International Journal of Biological Macromolecules 84 (2016) 79–86

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Controlling the rheology of gellan gum hydrogels in cell culture conditions

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ARTICLE INFO

Article history:
Received 22 September 2015
Received in revised form
10 November 2015
Accepted 2 December 2015
Available online 9 December 2015

Keywords:
Tissue engineering
Gellan gum
Alkaline phosphatase

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.

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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 [3–5].

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 $\rightarrow$4)-L-rhamnopyranosyl-(α−1$\rightarrow$3)-D-glucopyranosyl-(β−1$\rightarrow$4)-D-glucuronopyranosyl-(β−1$\rightarrow$4)-D-glucopyranosyl-(β−1$\rightarrow$. 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

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http://dx.doi.org/10.1016/j.ijbiomac.2015.12.007
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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

immobilisation within a thermoreversible and non-cytotoxic hydrogel [9–11].

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 [19–21]. 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 min at 20 °C. The amplitude of the ultrasonic waves used during the procedure was varied from 20 to 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 three replicates) flow time of the gellan solution at each concentration, t_0 is the flow time for the 10 mM NaCl solution, ρ is the density of the gellan solution at each concentration and ρ_0 is the density of the 10 mM NaCl solution. Due to the low concentrations used, $\rho/\rho_0 = 1$ [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 $[\eta]$ is taken as 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).

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