Gellan gum: A new biomaterial for cartilage tissue engineering applications

J. T. Oliveira,[1,2] L. Martins,[3] R. Picciochi,[1,2] P. B. Malafaya,[1,2] R. A. Sousa,[1,2] N. M. Neves,[1,2] J. F. Mano,[1,2] R. L. Reis[1,2]

[1]*3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal*

[2]*IBB - Institute for Biotechnology and Bioengineering, PT Associated Laboratory, Guimarães, Portugal*

[3]*Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal*

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Abstract: Gellan gum is a polysaccharide manufactured by microbial fermentation of the *Sphingomonas paucimobilis* microorganism, being commonly used in the food and pharmaceutical industry. It can be dissolved in water, and when heated and mixed with mono or divalent cations, forms a gel upon lowering the temperature under mild conditions. In this work, gellan gum hydrogels were analyzed as cells supports in the context of cartilage regeneration. Gellan gum hydrogel discs were characterized in terms of mechanical and structural properties. Transmission electron microscopy revealed a quite homogeneous chain arrangement within the hydrogels matrix, and dynamic mechanical analysis allowed to characterize the hydrogels discs viscoelastic properties upon compression solicitation, being the compressive storage and loss modulus of ~40 kPa and 3 kPa, respectively, at a frequency of 1 Hz. Rheological measurements determined the sol-gel transition started to occur at approximately 36°C, exhibiting a gelation time of ~11 s. Evaluation of the gellan gum hydrogels biological performance was performed using a standard MTS cytotoxicity test, which showed that the leachables released are not deleterious to the cells and hence were noncytotoxic. Gellan gum hydrogels were afterwards used to encapsulate human nasal chondrocytes (1×10^6 cells/mL) and culture them for total periods of 2 weeks. Cells viability was confirmed using confocal calcein AM staining. Histological observations revealed normal chondrocytes morphology and the obtained data supports the claim that this new biomaterial has the potential to serve as a cell support in the field of cartilage regeneration. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 93A: 852–863, 2010

Key words: hydrogel; natural origin; polysaccharide; cartilage; tissue engineering

INTRODUCTION

Tissue engineering has been proposed as a new method to address problems such as organ failure and tissue regeneration, being widely studied nowadays as a tool to tackle problems in a diverse range of tissues.[1–4] Such conditions pose serious health problems, being responsible for a decrease in people quality of life. Cartilage is one of the most studied tissues in this field giving the importance it has on mobility and locomotion. Because of its limited capacity for self repair, cartilage becomes an enormous constraint to normal everyday life once degenerated or traumatized. Structures that can provide support for specific cells to develop and generate a functional cartilaginous tissue are an important subject of study. Different types of natural and synthetic biomaterials have been processed using different techniques for this purpose. We are proposing in this work a new biomaterial-Gellan gum to be used in the engineering of cartilaginous tissues, even though its application may not be restricted to this tissue only, as it will be shown by the different 3D structures that can be obtained. Recent work performed by Smith et al. has also suggested the use of this biomaterial for tissue engineering applications.[5]

Correspondence to: J. T. Oliveira; e-mail: joao.oliveira@dep.uminho.pt

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Gellan gum is a linear anionic polysaccharide composed of tetrasaccharide (1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-β-D-glucose, 1,4-α-L-rhamnose) repeating units, containing one carboxyl side group, and was initially described by Moorhouse et al.[6,7] This material has a broad use in the food industry and biomedical fields, mostly due to its processing into transparent gels that are resistant to heat and acid stress. Two gellan gum forms exist, acetylated and deacetylated, being the latter the most common and commercially available form. Both form thermoreversible gels, varying in their mechanical properties from soft and elastic for the acetylated form to hard and brittle for the fully deacetylated polysaccharide.[8,9] Gellan gum can form gels in the following way: at high temperatures, Gellan gum is in the coil form; upon temperature decrease, a thermally-reversible coil to double-helix transition occurs, which is a prerequisite for gel formation. Afterwards, a structure composed of anti-parallel double helices self assembled to form oriented bundles, called junction zones, is formed. Untwined regions of polysaccharide chains, in the form of extended helical chains, link the junction zones, leading to the formation of a three dimensional network, that creates the gel.[10] These structural changes occurring to gellan gum molecules have been shown by different techniques. During the cooling process, for example, rheological and differential scanning calorimetry (DSC) studies revealed a first step increase of loss modulus that corresponds to the coil-helix transition, and a second step increase of loss modulus due to sol-gel transition.[11] The gelation of gellan gum solutions is strongly influenced by the chemical nature and quantity of cations present in the solution. The presence of cations is critical when a structurally stable gel is to be prepared.[10,12,13] In fact, at low Gellan gum concentrations, the helix formation and its partial aggregation may form an ordered structure, but this does not lead to gel formation because the number of helical aggregates does not give rise to a continuous network in the whole volume.[11] The main barrier are the carboxyl side groups that repulse each other by electrostatic interaction, therefore hindering the tight binding of helices and their cohesive aggregation.[9,14–16] The introduction of cations shields the electrostatic repulsion and thereby allows the tight binding and aggregation of helices.[11,17,18]

The gelation properties of Gellan gum are also influenced by the nature of the cations used, in which divalent cations promote the gelation much more strongly than monovalent cations.[11,12] In monovalent cations, the gelation is mainly a result of the screening of the electrostatic repulsion between the ionized carboxylate groups on the Gellan gum chains. In the case of divalent cations, the gelation and aggregation of Gellan occurs via a chemical bonding between divalent cations and two carboxylate groups belonging to glucuronic acid molecules in the Gellan chains, in adittion to the screening effect.[19] It was also suggested that different types of mono or divalent cations also influenced the viscoelastic behavior of Gellan gum solutions. K^+ was more remarkable than Na^+, and Ca^{2+} more than Mg^{2+}.[11] Gellan gum structures have excellent heat resistance properties because the formed junctions upon gelation can only be unzipped on heating at 120°C.[11] In the initial state, a junction zone in Gellan gum is estimated to be four double helices wide and five repeat units long, its length being increased to seven repeat units upon annealing.[10] In the solid state, the double helix structure adopted by Gellan gum has a similar arrangement to the double helix structure of iota carrageenan.[20] Previous studies indicate that solutions of deacetylated gellan gum behave as a pseudoplastic liquids, as evidenced by creep testing, and have little thixotropy.[13] Gellan gum advantageous use in the context of biomedical applications includes its lack of toxicity, processing under mild conditions, the ability to used as an injectable system in a minimally invasive manner, and also the structural similarity it presents with native cartilage glycosaminoglycans by the presence of glucuronic acid residues in their repeating unit.[21,22] The presence of this carbohydrate residue, which contains carboxylic groups, may confer added functions to this material. Some intellectual property associated with the application of this material in the medical field has already been disclosed, as its use for ophthalmologic purposes.[23,24]

This work tested for the first time gellan gum as a new biomaterial to be used in cartilage regeneration approaches. As shown here, gellan gum hydrogels are quite versatile in terms of processing and its materials properties reveal good prospects for their use as a cell encapsulating agents. Biological evaluation of their cytotoxicity and *in vitro* culturing of human nasal chondrocytes generated interesting results indicating that this new biomaterial may play a potential role in cartilage regeneration approaches.

MATERIALS AND METHODS

Versatility of Gellan gum: Processing into different structures (discs, membranes, fibers, particles, scaffolds)

Note: Unless otherwise stated the reagents were purchased from Sigma-Aldrich.

Gellan gum (G1910, Sigma, St. Louis, MO) was processed in different ways giving rise to various structures, therefore evidencing the versatility of this natural biomaterial. The processing involved temperature-dependent

and pH-dependent reactions. Regarding gellan gum discs and membranes production, the following methodology was used. Gellan gum powder was mixed with distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90°C, under which complete and homogeneous dispersion of the material was obtained. The solution was kept at this temperature during 20–30 min. Afterwards, $CaCl_2$ (Merck, DE) was added to obtain a final concentration of 0.03% (w/v) in the gellan gum solution and the temperature was progressively decreased to 50°C. Gellan discs were produced by casting the solution into cylindrical moulds and allowing it to rest at room temperature for 2–5 min and form a solid gel. The discs were then cut using a borer for final discs dimensions of Ø 6 ± 0.01 mm × 5.5 ± 0.46 mm height. Gellan gum membranes were produced by casting the solution into Petri dishes and allowing it to stand at room temperature for 2–5 min and form a solid gel. The Petri dishes were kept in an oven at 37°C for 90 min. Concerning the production of Gellan gum fibers and particles the methodology was as follows. Gellan gum powder was mixed with a NaOH 0.10*M* solution and stirred at room temperature with a final concentration of 4% (w/v). Gellan gum fibers were produced by extruding the gellan gum solution into a L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.2 mL/min, using a 21G needle. The gellan gum fibers formed were then washed in distilled water, pressed into cylindrical moulds, and dried overnight at 37°C. Gellan gum particles were produced by extruding the Gellan gum 4% (w/v) solution dropwise to an L-ascorbic acid 20% (v/v) solution under a constant flow rate of 0.8 mL/min, using a 21G needle. Gellan gum scaffolds were produced by immersing gellan gum 0.7% (w/v) (Ø 6 ± 0.01 mm × 5.5 ± 0.46 mm height) discs in liquid nitrogen for 1–2 min and quickly transferring them to a lyophilizator (Telstar Cryodos-80, Telstar, Spain) where they were lyophilized during 2 days. Lyophilized gellan gum 0.7% discs were further analyzed under micro-computed tomography (μ-CT) using a high-resolution μ-CT Skyscan 1072 scanner (Skyscan, Kontich, Belgium) using a resolution of 6.76 μm pixel size and integration time of 1.7 ms. The X-ray source was set at 70 keV of energy and 142 μA of current. Approximately 500 projections were acquired over a rotation range of 180° and a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was a 500 serial of 1024 × 1024 bitmap images. Representative data sets of 150 slices were segmented into binary images (CT Analyser, v1.5.1.5, SkyScan) with a dynamic threshold of 70–255 (gray values) that was applied to build the 3D models. 3D virtual models (height 1 mm × Ø 3 mm) of representative regions in the bulk of the hydrogels were created, visualized, and registered using image processing software (CT Analyser, v1.5.1.5 and ANT 3D creator, v2.4, both from SkyScan).

Transmission electron microscopy

Gellan gum discs were prepared for transmission microscopy analysis in the following way. Briefly, sections of 1 mm^3 were fixed in formalin-glutaraldehyde-osmium tetroxide for 2 h at room temperature and then washed 3 times in PBS. Semithin sections (1 μm) were cut from epon-embedded blocks and stained with toluidine blue. Ultrathin sections (600 Å) were cut in a ultratome (Reichert Ultranova Leica), mounted onto copper grids, stained with uranyl acetate (7 min) and lead citrate (5 min), and observed on a Zeiss 902A (50 Kv) electron microscope.

Dynamic mechanical analysis

Dynamic mechanical analysis (DMA) was conducted to characterize the mechanical behavior of Gellan gum hydrogel discs. Gellan gum 0.7% (w/v) discs (Ø 6 ± 0.01 mm × 5.5 ± 0.46 mm height) discs were subjected to compression cycles of increasing frequencies ranging from 0.1 to 10 Hz with constant amplitude displacements of 0.1 mm using a Tritec 2000 DMA (Triton Technology, UK). Storage and loss modulus were measured and experiments were conducted at room temperature. The total number of discs per assay were $n = 3$. The described values for the compression modulus were collected at a frequency of 1 Hz. Statistical analysis was performed using confidence intervals based on the experimental results, with a confidence level of 99%.

Rheological studies

Cone-Plate rheometry was conducted for gellan gum hydrogels in order to assess their rheological behavior dependence of temperature and time. For this purpose, gellan gum powder was mixed at room temperature with distilled water at a concentration of 0.7% (w/v) under constant stirring. The solution was heated to 90°C and kept at this temperature for 30 min. Afterwards, $CaCl_2$ was added to the Gellan gum solution at concentration of 0.03% (w/v) and rheological measurements were performed using a controlled stress cone-plate rheometer (Reometer Reologica, StressTech, Sweden). For each measurement, a volume of 2 mL of the Gellan gum solution was placed in the bottom plate of the rheometer and held at a constant temperature of 70°C. The polymer solution was allowed to rest for 1 min before starting the experiments. Measurements were performed by cooling each sample from 70°C to 25°C (at a cooling rate of −6°C/min) applying a constant shear stress of 0.1 Pa. Temperature, time, shear rate, and viscosity were constantly measured. The total number of repeats was $n = 3$ and confidence intervals were estimated, with a confidence level of 99%.

Cytotoxicity evaluation

To assess the possible cytotoxicity of the processed gellan gum hydrogels, MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium) test was used according to ISO/EN 10993 part 5 guidelines, which determines whether cells are metabolically active.[25] This cytotoxicity test is based on the bioreduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-

carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) (Cell Titer 96® Aq_{ueous} Solution Cell Proliferation Assay, Promega), into a brown formazan product by dehydrogenase enzymes in metabolically active cells, and is commonly used for cell viability evaluation. Latex rubber was used as positive control for cell death, due to its high cytotoxicity to cells, and culture medium was used as a negative control. A rat lung fibroblasts cell line-L929, acquired from the European Collection of Cell Cultures (ECACC), was used for the studies. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Biochrom, Berlin, Germany; Heat Inactivated) and 1% of antibiotic-antimycotic mixture.

The gellan gum hydrogel discs were incubated in culture medium for 24 h at 37°C with constant shaking, as well as latex. Cultured L929 cells were trypsinized using trypsin-EDTA (Gibco, Invitrogen Corporation) and plated at a density of 6.6×10^4 cells/well into 96-well micrometer plates (200 μL/well). The plates were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Afterwards, the medium was replaced by the extracts previously obtained, using culture medium as a negative control. After 72 h, the cell culture was incubated with MTS (using culturing medium without phenol red) for further 3 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium with MTS was then transferred to new wells. The optical density (OD) which is directly proportional to the cellular activity, being a measure of mitochondrial acitivity, was read on a multiwell microplate reader (Synergy HT, Bio-TeK Instruments) at 490 nm. Statistical analyses were conducted using a two-sample *t*-test assuming unequal variances for $n = 3$.

Isolation and expansion of human nasal chondrocytes

Nasal cartilage was harvested from the nasal septum of adult patients (40–65 years) undergoing reconstructive surgery. This was performed within the scope of a protocol established with the Hospital de S. Marcos, Braga, Portugal, approved by its Ethical Committee and always sampled upon patient informed consent. The human nasal septum cartilage free from all surrounding tissue was placed in a Petri dish containing sterile phosphate buffered saline (PBS) and cut into square slices of 5 mm and thickness between 2–3 mm. The pieces were washed in sterile PBS solution, immersed in 20 mL of trypsin-EDTA solution, and incubated for 30 min at 37°C on a rotator. Trypsin was removed and the pieces washed with basic DMEM. Then, 20 mL of filter sterilized collagenase type II solution (2 mg/mL) in basic medium was added, and the mixture incubated for ~12 h at 37°C on a rotator. The digested tissue and cell suspension solution was centrifuged at 200*g* for 7 min and the supernatant removed. The cell pellet was washed with PBS and the cells centrifuged as before. The procedure was repeated and the cells were ressuspended in PBS and counted using a hemocytometer. They were again centrifuged, the supernatant removed, and ressuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium, containing 10 m*M* HEPES buffer pH 7.4, 10,000 units/mL penicillin/10,000 μg/mL streptomycin, 20 m*M* L-alanyl glutamine, 1× MEM nonessential amino acids, and 10% (v/v) foetal bovine serum (FBS; Biochrom, Berlin, Germany; Heat Inactivated), supplemented with 10 ng/mL basic fibroblast growth factor (bFGF) (PeproTech, UK). Human nasal chondrocytes were plated into tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for expansion.[26]

Human nasal chondrocytes encapsulation in gellan gum and agarose hydrogels

Human nasal chondrocytes were expanded until an adequate cell number was obtained for cells encapsulation. Cells were encapsulated at passage 1 in gellan gum hydrogels and in agarose type VII (A6560; Sigma, St. Louis, MO) hydrogels, the latter being used as controls.

Regarding gellan gum the procedure was the following. Gellan gum powder was mixed with sterile distilled water under constant stirring at room temperature to obtain a final concentration of 0.7% (w/v). The solution was progressively heated to 90°C and kept at this temperature for 20–30 min. A sterile $CaCl_2$ solution was added to obtain a final concentration of 0.03% (w/v). The temperature was progressively decreased to 40°C and stabilized at this stage always under constant stirring. Human nasal chondrocytes were detached by trypsinization, mixed with expansion medium, and centrifuged at 200*g* for 7 min. The supernatant was removed and the cells were ressuspended in warm sterile PBS solution, counted using and hemocytometer, and finally centrifuged at 200*g* for 7 min. The supernatant was discarded and the cells pellet kept at the bottom of the falcon tube. The gellan gum 0.7% (w/v) with $CaCl_2$ 0.03% (w/v) solution was added to the cells pellet and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of 1×10^6 cells/mL. Gellan discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 1–2 min to form a solid gel, and then discs of Ø 6 ± 0.01 mm × 5.5 ± 0.46 mm height were cut using a borer.

Regarding the agarose hydrogels, the procedure is detailed elsewhere.[27] Briefly, a sterile agarose type VII low Tm 4% (w/v) solution prepared in sterile PBS was heated to 70°C for 30 s, until complete dissolution. The solution was stabilized at 40°C and added to a human nasal chondrocytes pellet prepared as described for the gellan gum encapsulation and the mixture ressuspended for complete homogenization of cells within the matrix with a final concentration of 1×10^6 cells/mL. Agarose discs with encapsulated human nasal chondrocytes were produced by casting this mixture into sterile cylindrical polystyrene moulds, allowing it to rest at room temperature for 20 min to form a solid gel, and then discs were cut using a borer. Both gellan gum and agarose hydrogels with encapsulated cells were cultured for 2 weeks with expansion medium under orbital rotation (50 rpm). Afterwards, expansion

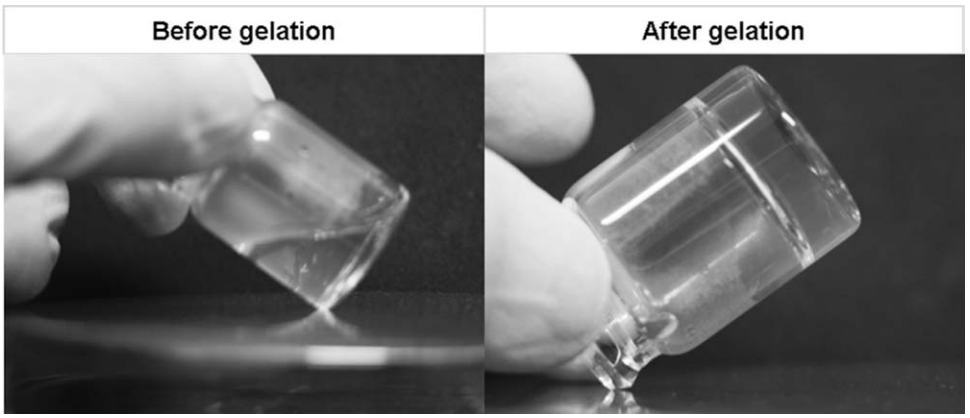

Figure 1. Sol-gel transition occurring in a Gellan gum solution containing $CaCl_2$.

medium was replaced for 6 weeks by differentiation medium to promote the formation of a chondrogenic phenotype. This medium presents the same composition as the expansion medium except for the bFGF which is replaced with 1 μg/mL of insulin and 50 μg/mL of L-ascorbic acid. The cells-hydrogel systems were returned to the orbital shaker and the culture medium was replaced every 2–3 days. The experiments were repeated three times independently.

Human nasal chondrocytes encapsulated in gellan gum and agarose hydrogels: Cell viability tests and histological analysis

Human nasal chondrocytes morphology in the two hydrogels used, gellan gum and agarose, was observed at 2 weeks of culture under optical microscopy. One representative sample of each type of support was observed at different magnifications using an optical microscope (Axiovert 40 CFL, Zeiss).

Cells viability at 2 weeks of culturing was assessed using calcein AM staining. Calcein AM (C3099, Invitrogen Corp.) is a fluorescence-based method for assaying cell viability and cytotoxicity in which the reagent is retained in cells that have intact membrane. Briefly, a calcein AM solution of 1/1000 was prepared in culture medium. One disc of each type of hydrogel with encapsulated human nasal chondrocytes was collected from the culturing plates and incubated in the calcein AM solution for 15–30 min at 37°C and afterwards washed in sterile PBS. The samples were observed under fluorescent microscopy (Zeiss HAL 100/HBO 100; Axiocam MRc5 (Zeiss)).

Concerning the histological analysis, hematoxylin-eosin and alcian blue staining were performed on 8 μm thickness sections of gellan gum and agarose discs collected at 2 weeks of culture using in-house methodology. The discs were fixated by immersion for 30–40 min in glutaraldehyde 2.5% (v/v) at 4°C, and washed in PBS. Histological processing was performed using Tecnhovit 7100® (Heraeus Kulzer GmbH, DE) and the technical details and procedure can be found in the commercial package. Sections were cut using a microtome Leica RM2155 (Leica Microsystems, Nusslock GmbH).

RESULTS AND DISCUSSION

Versatility of Gellan gum: Processing into different structures (discs, membranes, fibers, particles, scaffolds)

Gellan gum was processed into different shapes as shown in Figures 1 and 2. By using simple processing methodologies, involving temperature-dependent gelation (discs, membranes, scaffolds) [Fig. 2(A,B,E,F)] and pH-dependent gelation (fibers and particles) [Fig. 2(C,D)], all those structures were produced. This shows the versatility of this material to obtain different geometrical forms that can be used in a broad range of tissue engineering and drug delivery applications. Gellan gum hydrogels can be used to encapsulate cells and serve as supports for their development. Gellan gum can also be processed into fibers and generate a 3D structure onto which cells can be seeded and stimulated to proliferate, an approach that may also be explored by using membranes. A gellan gum solution, in which a specific drug is dispersed, can be processed into particles such as those shown in Figure 2(D) and used as a carrier for drug delivery applications. In fact, gellan gum has been used previously as an ocular drug delivery system.[28–30] Different parameters such as temperature, pH, polymer concentration, and ions nature can be adjusted to possibly improve the biological performance or confer certain functionality. The control of the sol-gel transition at physiological temperature and pH[31–33] renders this material the possibility to be used as an injectable system, which is a highly recommended approach in several situations.[34] The most relevant factor in the hydrogel forming ability of gellan gum is the presence of D-glucuronic acid molecules in the tetrasaccharide repeating unit of the polysaccharide. These monosaccharides possess carboxylic groups in their structure that form internal hydrogen bonds and stabilize the double helices.

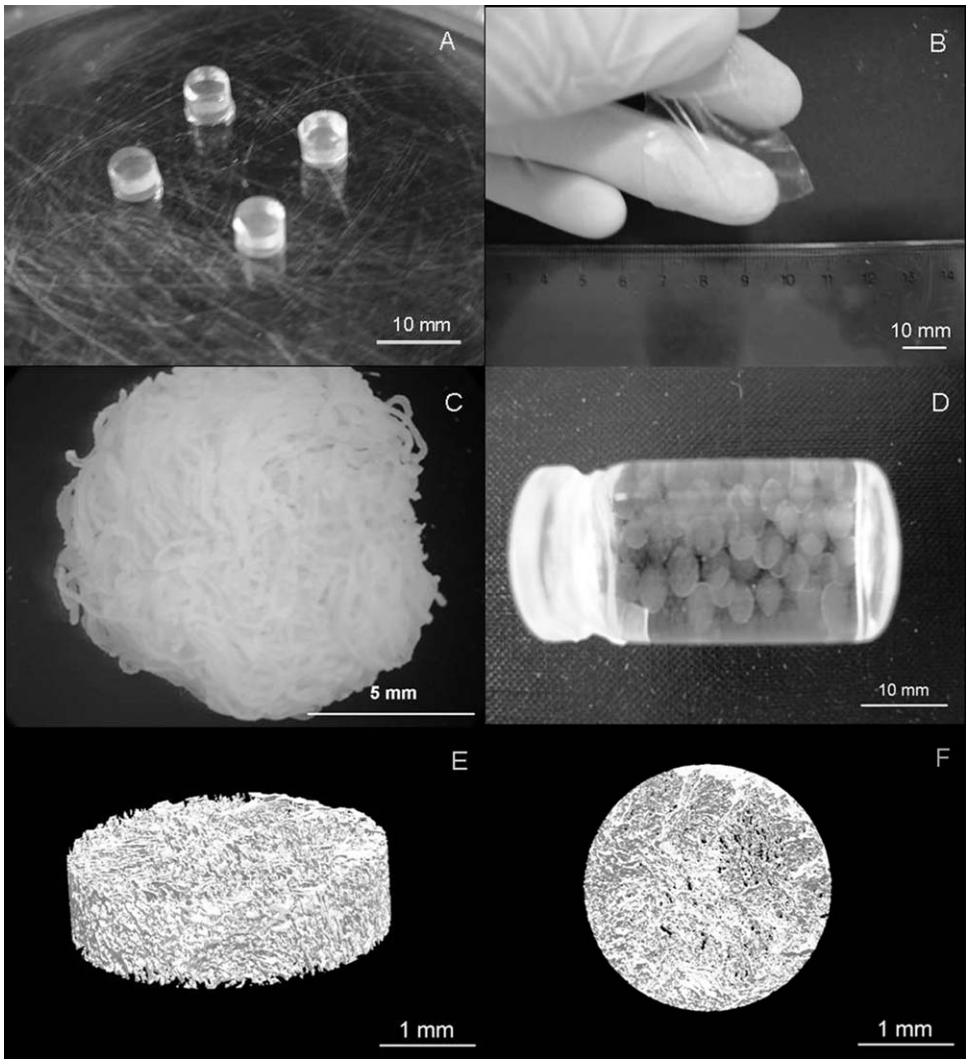

Figure 2. The versatility of Gellan gum structures that can be formed using simple polymer processing technologies: (A) discs; (B) membranes; (C) fibers; (D) particles; (E) and (F) 3D lyophilized scaffolds.

Nevertheless, carboxyl side groups that repulse each other by electrostatic interaction, hinder the tight binding of helices and their cohesive aggregation, affecting the formation of stable gels. The mono or divalent ions present in the solution play a key role in this matter. Their presence diminishes the repulsive energy between the carboxylic groups allowing the hydrogels to be formed. The variation in pH also affects the solubility of the material being this the main factor in the processing of gellan gum fibers and particles [Fig. 2(C,D)]. At a basic pH, such as the NaOH solution used in the experiments, the carboxylic groups present in each D-glucuronic acid residue should be in the anionic form, COO^-, and therefore soluble in solution. Once the pH is lowered, as upon extrusion into an L-ascorbic acid solution, the carboxylic groups become protonated, COOH, and the material turns insoluble.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed to have an insight on the ultrastructural morphology of the gellan gum hydrogels (Fig. 3). As described before for the gel state, gellan gum hydrogels constitute a matrix where double helices that originated from the coil form rearrangement in solution are widely present and distributed in a rather homogeneous fashion.[35] These give rise to junction zones by linking to a neighbor double helical molecule. The overall stability of the hydrogel network derives from the loose ends within the double helical molecules. These, together with the cationic anti repulsive effect allow obtaining a stable hydrogel when the temperature is decreased below the setting point. Previous work has already used TEM as a

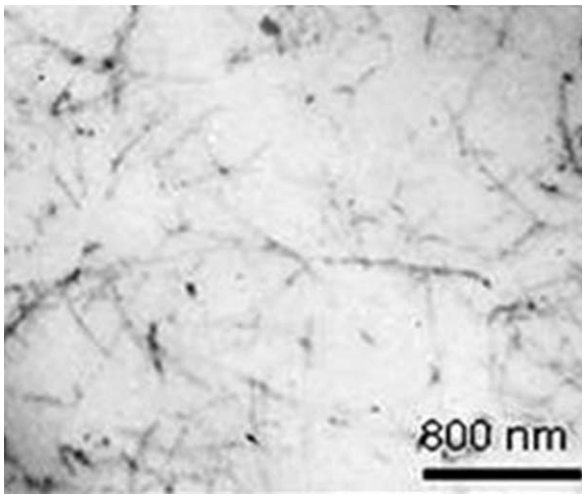

Figure 3. Transmission electron microscopy micrograph of a Gellan gum hydrogel showing a dense and homogeneous network structure at the ultrastructural level.

tool to characterize the ultrastructural properties of gellan-based hydrogels.[36] The authors showed that gellan gum forms strong gels at low ionic concentrations, being these highly homogeneous and constituted by a dense fibrous network structure. The work presented here confirmed this, being observed that gellan gum hydrogels provide a uniform matrix at a nanoscale throughout which cells could be encapsulated in a rather homogeneous way (Fig. 3).

Dynamic mechanical analysis

Living tissues exhibit clear viscolelastic properties and therefore it is important to characterize the solid-state rheological features of materials that are meant to be in contact with them. DMA has been used in our group to assess the viscoelastic properties of biomaterials, including natural-based hydrogels or highly hydrated systems.[37–40] In this work, gellan gum hydrogels were analyzed in the wet state throughout a physiological relevant frequency range. Both the storage (elastic) and loss (viscous) components of the complex modulus are shown in Figure 4. The storage modulus (E') is about one order of magnitude higher than the loss modulus (E'') indicating a clear elastic nature of the gel. However, it possesses some damping capability that may be useful to dissipate some cyclic mechanical energy that is imposed in an implantation scenario. Although some increase in E' is observed for increasing frequencies, the elastic properties of the biomaterial are quite stable, when compared with the viscous component. In fact, a clear increase in E'' is observed between 0.4 and 10 Hz, which suggests that the material exhibits higher dissipation capability for high frequencies. At

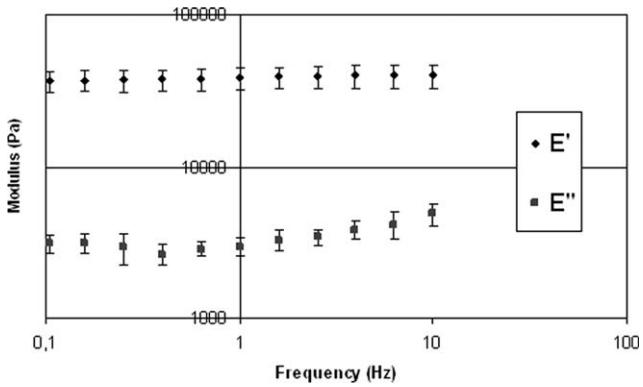

Figure 4. Dynamic mechanical analysis of Gellan gum hydrogels showing the storage (E') and loss (E'') modulus upon compression solicitation using different frequencies.

a frequency of 1 Hz, the compression modulus of the gels was estimated to be of 38.3 ± 6.3 kPa [38.2, 38.4 $_{t(0.01,2)}$] at room temperature. Even though this value is not optimal in terms of mimicking human articular cartilage mechanical properties,[41] it is higher or within the range of values found for hydrogels used in similar cartilage regenerative approaches.[42,43] The gellan gum support is conceived in this initial work to serve as a cell support due to its features, even though it may be optimized for being applied as an injectable system.[31–33] Cells encapsulation and extracellular matrix deposition may result in progressive increase of the mechanical properties of the 3D structures, as shown before for other systems.[42]

Rheological Studies

Rheological measurements were performed to determine the temperature range at which the

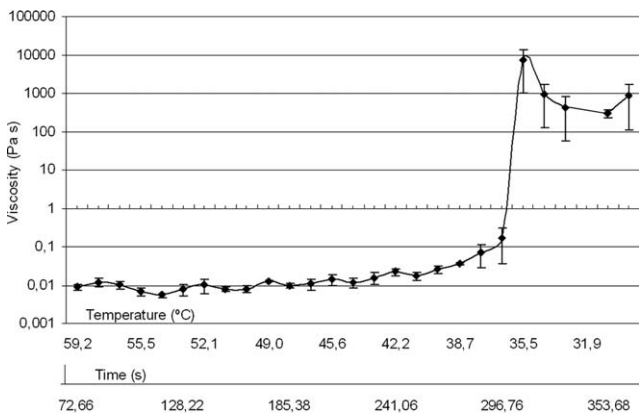

Figure 5. Rheological measurements of Gellan gum solutions. The upper x axis shows the relation between temperature and viscosity, while the bottom x axis shows the relation between time-length and viscosity.

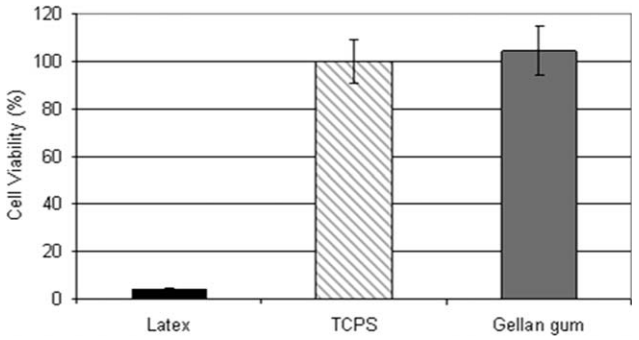

Figure 6. MTS cytotoxicity test performed to evaluate the possible cytotoxic effects of the leachables released by the gellan gum hydrogels. Results show the gels are noncytotoxic.

sol-gel transition occurred and the time-scale for gelling. Regarding gelation temperature, it is possible to state from the rheological measurements that it happens around 37°C (36.6 ± 0.05°C) [36.586, 36.588 $_{t(0.01,2)}$] (Fig. 5).

Concerning the time-scale for gelling, it is possible to observe from the graph on Figure 5 that it is of ~11 s (11.27 ± 0.40 s) [11.258, 11.275 $_{t(0.01,2)}$]. The results obtained for both temperature and time of gelation provide important information concerning subsequent experiments for cells encapsulation. The temperature at which the sol-gel transition occurs, and the overall residence time, is similar to other hydrogels used for the same purpose.[27,44] Gellan gum hydrogels allowed for a homogeneous cell suspension to be prepared at a temperature above the setting temperature of the gels. At such temperatures, the viscosity of the solution presents values near to zero, which enable it to be mixed with the cells, ressuspended to generate a uniform cells distribution, and then lower the temperature to allow gel formation and cells entrapment within the newly formed matrix. The quick gelling time may be useful in the use of gellan gum as an injectable system that could deliver cells through a minimally invasive procedure, although these kinetics can be modified.

Cytotoxicity evaluation

The MTS cytotoxicity test results showed that the gellan gum hydrogels did not cause any deleterious alteration to the metabolic activity of L929 cells and thus, were considered as noncytotoxic (Fig. 6). The results were comparable to those obtained with tissue culture polystyrene (TCPS), which was used as the negative control for cell death (differences are not statistically significant). The toxic effect of the positive control for cell death (latex) was clear, given the severe decrease on cell viability shown on the graph (Fig. 6).

Human nasal chondrocytes encapsulated in gellan gum and agarose hydrogels: Cell viability tests and histological analysis

Figure 7(A,B) show the human nasal chondrocytes efficiently encapsulated in the gels. This result is extremely important if the aim of the gellan gum supports is their use as cells encapsulating agents to be used in cartilage regeneration approaches. In fact, it is known that anchorage independent cells like chondrocytes exhibit good cell viability within hydrophilic scaffolds like hydrogels, and studies

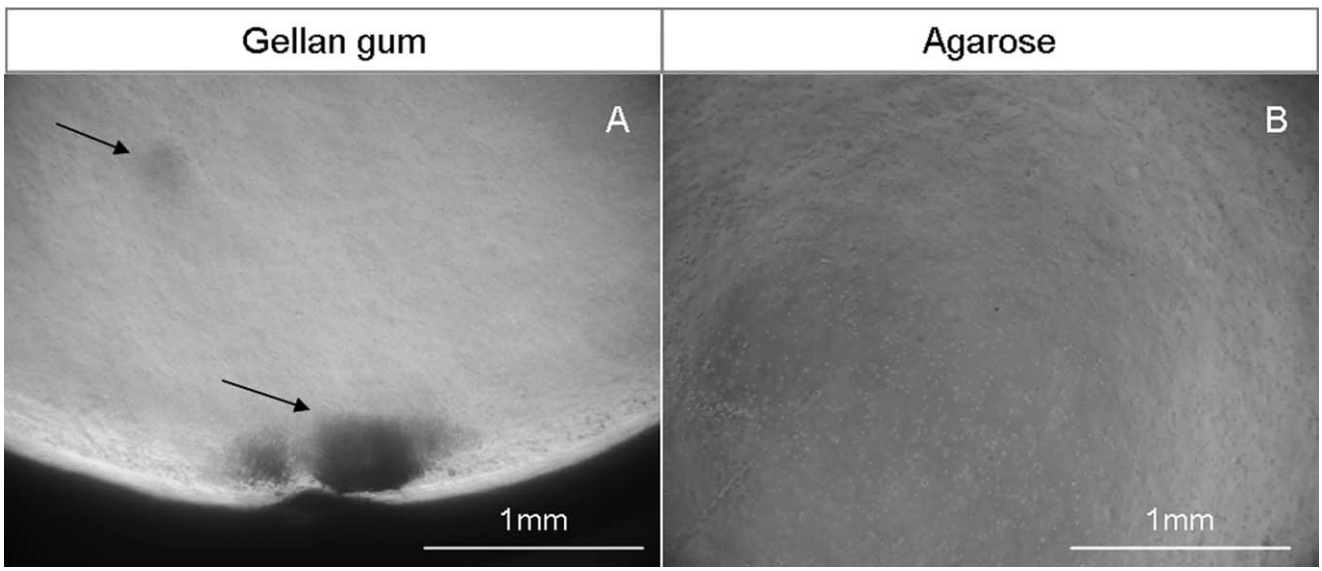

Figure 7. Optical microscopy images of human nasal chondrocytes encapsulated in gellan gum (A) and agarose (B) at 2 weeks of culture. The formation of human chondrocytes clusters was observed in gellan gum hydrogels (A, arrows).

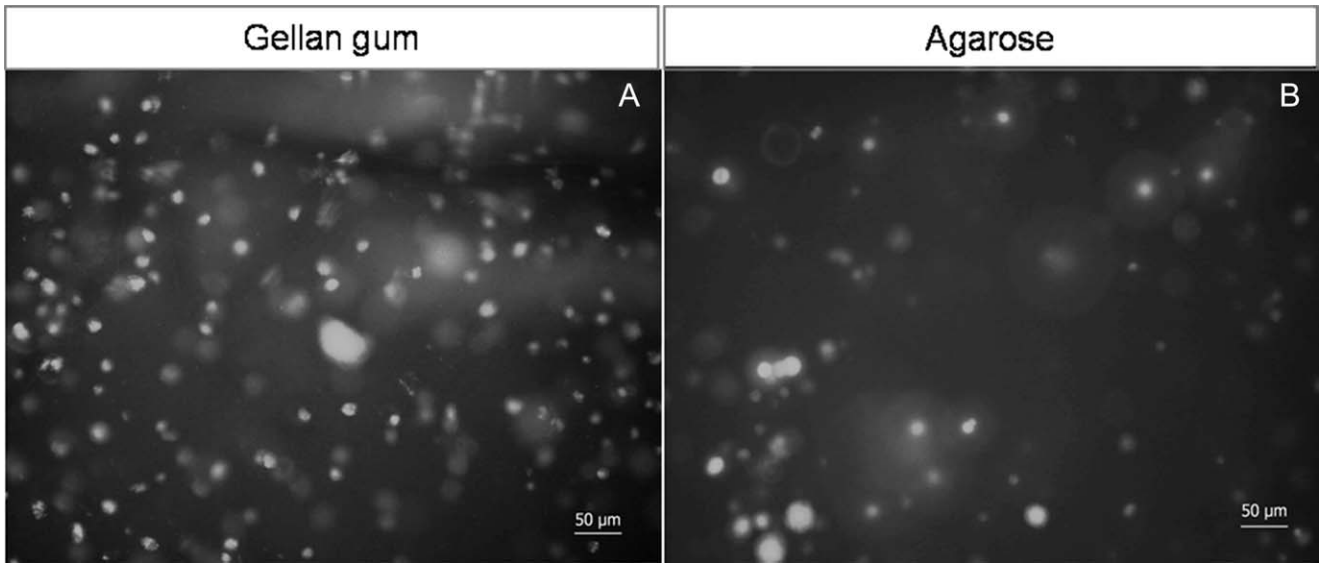

Figure 8. Calcein AM viability test of human nasal chondrocytes encapsulated in gellan gum (A) and agarose (B) hydrogels at 2 weeks of culture.

using human nasal chondrocytes revealed that this hydrophilicity facilitated the redifferentiation of de-differentiated chondrocytes.[34] This evidence opens interesting prospects for the performance of these new supports in cartilage regeneration along with the rather homogeneous distribution of the chondrocytes throughout the gellan gum hydrogels matrix (Fig. 7), which showed a round-shaped morphology typically present in the native human cartilaginous tissue. An interesting result was observed in gellan gum but not in agarose. In the first, the formation of chondrocytes clusters was frequently observed near 2 weeks of culture [Fig. 7(A)], a feature that was not noticed on the early periods of culture. Such structures may be indicative of cell proliferation in these clusters which may give a positive contribution towards the production of a hyaline-like cartilage matrix[45–47] The cells may use the gellan gum as a source of carbohydrates due to its polysaccharidic nature, a fact that may be even more interesting to study in an in vivo scenario. The scenario of cells using the matrix as a source of energy is possible although such hypothesis demand proper validation testing. Also, the formation of the chondrocytes clusters has been previously described in the literature as osteoarthritis related events.[47,48] This seems however unlikely because no typically hypertrophic cells with increased cell size were observed, but these assumptions should be confirmed in further studies.

Calcein AM fluorescence-based method was conducted to confirm chondrocytes viability. Results from samples collected after 2 weeks of culture are presented on Figure 8 showing the cells were viable in both hydrogels. These are also indicative of the adequacy of gellan gum for cartilage regeneration, because no apparent difference is noticeable when compared to agarose hydrogels.

Concerning the histological analysis, it is clear from the images that cells distribution within the two supports are similar, presenting uniform distribution and active states of division (Fig. 9, arrows). This indicates that gellan gum allowed adequate chondrocytes encapsulation while its network matrix permits cells to encompass active division. Moreover, alcian blue staining indicates that proteoglycans are being deposited to a small extent in the pericellular regions of some groups of cells, while maintaining the typical chondrocyte round-shaped phenotype (Fig. 10, arrows).

The data collected so far with gellan gum hydrogels showed that they possess suitable materials properties to be used as supports for chondrocytes development, such as the gelling at physiological conditions and their tested noncytotoxicity. Furthermore, they were able to efficiently encapsulate human nasal chondrocytes with a homogeneous distribution and maintain their viability for at least 2 weeks of culture. The overall data analysis suggests that this new biomaterial can have a high potential application in cartilage regeneration approaches and work is ongoing to further corroborate this hypothesis. Another aspect to look into is their potential use for other types of tissues or strategies, given its versatility in terms of processing and materials properties.

CONCLUSIONS

In this work, gellan gum has been presented as a new biomaterial for cartilage tissue engineering

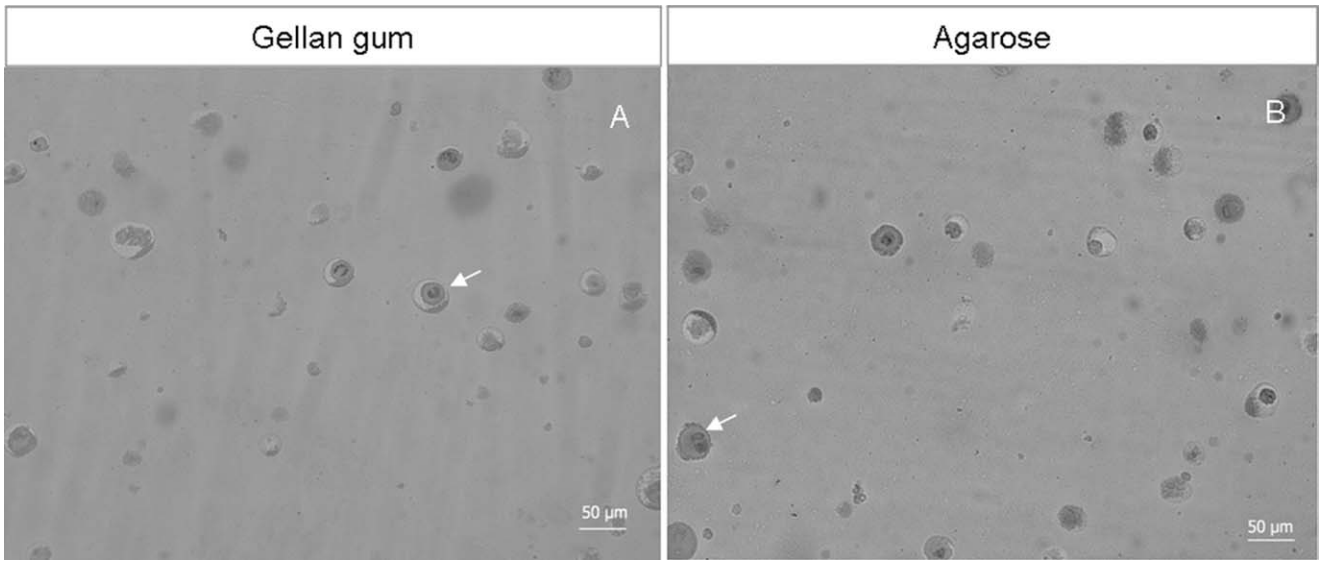

Figure 9. Hematoxylin-eosin staining of histological sections of gellan gum (A) and agarose (B) hydrogels at 2 weeks of culture. Human nasal chondrocytes present a typical round-shaped morphology and active cell division can be observed in both supports (arrows).

approaches. Gellan gum was shown to be very versatile in terms of processing, which can be controlled by both temperature and pH, forming structures with different shapes using simple polymer processing technologies. Discs, membranes, fibers, particles, and scaffolds were produced demonstrating the range of possible applications for this biomaterial. These may range from cell encapsulation technologies to drug delivery strategies, for example. An extensive characterization of Gellan gum discs to be used as cell supports indicated that they are suitable for fulfilling such functions, since a solution combining nonharsh reagents (gellan gum, calcium chloride and water) can be prepared, mixed uniformly with human nasal chondrocytes, and gelled near the body temperature in few seconds, enabling a high cell entrapment yield and homogeneous distribution. Gellan gum hydrogels presented viscoelastic properties within the range of other hydrogels used for cells encapsulation[42,43] and were shown to be noncytotoxic. Calcein AM staining showed cells were viable during the time of the experiments. Hematoxylin-eosin staining revealed that active cells division was occurring and alcian blue staining indicated that extracellular matrix components are being deposited to a small extent in some pericellular regions.

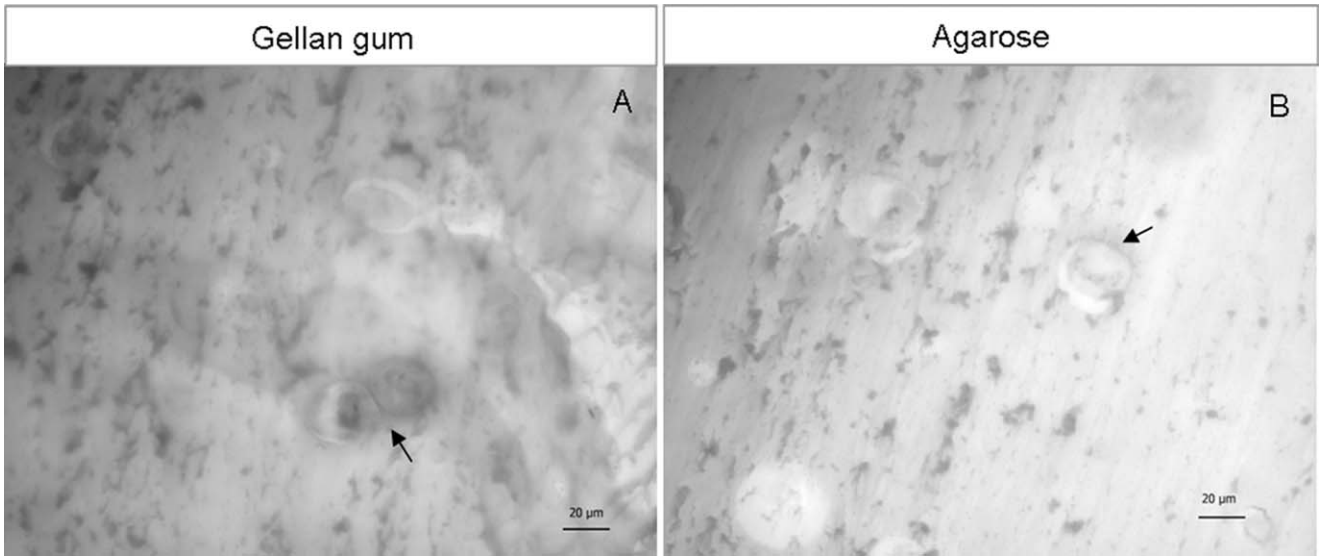

Figure 10. Alcian blue staining of histological sections of gellan gum (A) and agarose (B) hydrogels at 2 weeks of culture. Proteoglycans were detected in some pericellular regions in both supports (arrows).

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