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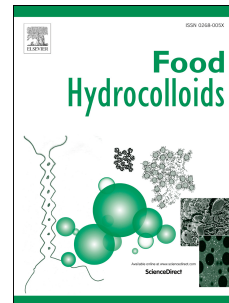
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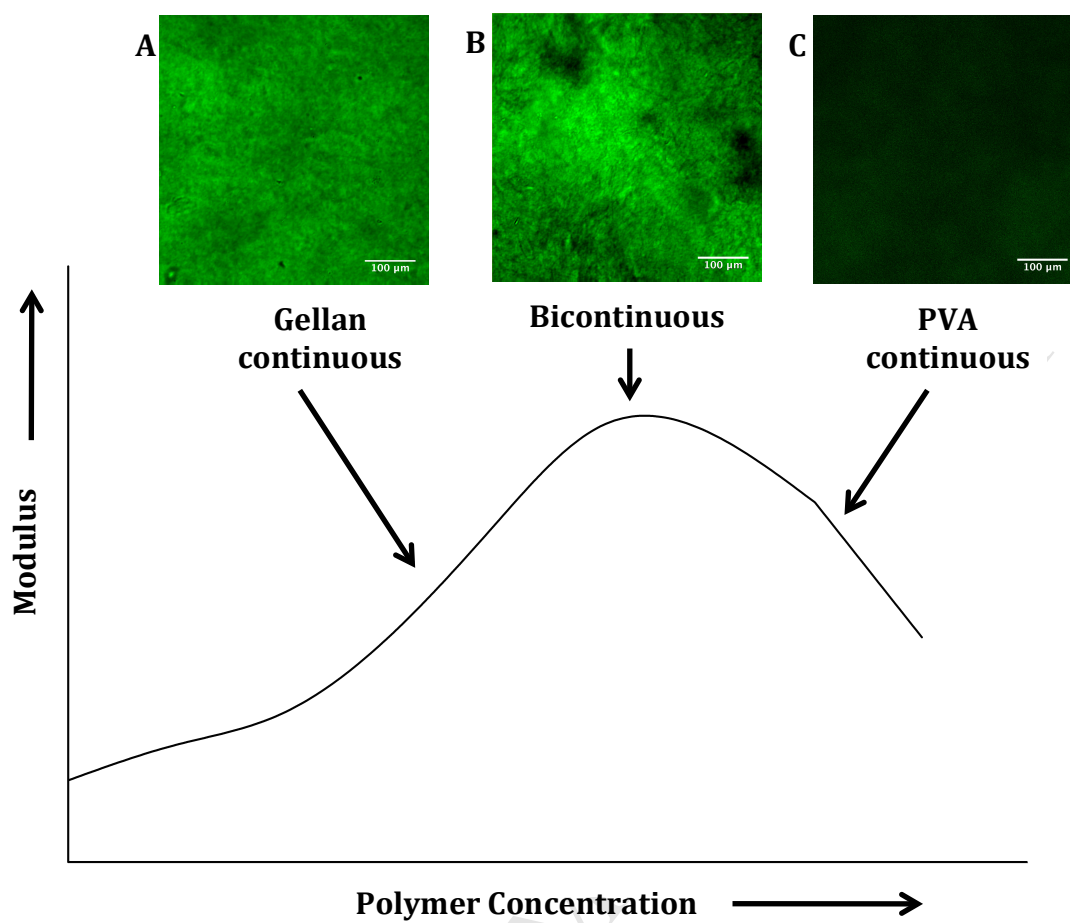
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1 **Development of 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) staining for the**
2 **characterisation of low acyl gellan microstructures**

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6 **Abstract**

7 Although hydrocolloids are used in a wide range of applications, understanding of microstructural
8 interactions in the past have often based solely on mechanical properties. Systems which contain
9 multiple polymers of similar properties are often, therefore, hard to fully understand since it is
10 difficult to distinguish visually between the different phases. As such, the development of a novel
11 staining method could aid our understanding of how microstructure relates to mechanical
12 properties.

13 This research has developed a method for the staining, and consequent visualisation, of low acyl
14 gellan gum using 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) without staining of a second
15 polymer (gellan or PVA).

16 The addition of DTAF on the gellan backbone was shown to affect mechanical properties, resulting in
17 stronger gels. The influence of changing the ratios of DTAF stained gellan, and unstained gellan
18 mixtures was also investigated. It was found; however, that these form phase separated networks.
19 In conclusion, DTAF modification does enable fluorescent staining of gellan and allows the
20 visualisation of microstructural interactions; however, since the modification influences the
21 mechanical properties of the material, this staining method would be best employed as a validation
22 method when used alongside other analytical techniques.

23
24 **Keywords**

25 Staining, gellan, DTAF, microstructure, visualisation, phase separation

26
27 **1. Introduction**

28 Hydrocolloids, which may be formed from polysaccharides and proteins, are versatile materials, and
29 thus have received a great deal of attention in the food (Nishinari, Miyoshi, Takaya, & Williams,
30 1996; Tang, Lelievre, Tung, & Zeng, 1994), pharmaceutical (Guo, Skinner, Harcum, & Barnum, 1998;

31 Osmalek, Froelich, & Tasarek, 2014) and tissue regeneration sector (Birdi, Bridson, Smith, Mohd
32 Bohari, & Grover, 2012; Hunt, Smith, Gbureck, Shelton, & Grover, 2010; Smith, Shelton, Perrie, &
33 Harris, 2007). The major attraction to using such materials is that their gelation may be manipulated
34 to suit a given application and their highly hydrated nature, which enables the diffusion of a range of
35 molecules through their matrix.

36 For many end applications, however, a single phase hydrocolloid system does not exhibit the
37 appropriate properties (I. Norton & Frith, 2001), such as strength, ability to self support, or stability.
38 The use of mixed polymer systems enables material properties from each polymer to be utilised, or
39 in some cases enhanced through new interactions or entanglements. Previous research in the area
40 has investigated mixed hydrocolloids with both natural and synthetic polymers for “improved”
41 mechanical properties, such as the addition of galactomannan to either agarose or k-carrageenan
42 (Morris, 1986), or the addition of poly (vinyl alcohol) to low acyl gellan (A. B. Norton, Hancocks, &
43 Grover, 2014). When two polymers are mixed, they interact with one another; this has a strong
44 influence on material properties. When studying such systems, microstructural changes (including
45 phase separation or the formation of interpenetrating networks) can be inferred through
46 mechanical testing. To develop a complete understanding of the systems, however, it would be
47 highly beneficial to visualise the microstructure exhibited by the polymer blends.

48 Due to the high water content, visualisation of polysaccharides is often difficult. As such, when using
49 a mixed polymer system, it is challenging to distinguish between the component polymers.
50 Therefore, there is a need to develop staining methods for polysaccharides.

51 Staining involves the addition of a compound that can give a colour change to the system, which can
52 then be seen using imaging methods such as light microscopy, or confocal scanning laser
53 microscopy.

54 Negative staining involves the component of interest being mixed or embedded into another
55 material which is visible during microscopy, resulting in a contrast in regions (Brenner & Horne,
56 1959). The areas of interest are consequently shown as black regions, embedded in a coloured
57 image. This has been extensively used for imaging viruses, tissue sections, and cell growth through a
58 hydrogel (Ho, Cool, Hui, & Hutmacher, 2010; Lawn, 1960; Park, Sugimoto, Watrin, Chiquet, &
59 Hunziker, 2005). Conversely, positively staining involves the component of interest being stained
60 using a material that is directly visualised using microscopy.

61 Mixed polymer systems are often challenging to stain, if the functional groups are similar in both
62 components. Staining has been shown to be successful when a polysaccharide is mixed with proteins
63 (Çakır et al., 2012); however, double polysaccharide systems often result in non-specific staining
64 across the system.

65 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) has been shown to have an affinity towards
66 proteins, carbohydrates and polysaccharides (Li, Dick, & Tuovinen, 2003; Russ, Zielbauer, Koynov, &
67 Vilgis, 2013). It has also been used to stain human articular cartilage (Buckley, Bergou, Fouchard,
68 Bonassar, & Cohen, 2010). Russ et al. (2013) stained agarose, within agarose/alginate and
69 agarose/xanthan systems, with the second polymers remaining unstained. This is one of the first
70 records of successful visualization of the agarose microstructure, highlighting the need for
71 developing a catalogue of novel methods to visualise such structures.

72 Within this study, a staining method was developed for low acyl gellan, when in a mixed polymer
73 system. Gellan has been shown to be phase separated when mixed with poly (vinyl alcohol), and
74 thus should exhibit distinct regions in micrographs. This research investigates the use of a non-
75 covalently bound (Toluidine Blue O), and a covalently bound stain (5-(4,6-dichlorotriazinyl)
76 aminofluorescein (DTAF)), and the affect of successful staining on the mechanical properties of the
77 bulk gel.

78

79 **2. Materials and Characterisation**

80 **2.1 Materials**

81 Low acyl gellan (Kelcogel[®], CP Kelco, UK) and Poly (vinyl alcohol) (PVA) (Sigma-Aldrich Company Ltd.,
82 UK) were employed in the gel systems reported in this study.

83 Toluidine Blue O (TBO) (Sigma-Aldrich Company Ltd., UK) and 5-(4,6-Dichlorotriazinyl)
84 Aminofluorescein (DTAF) (Life Technologies, UK) were used for staining gellan PVA systems.

85 DTAF powder was stored at -20 °C; once dissolved into the correct concentrations, solutions were
86 stored at 5 °C until required. Ammonium hydroxide (6.42 M) (Sigma-Aldrich Company Ltd., UK) and
87 hydrochloric acid (5 M) (Sigma-Aldrich Company Ltd., UK) were used to change the pH of the gellan.

88 All concentrations were calculated on a weight to weight (w/w) basis in double distilled water,
89 unless stated otherwise. All materials were used with no further purification. Gelation of all gels
90 occurred following temperature decrease, with no external cross-linking agents.

91

92 **2.2 Methods**

93 **2.2.1 Preparation of low acyl gellan gels**

94 Aqueous solutions of gellan were produced at 2%, at a temperature of approximately 80 °C, to
95 insure gellan was fully dissolved (Yamamoto & Cunha, 2007). Samples were poured into 30 ml

96 cylindrical sample pots (diameter 21 mm, height 80 mm), and left to gel at room temperature for a
97 minimum of 24 h. Mechanical testing of all gel samples was carried out immediately after this 24 h
98 period.

99 Samples for microscopy were mixed with varying concentrations of the secondary polymer, PVA, to
100 show single polymer staining. The materials were fabricated as previously reported (A. B. Norton et
101 al., 2014), as phase separation was already determined for these polymers. For this study, 5%, 10%,
102 12.5% and 15% PVA (w/w) were investigated (percentages were worked out according to the overall
103 volume mixed).

104

105 **2.2.2 Gellan stained with Toluidine Blue O (TBO)**

106 Toluidine Blue O was dissolved in distilled water, at 0.05% (w/w). 200 μ l of the Toluidine Blue O
107 solution was added to gellan PVA samples, at 80 °C. Approximately 5 ml of each sample was then
108 poured into petri dishes, and wrapped in foil, until analysed.

109

110 **2.2.3 Gellan stained with 5-(4,6-Dichlorotriazinyl) Aminofluorescein (DTAF)**

111 The natural pH of the gellan solutions was measured and recorded at pH 5.4. The pH was increased
112 to pH 9 - 10, through the dropwise addition of ammonium hydroxide prior to staining. 10 ml of DTAF
113 solution (400 μ M) was then added, and left to react for 5 h. The pH was then reduced to natural pH
114 of gellan, by the addition of hydrochloric acid.

115 Gellan gels, which were produced using this method, will be called "DTAF gellan" hereafter.

116 PVA was added to the system once the pH was reduced to gellan's natural pH. Approximately 5 ml of
117 each sample were poured into petri dishes, and wrapped in foil, until analysed.

118 Samples for mechanical testing were poured into 30 ml cylindrical sample pots (diameter 21 mm,
119 height 80 mm), and left to gel at room temperature for a minimum of 24 h. Mechanical testing of all
120 gel samples was carried out immediately after this 24 h period.

121

122 **2.2.4 Unstained gellan mixed with stained gellan**

123 For mixed stained and unstained gellan samples, 2% stained gellan was added to 2% unstained
124 gellan (at approximately 80 °C), in the required ratios, to give stained fractions between 0% and
125 100%. The pH of both gellan solutions was 5.4.

126 Samples for mechanical testing were poured into 30 ml cylindrical sample pots (diameter 21 mm,
127 height 80 mm), and left to gel at room temperature for a minimum of 24 h. Mechanical testing of all
128 gel samples was carried out immediately after this 24 h period.

129

130 **2.3 Characterisation Techniques**

131 **2.3.2 Light Microscopy**

132 Light microscopy (Brunel SP300-fl, Brunel Microscopes Ltd.) fitted with SLR camera (Canon EOS
133 Rebel XS, DS126 191) was used to image gellan PVA mixtures stained with Toluidine Blue O. Images
134 were processed using Image J.

135

136 **2.3.3 Confocal Scanning Laser Microscopy (CSLM)**

137 Confocal scanning laser microscopy (CSLM) (Lecia TCS-SPE, Lecia Microsystems Ltd., UK) was used
138 for DTAF gellan samples. Images were taken on a best focus plane, using argon laser, and 10x
139 magnification lens. Images were all processed using Image J.

140

141 **2.3.4 Mechanical Testing**

142 The mechanical properties of the DTAF Gellan gels were assessed by performing compressive testing
143 (5848 MicroTester, Instron, UK), using a 2 kN load cell, and 50 mm diameter stainless steel plate
144 covered with parafilm. Samples were cut into 20 mm length samples, with a diameter of 21 mm. The
145 compression rate was 20 mm/min, and the presented results are the mean of six or more replicates.

146 Compression force and change in sample height were then used to determine the stress (eq. 1) and
147 strain (eq. 2), true stress (eq. 3), true strain (eq. 4), of each sample.

$$148 \quad \delta_E = \frac{F}{A_0} \quad \text{Eq 1.}$$

$$149 \quad \varepsilon_E = \frac{H_0 - h}{H_0} \quad \text{Eq 2.}$$

$$150 \quad \delta_T = \delta_E (1 - \varepsilon_E) \quad \text{Eq 3.}$$

$$151 \quad \varepsilon_H = -\ln(1 - \varepsilon_E) \quad \text{Eq 4.}$$

152 where δ_E is Stress, F is compression force, A_0 is original area, ε_E is strain, h is compressed length of
153 sample, H_0 is original length of sample, and δ_T , ε_H are true stress and true strain respectively.

154 From the obtained true stress/true strain curves, the slope of the second linear region (strains over
155 ~ 0.1), leading to the subsequent failure of the structure, were used to calculate the bulk modulus of
156 each sample (A. B. Norton, Cox, & Spyropoulos, 2011; Nussinovitch, 2004).

157

158 **3. Method Development and Validation**

159 Previous research has shown that gellan mixed with PVA is a phase separated system (A. B. Norton
160 et al., 2014); therefore, distinct regions of each polymer should be seen in micrographs, with the
161 polymers producing continuous and included phases. Figure 1 shows low acyl gellan mixed with PVA,
162 in the presence of TBO. The addition of TBO physically coloured the system; however, this colouring
163 is a non-specific covering both polymers in the system. The use of this stain was unable to allow
164 discrimination between the component phases. Furthermore, it is unclear if the features seen in the
165 images are due to the polymers, or gelation artefacts. Therefore, it can be stated that a stain with
166 more selective binding properties is required to successfully stain gellan, which is itself a complex
167 structure.

168 The literature states that 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) is reactive at pH levels of
169 9 and above; therefore, it was hypothesised that this could be used in a double polymer system,
170 providing the second polymer was added at pH levels below 9. For this research, gellan was
171 increased in pH from pH 5.4, to above pH 9, using ammonium hydroxide; DTAF was then added and
172 left to react for 5 hours. Figure 2 shows confocal microscopy of gellan and PVA mixtures, in the
173 presence of DTAF. The images show a clear increase in black regions, when the concentration of PVA
174 is increased, which suggests that the black regions are PVA. The addition of the secondary polymer,
175 PVA, after the pH was decreased was shown to successfully avoid staining both polymers. Distinct
176 regions of colour also indicate that gellan PVA mixtures are phase separated, as previously stated.

177 In order to understand the affect of the presence of the DTAF stain had on the gellan structure,
178 mechanical testing was carried out on 2% gellan with DTAF in comparison with unstained 2% gellan.
179 Figure 3 shows the true stress verses true strain of 2% gellan gels, without and with DTAF, and then
180 mixed systems of unstained and stained gellan. As can be seen, the addition of the DTAF stain
181 affected the strength and stiffness of the resultant gel, with DTAF gellan being stronger; however is
182 more brittle than the control. This suggests that the addition of the DTAF in the gellan structure has
183 affected the side-by-side aggregation of the gellan, as a consequence of the molecular size of the
184 stain. However, the interaction between DTAF and the gellan causes a stronger interaction between
185 gellan chains than that observed for the unstained gellan, hence exhibiting behaviour similar to that
186 shown when gellan is crosslinked.

187 It was then hypothesised that mixing stained gellan with unstained gellan would reduce the
188 mechanical changes seen with DTAF gellan. As the ratio of stained gellan was increased, higher
189 stresses and failure points than those of the control gellan were observed (figure 3), until 40% of the
190 gellan in the system was stained. As the ratio of stained gellan was increased to 60%, the
191 stress/strain behaviour and failure stress decreased to similar levels observed for 20% stained
192 sample. A further decrease in stress/strain was observed for 80% stained gellan. Therefore, the
193 addition of the stained gellan to the unstained gellan structure increased the gel strength, until
194 further addition of stained gellan then disrupted the gellan microstructure as a consequence of
195 phase separation. This behaviour is typical for multicomponent gel systems, where the polymers
196 present cause phase separation, which can result in a weaker structure if there is little or no polymer
197 binding across the interface.

198 Bulk modulus, or elasticity, of gels needs to be considered when forming gels for particular
199 applications. Figure 4 shows the bulk modulus of 2% gellan gels, when the gellan concentration is a
200 ratio of unstained to stained gellan. The modulus of the gels increases with increase in stain, until
201 40% stained, when the bulk modulus then decreases. This increases when the quantity of stain in the
202 system is increased to over 80%.

203 It was hypothesised that the modulus would increase linearly with increasing ratios of stained gellan.
204 This would occur in a bi-continuous system. A linear relationship was observed when the stained
205 gellan was 40% or below in the system (as highlighted by the dashed line). This shows that at values
206 below 40% stained gellan, the system is bi-continuous. When the level of staining is increased to
207 60% and 80%, the phase-separated system occurs, with the stained gellan as the included phase.
208 This is indicated by values for 80% stained gellan being close to that of the 100% unstained gellan.
209 The trend shown in figure 4 is similar to that of an isostress/isostrain (or blending laws) shown in
210 two component composites (Clark, Richardson, Ross-Murphy, & Stubbs, 1983; McEvoy, Ross-
211 Murphy, & Clark, 1985).

212 Similar trends have also been observed when low acyl gellan is mixed with high acyl gellan
213 (Bradbeer, Hancocks, Spyropoulos, & Norton, 2014). Mixing low acyl gellan and high acyl gellan
214 should be considered as mixing two completely different polymers (due to their phase separating
215 nature), and thus shows similar considerations are required when using a stain on the gellan
216 backbone.

217

218 **4. Conclusions**

219 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) has been shown to successfully selectively stain
220 low acyl gellan, and can be processed to ensure that a secondary polymer remains unstained.
221 However, the addition of the DTAF within a gellan quiescent gel affects the mechanical properties of
222 the bulk gel. Using ratios of unstained gellan and stained gellan results in phase separation of the
223 polymers. Therefore, it is suggested that staining should only be used as a visualisation of an
224 investigated microstructure, and be one of many analytical methods. Furthermore, 100% staining
225 should be used for visualisation so that it is known that a second phase separation is not occurring
226 within the system. Future work could investigate the processing (i.e. time to stain, concentration of
227 stain), and how this affects the change in mechanical properties. This study left the stain to react for
228 a five hour period; however, if this was reduced, would reduced mechanical property changes be
229 seen.

230

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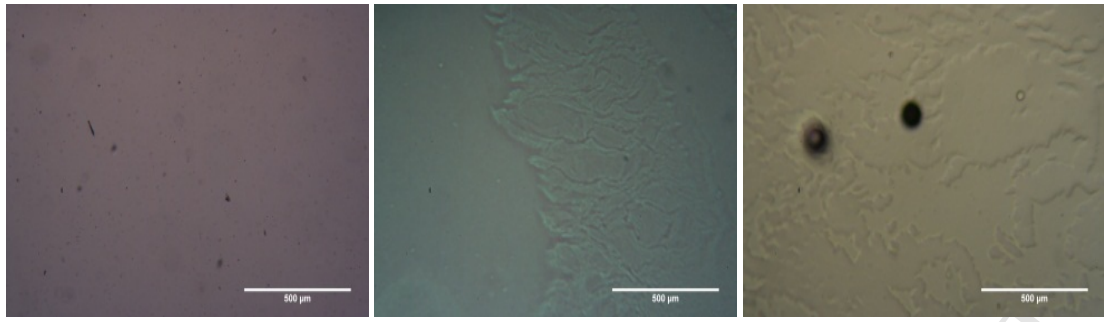
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- 305

Figure 1 – Microscope images of gellan PVA mixtures in the presence of Toluidine Blue-O: 2% gellan, 5% PVA (A), 2% gellan, 15% PVA (B), and 2% gellan, 20% PVA (C). (Gelation occurred through temperature decrease, when left at room temperature).

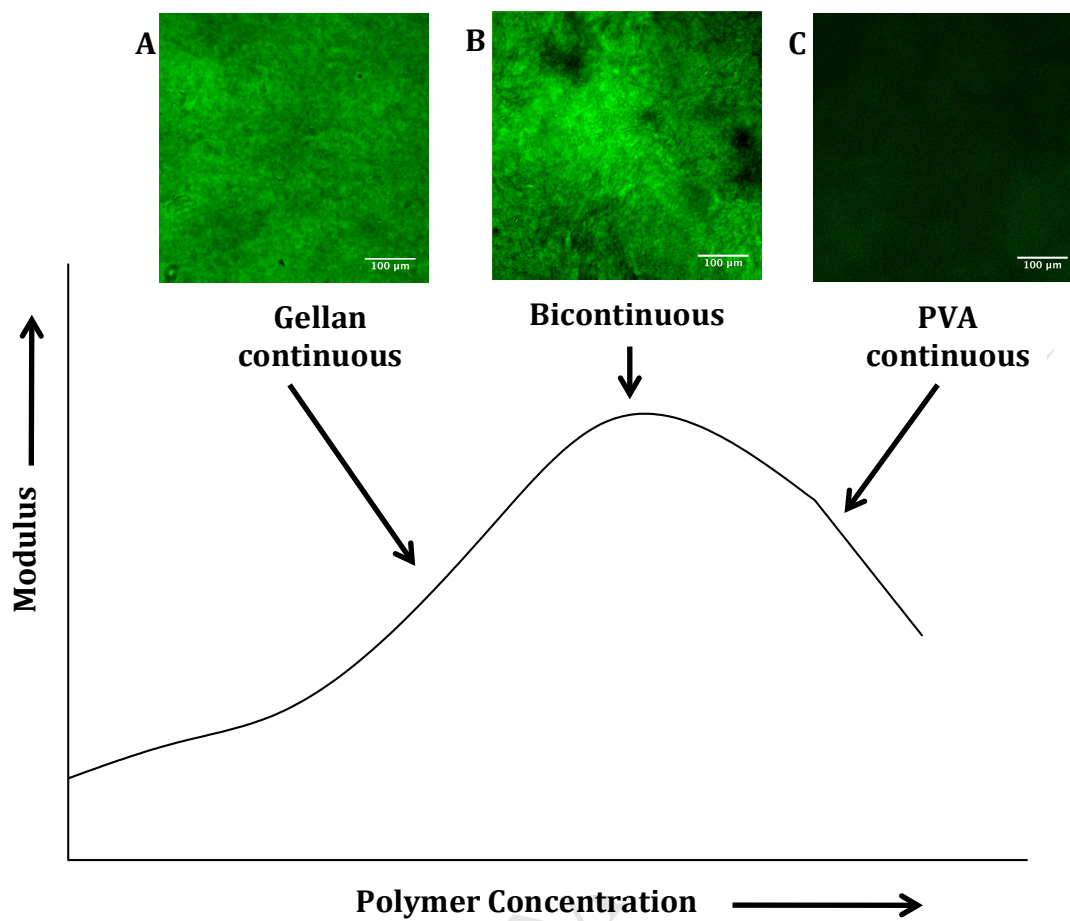
Figure 2 – Schematic of a modulus versus polymer concentration graph, showing a typical phase separation within a multicomponent gel system. Confocal microscopy images show quiescently set 2% gellan system with the addition of PVA, of varying concentrations ((A) 5% PVA, (B) 10% PVA, and (C) 15% PVA) in the presence of DTAF. Images show successful staining of the gellan polymer (shown in green), with PVA left unstained (black).

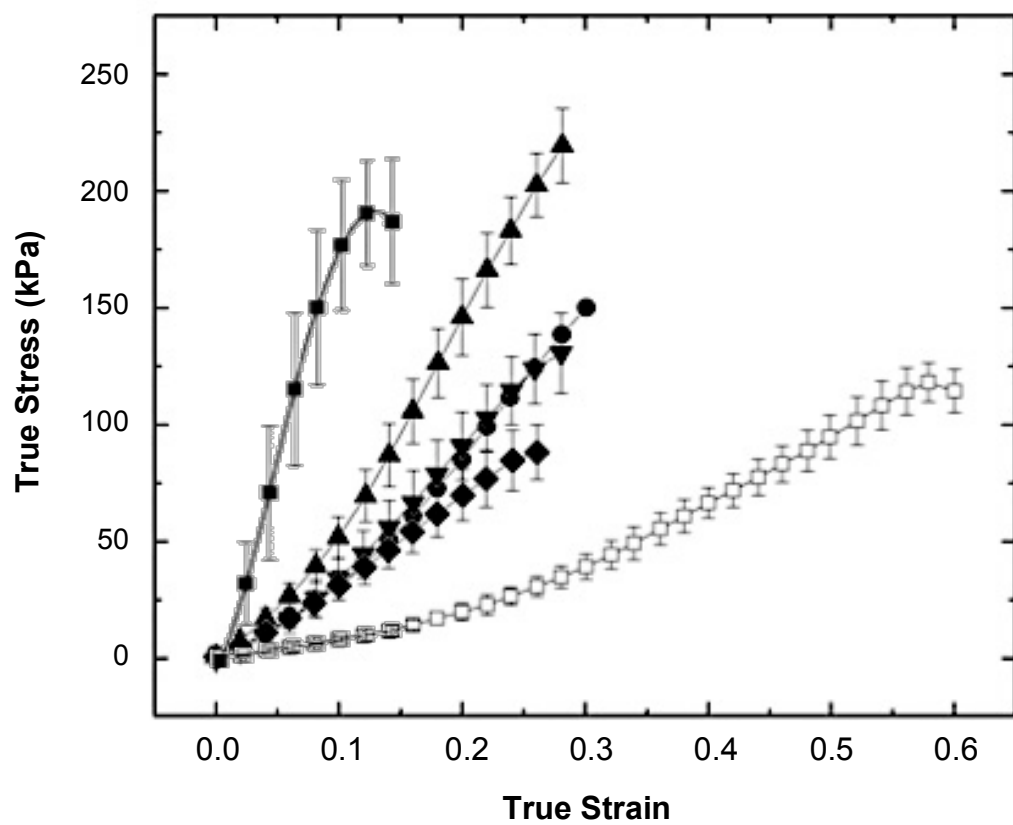
Figure 3 – True stress/true strain curves for DTAF gellan (at 2%) (■) and the control (unstained gellan) (at 2%) (□) and 2% low acyl with ratios of unstained to stained gellan present: 80:20 (●), 60:40 (▲), 40:60 (▼), and 20:80 (◆). Gelation occurred with temperature decrease. Error bars represent a single standard deviation.

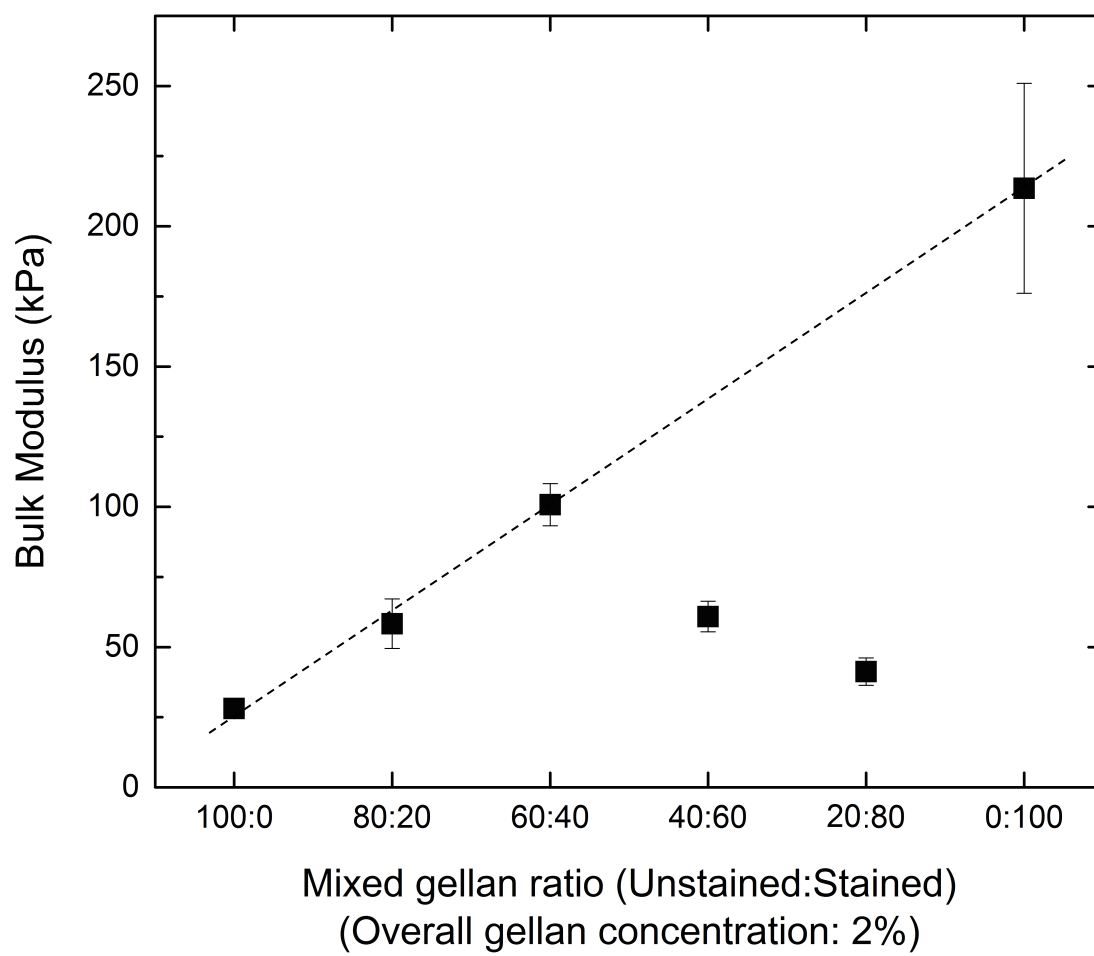
Figure 4 – Bulk modulus of 2% low acyl gellan, with ratios of unstained and DTAF stained gellan. Dotted line represents the hypothesised result of a linear change as ratios were changed. Gelation occurred with temperature decrease. Error bars represent a single standard deviation.



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Highlights

DTAF was shown to successfully stain low acyl gellan.

A secondary polymer present can be left unstained.

Mixing unstained and stained gellan resulted in a phase separating material.

Staining using DTAF is a successful method to confirm polymer interactions.