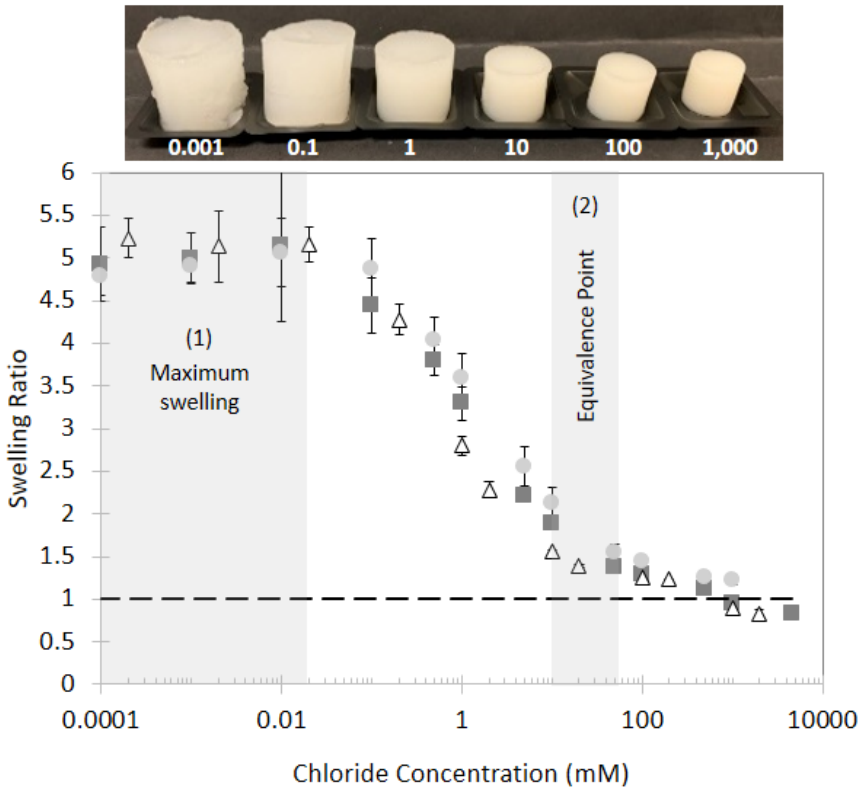
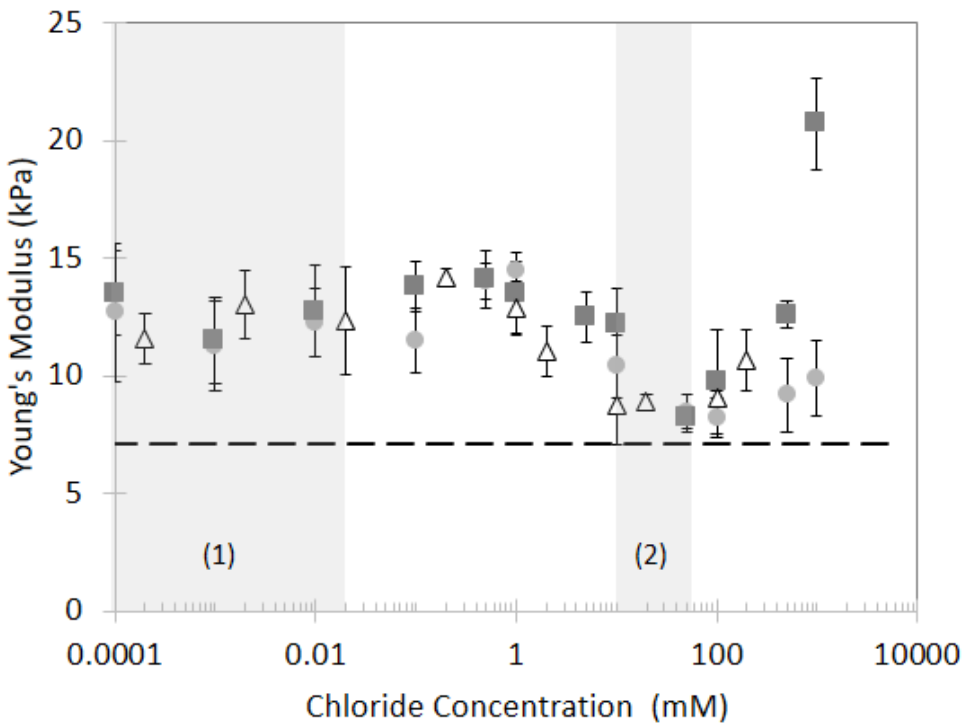


Figure 2. Influence of salt concentrations on swelling of 2% high acyl gellan gels after soaking in 150mL of the indicated solution of KCl (■), NaCl (●), and $CaCl_2$ (Δ) for 48 hours and images show the change in appearance for gellan soaked in KCl solutions. Maximum swelling occurred in zone 1 and the equivalence salt concentration was estimated between 10 and 50 mM salt in zone 2.

from 'salting-out' which is common for hydrocolloids at high ionic strengths. The lowest modulus of the soaked samples occurred in the equivalence salt (zone 2) where the modulus was similar to the fresh (not soaked) gellan gel. The increase in modulus at low ionic concentrations cannot be attributed to an increase in polymer or counter ion concentration as both parameters actually decreased during the swelling. Modulus increases at high degrees of swelling has been explained by a deviation from Gaussian behaviour caused from extensive stretching of the polymer chains (Djabourov, Nishinari, & Ross-Murphy, 2013; Skouri, Schosseler, Munch, & Candau, 1995). Additionally, it is possible new bonds were formed during swelling which will be examined by DSC in a following section.



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289 **Figure 3**. Influence of ionic effect on the Young's Modulus of 2% high acyl gellan gels after soaking in

290 150mL solutions of KCl (■), NaCl (●), and $CaCl_2$ (▲) compared to a fresh sample (dotted line) for 48

291 hours. Maximum swelling occurred in zone 1 and the equivalence salt concentration was estimated

292 between 10 and 50 mM salt in zone 2.

293

294 Strain to fracture of swollen gels was well correlated (R^2 = 0.96) to the ratio of swelling (Figure 4).

295 Images of the compressed and fractured gels are shown in (Figure 4). Water removal from gels

296 during compression (like a sponge) was not observed from the HA gellan gels as previously reported

297 for the LA gellan variant (Nakamura, Shinoda, & Tokita, 2001). The increased brittleness of gels (with

298 a lower strain to fracture) appears to be caused by the extension of polymer chains during swelling.

299 As swelling causes the network to expand, space between junction zones must get larger and the gel

300 more rigid, thus forming a network less able to deform without fracture.

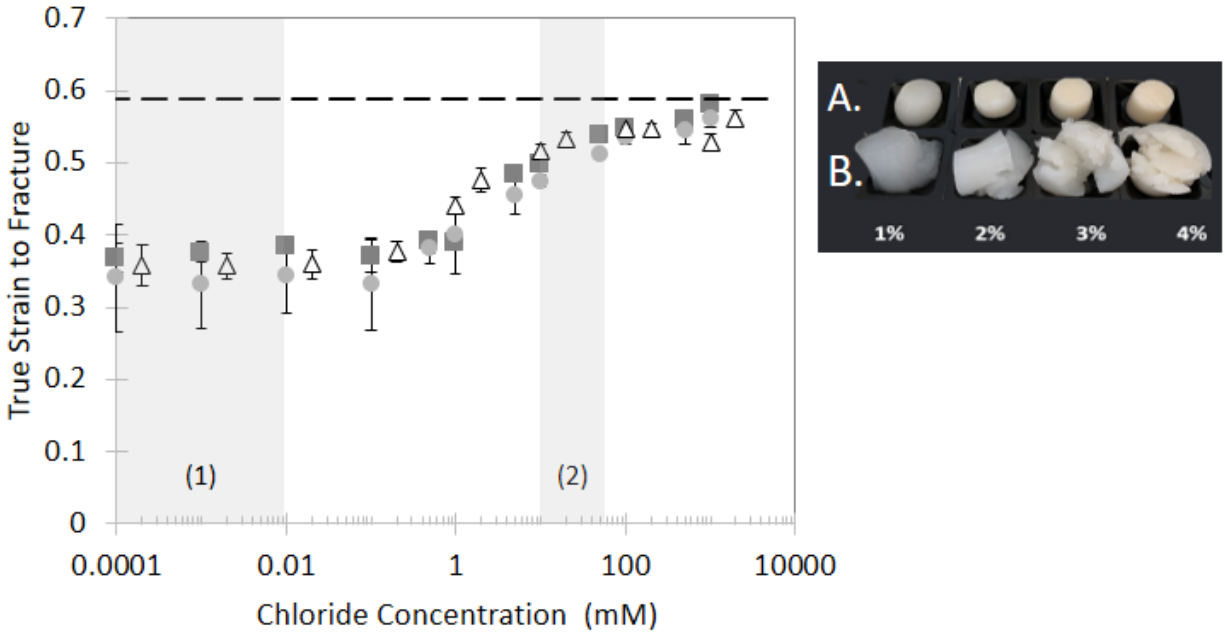

Figure 4. Changes in gel fracture of 2% high acyl gellan after soaking in 150mL of of KCl (■), NaCl (●), and $CaCl_2$ (Δ) compared to a fresh sample (dotted line). Maximum swelling occurred in zone 1 and the equivalence salt concentration was estimated between 10 and 50 mM salt in zone 2. Image shows the appearance of HA gellan gum gels at the indicated concentration (A) before treatment and (B) fractured gels after soaking in DI water.

Previous measurement of the change in modulus of soaked HA gellan gels did not account for the salt concentration. For a 2% HA gellan gel, De Silva et al. (De Silva, Poole-Warren, Martens, & in het Panhuis, 2013) found no change in the Young's Modulus or strain to fracture for a gel submerged in PBS for up to 14 days. Both an increase and a decrease in modulus were observed for a 0.4% HA gellan in a comparison of different bodily fluids (Osmałek, Froelich, Jadach, & Krakowski, 2018). Applying the effect of ionic concentration from the current work, the discrepancy between these authors work likely originates from differing ionic strengths.

Here there are likely two factors at play in changes to the modulus with the varying ionic strengths: Donnan effect swelling and salting out of the polymer. Between 0 and 10mM the changes are driven

317 by an ionic-imbalance salt swelling (the Donnan effect). This range is characterized by high swelling,
318 increased modulus, and the most brittle gels. The range from 0.1 to 10mM is roughly the linear
319 region of the sigmoidal curve where the greatest change in swelling ratio with salt was observed
320 (largest slope). The modulus did not vary with swelling ratio. Between 10 and 50mM was the
321 equivalence point of ionic concentration inside and outside the gel. Swelling ratio at this point is not
322 zero though, as swelling was still driven by the other contributions (Equation 1). At the ionic
323 equivalence point, the Young's Modulus is at a minimum. Matching the internal and external
324 solution properties resulted in the smallest changes from the original gel. At even higher ionic
325 concentrations (greater than 100 mM), salting-out of the polymer likely had caused the increased
326 modulus. Molecular origins of this higher modulus at low ionic strength (<10 mM) were examined
327 next.

328 **3.3. Characterization of gel network changes**

329 The increased gel strength observed upon submersion in low ionic strength solutions was
330 hypothesized to be caused by hydrophobic-driven helix formation. A low correlation to the swelling
331 itself (R^2 = 0.11) suggested an alternative mechanism to just "stiffening of chains" as proposed for
332 the LA gellan (Hossain & Nishinari, 2009). The effective concentration was lower and the salt
333 concentration was lower; both of these are typically thought to drive gelation. Therefore a different
334 mechanism must have caused the increased modulus. First, the networks of swollen and standard
335 gels were compared with DSC and then mixed solvents were compared to understand solubility
336 characteristics of the gels.

337 **3.3.1. DSC**

338 To examine changes in the network during swelling of gels, fresh (no soaking in water) and swollen
339 gels were examined by DSC. Thermographs of the heating curves are shown in Figure 5 and the
340 enthalpy and transition temperatures are shown in Table 2. Enthalpies were normalized to the
341 weight of the polymer to account for the differences in concentration between the swollen samples

342 and fresh gel. An endothermic peak between 65 and 76 °C is known to be the helix coil transition of
343 HA gellan gum (Huang, Singh, Tang, & Swanson, 2004; Mazen, Milas, & Rinaudo, 1999; Murillo-
344 Martínez & Tecante, 2014). A peak representing this helix to coil transition was observed for each of
345 the samples with some variation in melting temperature due to the differences in salt. Ionic
346 concentration is well known to effect the gelation temperature of HA gellan gum (Flores-Huicochea,
347 Rodríguez-Hernández, Espinosa-Solares, & Tecante, 2013; Huang, Singh, Tang, & Swanson, 2004;
348 Mazen, Milas, & Rinaudo, 1999). The decreased melting temperature of the sample soaked in DI
349 water was indicative of the lower salt environment. Alternatively, soaking in 50 mM KCl resulted in a
350 higher melting temperature (4 °C). This concentration was an estimation of the equivalent
351 concentration (between 10 and 50mM KCl) and in practice the selected 50mM KCl was marginally
352 higher than the gel itself. The higher melting temperature was a reflection of this greater salt.

353 Comparing enthalpies of melting for gel networks gives an indication of internal energy associated
354 with the helix coil transition. Differences in melting temperature were taken into consideration by
355 calculating the entropy and Gibbs free energy (ΔG) associated with each melting event. In the
356 soaked samples there was considerable sample to sample variability in temperature (suggested by
357 the large error bars). The µDSC technique utilizes only a small (~750 mg) portion of sample and this
358 high variability would be consistent with heterogeneity within the gel. Due to the lack of helix
359 aggregation, there is little cooperation between helicies and DSC peaks are normally wide (Morris,
360 Nishinari, & Rinaudo, 2012). Gels soaked in DI water resulted in greater enthalpy and ΔG compared
361 to a fresh gel (enthalpy of 45 J/g compared to 27 J/g from Table 2 $p < 0.05$). Soaking in 50 mM KCl
362 did not result in a significant change in the enthalpy ($p > 0.05$) but did cause an increase in ΔG
363 compared to the fresh gel. The greater ΔG could be explained by the greater salt content (Mazen,
364 Milas, & Rinaudo, 1999). Generally, salt environments can be expected to drive further helix
365 crosslink formation (Mazen, Milas, & Rinaudo, 1999). The same is not expected of submersion in DI

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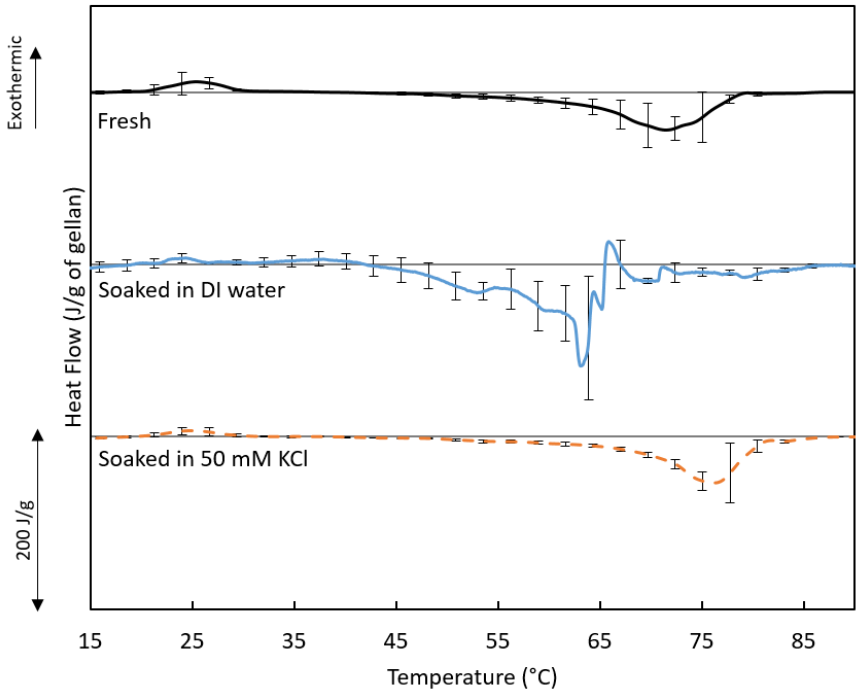

Figure 5. DSC heating thermographs for 2% high acyl (HA) gellan gum fresh (without any treatment) and after soaking in deionized (DI) water or 50 mM KCl for 48 hours. Swelling took place outside of the DSC cells and changes in HA gellan concentration were accounted by normalizing to total the polymer weight in each sample. Error bars represent the standard deviation of five replicates where the deviation was mainly from differences in temperatuare ranges for 'soaked in DI water' and differences in area between 'fresh' and 'soaked in 50mM KCl' samples.

water. The process of swelling and the lower salt environment appeared to decrease the solubility of HA gellan and drive helix formation. These new bonds may have been the cause of the network strengthening (increased modulus) observed during swelling. Alternatively, extensive stretching of chains, past the point of a Gaussian assumption, has also led to a higher modulus (Skouri, Schosseler, Munch, & Candau, 1995). Although the enthalpy of HA gellan (per gram) increased during swelling, the effective concentration decreased by 4-5x causing much complexity for assigning an origin of behaviour. It is likely that both factors were influential in the change of modulus.

382 **Table 2**. Enthalpy of melting (J/g of polymer) and peak melting temperatre (°C) for 2% high acyl
383 gellan gum from Figure 5 and calculated entropy and Gibbs free energy (ΔG). Values were
384 normalized to the grams of gellan in each sample and are reported as the average with one standard
385 deviation. Means were compared for each column and different lettering is indicative of a significant
386 difference between sample means.

	Exothermic Peak		Endothermic Peak			
	Enthalpy (J/g)	**Peak Temperature (°C)**	**Enthalpy of melting (J/g)**	**Peak Temperature (°C)**	**Entropy (J/g·K)**	**ΔG**
Fresh	4.2 ± 2.6 [a]	26.1 ± 1.2 [a]	27.1 ± 8.7 [a]	72.1 ± 2.2 [b]	0.079	4.1
Soaked in DI water	*	*	45.4 ± 0.5 [b]	61.0 ± 4.0 [a]	0.136	5.6
Soaked in 50mM KCl	2.5 ± 1.7 [a]	24.4 ± 1.3 [a]	28.5 ± 6.9 [a]	75.8 ± 1.5 [c]	0.082	4.6

387 * Indicates peak was not significantly different than baseline

388

389 An unusual exothermic peak was observed in the fresh sample but not present in the gel soaked in
390 DI water. A slow-cooled (1 °C/min in the µDSC) HA gellan gel also did not exhibit this exothermic
391 transition. A pre-melting step is common in DSC analysis and may be the reason this peak has not
392 been previously reported. It is proposed the peak represents an ordering or semi-crystallization of
393 the amorphous chains in aggregates. Dissolution of HA gellan chains is not complete after 2 hours of
394 heating and some aggregates of up to 10 chains is expected to be present in the sample (Shinsho et
395 al., 2020). When soaking the gels in water, the aggregates must have dissolved and correspondingly
396 the exothermic peak was not present. A much lower effective concentration and lower ionic solution
397 are reasonable to have caused the breakup.

398 **3.3.2. Temperature dependence of modulus**

399 Rheological tests were used to measure the effect of temperature on the modulus to elucidate the
400 importance of each thermal transition. During heating, the modulus gradually decreased with a
401 steep drop approaching the melting temperatures (Figure 6). Melting temperature by µDSC of 72
402 and 61 °C for fresh gellan gum and soaked in DI water respectively were in good agreement with the
403 rheologically determined gel melting points. Consistent with theory, the helix-coil transition
404 temperature from DSC aligns with the sol-gel transitions temperature measured with rheology
405 (Flores-Huicochea, Rodríguez-Hernández, Espinosa-Solares, & Tecante, 2013; Yang et al., 2019).
406 Aggregate ordering at 25 °C did not result in a measureable shift during the heating ramp and
407 suggested minimal contribution to the gel modulus (Figure 6).

408 From these experiments, it was hypothesized the further helix formation was driven by a
409 hydrophobic effect. Orientation of the HA gellan molecule during double helix formation has the
410 glyceryl groups internal to the double helix (Morris, Gothard, Hember, Manning, & Robinson, 1996).
411 Hydrogen bonds are known to occur within the helix (Chandrasekaran, Radha, & Thailambal, 1992)
412 but hydrophobicity of the glyceryl groups may also contribute to the stability of the helix. Increased
413 stability of helices of HA over LA gellan was demonstrated to be from the glyceryl groups internal to
414 the helix (Morris, Gothard, Hember, Manning, & Robinson, 1996). Hydrophobic interactions have
415 been suggested to contribute to chain associations of HA gellan (Tako, Teruya, Tamaki, & Konishi,
416 2009) but were not examined. The following section will use varying solvents to probe a
417 hypothesized hydrophobic-driven helix-coil transition.

418 **3.3.3. Mixed solution swelling**

419 Mixed solvents of water with glucose and ethanol were used to examine the solvent effects on
420 swelling ratio, Young's Modulus, and strain to fracture (Figure 7). Without added salts, increasing
421 ratios of organic solvents resulted in decreasing swelling ratios following a continuous swelling
422 pattern (Figure 7A). This is explained by the lower dielectric constant of ethanol and glucose than

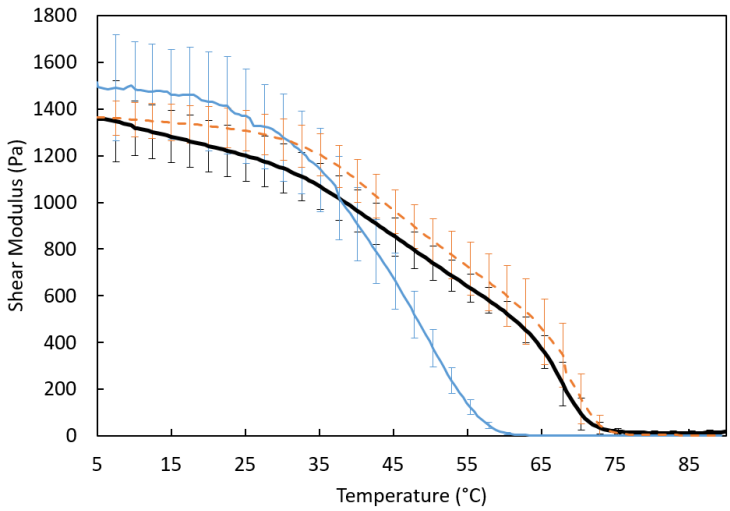

Figure 6. Temperature dependence of storage modulus for 2% high acyl gellan (black) and after soaking in deionized water (blue) and 50mM KCl (dashed orange) utilizing small deformation rheology with controlled heating.

water and shown by the high correlation (R^2 = 0.99 for ethanol and R^2 = 0.97 for glucose) between ratio of swelling and dielectric constant of the mixture (Wyman, 1931). For up to 30% of either solvent, there was little effect of the solvent concentration on the modulus, and a slight (20-30%) increase for ethanol compared to pure water. Similar to the effects of salt, the correlation between swelling ratio and modulus was low (R^2 = 0.12 for glucose and R^2 = 0.71 for ethanol) emphasizing a differing underlying mechanisms. Greater ratios of ethanol led to an increasing in modulus but a decrease for glucose. Although not shown on the graph, a 50% ethanol solution resulted in a modulus of 56,000 Pa modulus and was too large to include in Figure 7. Ethanol is thought to decrease swelling and increase the modulus by a de-hydration of the polymer chains (Cassanelli, Norton, & Mills, 2018b). For pectin, the greatest gel strength (rupture force) was also at the point of greatest hydrophobic interactions at 23% ethanol (w/w) (Oakenfull & Scott, 1984). The balance between solubility and molecular interactions are likely both contributing here. At high concentrations of ethanol there was likely a dehydration-based stiffening, while at low concentrations little change was observed.

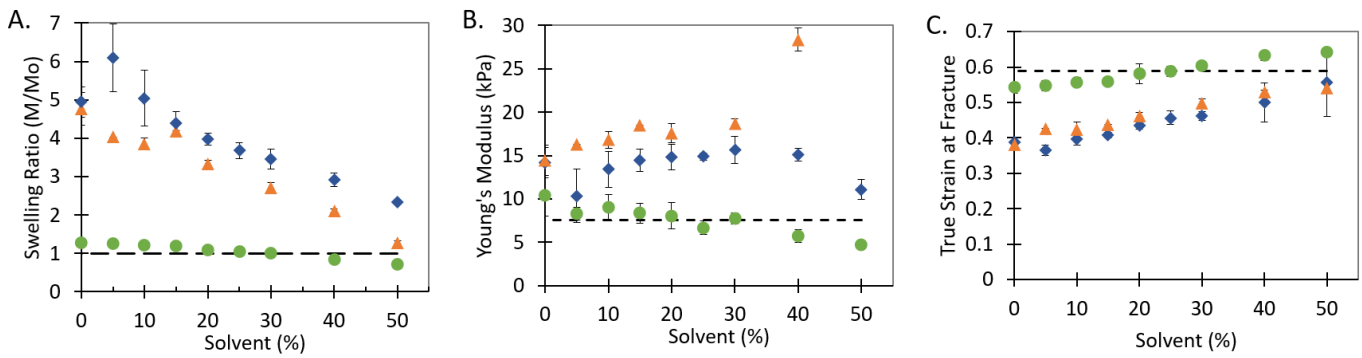

Figure 7. Solvent effects on swelling ratio (A), Young's Modulus (B), and strain at fracture (C) of 2% high acyl gellan gels after soaking in 150mL of water mixed with the indicated percentage of glucose (♦), glucose plus 100mM KCl (●), and ethanol (▲) for 48 hours and compared to a fresh gel (dotted line).

To view the effect of glucose without the swelling contribution, a 100 mM KCl concentration was kept constant while changing the ratio of glucose. As intended, the swelling ratio for the glucose mixed solvent with KCl was near to one for every concentration of glucose, although the swelling did decrease with glucose ranging from 1.3 to 0.72 (Figure 7A). When minimizing the swelling, glucose was shown to decrease the modulus and increase the strain to fracture. However there was high correlation between the swelling and modulus (R^2 = 0.88) suggesting swelling was still playing a role in the modulus. For both glucose and ethanol, at low ratios there was little impact of solvent changes on the modulus, but there was a decrease in swelling from the decreasing dielectric constant.

Interdependencies between swelling, modulus, and solvent properties are clear from the cumulation of results. The observed swelling of HA gellan gum was consistent with both a swelling process and a de-solvation process. A reduction in salt ions in the surrounding environment appeared to cause both an influx of water by the Donnan effect and a helix-coil transitions. Further testing may allow a better understanding of the properties, but what does seem clear is an importance of both hydrophobic interactions and hydrogen bonds to the gelation of HA gellan gum.

461 **3.4. Importance of swelling in release**

462 If HA gellan is submerged in water prior to use, the texture and water ratios would be vastly
463 different during utilization as shown in section 3.2. Even if the gel was not modified prior to use,
464 during digestion or tissue application the solvent properties of the environment would dictate how
465 the gel responds. It is therefore important to measure the influence of swelling on functionality of
466 the gel. For designing food and drug biomaterials, the effect of the characterized swelling on release
467 of an active molecule is crucial.

468 A small and uncharged molecule, glucose, was chosen as the drug of interest for these experiments.
469 Release from a similar gelling agent (LA gellan) which displayed a lower ratio of swelling was
470 included as a reference material. Comparison of swelling of the polymers was shown in Figure 1 and
471 the swelling of LA gellan was small (1.1 swelling ratio at 300 minutes). Release profiles of 30%
472 glucose from 2% HA gellan and LA gellan are shown in Figure 8. In DI water, release from HA gellan
473 was considerably slower than LA gellan (at 120 minutes LA gellan was 38% greater) (Figure 8 filled
474 symbols). Under these low ionic conditions, HA gellan swelled considerably during the release
475 experiment (at 120 and 300 minutes a 1.9 and 2.4 swelling ratio respectively). It was hypothesized
476 that swelling decreased release rates of glucose and was tested by conducting the glucose release
477 experiment in ionic conditions that would minimize swelling. Under these ionic conditions (50 mM
478 KCl), the difference between HA and LA gellan was only 17% at 120 minutes (Figure 8 empty
479 symbols). When swelling was inhibited in HA gellan, the release was quicker and more similar to that
480 of the control gel (LA gellan). However, there was still some swelling (1.1 at 120 minutes) which may
481 explain the remaining difference. LA gellan also displayed a small increase in release rate with KCl
482 which corresponded to a decrease in swelling ratio from 1.15 to 1.04 at 120 minutes. Release from
483 HA gellan was quicker when swelling was reduced (in 50 mM KCl) and very similar to LA gellan at
484 comparable swelling ratios. Thus, it was concluded that swelling of HA gellan caused the decreased
485 release rate and the mechanism was subsequently investigated.

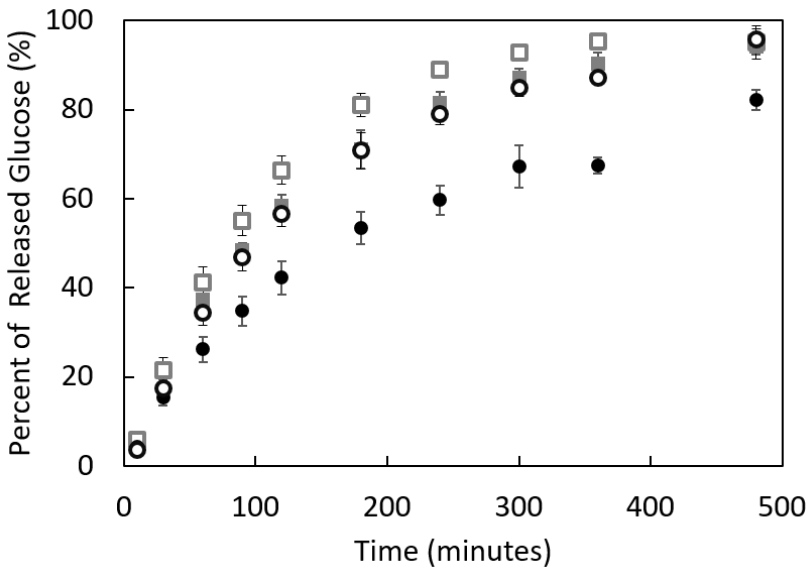

Figure 8. Release of glucose from gels prepared with 30% glucose and 2% high acyl gellan (●) and 2% low acyl gellan (■) into a bulk phase of deionized water (filled symbols) and 50mM KCl (unfilled symbols ○ and □ respectively).

Mesh size of a gel network allows prediction of how compounds of interest would move through the gel. Molecules considerably smaller than the mesh size display little effect to the diffusion through the network, while larger molecules should become trapped in the pores and the release inhibited (Lin & Metters, 2006; Mills, Spyropoulos, Norton, & Bakalis, 2011). Pore size of typical hydrogels in literature range from 100 to 5 nm (Lin & Metters, 2006). Freeze dried HA and LA gellan gum have reported average pore sizes 800-1100 µm and 400-600 µm respectively (Cassanelli, Norton, & Mills, 2018b). Swelling polymers must have structural flexibility and low crosslink density to allow this rearrangement (Moe, Elgsaeter, Skjåk-Bræk, & Smidsrød, 1993). From this understanding, the pore size of both HA and LA gellan is estimated to be much larger than glucose (~1 nm) so trapping in the network pores was unsupported. Additionally, during swelling pores would have increased in size and had the opposite of the observed effect; instead the swelling actually slowed the release.

502 Network dimensions of these gels suggest glucose was not sterically inhibited during release, so
503 another method was investigated.

504 In large-pored gels, the effect of a gel network is principally to prevent convection-based mixing so
505 that diffusion is the driving force through the network (Mills, Spyropoulos, Norton, & Bakalis, 2011).
506 Examining the slower release based on the principles of diffusion suggests two possible mechanisms.
507 First, the swelling of the polymer would increase the distance a glucose molecule must travel within
508 the gel to reach the edge. Second, swelling (corresponding to an increase in volume and mass)
509 would lower the effective concentration of glucose inside the gel. Both of these would have caused a
510 lower flux and the slower measured release of glucose from the HA gellan. The decreased flux of
511 glucose due to swelling was confirmed by the release profile quickening when swelling was
512 inhibited. Increasing dimensions of HA gellan during swelling slowed release of the small molecule
513 glucose.

514 **4. Conclusion**

515 In low ionic strength environments, HA gellan gum gels displayed high swelling and an increased
516 modulus caused by an osmotic imbalance and the effect of slowed release of a model drug was
517 demonstrated. Unlike typical polymers for which swelling increases release of a molecule entrapped
518 in the gel network, the swelling of HA gellan caused a slower release. Magnitude of swelling and the
519 speed generated this unique impact. The unusual swelling ability also provided a structural
520 implication consistent with the previously proposed gelation mechanism of a loose and fibrous
521 network without helix aggregation. Additionally, the swell-strengthening behaviour highlighted the
522 contribution of hydrophobic interactions in helix formation for this hydrocolloid. This work begins to
523 suggest that swelling of HA gellan could be harnessed to capture temperature sensitive compounds
524 into a prefabricated gel under suitable conditions. The high degree of swelling and gel hardening,
525 and the tunability of such with ionic environment, provide many opportunities to use HA gellan as a
526 functional ingredient in biopolymer gels.

Acknowledgements. This research was partially funded by the Engineering and Physical Sciences Research Council [grant number EP/K030957/1], the EPSRC Centre for Innovative Manufacturing in Food.

CRediT Author Statement. Kelsey M. Kanyuck: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft**. Tom B. Mills:** Supervision, Writing - review & editing**. Ian T. Norton:** Supervision, Funding acquisition, Writing - review & editing. **Abigail B. Norton-Welch:** Supervision, Funding acquisition, Writing - review & editing.

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