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An Initial Evaluation of Gellan Gum as a Material for Tissue Engineering Applications

ALAN M. SMITH,^{1,2} RICHARD M. SHELTON,¹ YVONNE PERRIE²
AND JONATHAN J. HARRIS^{1,*}

¹*Biomaterials Unit, School of Dentistry, University of Birmingham
St Chad's Queensway, Birmingham, UK*

²*Medicines Research Unit, School of Life and Health Sciences
Aston University, Aston Triangle, Birmingham, UK*

ABSTRACT: Alpha-modified minimum essential medium (α MEM) has been found to cross-link a 1% gellan gum solution, resulting in the formation of a self-supporting hydrogel in 1:1 and 5:1 ratios of polysaccharide: α MEM. Rheological data from temperature sweeps confirm that in addition to orders of magnitude differences in G' between 1% gellan and 1% gellan with α MEM, there is also a 20°C increase in the temperature at which the onset of gelation takes place when α MEM is present. Frequency sweeps confirm the formation of a true gel; mechanical spectra for mixtures of gellan and α MEM clearly demonstrate G' to be independent of frequency. It is possible to immobilize cells within a three-dimensional (3D) gellan matrix that remain viable for up to 21 days in culture by adding a suspension of rat bone marrow cells (rBMC) in α MEM to 1% gellan solution. This extremely simple approach to cell immobilization within 3D constructs, made possible by the fact that gellan solutions cross-link in the presence of millimolar concentrations of cations, poses a very low risk to a cell population immobilized within a gellan matrix and thus indicates the potential of gellan for use as a tissue engineering scaffold.

KEY WORDS: gellan, hydrogel, bone, cell viability, three-dimensional.

*Author to whom correspondence should be addressed. E-mail: j.j.harris@bham.ac.uk
Figures 1 and 4 appear in color online: <http://jba.sagepub.com>

INTRODUCTION

B iopolymers have received attention as tissue engineering (TE) substrates in the past with several studies examining materials, such as alginate, chitosan, and gelatin as cell scaffolds for both two-dimensional (2D) and three-dimensional (3D) cell culture [1–5]. An advantage in using hydrogel-forming biopolymers for the creation of 3D culture matrices is that the high water content of such materials facilitates the diffusion of nutrient and signaling molecules both to and from the cells seeded within and upon a hydrogel matrix.

Three-dimensional (3D) cell culture as a precursor to tissue creation is necessary because cells do not spontaneously assemble into complex 3D structures after seeding on a flat surface; some form of external support is required. The ability to maintain cells in a viable state, allowing nutrient/waste exchange is a key requirement of any scaffold material employed in this supporting role. The biological relevance of 3D culture as opposed to conventional 2D culture was highlighted by Hishikawa et al. [6] who demonstrated that the spatial relationship between cells seeded within a 3D matrix had a direct bearing upon gene expression.

Many polysaccharide solutions may be converted into gels by exposure to cations. Alginate is cross-linked by Ca^{2+} ions to form a gel with physical properties for a given ratio of guluronic acid: mannuronic acid being determined by the initial solution strength and the concentration of cations available for cross-linking. A variety of studies have demonstrated that incorporation of cells into a liquid solution of alginate, which is then dripped into a salt solution, can result in the creation of alginate beads with immobilized cells that remain viable within the 3D construct [7]. However, while such approaches have demonstrated that cells may be maintained in a viable state, there exists the risk that viability may be compromised through exposure of cells to relatively high ionic concentrations during and after cross-linking to obtain gels of suitable strength (as might be necessary where constructs of greater complexity than a bead are required). Other routes to cell immobilization have explored the possibility of thermal gelation phenomena in alternative polysaccharides and again, although the extent of cell viability has been encouraging it would intuitively be advantageous to avoid exposure of cells to fluctuations in temperature in order to maximize the chance of survival.

Gellan gum is an extracellular polysaccharide, secreted by the bacteria *Sphingomonas paucimobilis* (formerly *Sphingomonas elodea*) during

aerobic fermentation [8] that has been extensively employed within the food industry as a stabilizer and thickening agent and is commonly referred to in this application (within the European Union) as E418. At elevated temperatures (e.g., $\approx 80^{\circ}\text{C}$ for a 1% solution) the polymer exists in solution as a disordered coil which is converted to a double helix structure [9,10] when cooled. This helical structure is not a true gel network although in this ordered state gellan can exhibit 'weak gel' characteristics. Formation of a true gel network is reliant upon aggregation of helical sequences, which on the basis of current evidence [11] can occur via different mechanisms dependent on the cation used in cross-linking; divalent cations, such as Ca^{2+} and Mg^{2+} form direct bridges by site binding between pairs of double helices, monovalent ions, such as Na^{+} and K^{+} suppress repulsion by binding to the surface of the helices balancing the negative charge of the carboxyl groups. The concentrations required to induce gelation are therefore different and depend on the gellan concentration, but typically, concentrations of $\approx 5\text{ mM Ca}^{2+}$ and Mg^{2+} , $\approx 100\text{ mM}$ for Na^{+} and K^{+} would be required for cross-linking.

In addition to various forms of gellan used within the food industry, gellan gum is commercially available in a form that is guaranteed to be free of endotoxin allowing for its use in medical formulations. The main medical application to date has been for controlled drug delivery both as an encapsulating agent [12] and as an active ingredient in an ophthalmic formulation that gels on exposure to the tear film, thus ensuring the medications remain suitably located in the eye [13]. Gellan has been used in plant tissue culture [14,15], bacterial culture [16] and has been evaluated as a component of a composite material for mammalian TE [17,18]. However, there have been no reports of gellan being used exclusively for the immobilization and culture of mammalian cells. The present study investigated whether exposure of gellan gum to αMEM would result in gelation, with a view to possible application in TE where cell immobilization within a synthetic extracellular matrix was required.

MATERIALS AND METHODS

Preparation of Polysaccharide Solutions

Endotoxin-free low-acyl gellan gum (Gelrite, CP Kelco Inc, USA, Lot#: 4I1690A) was dissolved in deionized water (dH_2O) at 80°C with continuous stirring to a solution strength of 1% wt/wt.

Cell Isolation and Incorporation into Gellan Constructs

Rat bone marrow cells (rBMC) were isolated and cultured according to the method described by Maniatopoulos et al. [19]. Briefly, femora from mature albino Wistar rats (around 120 g in weight) were dissected out removing as much as adherent soft tissue as possible. The epiphyses were removed and the femora were repeatedly flushed with minimum essential medium (α MEM) (Sigma, UK) containing 10% fetal bovine serum (FBS), 2.5% HEPES, 10% penicillin/streptomycin (P/S), and 1% amphotericin. Cells were then incubated in a 75 mL flask using supplemented α MEM medium containing 10% FBS, 2.5% HEPES, 1% P/S in a humidified atmosphere of 95% air, and 5% CO₂ at 37°C. After 7 days of primary culture, cells were dissociated with 0.25% trypsin-0.02% EDTA (Sigma, UK). The cells were centrifuged at 1500 rpm (400 g) for 5 min before resuspension at a density of 2.3×10^5 cells/mL in α MEM and mixed with 1% gellan gum followed by light agitation to promote mixing of the two solutions.

Assessment of Rheological Properties

Rheological analysis was carried out in the linear viscoelastic region using a 1°/55 mm cone and plate geometry mounted on a Malvern Gemini Rheometer (Malvern Instruments, UK) fitted with Peltier plate thermal control. Temperature sweeps were conducted at a rate of 1°/min from 60 to 10°C, with a fixed oscillation frequency of 1 rad/s and a strain of 1%, while frequency sweeps to assess the mechanical properties of the material at varying strain rates were conducted at an isotherm of 37°C, in auto-stress mode with a fixed strain of 1%.

Assessment of Cell Viability

Cell viability was evaluated using a Live/Dead Viability/Cytotoxicity Kit [L-3224] (Molecular Probes, Invitrogen, USA) following culturing rBMC immobilized in 1 mL gellan matrix for periods of up to 21 days. This stain reflects the uptake of calcein AM and conversion to fluorescent calcein (green) by intracellular esterases in viable cells and the binding of ethidium bromide (red) to the DNA of cells with damaged plasma membranes.

RESULTS

Construct Formation

Where α MEM and gellan were mixed in equal quantities prior to extrusion through an aperture of 1.7 mm a mechanically unstable composition resulted which rapidly dissociated into isolated lumps of gel in an excess of α MEM. The proportions were varied before identifying 0.2 mL cell suspension in 1 mL 1% gellan solution (1:5 volume ratio) as an appropriate mixing regime to produce self-supporting constructs. Mixing of the gellan solution with cell suspension was carried out thereafter within a 5 mL disposable syringe at 37°C, the mixture being subject to vibratory mixing before being extruded into an excess of α MEM to form self-supporting cylindrical structures as illustrated in Figure 1.

Rheological Characteristics

Measurements of storage (G') and loss (G'') modulus in samples of 1% gellan are provided in Figure 2 which depicts the changes in rheological

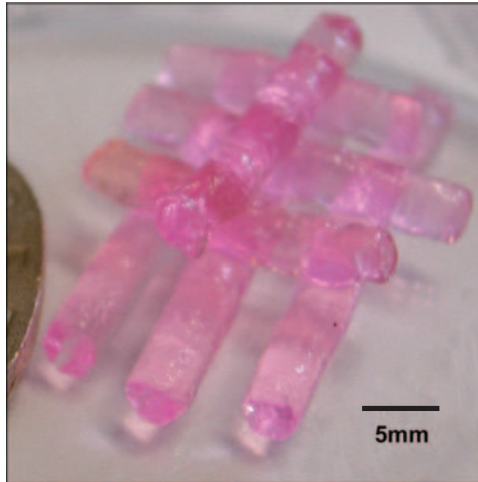


Figure 1. Construct creation. Photograph illustrating the self-supporting nature of gellan cylinders created through extrusion of 1% gellan gum solution from a 5 mL syringe (aperture 1.7 mm) into α MEM. The cylinders produced were manually positioned in this example, demonstrating that cylinders were not only self-supporting, but were sufficiently robust to permit handling with forceps.

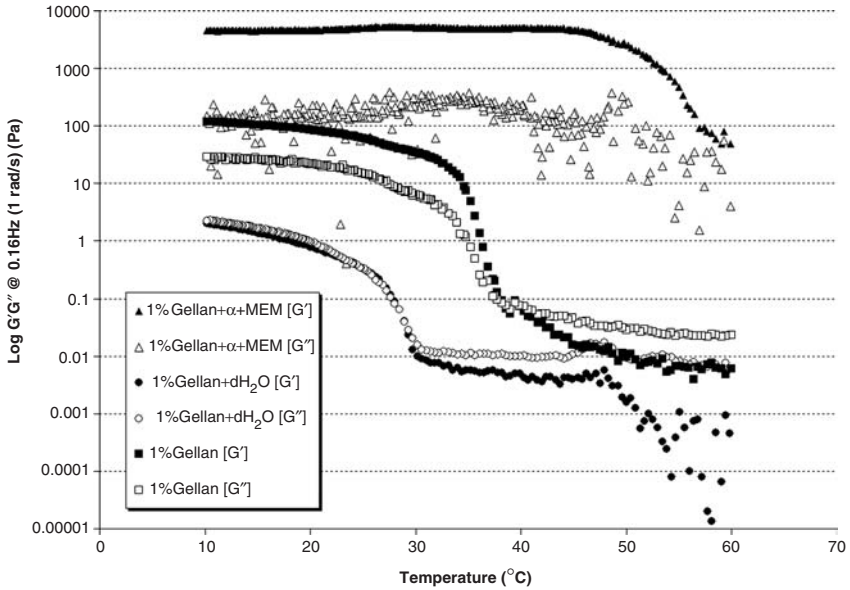


Figure 2. Rheology: temperature sweeps. The elastic (storage) and viscous (loss) moduli (G' and G'' , respectively) of a 1% gellan solution as compared with a specimen containing equal volumes of 1% gellan and α MEM and another containing equal volumes of 1% gellan and distilled water (dH₂O). Of significance is that where 1% gellan was supplemented with an additional liquid the same volume of liquid was used in both cases; however, in the case of α MEM the ionic concentration was sufficient to outweigh the dilution that took place.

behavior on cooling of 1% gellan, 1% gellan with an equal volume of α MEM, and a control specimen comprising a 1:1 mixture of 1% gellan with distilled water (dH₂O). Mechanical spectra derived from frequency sweeps are shown in Figure 3, illustrating behavior consistent with a solution (1% gellan with dH₂O), a weak gel (1% gellan), and a true gel in which G' is independent of frequency (1% gellan with α MEM).

Cell Viability

Constructs arising from 1:1 mixtures of gellan and cells suspended in α MEM exhibited nearly 100% viability at 3, 6, 10, and 21 days (10 day culture illustrated in Figure 4). Although occasionally cells fluorescing red were visible amongst the predominance of green fluorescence, they were isolated and were only identified following lengthy and detailed examination of the constructs and did not correspond to any particular location within the constructs. Validation of the live/dead assay in terms

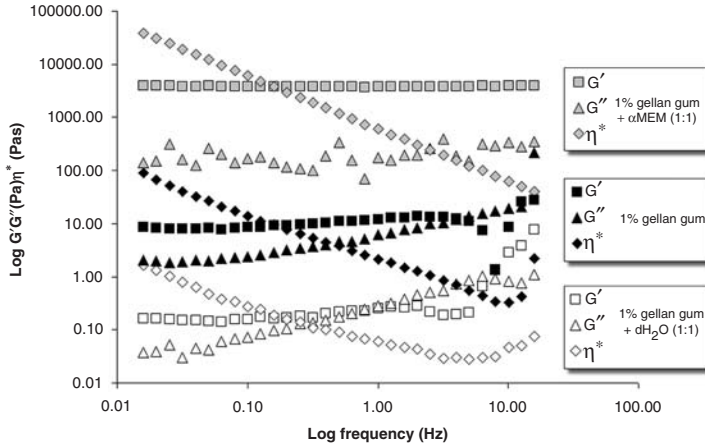


Figure 3. Rheology: frequency sweeps. Mechanical spectra illustrating (A) solution behavior of a specimen of 1% gellan mixed with an equal volume of dH₂O; (B) the weak gel characteristics of a 1% gellan solution; and (C) the true gel characteristics of a specimen comprising 1% gellan with an equal volume of αMEM. All measurements were carried out at 37°C. Elastic modulus (G'), storage modulus (G'') and complex viscosity (η^*) are plotted against the oscillating frequency for each specimen analysed.

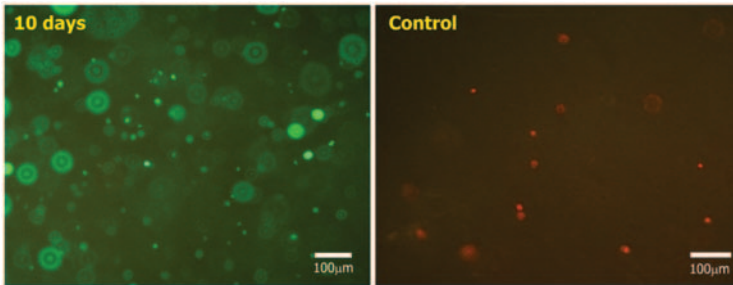


Figure 4. Cell viability. Photomicrographs obtained using fluorescence microscopy illustrating (left) rBMC immobilized within a gellan gum matrix, compared with the experimental control (right) in which rBMC immobilized within a gellan gum matrix were fixed with ethanol prior to performing the assay. Exclusively green fluorescence (left) indicated intracellular esterase activity while red fluorescence (right) indicated binding of the ethidium bromide homodimer with the nucleus of cells with disrupted plasma membranes. This result confirmed that a gellan gum matrix does not inhibit the diffusion of the ethidium bromide components of the assay to cells within the matrix.

of ensuring that the gellan construct did not inhibit the diffusion of the ethidium bromide component to entrapped cells was carried out by immersing a portion of a construct in 70% ethanol for 15 min to bring about cell death. The assay was then performed and on inspection was

found to have resulted in exclusively red staining being observed under fluorescence microscopy (Figure 4) indicating a lack of integrity of the plasma membrane highlighted by binding of the ethidium bromide homodimer with the nucleus of affected cells, in turn supporting the veracity of the assay when used upon 1% gellan gum constructs containing cells.

Self-supporting cylindrical constructs arising from 1:5 mixtures of α MEM: gellan demonstrated a more mixed response when assayed, showing an increased tendency for cell aggregation in comparison to the 1:1 mixtures, as might be expected given the cell density of the suspension employed was identical in both cases (2.3×10^5 cells/mL). Some additional 1:5 constructs were seeded with substantially increased cell density of 7.9×10^6 cells/mL, equating to a total of 1.58×10^6 cells per 1.2 mL gellan construct. In this case a more aggressive vibratory mixing regime was employed wherein an air bubble was incorporated into the mixture to act as a mixing ball, in an attempt to improve the distribution of cells within the construct. Although live cells were visible throughout the 1:5 constructs, the density of cells within the gellan matrix was much reduced (initial suspension density 2.3×10^5 cells/mL). In addition, evidence of cell death was observed, typically in areas where multiple cells were clustered together in a relatively small volume. However, where the higher seeding density (7.9×10^6 cells/mL) was employed in conjunction with vigorous mixing this problem appeared to be eliminated; such constructs exhibited even cell distribution and a similar predominance of green fluorescence as had been observed for the original 1:1 mixtures.

DISCUSSION

Construct Formation

The mechanical instability of constructs comprising 1:1 gellan: α MEM is likely to have arisen from disruption of helical junction zones within the gel matrix during extrusion due to the shear forces that would have been induced within the polysaccharide matrix. In contrast, reduction of the availability of cations resulting from a reduced volume of α MEM added to gellan (0.2 mL in 1 mL, respectively) allowed formation of gelled cylinders on extrusion into excess α MEM. A possible reason for the difference was due to the gellan being in the ordered form but not fully aggregated in the 1:5 mixture, thus a reduced proportion of junction zones were disrupted on shearing. At a 1:1 ratio full aggregation had occurred, which could not be recovered post-shear

in spite of an excess of α MEM being present; it is inferred that cations had occupied all available binding sites on the gellan molecule. Where reduced quantities of α MEM were added followed by mixing, a heterogeneous mixture of gelled regions (aggregated gellan helices) within a solution of ordered but not aggregated gellan helices was formed. It is proposed that subsequent extrusion into excess α MEM resulted in aggregation of polymer helices through ionotropic cross-linking induced as the fresh supply of cations began to diffuse into the partially formed construct, entrapping the previously aggregated helices in a gelled matrix. Indirect evidence existed to support this suggestion as a degree of cell aggregation is noted in 1:5 mixtures that was absent in the 1:1 mixtures, where the distribution of cells was considerably more uniform throughout the construct. This was probably related to the fact that cells were introduced as a suspension in α MEM and so became entrapped within a cation-rich region of gellan that was subsequently shattered upon mixing and dispersed through the ungelled remainder, as discussed above. However, with the use of a more aggressive mixing regime involving an incorporated air bubble these problems were eliminated; cell distribution improved to a point where no differences could be seen between the 1:1 and 1:5 constructs.

Cross-linking of gellan with α MEM occurred as a result of the availability of cations within α MEM, which as supplied by Sigma Aldrich UK were present in the following concentration: NaH_2PO_4 (1 mM), NaCl (116 mM), KCl (5.4 mM), MgSO_4 (0.75 mM), and CaCl_2 (1.8 mM). It is presently unclear precisely which cations were responsible for gelation; however, it has previously been demonstrated that all of those listed as constituents of α MEM are capable of causing gelation of 1% solutions of gellan [20], with stronger gels resulting from gel formation in the presence of Ca^{2+} and Mg^{2+} as opposed to the monovalent cations, such as Na^+ and K^+ .

Rheological Characteristics

Temperature sweeps (Figure 2) illustrated shifts in the temperature at which the onset of gelation occurred within each sample. In the case of 1% gellan and 1% gellan diluted with dH_2O the several orders of magnitude increases in G' were consistent with disordered-to-ordered transitions within the polysaccharide chains, while aggregation of ordered helices occurred where cations were present from the addition of α MEM, pushing the gelation temperature above 50°C . With the addition of dH_2O the increased dilution of the gellan solution (effectively creating a 0.5% gellan solution) resulted in a reduction in the

temperature at which increases in G' were observed, those increases being consistent with the adoption of helical conformations by polysaccharide chains. Where α MEM was added to gellan an identical volume to that of dH_2O in the control was employed, hence in effect the system was diluted to an equal extent in the case of α MEM as originally for dH_2O . However, the supply of cations from α MEM was sufficient to induce aggregation of helical domains (cross-linking) which outweighed the dilution that took place in terms of the response of the material to applied strain. The mechanical spectra illustrated in Figure 3 supported this finding; gellan with α MEM in a 1:1 ratio had characteristics of a true gel, namely that G' was clearly dominant over G'' and was independent of the frequency of oscillation. In contrast, the mechanical spectra of 1% gellan suggested the formation of a weak gel that arose through the adoption of helical domains within gellan chains, but no subsequent aggregation due to a lack of available cations. The frequency sweep of gellan with dH_2O exhibited no gel characteristics whatsoever, instead this mechanical spectrum was typical of a solution of entangled polymer chains. Noticeable for both 1% gellan and 1% gellan + dH_2O were some significant fluctuations in measured G' and G'' values where frequency < 5 Hz, commensurate with spurious interpretations of inertia during rheological testing, probably arising from the combination of relatively high frequencies of oscillation and relatively low viscosities of the specimens under test.

Cell Viability

Three-dimensional cultures based on 1:1 mixtures assayed for intracellular esterase activity or damaged plasma membranes revealed the presence of live cells at all depths within the construct, for all culture times. In contrast, some cell death was observed where cell clustering had occurred in the 1:5 gelled mixtures, which was eliminated on mixing as has been previously discussed. The difference in the spatial distribution of cells within the two types of construct before the more aggressive mixing regime was adopted is likely to be behind the observed differences in viability, as it is well known that cell aggregates are prone to apoptosis.

In general terms the survival of cells within gellan constructs is highly encouraging with respect to the future prospects for the use of gellan gum for TE purposes. However, in isolation live/dead assays provide a rather limited assessment of the impact on cells of immobilization in a gellan matrix and certainly do not address the question of how a

mammalian host would respond to the implantation of cell-loaded gellan constructs, which is a long-term aim. However, at the present stage the authors are primarily interested in ways in which culture of cells in three dimensions can be achieved for the purposes of *in vitro* study, and to this end gellan exhibits some promise.

GENERAL DISCUSSION

The potential for gellan in TE goes far beyond what can be achieved in terms of cell viability in a 1% solution cross-linked with α MEM. Variations in the structure of the tetrasaccharide molecule (i.e., high or low acyl forms, which may be mixed in a range of ratios) provide for extensive tailoring of rheological characteristics; the respective formation of everything from soft elastic gels to firm brittle gels. Consequently, there is scope for creating culture matrices of varying gel texture that will allow exploration of the response of cells to the texture of a 3D environment in which they are immobilized along the lines of recent studies [21–24], just as the response of cells to textured surfaces has been extensively explored with 2D cultures [25–28]. Control over rheological properties suggests that gellan could be used both in injectable and indirect (*in vitro* culture followed by implantation) approaches to TE, with injectable routes taking advantage of the gelation behavior of gellan under physiological conditions. Inspection of immobilized cells to considerable depths using conventional microscopy is aided by the excellent optical clarity of gellan gels and the capacity to process a cell loaded construct using polymerase chain-reaction (PCR) to identify markers of gene expression could be greatly aided by the fact that gellan gum appears not to inhibit PCR [29] unlike other polysaccharides, such as alginate and agar [30,31].

CONCLUSIONS

Gellan gum represents a potentially useful addition to the range of polysaccharide hydrogels that may be employed as tissue engineering (TE) substrates, particularly given that media-driven gelation or gelation under physiological conditions represents a very low risk route to a cellular payload. There is considerable scope for further work to more fully explore the potential of gellan gum for TE and to exploit the wider research possibilities offered by this extremely simple route to cell entrapment in a 3D hydrogel matrix.

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