Controlling the Rheology of Gellan Gum Hydrogels in Cell Culture Conditions

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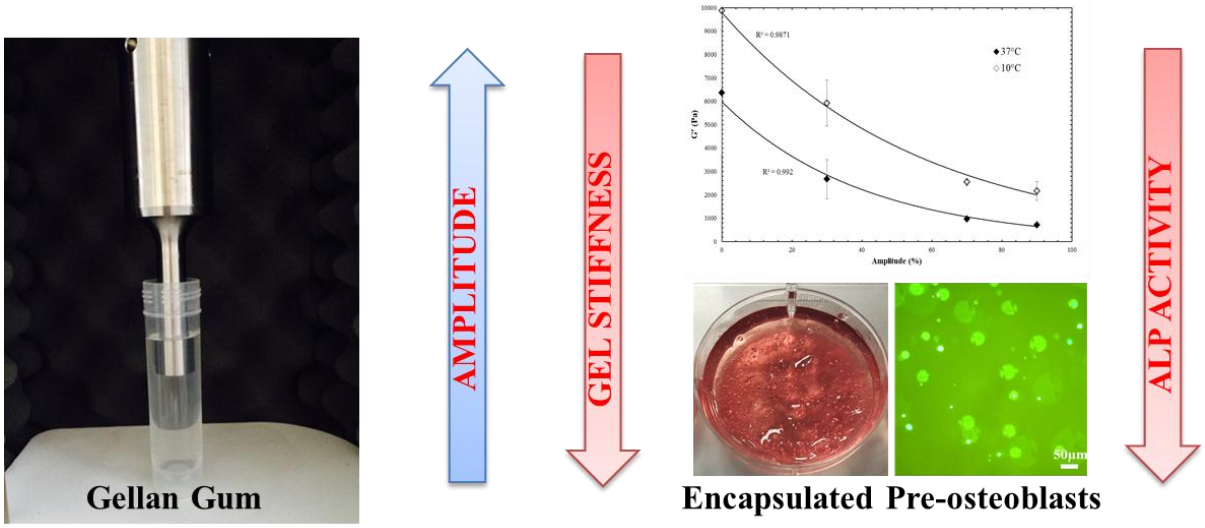
Abstract

Successful culturing of tissues within polysaccharide hydrogels is reliant upon specific mechanical properties. Namely, the stiffness and elasticity of the gel have been shown to have a profound effect on cell behaviour in 3D cell cultures and correctly tuning these mechanical properties is critical to the success of culture. The usual way of tuning mechanical properties of a hydrogel to suit tissue engineering applications is to change the concentration of polymer or its cross-linking agents. In this study sonication applied at various amplitudes was used to control mechanical properties of gellan gum solutions and gels. This method enables the stiffness and elasticity of gellan gum hydrogels cross-linked with DMEM to be controlled without changing either polymer concentration or cross-linker concentration. Controlling the mechanical behaviour of gellan hydrogels impacted upon the activity of alkaline phosphatase (ALP) in encapsulated MC3T3 pre-osteoblasts. This shows the potential of applying a simple technique to generate hydrogels where tissue-specific mechanical properties can be produced that subsequently influence cell behaviour.

Key Words: Tissue engineering, Gellan gum, Alkaline Phosphatase.

Graphical Abstract



1. Introduction

Tissue engineering is growing evermore as a method for addressing the degradation/failure of tissues and organs. Part of the increased demand for such methods comes from the improved life span of the global population as a result of medical advancements and improvements in technologies to prolong life. Tissue and organ donation is currently a method that is heavily relied upon to treat patients with diseased/degraded tissue but donor availability is vastly outpaced by patient demand [1].

In order to engineer tissues three dimensional culture is required, as the orientation of cells in a tissue has a very integral effect on their phenotype/behaviour. As a result, using polysaccharide cell culture scaffolds has become a very popular method for engineering tissues *in vitro* [2].

One particular class of substrates that have shown promise are hydrogels. Within this area of study, a lot of interest has been in using hydrogel structures to engineer complex tissues from mesenchymal stem cells (MSC's) due to their multipotency. It is possible to encapsulate MSC's within a hydrogel and trigger differentiation into a variety of tissue types by stimulating the cells with specific differentiation cues [3] [4] [5].

Hydrogels used for tissue engineering are often over 30% water [6]. They are excellent tools for tissue engineering because they contain a microstructure similar to that of extracellular matrix (ECM). They essentially provide a scaffold around which tissues can be engineered with an architecture that is reflective of the native tissue being targeted. This ability is essential to successfully applying tissue engineering as a medical method [7].

The high water content of hydrogels makes them extremely porous. This facilitates diffusion of differentiation cues and essential cell nutrients through the structures.

Gellan gum (gellan) is a polysaccharide that has generated lots of interest in pharmaceutical and biomedical applications due to its advantageous physicochemical properties [8]. It is produced by the bacterium *Sphingomonas elodea* which is a Gram negative bacterium. It has a chemical structure comprised of a repeating tetrasaccharide unit consisting of → 4)-l-rhamnopyranosyl-(α-1 → 3)-d-glucopyranosyl-(β-1 → 4)-d-glucuronopyranosyl-(β-1 → 4)-d-glucopyranosyl-(β-1 →. The polymer carries a net negative charge due to the carboxylic groups present in the glucaronic acid residues which is central to the ability of gellan to form hydrogels. In order to form strong, durable hydrogels cations are used, with divalent cations creating stronger gels than monovalent cations. It has been previously shown however, that the range and concentration of ionic species present in Dulbecco's Modified Eagles Media (DMEM) (Table 1) are sufficient for gellan gelation and therefore provides a very simple method of 3D cell immobilisation within a thermoreversible and non-cytotoxic hydrogel [9] [10] [11].

The gelation mechanism is a two part process that occurs when a hot solution of gellan is cooled. Initially, molecules of gellan undergo a very rapid phase transition from random coiled structures to more ordered double helices [12] [13]. This occurs in gellan regardless of whether or not ions are added. The addition of cations however, results in a second phase of the process where helices aggregate and form a complex 3D network [14].

The modulus and elasticity of a gel are key to the successful engineering of a tissue. Certain tissue types develop much more efficiently in stronger, stiffer gels i.e. gels with a much

higher elastic modulus (G′) while other tissue types are more suited to weaker, softer gels with a lower G′ value. This has previously been demonstrated in mesenchymal stem cell differentiation [15]. For example, it has been shown that engineering of bone tissue requires a strong and relatively stiff gel [16]. Conversely, the engineering of tissues such as cardiac or corneal tissue requires a matrix to exhibit a much more soft and elastic environment [17] [18]. In order to optimise a hydrogel for culturing a specific tissue these factors must therefore be considered. Therefore, by tailoring the mechanical properties of a hydrogel to suit that of the tissue to be cultured, an *in vitro* mechanical behaviour can be produced that is more reflective of the *in vivo* environment. Generally, the main ways to achieve variations in mechanical properties of hydrogels are either through varying the concentration of crosslinking agents or varying the polymer concentration. This can also impact on other factors however such as porosity and permeability as well as the osmotic environment. A method of tuning mechanical properties without varying the formulation would be beneficial to the field.

Mouse calvaria-derived/MC3T3-E1 cells are widely used in bone culture models. As osteoblast pre-cursors, MC3T3's have the ability to express multiple markers associated with osteogenic differentiation [19] [20] [21]. One such marker is the expression of alkaline phosphatase/ALP which plays a key role in the deposition of calcium mineral deposits during osteogenesis. ALP promotes mineralisation by decreasing extracellular concentrations of pyrophosphate and increasing the concentration of inorganic phosphate [22]. A colorimetric assay based on hydrolysis of p-nitrophenyl phosphate to p-nitrophenol by ALP can be used to determine its activity [23]. Since matrix stiffness is integral to the success of tissue culture, varying mechanical properties could have an effect on expression of differentiation markers.

In this study sonication was investigated as a mechanism to tune mechanical properties of gellan gum hydrogels crosslinked with cell culture media without altering concentrations used in the gelation process. The effect of tuning matrix stiffness via sonication on ALP activity and viability of encapsulated MC3T3 cells was investigated.

2. Materials and Methods

2.1. Materials

Cell culture plastics were purchased from Sigma-Aldrich (UK). Differentiation supplements and TrypLE™ dissociation enzyme were purchased from Thermo Fisher Scientific (UK). Gellan gum was purchased from Kalys (UK). All other reagents and cell culture media and supplements were purchased from Sigma-Aldrich (UK) and used without further purification.

2.2. Preparation of gellan solutions

Batches of 200 mL low acyl gellan (Kalys, UK) were prepared at a concentration of 1% w/w by dispersing the powdered low acyl gellan gum in deionised water at 85 °C while stirring at 900 rpm until fully dissolved. Once in solution any water lost to evaporation was replaced and the sample was allowed to quiescently cool to room temperature.

2.3. Sonication of gellan solutions

The 1 % w/w prepared solution of gellan gum was split into 20 mL aliquots for sonication.

Sonication was conducted using a Sonics Vibra Cell 20 kHz ultrasonic probe.

Samples were sonicated for 5 min with a 1 s on/off pulse resulting in a total run time of 10 minutes at 20°C. The amplitude of the ultrasonic waves used during the procedure was varied

from 20-100% between the samples i.e. a sample was only ever sonicated at one specific amplitude.

2.4. Intrinsic viscosity

The intrinsic viscosity of all samples was determined using an Oswald viscometer (Rheotek, UK). The viscometer was immersed fully in a water bath at 25 °C to ensure all samples were tested at a constant temperature. Triplicate samples with concentrations ranging from 0.02% to 0.06% gellan gum were prepared in 10 mM NaCl solution; used to counteract the electro-viscous effect that is known to occur with gellan gum prepared in deionised water [24]. The relative, η_{rel} and specific viscosities, η_{sp} were calculated as described in equations 1 and 2, respectively:

$$\eta_{rel} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) \quad (1)$$

$$\eta_{sp} = \eta_{rel} - 1 \quad (2)$$

where t is the average (of 3 replicates) flow time of the gellan solution at each concentration, t_o is the flow time for the 10 mM NaCl solution, ρ is the density of the gellan solution at each concentration and ρ_o is the density of the 10 mM NaCl solution. Due to the low concentrations used, $\rho/\rho_o = 1$ [25].

Measurements were extrapolated to infinite dilution using both equations 3 [26] and 4 [27]:

$$\frac{\eta_{sp}}{c} = [\eta](1 + K_H[\eta]c) \quad (3)$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K[\eta]c) \quad (4)$$

where the intrinsic viscosity [η] is taken as the is the mean of the intercepts from equations (3) and (4) and K_H and K_K are the Huggins and Kraemer constants respectively [26-27].

2.5. Viscosity Measurements

Dynamic viscosity of 0.5% w/w gellan samples was measured under an increasing shear rate from 0.1 s^{-1} to 1000 s^{-1} using a 2° / 55 mm cone and plate geometry and an isothermal temperature of 20 °C using a Bohlin Gemini rheometer (Malvern, UK). The concentration of 0.5% w/w was used to remain consistent with the concentrations used in the cell culture experiments (Section 2.7).

2.6. Preparation of samples for oscillatory rheological analysis during cooling

For rheological analysis of gellan gum mixed with Dulbecco's Modified Eagles Media (DMEM - Sigma, UK) 1% solutions of each sample were first immersed in at water bath at 95°C in sealed bottles (to prevent water loss through evaporation) to ensure the gellan was in the disordered form. Samples were then mixed with unsupplemented DMEM (which provides the gel promoting ions) in a 1:1 ratio resulting in final concentrations of 0.5% gellan gum. Samples were then placed immediately back into the water bath to prevent gelation prior to rheological analysis.

2.7. Oscillatory rheological analysis

Oscillatory rheological analysis was used to measure the effect of sonication on the mechanical properties of the gellan. Stress sweeps were used to determine the extent of the linear viscoelastic region (LVR) and the critical onset of non linearity, cooling scans were

performed to elucidate any differences in gelation temperature and frequency sweeps were performed to assess the effects on the mechanical spectra.

2.7.1 Determination LVR and the critical onset of non linearity

1% solutions of un-sonicated gellan and gellan sonicated at 100% amplitude as described in section 2.2 were gelled in a 1:1 ratio with DMEM. The gels were set for 30 minutes at 4°C in 6 well plates (Sigma, UK) prior to analysis on a Bohlin Gemini rheometer.

Stress sweeps were conducted at a constant temperature of 25°C using a 25 mm parallel plate geometry (serrated to prevent slippage) with a gap of 1 mm which equated to the thickness of the loaded gel. Shear stress was increased from 1 to 1000 Pa at an oscillation frequency of 10 rad s^{-1} and the resulting moduli (G′ and G″) were measured.

2.7.2 Cooling scans of gellan samples

Analysis of gel modulus during cooling was carried out within the liner viscoelastic region using a Kinexus rheometer (Malvern, UK) fitted with Peltier plate thermal control. A 2°/55 mm cone and plate geometry was used and temperature sweeps were conducted at a cooling rate of 2 °C/min from 90 to 10°C. The oscillation frequency was fixed at 10 rad s^{-1} with a constant strain of 0.5%. The strain amplitude chosen was within the linear viscoelastic region of the sample determined by amplitude sweeps and moisture evaporation was minimised during the measurements by coating the periphery of the sample with light silicone oil and using a solvent trap on the geometry.

2.7.3 Frequency sweeps in cell culture conditions

Hydrogels were prepared as in cell culture conditions and then mechanical spectra measurements were performed to show variations in the gel strength. 1% low acyl gellan was mixed 1:1 with DMEM (3 mL with 3 mL) in a 6 well plate at 37°C resulting in a final gellan concentration of 0.5 %w/w. Plates were shaken lightly to promote mixing and left to form gels for 30 minutes. Frequency sweeps were conducted on a Kinexus rheomter with a 20 mm

parallel plate geometry. Elastic and viscous moduli were measured at frequencies of 1-100 rad s^{-1} with a constant temperature of 37°C.

2.8 Cell Culture

2.8.1 Encapsulation of MC3T3's in sonicated gellan

MC3T3 pre-osteoblastic cells were cultured in T75 flasks until confluent in supplemented DMEM (10% FBS, 2.5% L-glutamine, 2.5% HEPES buffer and 1% penicillin/streptomycin – Sigma UK). Media was aspirated and cells were washed with 10 mL DPBS before being trypsinised with 2 mL TrypLE™ (Life Technologies) and incubated at 37°C for 5 minutes. The reaction was stopped with 8 mL of supplemented DMEM. Cells were counted, centrifuged at 1000 RPM for 3 minutes, resuspended at a density of $2x10^6$ cells/mL and mixed with gellan in a 1:10 ratio of cells to gellan. Gellan (1 %w/w) was then mixed with DMEM in a 1:1 ratio to trigger gelation to a cell loaded construct. Gellan exposed to three different amplitudes of sonication was used for encapsulation (0%, 50% and 100%). Cells were cultured for 7 days using supplemented DMEM and media was changed every 3 days. The resulting hydrogels can be seen in Fig 7.

2.8.2 Live/dead staining

A fluorescent staining protocol using calcein AM and propidium iodide was used to analyse the proportion of live and dead cells within each gel sample. Media was aspirated from the surface of each gel before washing with DPBS. Fresh media was added along with 5 µl calcein AM and 15 µL propidium iodide. Samples were incubated for 30 mins at 37°C before visualisation with fluorescence microscopy.

2.8.3 Alkaline phosphatase activity

To measure activity of alkaline phosphatase in the encapsulated cells a StemTAG™ colourmetric alkaline phosphatase assay kit was used (Cambridge Bioscience). Excess media

was aspirated from the gel surface before washing with 5 ml cold DPBS. Cells were lysed at 4°C for 20 minutes with cell lysis buffer. The solution was removed and centrifuged at 12,000 Xg for 10 minutes and the supernatant was removed as cell lysate. 50 µL of lysate from each sample was added to a 96-well plate in triplicate. The reaction was initiated with the addition of 50 µL of StemTAG™ AP Activity Assay Substrate (p-nitrophenyl phosphate) and incubated for 30 minutes at 37ºC. Absorbance was read at 405 nm and compared with a standard curve of absorbance vs p-nitrophenol concentration. Results were standardised to concentration of p-nitrophenol yielded per 1000 cells by incorporating cell counts obtained by an MTT assay.

2.8.4 Statistical Analysis

Data was analysed for statistical differences with Microsoft Excel using a combination of ANOVA and the Student–Newman–Keuls post-hoc test for comparison of means. A p-value of <0.05 was considered indicative of a significant difference.

3. Results

3.1. Determination of intrinsic viscosity

It has been previously shown that sonication can be used to lower the molecular weight and intrinsic viscosity of gellan gum [28]. To evaluate the effects of this sonication protocol on such properties, measurements of the intrinsic viscosity of sonicated and un-sonicated gellan were taken (Fig. 1). As sonication amplitude increased, the intrinsic viscosity of the gellan was subsequently decreased. Prior to sonication, the gellan was displaying an average intrinsic viscosity of 12.93 dL/g which decreased exponentially as a function of sonication amplitude, to as low as 4.99 dL/g in the most highly sonicated samples.

3.2. Viscosity Measurements

In order to determine the effects of sonication on the dynamic viscosity of gellan solutions, steady shear viscosity measurements were performed as a function of shear rate. This determined both the dynamic viscosity of the samples and the non-Newtonian behaviour. Samples were run at a concentration of 0.5% and all showed shear thinning behaviour (Fig. 2). The differing factor between each sample was the sonication amplitude applied. Increasing the amplitude of the sonication resulted in a decrease in the viscosities of the samples which was most notable at lower shear rates (Fig 2).

3.3. Oscillatory Rheology

3.3.1 Temperature sweeps

To assess the effect of sonication on the gelation properties of gellan, dynamic measurements of viscoelastisity were performed on cooling from 90°C to 10°C. Fig. 3 shows cooling scans of 0.5% gellan gels both before and after various amplitudes of sonication. Un-sonicated gellan had an average G′ value of 9.88×10^3 Pa at 10°C (after complete gelation of the gel). Sonication resulted in a G′ value at 10°C that was significantly lower. Despite changing the modulus of the gellan by sonication, gelation temperature remained unchanged at ~46°C across all samples. Sonication amplitude was shown to reduce the G′ value at 10°C, and at 37°C (Fig. 4). Therefore, by varying the amplitude of sonication, the moduli of gellan hydrogels could be tuned (Fig. 4 and Table 2).

3.3.2 Stress Sweeps

Stress sweeps were performed to assess the effects of sonication on the LVR and to determine the critical stress the gellan networks begin to break down. Fig. 5 shows the results from stress sweeps of the two samples. Hydrogels made from un-sonicated gellan (Fig. 5A) presented a significantly larger linear region in the presence of increasing shear stress

compared with hydrogels made from sonicated gellan (Fig. 5B). Furthermore, un-sonicated gellan displayed a higher critical stress than sonicated gellan with an initial gradual decline of modulus at a shear stress of approximately 65 Pa. The same decline took place at a shear stress of around 37 Pa in the sonicated samples. The final breaking point where the polymer underwent full deformation was at around 863 Pa in the un-sonicated samples and around 282 Pa in the sonicated samples. These results are summarised in Table 3.

3.3.3 Frequency sweeps in cell culture conditions

When gelled in cell culture conditions, gellan samples showed a decrease in modulus with increasing sonication amplitude (Fig. 6). The average modulus was not as high as when gels were prepared as in section 2.5 but a pronounced difference was still observed. For example, at a frequency of 30 rad s^{-1}, the average modulus was 1091 Pa for un-sonicated gellan (Fig. 6A). When sonicated at 50% amplitude this reduced to an average of 857 Pa (Fig. 6B) and further decreased to an average of 551 Pa for gellan sonicated at 100% amplitude (Fig. 6C). This equates to a nearly 50% reduction in matrix stiffness.

3.4 Cell Culture

3.4.1 Encapsulation of MC3T3's

Cell-loaded hydrogels were successfully created with all gellan samples (Fig. 7). However, as the amplitude of sonication applied increased, hydrogels became more uniform in appearance.

3.4.2 Live/dead assay

All samples showed a high proportion of encapsulated cells remained live for the duration of the study (Fig. 8). This is indicated by the green appearance of cells in the images. Cells appeared uniformly dispersed throughout the matrices.

3.4.3 Alkaline phosphatase activity

Fig. 9 shows the average p-nitrophenol concentration generated per 1000 cells for each sample. Cells encapsulated in un-sonicated gellan yielded the highest concentration of p-nitrophenol (0.137 nmol) with the lowest concentration being produced by cells encapsulated in 100% sonicated gellan (0.058 nmol). Results for all samples were shown to be significantly different ($P<0.05$).

4. Discussion

The ability to control and tune the mechanical behaviour of biopolymer materials has been of particular interest in tissue engineering over the past decade since it was found that matrix stiffness can directly influence stem cell differentiation [15]. Here we have shown that various mechanical properties of gellan gum (as a solution and as a hydrogel when crosslinked with DMEM) can be modified by applying varying amplitudes of sonication. The reduction in intrinsic viscosity is perhaps the most significant factor when it comes to explaining the mechanism behind modification of mechanical properties and the ability to tune functional mechanical behaviour. As previously stated, gelation of gellan involves both formation of ordered helices and subsequent aggregation of the helices into a complex 3D structure strengthened by ionotropic crosslinking between polymer molecules. The intrinsic viscosity of a polymer is essentially the capability of that polymer to enhance the viscosity of a solution in which it is dissolved and is therefore, related to molecular weight such that it is

often used in the determination of molecular weight [25]. A decrease in molecular weight therefore usually results in a decrease in intrinsic viscosity and vice versa.

Increasing the amplification of sonication resulted in a reduced intrinsic viscosity and therefore, it can be inferred that there was a reduction in molecular weight (Fig. 1). This supports work conducted previously by D'Arrigo *et al.* (2012) [24] who showed that sonication of gellan gum reduces the molecular weight of gellan without altering its chemical structure. Furthermore, Taylor *et al.* (2012) [28] showed that there was in increase in gellan oligomers following sonication at only 30% amplitude which resulted in a reduction in apparent viscosity, as a result of reduced intermolecular entanglement [28]. In the present study it was shown that by varying sonication amplitude from 0% to 100% a reduction in viscosity can be tuned to the level required for the application (Fig 2), from small reductions in viscosity at low amplitudes to dramatic reductions and an almost complete loss of non-Newtonian behaviour at 100% sonication amplitude.

Modulation of sonication amplitude can also control the stiffness of resulting hydrogels crosslinked in DMEM. When gellan solutions cool they undergo a two-part gelation mechanism that is normal for gellan [29] but a sample of subjected to higher amplitudes of sonication produced a weaker gel (Fig. 3-5). Since intrinsic viscosity results infer a decrease in molecular weight, reduction in gel modulus is likely attributable to a decrease in polymer chain length resulting in fewer crosslinks between each molecule. The exponential reduction in elastic modulus from 10000 Pa in the un-sonicated gellan to ~3000 Pa when sonicated at 90% amplitude (Fig. 4 and Table 2) highlights how gel stiffness (when crosslinked with DMEM) can be controlled and predicted. The modulus therefore, can be controlled to suit an application by simply applying varied amplitudes of sonication.

In addition to tuning both the modulus and viscosity of the polymer, sonication also affects the elasticity of hydrogels. This is shown in Fig. 5 where hydrogels formed from sonicated gellan displayed a smaller LVR and a lower critical stress (Fig. 5A) than hydrogels formed from gellan that was not subjected to sonication (Fig. 5B). Frequency sweeps shown in Fig 6 also highlight a reduction in elasticity as a function of increasing sonication amplitude. This change adds a new dimension to the sonication protocol whereby elasticity of a hydrogel can also be tuned along with modulus and viscosity to further mimic matrix properties for the tissue to be cultured. Again, reductions in intrinsic viscosity present the most likely explanations for this. Since sonicated gellan has a lower intrinsic viscosity and therefore a lower hydrodynamic volume, the distance of molecular interactions would be expected to be reduced. Therefore, cross-linked hydrogels formed from sonicated gellan pack together in a tighter structure, due to the smaller molecular size and reduced length of interactions. This results in a gel that is significantly less elastic compared with un-sonicated gellan where interaction length is greater.

In cell culture conditions, a similar trend was seen between sonication amplitude and gel stiffness (Fig. 6). Un-sonicated gellan showed the highest modulus (Fig. 6A) while gellan sonicated at maximum amplitude showed the lowest with a ~50% decrease in gel stiffness (Fig. 6C). However, gels formed in culture conditions showed a lower modulus than when gelled under conditions outlined in section 2.5. Results in Fig 6 are far more reflective of the mechanical behaviour of each sample under cell culture conditions than those in Fig 3 and 4 as gelation was triggered at 37°C whereas the results shown in Fig 3 and 4 were obtained by mixing gellan with DMEM at 90°C and slowly cooling to form a stable hydrogel. This difference in approach is likely the cause of changes in mechanical behaviour. The onset of gelation shown in Fig. 3 takes place at ~46°C so when DMEM is mixed at 37°C (as occurs

when preparing for cell culture) gelation is triggered instantly as the gellan comes into contact with the ions in DMEM. Moreover, when gelled in cell culture conditions samples are gently stirred to promote mixing and as a result imparts a small shear force during the sol-gel transition. This results in a suspension of gel particles, otherwise known as a fluid gel [30] [31], rather than a continuous bulk gel. A consequence of this is that the collective strength of the structure is reduced. Interestingly, sonication also seemed to affect physical appearance of cell loaded gellan structures (Fig. 7). Gels formed from sonicated gel appeared to be more uniform. This is likely due to reduced viscosity of gellan solutions as a result of sonication promoting more homogeneous mixing with DMEM.

Changes to rheological properties did not appear to impact on viability in cell loaded hydrogels. Similarly sonication does not lead to any potentially harmful contamination of polymer sample with cytotoxic metal particles that may have been released from the sonic probe. It is worth noting that the duration of sonication for each sample was relatively short reducing the chances of such contamination. Both are indicated by results in Fig 8 where all samples yielded a very high proportion of cells emitting green fluorescence as a result of a calcein AM/propidium iodide assay. This is indicative of live cells as a green fluorescence is emitted when live cells cleave calcein AM to calcein with the use of intracellular esterases [32]. A presence of dead cells would be indicated by red fluorescent signals as a result of propidium iodide penetrating compromised membranes and binding to nucleic acids in dead/dying cells [33].

A decrease in p-nitrophenol concentration with increasing sonication amplitude (Fig. 9) is indicative of reduced p-nitrophenyl phosphate hydrolysis and thus a decrease in alkaline phosphatase activity. As a key marker for osteogenic differentiation, ALP activity levels can

indicate the extent of osteogenesis within a tissue engineering construct [20]. The most common method to promote osteogenic differentiation is to supplement the cell culture media with specific differentiation promoting reagents. In this study, however, no differentiation supplements were added. Any differentiation therefore, would more likely be a result of mechanical stimuli from the surrounding gel matrix.

Rheological measurements showed that sonicating gellan resulted in structures with lower moduli. Reducing the modulus of the structures appeared to cause a reduction in ALP activity in encapsulated MC3T3's. Interestingly, the extent of reduction for both seems to be of similar proportion, with ALP activity and matrix stiffness decreasing by ~50% in gellan sonicated at 100% amplitude. This could be indicative of a mechanism whereby sonication reduced matrix stiffness and had a negative impact on osteogenic differentiation. A higher amplitude of sonication resulted in a further decrease in ALP activity for encapsulated cells. This seems to correspond with previous work showing how matrix stiffness can impact differentiation [15]. Osteogenesis requires a more stiff and rigid culture environment so a decrease in gel modulus can have a negative impact on osteogenic potential. While data appears to show a negative trend, the reduction in ALP activity as a result of gellan sonication acts as a proof of concept for the level control that can be exhibited over mechanical properties of a gellan scaffold using sonication. ALP assay results show that reductions in scaffold matrix stiffness were significant enough to have an impact on cell culture conditions. This provides potential for the use of sonication in tuning gel properties to optimise a structure for culture of specific tissues.

Changes in mechanical properties can be achieved without altering the concentration of polymer or crosslinking ions and thus there is little impact on the osmotic environment within a construct.

5. Conclusions

Application of a simple sonication method was shown to reduce the viscosity and elasticity of gellan gum hydrogels crosslinkied with DMEM. ALP activity of encapsulated MC3T3 cells showed variance as a result of changes in scaffold properties via sonication and cell viability was good in all constructs. This was achieved without changing the concentrations of the polymer or the gelling agents and was a direct result of the sonication method. This further supports the potential of gellan gum for tissue engineering applications as the ability to tune mechanical properties is integral to the success of such procedures.

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Table 1 – Ionic composition of DMEM.

Salt	Concentration (mM)
NaH_2PO_4	1.0
NaCl	116.0
KCl	5.4
$MgSO_4$	0.75
$CaCl_2$	1.8

Table 2 – The effect of sonication amplitude on the modulus and gelation temperatures of 0.5% gellan gum gels.

Sonication Amplitude (%)	G' at 37 °C (Pa)	G" at 37 °C (Pa)	G' at 10 °C (Pa)	G" at 10 °C (Pa)	Gelation Temperature (°C)
0	9880.00	119.00	6370.00	230.00	46
30	5930.00	51.12	2670.00	113.76	46
70	930.15	18.73	2494.50	55.10	46
90	524.90	15.75	1944.00	67.19	46

Table 3 – The effect of sonication amplitude on the modulus of 0.5% gellan gum gels in response to increasing stress.

Sample	Initial G′ (Pa)	Required Stress to Lower Modulus (Pa)	Required Stress for Major Deformation (Pa)
Un-sonicated Gellan	6555.33	65	863
Sonicated Gellan	2594.00	37	282

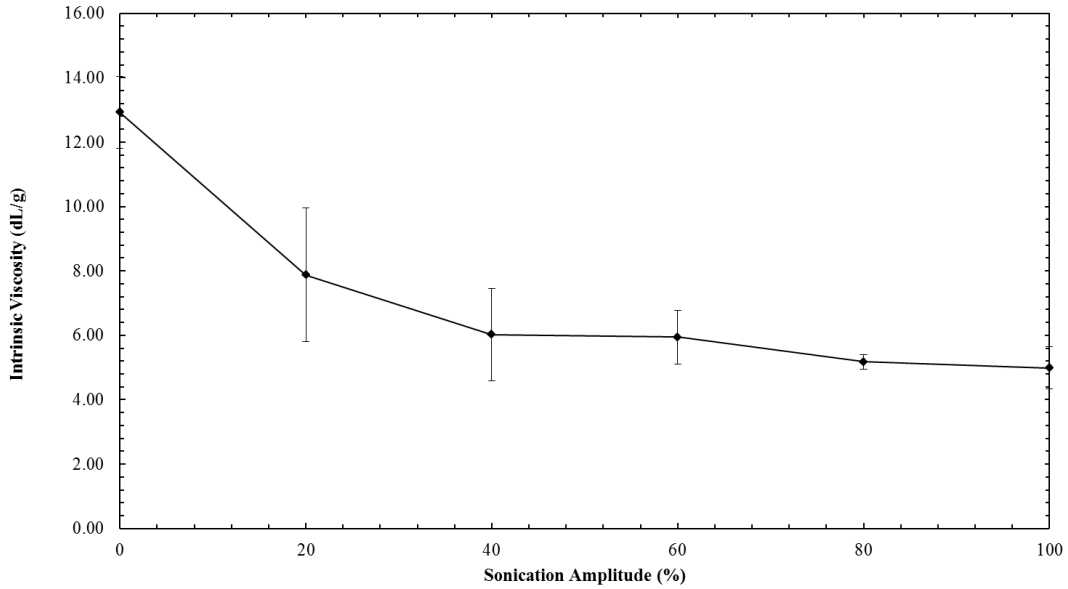

Fig. 1 – The effects of increasing the amplitude (%) of sonication on the intrinsic viscosity of low acyl gellan gum.

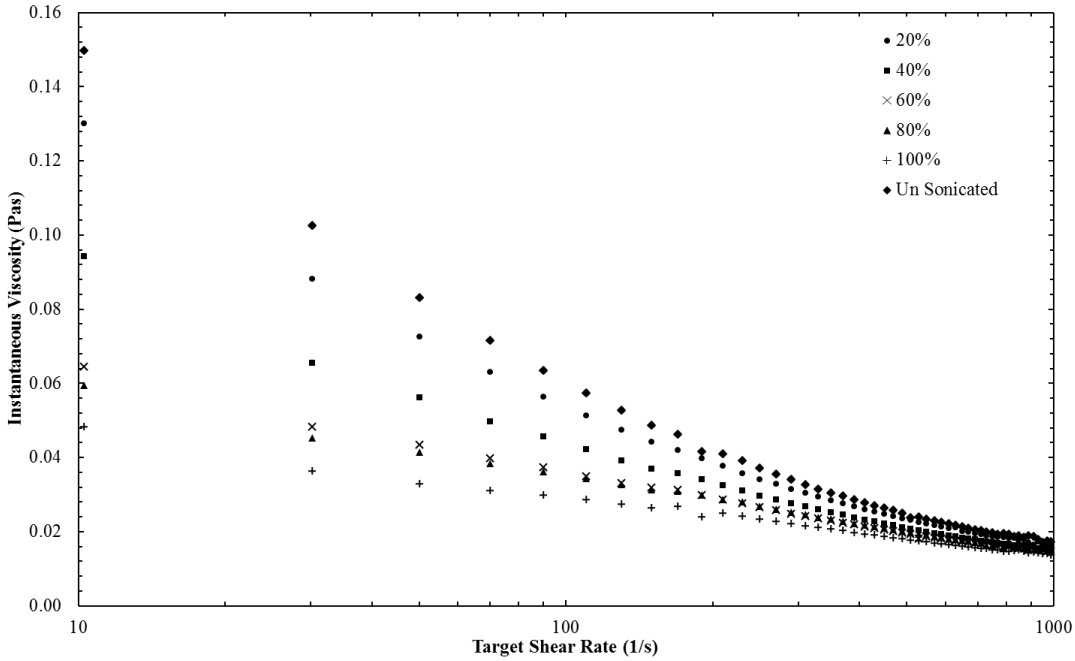

Fig. 2 – The effects of increasing the amplitude (%) of sonication on the shear thinning of 0.5% low acyl gellan gum to a target shear rate of 1000.

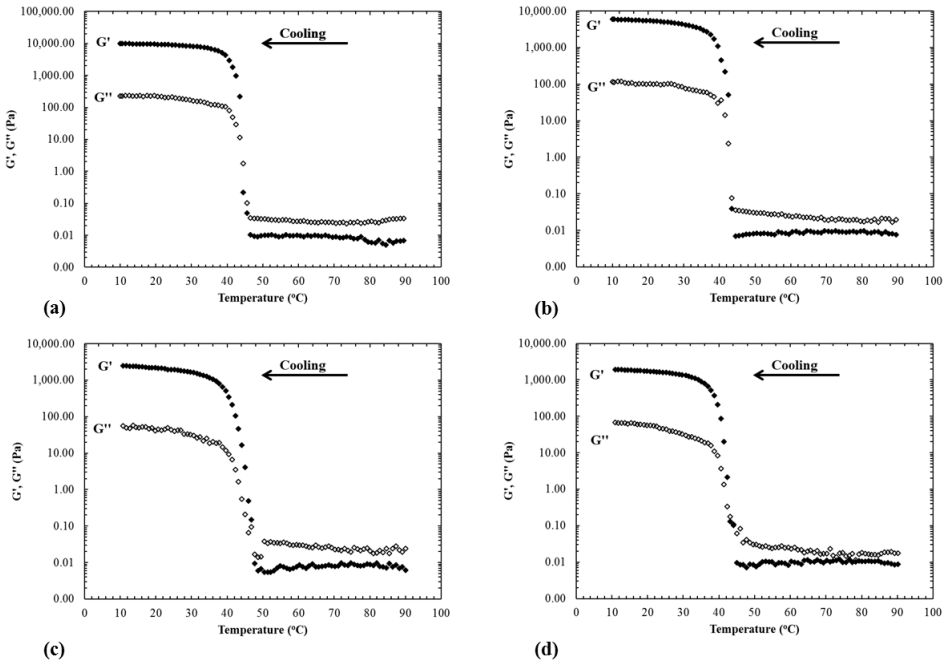

Fig. 3 – Oscillatory cooling scans displaying both the elastic modulus (G′) and viscous modulus (G**"**) of 0.5% low acyl gellan hydrogels gelled using DMEM after no sonication (a), 30% amplitude sonication (b), 70% amplitude sonication (c) and 90% amplitude sonication (d).

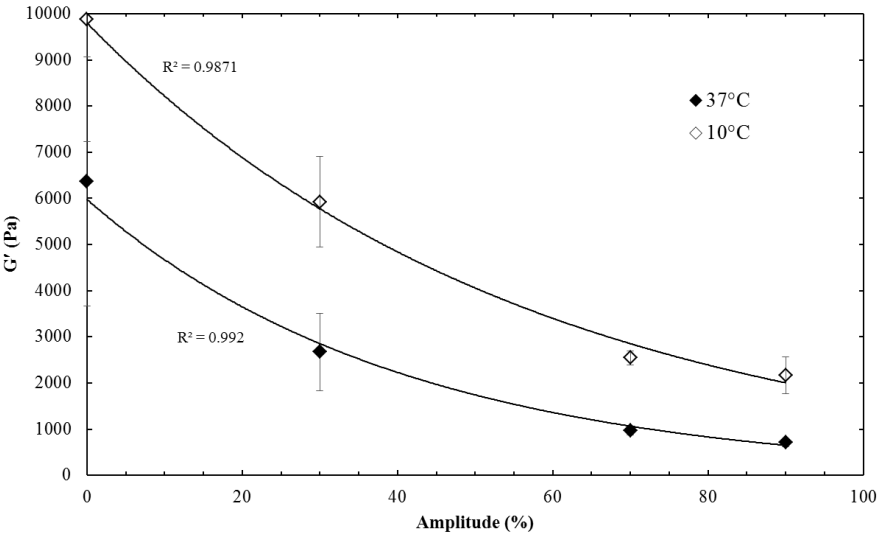

Fig. 4 – A comparison of the G′ of 0.5% low acyl gellan gum hydrogels at 10 °C and 37°C with no sonication, sonication at 30% amplitude, sonication at 70% amplitude and sonication at 90% amplitude.

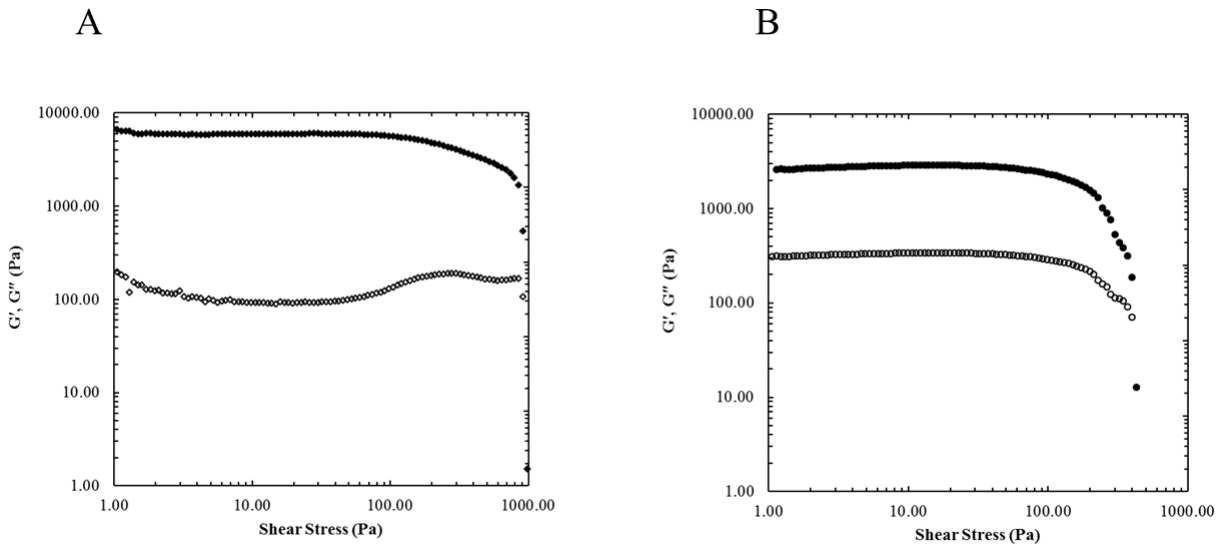

Fig. 5 – Stress sweeps highlighting the critical stress required for polymer network breakdown in 0.5% gellan hydrogels sonicated at A) 0% and B) 100%

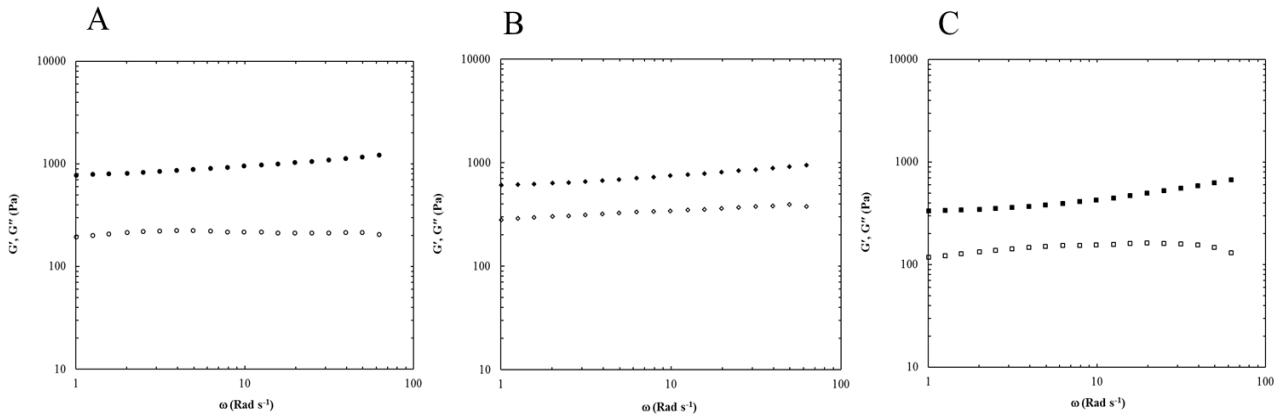

Fig. 6 – Mechanical Spectra measured at 37°C showing the variation in G′ and G″ as a function of frequency for 0.5% gellan hydrogels sonicated at A) 0% B) 50% and C) 100%

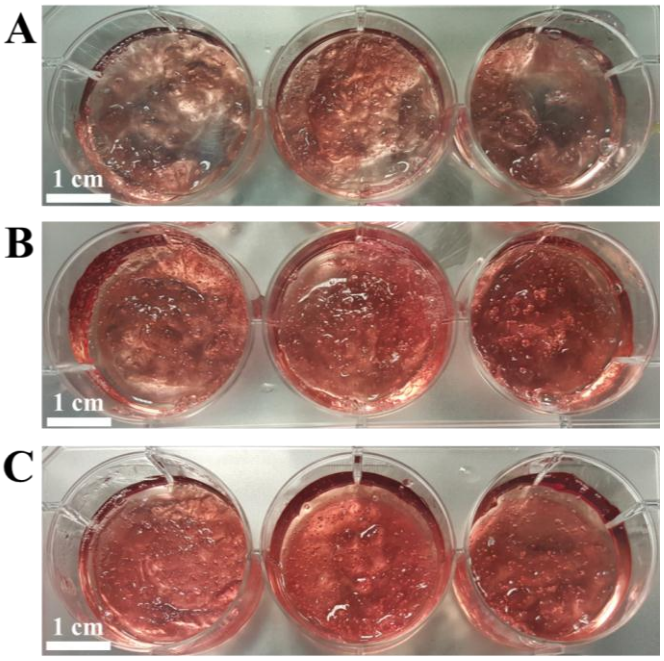

Fig. 7 – Physical appearance of cell-loaded hydrogels made from A) un-sonicated gellan, B) gellan sonicated at 50% amplitude and C) gellan sonicated at 100% amplitude gelled with DMEM in cell culture conditions.

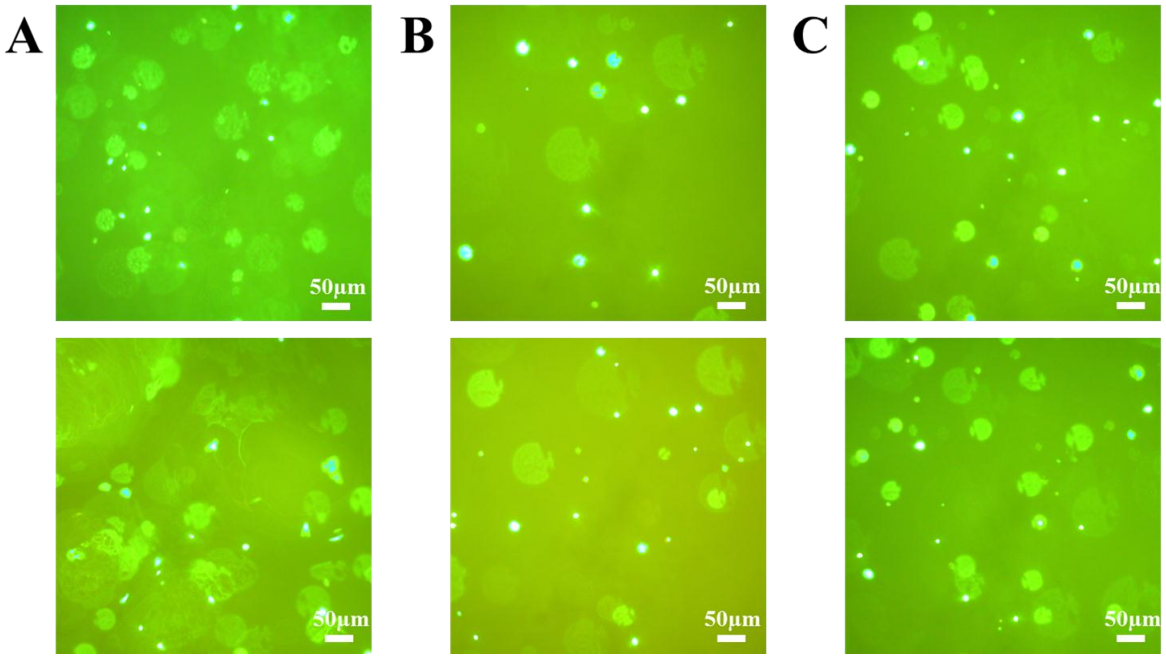

Fig. 8 – Live/dead staining results for MC3T3 preosteoblasts cultured for 7 days in A) un-sonicated gellan, B) gellan sonicated at 50% amplitude and C) gellan sonicated at 100% amplitude.

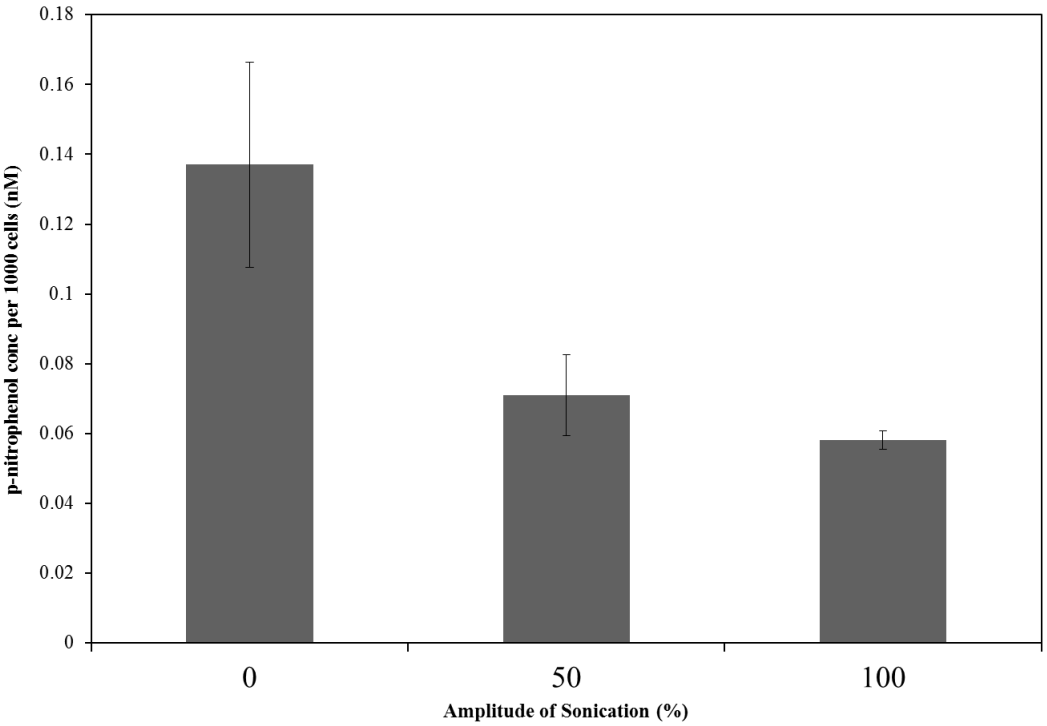

Fig. 9 – The effect of sonication on concentration of p-nitrophenol produced per 1000 encapsulated MC3T3-E1 cells.