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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.

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

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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

2.3.1. 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.

2.3.2. 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 10× magnification lens. Images were all processed using Image J.

2.3.3. 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