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Enhanced gelation properties of purified gellan gum

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Keywords
Gellan gum, purification, gelation, impedance

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Enhanced gelation properties of purified gellan gum

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Abstract

Gellan gum is a hydrogel-forming polysaccharide when combined with monovalent or divalent cations such as sodium, magnesium, potassium or calcium. Commercially, gellan gums are sold with trace amounts of these cations, which have been proven to affect the gelation and mechanical properties of the resultant hydrogels. A new method based on impedance analysis for determining the gel transition temperature of purified and unpurified gellan gum is presented. The sodium salt form of gellan gum is shown to have lower dissolution and gel transition temperatures.

Keywords: gellan gum; purification; gelation; impedance

1. Introduction

Gellan gum is an anionic polysaccharide hydrogel-forming polymer produced from the bacteria Sphingomonas elodea1. Structurally, it comprises a tetrasaccharide repeat unit of two β-D-glucoses, one β-D-glucuronate, and one α-L-rhamnose2 (Figure 1). Gellan gum is available commercially under the trade names Gelrite™ and Kelcogel™ in “high acyl” and “low acyl” forms with the high acyl form being the native state3. The low acyl gellan gum is prepared via alkali treatment of the native gellan gum and is distinctively different in its gelation behaviour and mechanical properties4 - high acyl gellan gum will form a gel upon cooling from 65°C creating a flexible, soft hydrogel while low acyl gellan gum will form a gel upon cooling below 40°C creating a rigid and brittle hydrogel5.

In recent years, low acyl gellan gum has become an attractive biopolymer for applications in tissue engineering as a cellular scaffold because it resembles the natural extracellular matrix.
Gellan gum, like many anionic polysaccharides, forms a physical gel by undergoing a random coil to double helix transition upon cooling. Stronger gels are formed if cations are present during the sol-gel transition. In this case, divalent cations form particularly strong gels through the aggregation of helixes and monovalent cations form intermediate strength hydrogels through electrostatic interactions with carboxylate groups. The presence of divalent cations also inhibits the ability of the un-hydrated gellan gum to become hydrated. In the food industry, it is common practice to add calcium sequestrants (citrates and phosphates) to water to improve the ability of low acyl gellan gum to be hydrated. The presence of cations in commercially provided gellan gum is ordinarily minimal and may not impede their use in food and pharmaceutical applications. However, very small amounts of calcium present in commercial gellan gums may still affect the more sophisticated chemistries used to modify gellan gum for tissue engineering applications. Calcium may also affect the gel transition temperature so significantly that it precludes it from being utilised in rapid prototyping technology.

A method for the rapid purification of gellan gum was established two decades ago which employed an ion-exchange resin to capture the cations present in commercial gellan gum. They reported that after purifying the gellan gum of divalent cations, the acid form gellan gum could be converted to a monovalent salt using a corresponding hydroxide salt. Sodium or potassium gellanate salts were able to be hydrated at much lower temperatures and formed gels of comparable strengths to un-purified gellan gum hydrogels.

The research reported herein elaborates on Doner’s purification method, and provides quantitative information regarding the concentration of sodium, magnesium, potassium, and calcium ions before and after purification; the temperature of hydration and gel transition temperature; and the mechanical properties of hydrogels prepared from purified and un-purified gellan gum solutions.

In this paper, we present a new method based on impedance analysis for determining the gel transition temperature of purified and un-purified gellan gum. Rheological and mechanical compression results are reported.

Figure 1: a) The tetrasaccharide repeating unit of acid-form gellan gum. When deacylated, the indicated acetyl and glyceryl units are removed. b) Photograph of the PVC mould and a typical gellan gum hydrogel sample used for mechanical testing.
2. Materials and Methods

All reagents used in these experiments were AR Grade or better with inorganic contaminants present at 0.01% or lower. Deionised water (DI water) was prepared using a combination reverse osmosis and ion exchange filter (Millipore, Australia) to a resistivity of 18.2 MΩ cm. All glassware was soaked in 10% (v/v) hydrochloric acid (Ajax Finechem, Australia) for 24 hours prior to use to minimise contamination of the purified products and also to minimise contamination during inorganic elemental analysis. Purified gellan gum was prepared from low acyl gellan gum (GELZAN-CM, Lot #1/1443A, CP Kelco, Singapore, pKa = 3.5 ± 0.2, molecular weight ~200 kDa, 0.2% remaining acyl substituents) which was generously gifted from CP Kelco.

2.1. Moisture content

The moisture content of un-purified gellan gum was determined using an infrared moisture determination balance (AD-4712, A&D Company Ltd., Australia). A 5 g sample of gellan gum was heated on the balance to 80°C and weighed periodically until a steady mass was attained.

2.2. FTIR spectroscopy

FTIR spectroscopy of dried samples was performed using a diamond attenuated total reflectance spectrometer (IRAffinity-1, Shimadzu, Japan) with 2 cm⁻¹ resolution and Happ-Genzel apodisation.

2.3. Purification of gellan gum

Gellan gum (3 g) was dissolved in 300 mL of DI water at 80°C whilst being stirred by an overhead mixer (RW 20, IKA, Australia) for 10-15 minutes at 300 rpm. Once dissolved, the gellan gum solution was cooled to 60°C before adding 8 g of cation exchange resin (50WX8 DOWEX, Sigma Aldrich, USA). The mixture was stirred for 30 minutes at 60°C before stirring was stopped and the resin was allowed to settle (pH = 2.2). The supernatant was then decanted and filtered (grade 165, Filtech, Australia) into a chilled reservoir containing 300 mL of 2-propanol (Ajax Finechem, Australia) with rapid stirring whereupon fibrous agglomerates of acid-form gellan gum (A-GG) precipitated. The A-GG was recovered from the 2-propanol solution using vacuum assisted filtration and freeze-dried (Alpha 1-2LDplus, Christ, Germany) for 48 hours to remove residual water and 2-propanol. The purified gellan gum sodium salt (Na-GG) was prepared in the same manner described above excepting that the supernatant, after being decanted, was neutralised with ~10 mL 0.4 M standardised NaOH solution until the pH of the solution reached 7.4 (826 pH-Mobile pH meter, Metrohm, Australia). After being neutralised, the supernatant was precipitated into 2-propanol and freeze dried as previously described.

2.4. Atomic absorption spectroscopy

Atomic absorption spectrometry of gellan gum samples was performed using a flame atomisation atomic absorption spectrometer (flame-AAS, Spectra AA 220FS, Varian, Australia) with hollow cathode lamp light sources for sodium (589.6 nm), potassium (769.9
nm), calcium (422.7 nm) and magnesium (285.2 nm), and an air/acetylene oxidant/fuel mixture.

Samples, blanks and calibration standards were prepared with a 5% (v/v) sulfuric acid matrix with CsCl (Sigma Aldrich, USA) added as ionisation suppressant. Calibration standards were prepared from certified multi-element standard solution (Lot #A2-MEB2366019, Inorganic Ventures, Australia). Samples were digested prior to analysis as follows: Approximately 1.00 g of sample was weighed and transferred quantitatively into a 150 mL Erlenmeyer flask. 5.0 mL of concentrated sulfuric acid (Ajax Finechem, Australia) was then added, followed by heating until the solids dissolved and the solution turned dark brown and started to fume. Hydrogen peroxide (30% v/v, Ajax Finechem, Australia) was then added drop-wise until the solution turned clear. The solution was then allowed to cool to room temperature before having 16 mL of 12.6 mg/mL CsCl solution was added. The solution was then diluted to 100 mL with DI water, mixed thoroughly, and measured within the day.

2.5. Gel transition temperature

The gel transition temperature for solutions of GG and Na-GG was assessed using a custom-designed electrical impedance instrument as well as a rheometer (Physica MCR-301, Anton Paar, Australia) in rotation mode. For electrical impedance analysis, hot gel solutions were poured into rectangular plastic troughs (1 cm x 1 cm x 4.5 cm) with reticulated vitreous carbon foam electrodes (foam structure with 20 pores per inch, relative density 3% or void volume 97%, resistivity 0.323 Ω.cm, ERG Aerospace, USA) placed at either end such that the distance between the electrodes was 2.5 cm. The impedance analysis was performed by applying 1 V peak voltage and alternating current signals from a waveform generator (U2761A, Agilent, USA) to a circuit comprised of the gel sample cell and a 10 kΩ resistor in series while the temperature was measured simultaneously with a digital thermometer probe (Jaycar Electronics, Australia). Impedance across the gel sample cell for frequencies ranging from 100 Hz to 100 kHz were measured as the gel cooled using an oscilloscope (U2701A, Agilent, USA). The gel transition temperature is defined as the point where the slope of the impedance divided by the temperature of the gel changes from one constant to another.

For rheometry based measurements, hot gel solution was sandwiched between a temperature controlled stage (Jaluba, AWC 100) and 50 mm diameter and 1° cone (CP50-1, Anton Paar, Australia) with a gap length of 0.097 mm. The sample was then sheared in rotation at a shear rate of 100 s⁻¹, while the temperature controlled stage was cooled from 70°C to 20°C at a rate of 5°C.min⁻¹. The temperature of gelation was determined graphically, as the temperature at the point where viscosity started the increase dramatically.

2.6. Compressive mechanical analysis

Hydrogel samples for mechanical analysis were prepared by dissolving gellan gum in DI water at 80°C for 30 minutes with gentle stirring followed by addition of 1 M NaCl solution to a concentration of 100 mM or 0.5 M CaCl₂ solution to a concentration of 5 mM. The hot gel solutions were then poured into moulds (17.5 mm diameter x 10 mm high, PVC rings) and cooled to 21°C to form firm hydrogels (Figure 1b). Samples were compressed at a rate of 1 mm.min⁻¹ at 21°C using a universal mechanical testing apparatus (EZ-S, Shimadzu, Japan).
The resulting stress-strain data was used to determine the compressive failure strain ($\varepsilon_c$), compressive secant modulus over 20% - 30% strain ($E_c$), compressive failure stress ($\sigma_c$) and compressive strain energy to failure ($U$).

The swelling ratio ($SW$) of hydrogels were calculated as the mass of the swollen hydrogel ($m_s$) divided by the mass of the dried hydrogel ($m_d$).

$$SW = \frac{m_s}{m_d}, \quad (1)$$

2.7. Statistical treatment of data

Unless otherwise stated, the errors presented herein are the standard deviation of at least three measurements.

3. Results

3.1. Purification of gellan gum

As supplied, the un-purified gellan gum (GG) was a beige coloured powder which dissolved slowly at a concentration of 1% (w/v) at 80°C in DI water to a produce clear, pale yellow solution from which hydrogels of gellan gum could be prepared by cooling the solution to room temperature (21°C). The moisture content of un-purified GG was found to be 1.1 ± 0.2%.

A procedure based on the method described by Doner$^{13}$ was used to purify the gellan gum to produce the acid-form gellan gum (A-GG) with an average yield of 58 ± 2%. This indicates that a significant amount of A-GG may have been lost during transferring and filtration. After drying, the A-GG was in the form of a dry, paper-like fibrous mat which was beige in colour. The solubility of the A-GG in DI water was especially poor and would not dissolve at a concentration of 1% (w/v) at 80°C like GG did. We suggest that this may be because the neutral acid form of gellan gum is less polar than the carboxylate salt form which is present to some extent in commercial gellan gum. Review of the FTIR spectra of GG and A-GG indicates that the peak at 1740 cm$^{-1}$ is only apparent in the A-GG spectrum which is typical of the carbonyl stretching mode of carboxylic acids. This peak is replaced by one at 1600 cm$^{-1}$ in the GG and Na-GG spectra which is typical of the carbonyl stretching mode of a deprotonated carboxylic acid. This suggests that during the purification process, GG became acidified and the cations were stripped away from the carboxylate groups (Figure 2). This hypothesis is also supported by the observation that addition of a small amount of sodium hydroxide (which changes the acid form gellan gum to the sodium carboxylate salt of gellan gum) drastically improved the solubility and clarity of A-GG solutions.

Acid-base titration of A-GG using standardised potassium hydroxide solution (0.01 M, Sigma Aldrich, USA) were conducted to determine the number of cation binding sites available to interact with cations such as Na$^+$, Mg$^{2+}$, K$^+$ and Ca$^{2+}$. The number of cation binding sites was determined to be 1.27 ± 0.08 mmol.g$^{-1}$ which is marginally less than the theoretical number cation binding sites (1.55 mmol.g$^{-1}$).

The purified sodium salt form gellan gum (Na-GG) was prepared with an average yield of 64 ± 6% which is comparable to that previously reported$^{4,13}$. In contrast to A-GG, the dried
product was white and dissolved very quickly and easily at a concentration of 1% (w/v) at 80°C.

![ATR-FTIR spectra of GG, Na-GG and A-GG.](image)

Figure 2: ATR-FTIR spectra of GG, Na-GG and A-GG.

3.2. **Elemental analysis of impurities**

Elemental analysis of A-GG indicates that the purification method effectively removed Na, Mg, K and Ca from GG. The concentration of Na, Mg, K and Ca in GG, A-GG and Na-GG were measured using Flame-AAS (Table 1). The analysis revealed that an appreciable amount of Na, K and Ca was present in the commercial GG which accounts for approximately 6.4% of the dry weight of GG and would be expected to occupy a large proportion of the available cation binding sites. Of particular significance is the concentration of Ca in commercial GG (0.34 ± 0.06 mmol.g⁻¹) which is known to form strong associations with the cation binding sites in gellan gum².

The concentration of Na, Mg, K and Ca present in the A-GG and Na-GG samples were significantly lower than those found in GG (Table 1) which attests to the efficacy of the purification methods. The concentration of Mg and Ca in A-GG and Na-GG were both reduced to below the limit of detection for the method (0.013 mmol(Mg).g⁻¹ and 0.01 mmol(Ca).g⁻¹, respectively). With respect to Na-GG, Mg²⁺ and Ca²⁺ were replaced mainly by Na⁺ and to a smaller extent K⁺; the aggregated concentration of Na and K (1.35 ± 0.05 mmol.g⁻¹) approximately equals the previously measured number of cation binding sites (1.27 ± 0.08 mmol.g⁻¹). At present it is not clear why the concentration of K increases during the conversion of A-GG to Na-GG.
Table 1: Concentration of metal cations in GG, A-GG and Na-GG (±SD). *These concentrations were below the limit of detection for these elements.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>GG</th>
<th>A-GG</th>
<th>Na-GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol (Na).g⁻¹</td>
<td>0.24 ± 0.06</td>
<td>0.0044 ± 0.0004</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>mmol (Mg).g⁻¹</td>
<td>0.048 ± 0.003</td>
<td>&lt; 0.013*</td>
<td>&lt; 0.013*</td>
</tr>
<tr>
<td>mmol (K).g⁻¹</td>
<td>1.14 ± 0.01</td>
<td>0.0240 ± 0.0006</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>mmol (Ca).g⁻¹</td>
<td>0.34 ± 0.6</td>
<td>&lt; 0.01*</td>
<td>&lt; 0.01*</td>
</tr>
</tbody>
</table>

3.3. **Dissolution temperatures**

Purified Na-GG was observed to dissolve at lower temperature than GG or A-GG. The temperature at which 1% (w/v) mixtures of GG, A-GG and Na-GG would dissolve was examined by slowly warming the mixtures from 5°C to the temperature where solutions ceased to be turbid. According to technical data from CP Kelco⁵, GG requires heating to approximately 80°C for full dissolution¹⁵, however we observed that the 1% (w/v) solution of GG went from turbid to clear at a temperature of 57 ± 3°C. A-GG was observed to be completely insoluble even at temperatures up to 90°C; this observation was also noted in Doner’s previous experiments⁴,¹³. It was possible to prepare a clear solution of A-GG by boiling at ~ 96°C, however it is likely this occurred because the gellan gum was depolymerising to a lower molecular weight. Na-GG was observed to dissolve at a temperature of 39 ± 4°C.

3.4. **Impedance analysis of gel transition temperatures**

A new method using a custom-designed electrical impedance apparatus was used for determining the gel transition temperature of 1% (w/v) GG and Na-GG with no added cross-linker, 100 mM Na⁺, and 5 mM Ca²⁺ (these concentrations are typical cross-linker concentrations for the formation of firm gellan gum hydrogels²,¹⁶). In this method, a gel solution was placed in between two electrodes and slowly cooled while the electrical impedance and temperature were measured simultaneously. As the solution cools, the ion mobility decreased and the electrical impedance increased at a linear rate. The gelation event is apparent by a distinct change in the rate of change of electrical impedance, i.e. from 14 ± 1 Ω °C⁻¹ to 60 ± 3 Ω °C⁻¹ (Figure 3a). Using this method, the temperature of gelation for 1% (w/v) Na⁺ and Ca²⁺ cross-linked GG and Na-GG were determined (Table 2).

The gel transition temperature of Na-GG was observed to be lower than that of GG, regardless of the cross-linking cation used. Samples which were cross-linked with 100 mM sodium possessed the highest gel transition temperature of 46 ± 2°C and 45 ± 2°C for GG and Na-GG, respectively. Gellan gums which were cross-linked with 5 mM calcium possessed an intermediate gel transition temperature of 37 ± 2°C and 36 ± 2°C for GG and Na-GG,
respectively. Gellan gums which were not cross-linked with cations also exhibited a gel transition at 30 ± 2°C and 29 ± 2°C (GG and Na-GG, respectively).

For comparison, the gel transition temperature was also measured using a rheometry based method. In the rheometric analysis, samples of gel solution were sandwiched between a temperature controlled stage and a rotating cone. While the temperature dropped, the viscosity of the gel solution was measured and the gelation event was apparent when the viscosity increased dramatically (Figure 3b). There was excellent agreement between the two methods (Table 2). This indicates the validity of the impedance method.

Table 2: Gel transition temperatures of GG and Na-GG with either Na⁺, Ca²⁺ or no added cross-linking ions. T_{impedance} and T_{rheometric} indicate the gel transition temperatures determined using the electrical impedance method and rheometric method, respectively. *T_{impedance} could not be determined for those samples as the gel transition temperature was below the minimum measureable temperature.

<table>
<thead>
<tr>
<th>Added cross-linker</th>
<th>$T_{impedance}$</th>
<th>$T_{rheometric}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG None</td>
<td>*</td>
<td>29.5 ± 2.0</td>
</tr>
<tr>
<td>GG Na⁺</td>
<td>44 ± 1</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>GG Ca²⁺</td>
<td>37 ± 1</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Na-GG None</td>
<td>*</td>
<td>28.5 ± 2.0</td>
</tr>
<tr>
<td>Na-GG Na⁺</td>
<td>44 ± 3</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Na-GG Ca²⁺</td>
<td>36 ± 1</td>
<td>35.5 ± 2.0</td>
</tr>
</tbody>
</table>
Figure 3: Gel transition temperature determinations of a typical Na-GG solution cross-linked with 5 mM Ca$^{2+}$. a) average electrical impedance between 100 Hz and 100 kHz as a function of temperature b) viscosity at 100 s$^{-1}$ as a function of temperature. Straight lines are linear fits of the data.
3.5. Mechanical analysis

Hydrogels prepared from Na-GG were observed to be weaker and less stiff than those prepared from GG. The purified form of gellan (Na-GG, without added crosslinkers) did not form gels that could be analysed. The mechanical properties of hydrogels prepared from 1% (w/v) solutions GG and Na-GG which were cross-linked with either 100 mM Na\(^+\) or 5 mM Ca\(^{2+}\) were examined using compressive mechanical analyses (swelling ratio of all hydrogels was 101). Calcium cross-linked hydrogels were observed to have higher compressive stress to failure and secant moduli than sodium cross-linked hydrogels for both purified and unpurified gellan gums (Figure 4, Table 3). In Doner’s previous report\(^{13}\), it was remarked that hydrogels prepared from the purified Na-GG were of comparable strength to the unpurified gellan gum. However, Doner used different brands/grades of gellan gum (Phytagel from Sigma Chemical Company and GelGro from ICN Biochemicals) which may have had different degrees of acetylation and molecular weights which would be expected to effect the gelation behaviour and gel strength\(^{13}\). Also, the cross-linker concentrations stated are the nominal concentrations resulting from direct addition of Na\(^+\) or Ca\(^{2+}\) solution; the concentration of Na\(^+\) and Ca\(^{2+}\) which were already present in GG were not taken into consideration for these measurements.

![Figure 4: Typical compressive stress-strain curves of hydrogels prepared from GG and Na-GG prepared using 100 mM Na\(^+\) and 5 mM Ca\(^{2+}\) nominal cross-linker concentrations.](image)

Table 3: Compressive mechanical properties of hydrogels prepared from GG and Na-GG (100 mM Na\(^+\) and 5 mM Ca\(^{2+}\) nominal cross-linker concentrations were used). $SW$ – swelling ratio, $\varepsilon_c$ - compressive failure strain, $E_c$ - compressive secant modulus over 20%-30% strain, $\sigma_c$ - compressive failure stress and $U$ - compressive strain energy to failure, *where samples fractured for less than 30% strain, the average modulus over 0-25% strain is presented.
<table>
<thead>
<tr>
<th></th>
<th>SW</th>
<th>$\sigma_c$ (kPa)</th>
<th>$\varepsilon_c$ (%)</th>
<th>$E_c$ (kPa)</th>
<th>$U$ (kJ.m$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (Na$^+$ cross-linked)</td>
<td>101 ± 2</td>
<td>30 ± 5</td>
<td>34 ± 3</td>
<td>114 ± 5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>GG (Ca$^{2+}$ cross-linked)</td>
<td>101 ± 2</td>
<td>130 ± 20</td>
<td>61 ± 3</td>
<td>108 ± 2</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Na-GG (Na$^+$ cross-linked)</td>
<td>101 ± 2</td>
<td>10 ± 1</td>
<td>35 ± 2</td>
<td>41 ± 2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Na-GG (Ca$^{2+}$ cross-linked)</td>
<td>101 ± 2</td>
<td>19 ± 1</td>
<td>25 ± 1</td>
<td>78 ± 3*</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

4. Conclusions

In this paper, a new impedance method for determination of gel transition temperature for commercial gellan gum and purified gellan gum was presented. Commercial gellan gum was shown to possess inorganic cations which affected its dissolution and gelation behaviour as well as the mechanical properties of resultant hydrogels. Purified gellan gum was dissolved fully at approximately 39 ± 4°C and could form a firm hydrogel at approximately the same temperature (36 ± 2°C) in the presence of Ca$^{2+}$ ions. The gel transition temperatures for both commercial and purified gellan gum was higher for gels cross-linked with Na$^+$ compared to Ca$^{2+}$. This paper demonstrates that impedance analysis can be used to provide insights into the gelation behaviour of gel materials containing ions.

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