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DOI:
10.1016/j.foodhyd.2015.03.025

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Document Version
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Citation for published version (Harvard):
Norton, A, Hancocks, R, Spyropoulos, F & Grover, L 2016, 'Development of 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) staining for the characterisation of low acyl gellan microstructures', Food Hydrocolloids, vol. 53, pp. 93–97. https://doi.org/10.1016/j.foodhyd.2015.03.025

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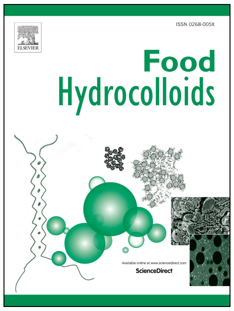

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PII: S0268-005X(15)00147-2

DOI: 10.1016/j.foodhyd.2015.03.025

Reference: FOOHYD 2941

To appear in: *Food Hydrocolloids*

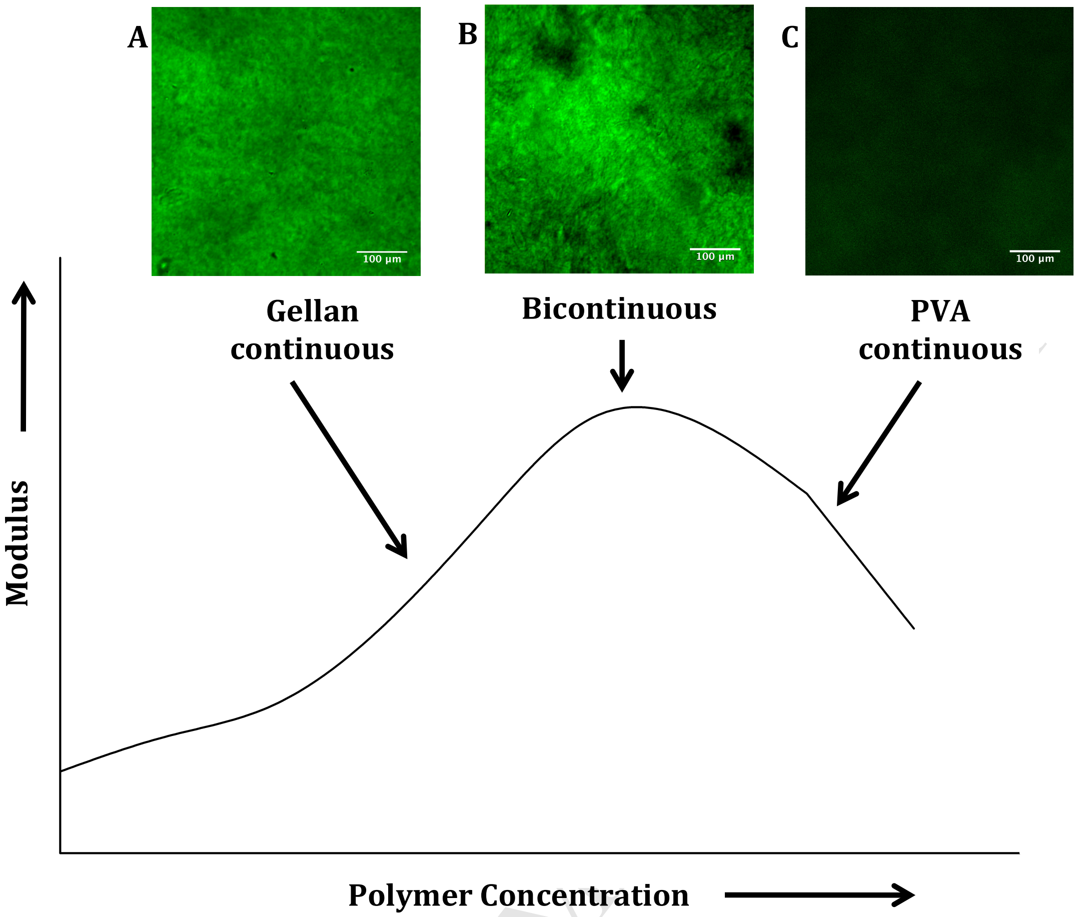
Received Date: 30 September 2014

Revised Date: 22 March 2015

Accepted Date: 30 March 2015

Please cite this article as: Norton, A.B, Hancocks, R.D, Spyropoulos, F., Grover, L.M, Development of 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) staining for the characterisation of low acyl gellan microstructures, *Food Hydrocolloids* (2015), doi: 10.1016/j.foodhyd.2015.03.025.

Graphical Abstract



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

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Abstract

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

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

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

Keywords

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and Characterisation

2.1 Materials

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

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

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

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

2.2 Methods

2.2.1 Preparation of low acyl gellan gels

Aqueous solutions of gellan were produced at 2%, at a temperature of approximately 80 °C, to insure gellan was fully dissolved (Yamamoto & Cunha, 2007). Samples were poured into 30 ml cylindrical sample pots (diameter 21 mm, height 80 mm), and left to gel at room temperature for a minimum of 24 h. Mechanical testing of all gel samples was carried out immediately after this 24 h period.

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

2.2.2 Gellan stained with Toluidine Blue O (TBO)

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

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

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

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

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

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

2.2.4 Unstained gellan mixed with stained gellan

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

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

2.3 Characterisation Techniques

2.3.2 Light Microscopy

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

2.3.3 Confocal Scanning Laser Microscopy (CSLM)

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

2.3.4 Mechanical Testing

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

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

$$\delta_E = \frac{F}{A_\circ} \qquad \text{Eq 1.}$$

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

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

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

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

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

3. Method Development and Validation

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

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

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

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

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

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

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

4. Conclusions

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

5. Acknowledgements

The authors would like to thank both Dr. Hamilton and Dr. Watson for scientific discussions and advice. The authors would also like to acknowledge EPSRC for funding this research (EP/K502984/1). The MicroTest 5848 mechanical tester (Instron, UK) and Confocal Microscope used in this research was obtained, through Birmingham Science City: Innovative Uses for Advanced Materials in the Modern World (West Midlands Centre for Advanced Materials Project 2), with support from Advantage West Midlands (AWM) and part funded by the European Regional Development Fund (ERDF).

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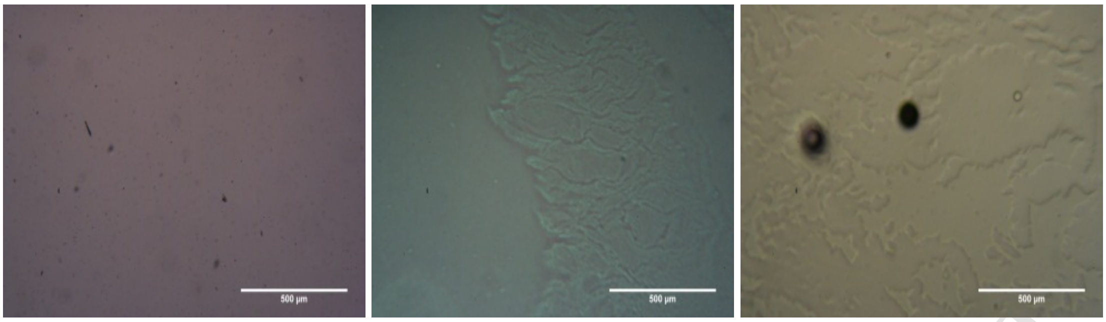
Figure 1 – Microscope images of gellan PVA mixtures in the presence of Toluidine Blue-O: 2% gellan, 5% PVA (A), 2% gellan, 15% PVA (B), and 2% gellan, 20% PVA (C). (Gelation occurred through temperature decrease, when left at room temperature).

Figure 2 – Schematic of a modulus verses 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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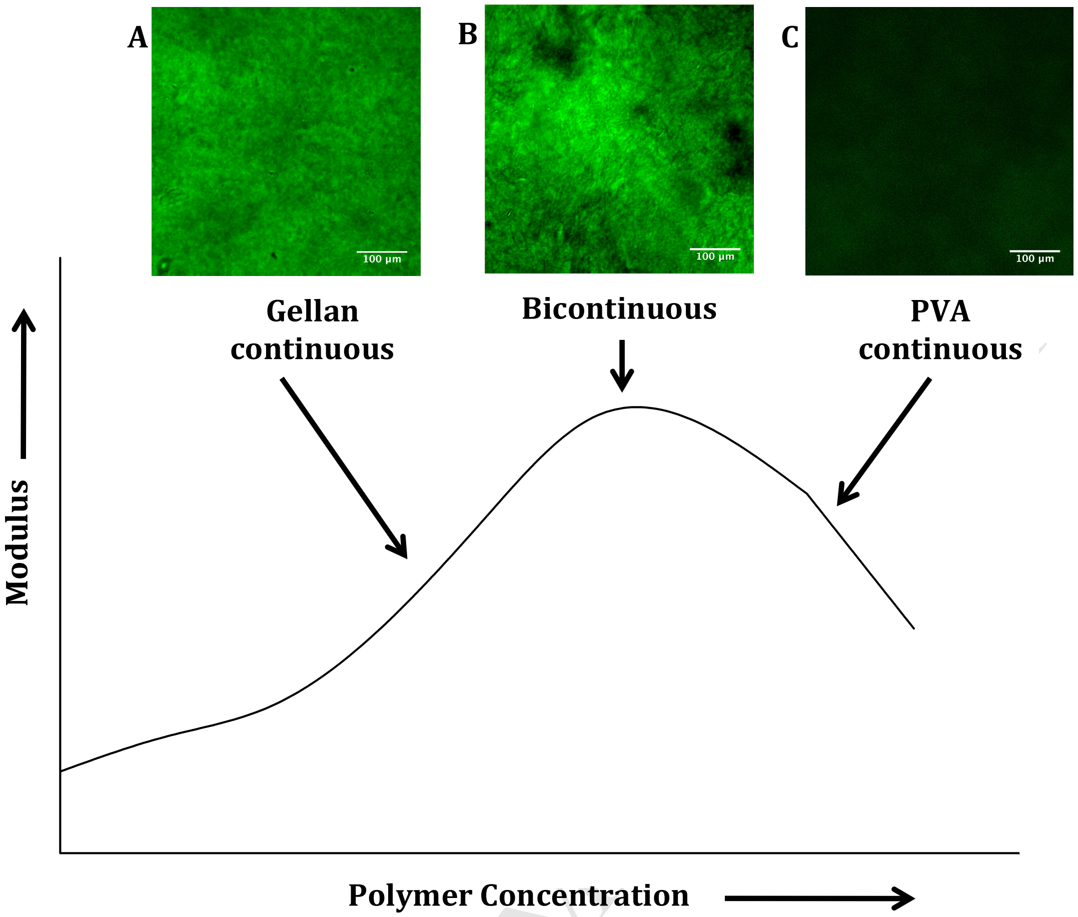
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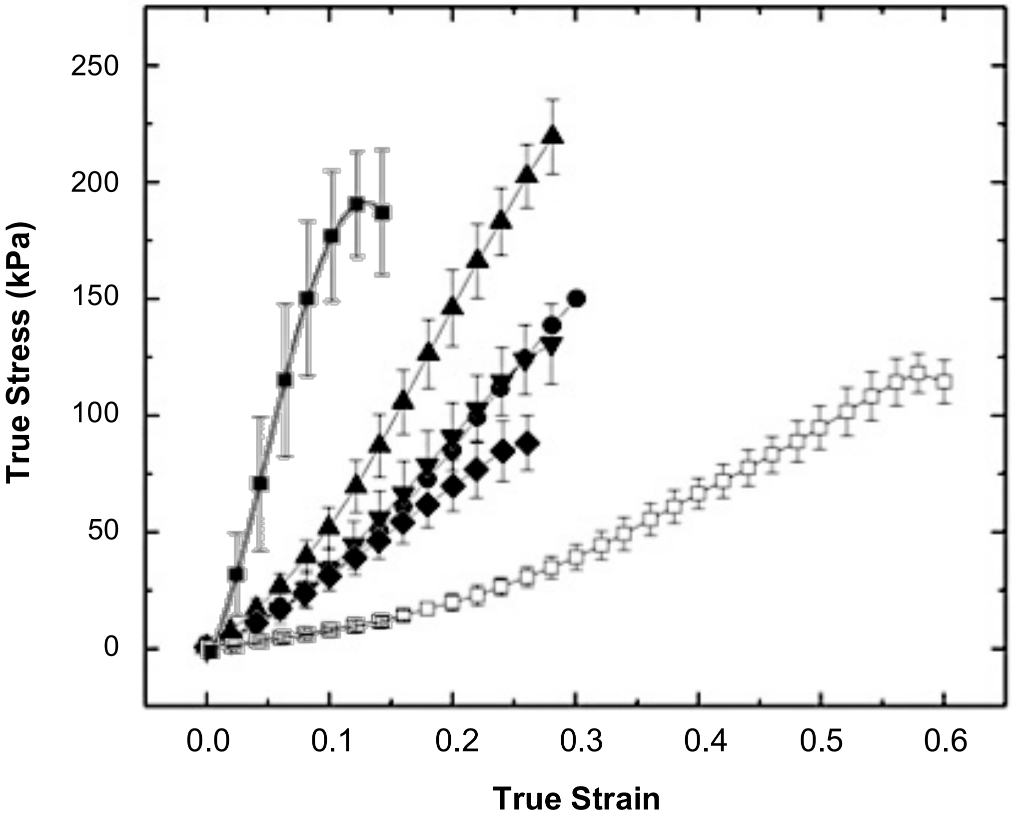
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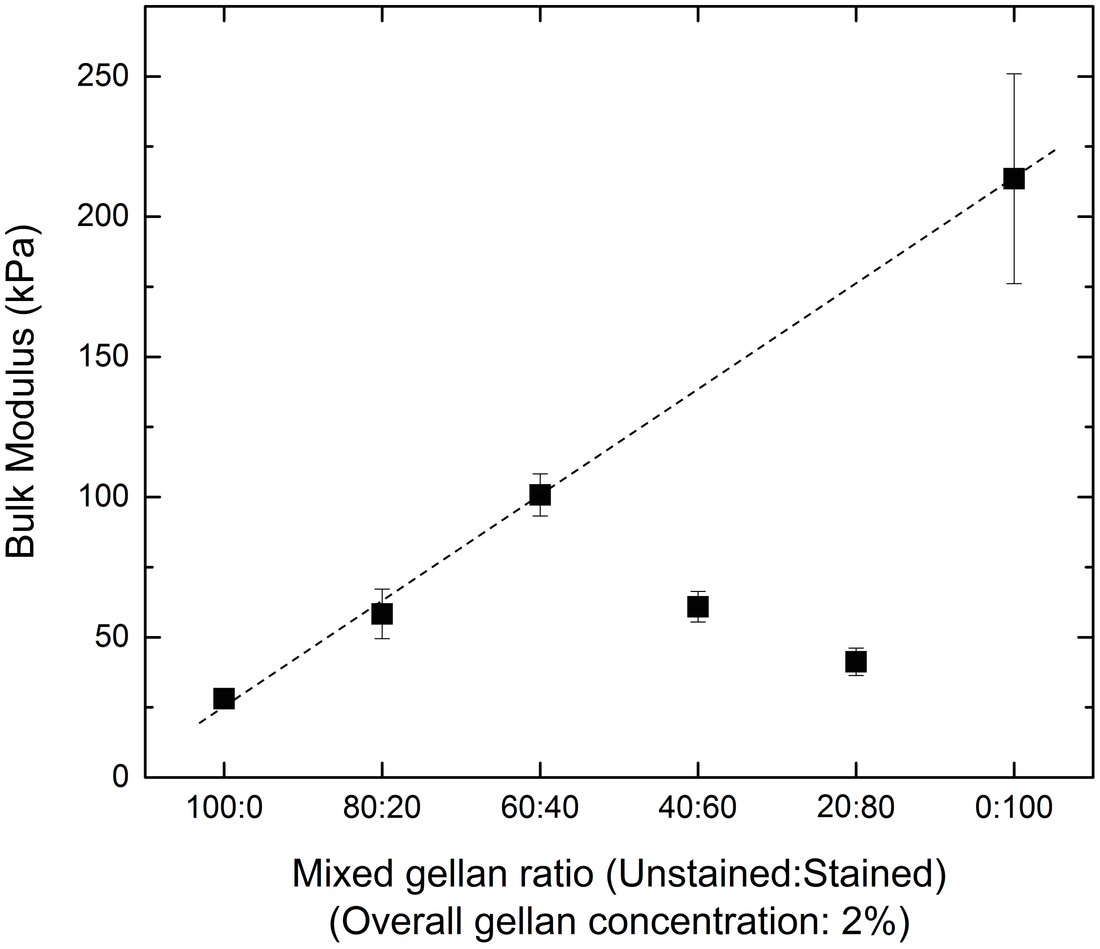
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Mixed gellan ratio (Unstained:Stained)
(Overall gellan concentration: 2%)

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.