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An injectable chitosan/chondroitin sulfate hydrogel with tunable mechanical properties for cell therapy/tissue engineering

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Abstract

Chitosan (CH) hydrogels with remarkable mechanical properties and rapid gelation rate were recently synthesized by combining sodium hydrogen carbonate (SHC) with another weak base, such as beta-glycerophosphate (BGP). To improve their biological responses, in the present study, chondroitin sulfate (CS) was added to these CH hydrogels. Hydrogel characteristics in terms of pH and osmolarity as well as rheological, mechanical, morphological and swelling properties were studied in the absence and presence of CS. Effect of CS addition on cytocompatibility of hydrogels was also assessed by evaluating viability and metabolic activity of encapsulated L929 fibroblasts. New CH hydrogels containing CS were thermosensitive and

injectable with pH and osmolality close to physiological levels and enhanced swelling capacity. Encapsulated cells were able to maintain their viability and proliferative capacity up to 7 days and CS addition improved viability of the cells, particularly in serum free conditions. Addition of CS showed a reducing and dose-dependent effect on the mechanical strength of the hydrogels after complete gelation. This work provides evidence that CH-CS hydrogels prepared with a combination of SHC and BGP as a gelling agent have a promising potential to be used as thermosensitive, injectable and biocompatible matrices with tunable mechanical properties for cell therapy applications.

Keywords:

Hydrogel; Chitosan; Chondroitin sulfate

1. Introduction

Cell therapy aims to regenerate tissues and organs by providing cells and/or bioactive substances that are entrapped within matrices [1]. Scaffolds intended for such applications are designed to mimic the native extra cellular matrix (ECM) as much as possible [1] and to deliver cells efficiently and specifically to the target site, avoiding the weaknesses of traditional cell therapy, such as limited cell retention and poor cell survival [2].

In this regard, injectable hydrogels have received much attention because of their interesting properties such as ability to retain high quantities of water, insignificant frictional irritation with the native tissue and minimally invasive delivery into the body [3]. Moreover, the three-dimensional and porous structure of such hydrogels make an ideal network which holds the cells in place while allowing the transport of nutrients, wastes, and other essential molecules [4].

Chitosan (CH), a natural linear copolymer of β-linked D-glucosamine and N-acetyl-D-glucosamine obtained from deacetylation of chitin, is broadly used for the synthesis of injectable hydrogels due to its interesting properties such as biocompatibility, biodegradability mucoadhesivity and antimicrobial and fungicidal activities [5-7]. Furthermore, it is possible to form thermosensitive hydrogels when mixing CH acidic solution with a weak base such as beta-glycerophosphate (BGP) [8, 9]. Recently, our team has shown the possibility of significantly increasing both mechanical properties and cytocompatibility of these hydrogels by combining BGP with another weak base, namely sodium hydrogen carbonate (SHC) [10, 11].

In this work, we hypothesized that the potential of these hydrogels for cell therapy applications can be further improved by their association with glycosaminoglycans (GAGs). GAGs are a key element of the extracellular matrix (ECM) and perform vital roles in cell signaling and cell-cell communication in all animal species [12]. In this regard, chondroitin sulfate (CS), a polysaccharide containing repeating units of glucuronic acid and galactosamine, is of particular interest. It is the most abundant GAG in the body and exists in a large number of structural proteoglycans found in many tissues, such as skin, cartilage, tendons, heart valves and central nervous system [13]. CS plays important roles such as providing resistance to compression forces and triggering key mechanisms involved in vascular repair and cell migration [14, 15]. It also presents anti-oxidant [16] and anti-apoptotic activities [14, 17] and plays important roles in the immune response such as growth factor activity regulation and recruitment of leukocytes [18]. It has a regulatory effect on the function of growth factors, namely fibroblast growth factor-2 and transforming growth factor-β [19-21] and is also involved in chondrogenesis and bone formation [22]. However, the high solubility of CS in physiological conditions limits its availability to the site of administration [23]. To solve this problem, CH-CS hydrogels can be easily formed since the cationic nature of CH facilitates the entrapment of the highly anionic GAGs [24].

In the present study, we developed a thermosensitive and injectable hydrogel composed of CH and CS through a relatively easy and fast method and studied the effect of CS addition on the hydrogel properties. The dose-dependent effects of CS were studied in terms of pH, osmolality, rheological, mechanical, morphological and swelling properties of hydrogels. Moreover, L929 fibroblasts were entrapped within hydrogels to evaluate the effect of CS addition on the viability and proliferation of cells for up to 7 days in presence or absence of serum.

2. Materials and methods

2.1. Materials

Shrimp shell chitosan (Kitomer, PSN 326-501, Premium Quality, Mw 250 kDa, DDA 94%) was purchased from Marinard Biotech (Rivière-au-Renard, QC, Canada). β-Glycerol phosphate disodium salt pentahydrate ($C_3H_7Na_2O_6P \cdot 5H_2O$, hereafter BGP) and chondroitin sulfate A sodium salt from bovine trachea were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium hydrogen carbonate $NaHCO_3$ (sodium bicarbonate, hereafter SHC) was purchased from

MP Biomedicals (Solon, OH, USA). Other chemicals were of reagent grade, and were used without further purification.

2.2. CH hydrogel preparation

CH was first purified using NaOH and sodium dodecyl sulfate as previously described [10]. CH was then freeze-dried, ground and sieved to obtain purified CH powder which was solubilized in HCl (0.1 M) at 3.33% (w/v). The solution was sterilized by autoclaving (20 min, 121°C) and stored at 4°C.

Gelling agents (GA) were either BGP alone, as a negative control, or a mixture of BGP with SHC (Table 1), chosen based on our previous work [11]. CS was dissolved in the GA at different concentrations, to reach 0 to 1% w/v in the final hydrogel. The solutions used for cell culture were then sterilized by filtration through 0.22 μm filters.

Hydrogels were prepared in two steps. First, the acidic CH solution was mixed with the GA solution (with and without CS) at a 3:1 (v/v) ratio at room temperature (RT) using two syringes and a Luer Lock connector (Figure 1). The CH-GA (with and without CS) mixture was then combined at a 4:1 (v/v) ratio with a third syringe containing cell culture media (DMEM:F12; Gibco Life technologies, USA) supplemented with 10% v/v fetal bovine serum (FBS; gibco, Life technologies, USA) and 100 U/mL penicillin, 100 g/mL streptomycin (P/S; Wisent Inc. Canada) with or without cells (10^6 cells/mL in the final hydrogel).

Table 1 summarizes the final composition of each tested hydrogel. To avoid repetition, only hydrogels containing 0, 0.5 and 1% w/v CS are presented in Table 1. Since CH concentration was kept constant at 2% (w/v) in all formulations, in this paper the name of the hydrogels will be based on the final molar concentration of the GA components in the formulation (e.g. SHC0.075BGP0.1 corresponds to a gel containing 2% (w/v) of CH, 0.075M SHC and 0.1M BGP). The concentration of CS present in the hydrogel is added at the end (e.g. BGP0.4+1%CS corresponds to a BGP0.4 hydrogel containing 1% w/v of CS).

2.3. Hydrogel characterization

2.3.1 pH and osmolality measurements

The pH and osmolality measurements were carried out at RT on hydrogel filtrates, obtained by pressing the hydrogels with 0.45 µm filters after 24h incubation at 37°C, using a Denver Instrument UltraBasic pH meter and an osmometer (Advanced® Micro Osmometer, Model 3300, Advanced Instruments Inc.) respectively.

2.3.2. Rheological properties

Rheological properties were investigated using an Anton Paar instrument (Physica MCR 301, Germany). Strain and frequency sweeps were first performed on fully formed gels to identify the linear viscoelastic region (LVE) [25]. To do so, hydrogel solution (2mL) was left to gel at 37°C for 24h in a 12-well plate (diameter=20mm) before testing the sample with a plate-plate geometry (PP25/P2). A strain sweep from 0.1 to 100% was conducted at a frequency of 1Hz. Afterwards, frequency sweeps from 0.01 to 100Hz were conducted at LVE strain amplitude determined in the preceding section.

The gelation kinetic was then studied with a coaxial cylinder geometry (CC10/T200). The evolution of the storage (G') and loss (G") moduli was measured in the LVE, at a constant strain of 5% and constant frequency of 1Hz, immediately after mixing the hydrogel components. The gelation kinetic was studied during isotherms at 37 °C for 1h. In addition, we followed the evolution of G' during isotherms at 22 °C followed by sudden increase at 37 °C, in order to confirm the thermosensitive properties of the hydrogels and mimic the temperature change occurring when the gel is prepared at RT followed by in vivo injection.

2.3.3. Mechanical properties

Viscoelastic properties of hydrogels after complete gelation, as a function of CS concentration were characterized using ElastoSens^{TM}Bio (Rheolution Inc), a novel technique using the dynamic displacement response of a material to a low amplitude vibration. This non-destructive and contactless technique was previously explained in detail and validated for measuring the mechanical properties of CH hydrogels [26]. Briefly, hydrogel solution (2 mL) was added into a detachable sample holder containing an elastic membrane at its bottom and incubated at 37 °C for 24h. PBS was added after 1 hour to avoid sample drying and removed just prior to measurements.

Free resonances of the samples in the LVE were detected between 20 Hz and 200 Hz. In this method, a laser is used to measure the displacement of the material during the vibration. This data is then converted to storage (G') and loss (G'') moduli by a theoretical model that takes into account the volume of the sample, the properties of sample holder membrane and the excitation signal.

2.3.4. Scanning electron microscopy (SEM) and optical microscopy

Morphology of freeze-dried hydrogels was investigated by SEM (Hitachi S-3600). Samples were left to gel for 24h at 37 °C, lyophilized, transversally cut using a surgical blade, deposited on double-coated carbon conductive tape and coated with a gold layer using an Emitech K550X sputter coater (Quorum Technologies Ltd).

In addition the porosity of the hydrated hydrogels was studied by optical microscopy (Leica DM LB2). A drop (0.2 mL) of hydrogel was placed on a glass microscope slide, covered by a coverslip and observed after 24h incubation at 37°C.

2.3.5. Fourier-transform infrared (FT-IR) spectroscopy

FTIR spectra were obtained for CS powder and freeze-dried SHC0.075BGP0.1 hydrogels containing CS (1% w/v) or not. FTIR spectra were recorded at wavenumbers ranging from 4000 to 400 cm^{-1} and at a 2 cm^{-1} resolution with 32 scans, using a Nicolet 6700 FTIR spectrometer (ThermoElectron Corporation). The pellets were prepared by the compression of homogenous mixtures of KBr and powder of each sample in flat-faced punches.

2.3.6. Swelling test

The swelling properties of hydrogels were determined using lyophilized samples. Weight of the samples was determined after immersion in PBS at RT at different time intervals once the excess liquid was removed from the surface (W_{wet}). The samples were put back in fresh PBS afterwards. At each time point, the swelling percentage was expressed as follows: Swelling (%) = $(W_{wet}-W_{dry})/W_{dry} \times 100$ where W_{dry} is the initial mass of the lyophilized samples.

2.3.7. Injectability

Injectability of hydrogels was studied by measuring the maximal force required to extrude hydrogels through a small diameter catheter (length = 150 cm and inner diameter = 0.54 mm, Fas

TrackerTM-18 MX Microcatheter, Boston Scientific) using an ElectroForce® 3200 instrument (Bose Corporation, USA) equipped with a 220 N load cell. After preparation, the hydrogel solution was placed in a 1 mL syringe connected to the catheter immersed in saline at 37 °C. The force required to extrude the gel was monitored immediately or after 5 min in catheter at 37 °C, and the maximum forces recorded for the various formulations were compared.

2.3.8. CS release from hydrogels

CS release from hydrogels was studied using dimethylmethylene blue (DMMB) assay [27]. Briefly, after 24h gelation at 37°C in cylindrical molds (2mL each), gels were immersed in PBS (7mL) and incubated at 37°C. Hydrogels without CS were used as controls. At different time points, the liquid was retrieved and replaced by fresh PBS. A volume of 100 µL of the solution was tested for CS concentration by mixing with 1 ml of DMMB color reagent containing 38 x 10^{-5} M 1,9-dimethylmethylene blue, 1 M guanidinium chloride, 0.2% formic acid, 2.9 x 10^{-5} M sodium formate and 4.8% ethanol. The absorbance was immediately read at 525 nm with a Varian Cary 300 Bio UV-vis spectrophotometer and CS concentration was extrapolated using a standard curve.

2.4. Cell experiments

2.4.1. Cell culture

L929 mouse fibroblast cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM: F12) supplemented with 10% v/v FBS and 1% v/v P/S and used between passages 3 to 15. Cells were mixed with hydrogel solutions as described previously and a volume of 0.5 mL of the mixture was deposited in 48-well plate culture dishes and left to gel at 37°C in an incubator. After 5 min, cell culture medium (0.5 mL per well) was added on top. Culture media was changed every 3 to 4 days.

For serum free experiments, hydrogel preparation was carried out in a similar manner except the culture medium added on top of hydrogels did not contain FBS. Moreover, media was changed after 4h incubation in order to remove traces of FBS diffused out of hydrogels.

2.4.2. Alamar blue assay

Metabolic activity of entrapped cells was evaluated using Alamar Blue (AB) assay (Resazurin Cell Viability Assay Kit, Biotium, USA) after 1, 3 and 7 days. To optimize the access of AB solution to the entrapped cells, it was mixed with the hydrogel by gently pipetting up and down with a 1000 µl pipette (P1000, Eppendorf). After 3h of incubation at 37 °C, 100 µl of supernatant was transferred to a 96-well plate and fluorescence emission intensity was quantified using a microplate fluorescence reader (λex 560 nm, λem 590 nm, BioTek Instruments Inc., Synergy 4, USA)

2.4.3. LIVE/DEAD assay

Cell viability after 1 and 7 days entrapment was investigated by Live/Dead assay using 1 µM calcein AM and 2 µM ethidium homodimer-3 in DMEM supplemented with 10% (v/v) FBS to stain dead cells in red and live cells in green (Life technologies, USA). After staining, hydrogels were washed with DMEM without FBS and observed under fluorescent microscopy (Leica DM IRB) at 5x magnification.

2.5. Statistics

Results are expressed as mean ± standard deviation (SD). One-way or two-way ANOVA and Tukey's multiple comparison tests were used as appropriate. $P<0.05$ was considered to be statistically significant. Statistical values were obtained using GraphPad Prism 6.0 software.

3. Results

3.1. Hydrogel formation, pH and osmolarity

Simple mixing of the CH acidic solution with any of the GA formulations (with or without CS) formed a solution with physiological pH, which gelled at 37°C (Figure 1). Table 1 summarizes the composition, pH and osmolality values of the hydrogels. After 24h gelation, all hydrogels possessed pH close to the physiological level. Addition of CS, even at the highest concentration, did not have a significant effect on the pH. Hydrogels synthesized using BGP0.4 were hypertonic (>800 mOsmol/L) while the osmolality of the hydrogels made with SHC0.075BGP0.1 were close to the physiological range (400-430 mOsmol/L). It should be noted that part of this value (estimated to 158 mOsmol/L) is due to culture media used to encapsulate the cells in the

hydrogels [11]. Addition of CS did not have a significant effect (no statistical difference) on the osmolality of the hydrogels (Table 1).

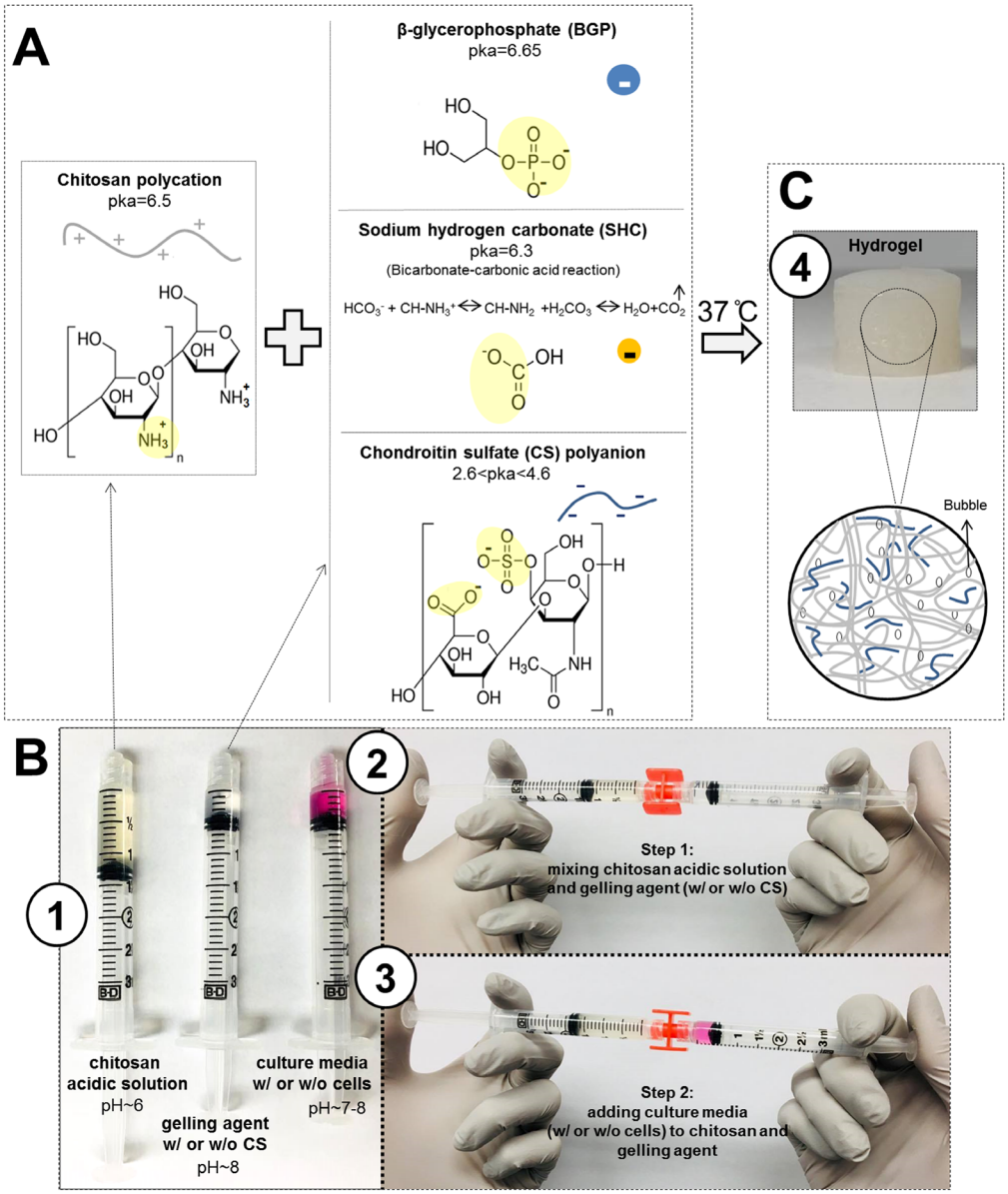

Figure 1. Schematic overview (not drawn to scale) of (A) chemical structure of the components, (B) preparation steps, and (C) structure of CH-CS hydrogels formed with BGP and SHC as gelling agents.

Table 1. Composition, pH and osmolality of the tested hydrogels

Hydrogel	[CH] (% w/v)	[GA] (M) BGP	[GA] (M) SHC	[CS] (% w/v)	pH after 24h	Osmolality (mOsmol/L)
BGP0.4	2	0.4	-	-	6.9 ± 0.2	829 ± 29
BGP0.4+0.5%CS	2	0.4	-	0.5	6.9 ± 0.1	844 ± 37
BGP0.4+1%CS	2	0.4	-	1	6.9 ± 0.1	858 ± 27
SHC0.075BGP0.1	2	0.1	0.075	-	6.7 ± 0.1	406 ± 13
SHC0.075BGP0.1+0.5%CS	2	0.1	0.075	0.5	7.1 ± 0.3	415 ± 16
SHC0.075BGP0.1+1%CS	2	0.1	0.075	1	7.1 ± 0.3	422 ± 14

3.2.Rheological properties of the CH-CS hydrogels

Strain sweeps (Figure S1 in supplementary data) and frequency sweeps (Figure S2 in supplementary data) were first performed on the fully formed gels to determine the LVE. Results of BGP0.4 hydrogels with and without CS are shown in the supplementary data (Figure S1 and S2) as representative values. Strain of 5% was chosen for further rheological characterizations based on linear behavior of storage modulus (G'). Frequency sweep results at 5% strain confirmed gel behavior (G' reaching a plateau value) up to 100 Hz for all hydrogels and a frequency of 1 Hz was chosen for the next tests based on good torque signal.

Time sweep analyses at the chosen strain and frequency (5% and 1 Hz) were performed to study the gelation kinetic of hydrogels prepared with or without CS (1% w/v) (Figure 2) and to demonstrate their thermosensitive properties (Figure 3). As shown in Figure 2, G' rapidly increased as a function of time at 37°C and didn't reach a plateau, indicating that the equilibrium state was not reached for any of the hydrogels after 1h at 37°C. The gelation time (time where G' = G'' according to the approach from Winter and Chambon 1986 [28]) was less than 15sec for BGP0.4 hydrogels. Addition of CS (1% w/v) decreased G' values (700 Pa at 1 h versus 1200 Pa in the absence of CS) and slightly slowed down gelation kinetics as the crossover of G' and G'' was observed at around 90 sec. Very similar effects were observed for SHC0.075BGP0.1 hydrogels, despite significantly higher G' values (reaching to more than 1500 and 4000 Pa after 1h in the presence or absence of CS respectively).

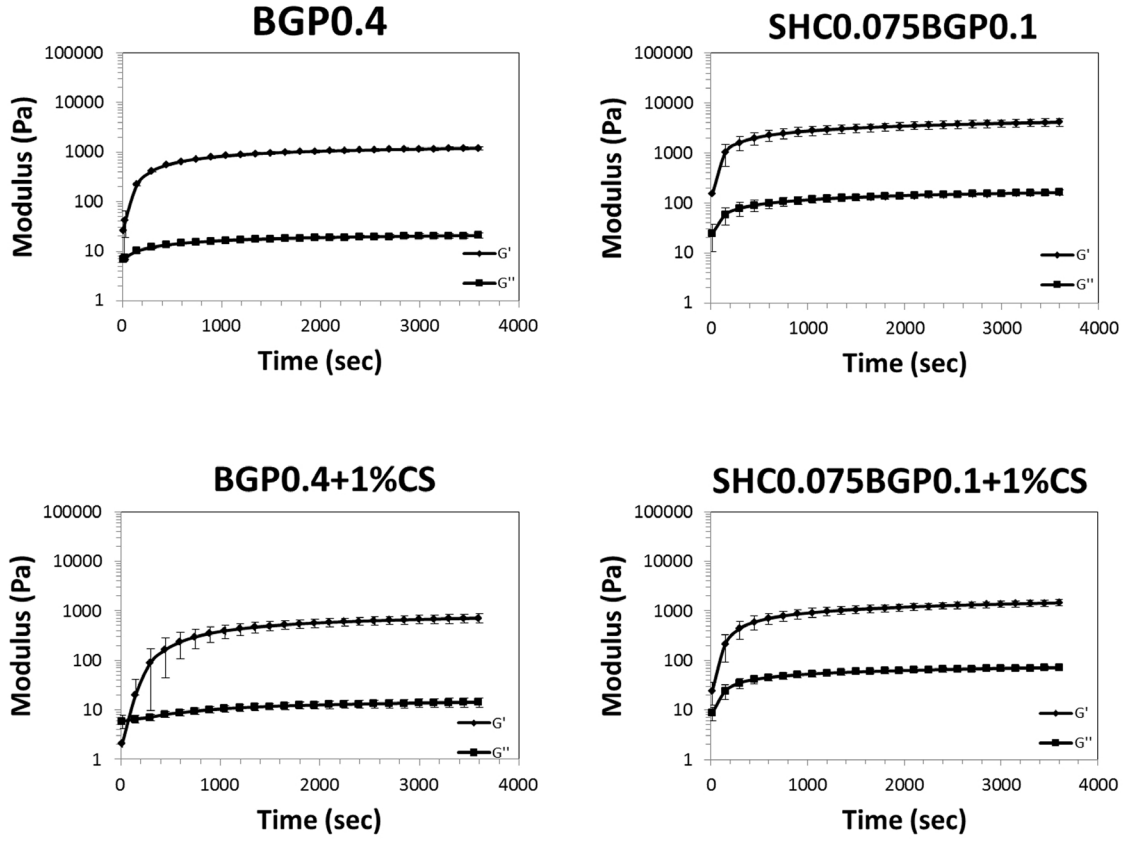

Figure 2. Effect of CS on the rheological properties of hydrogels. Evolution of the storage (G') and loss (G'') moduli with time at 37 °C monitored in the LVE (strain 5%, frequency 1 Hz) immediately following preparation (n≥3).

As shown in Figure 3A, SHC0.075BGP0.1 hydrogels presented relatively stable G' values during isotherms at 22°C which were followed by sudden increase at 37°C confirming thermosensitive properties of these hydrogels. Similar behavior was observed for CH-CS gel, despite a less drastic increase of G' when temperature was raised to 37°C. The same trend was observed for BGP0.4 hydrogels (data not shown). A closer look at the evolution of G' and G''of SHC0.075BGP0.1 hydrogels containing different concentrations of CS during isotherms at 22°C is presented in Figure 3B. Addition of CS resulted in more rapid gelation as G' intersected G'' after 10 min in the absence of CS, while the intersection occurred after 1 min and less than 15 sec in presence of 0.5 and 1% w/v CS respectively.

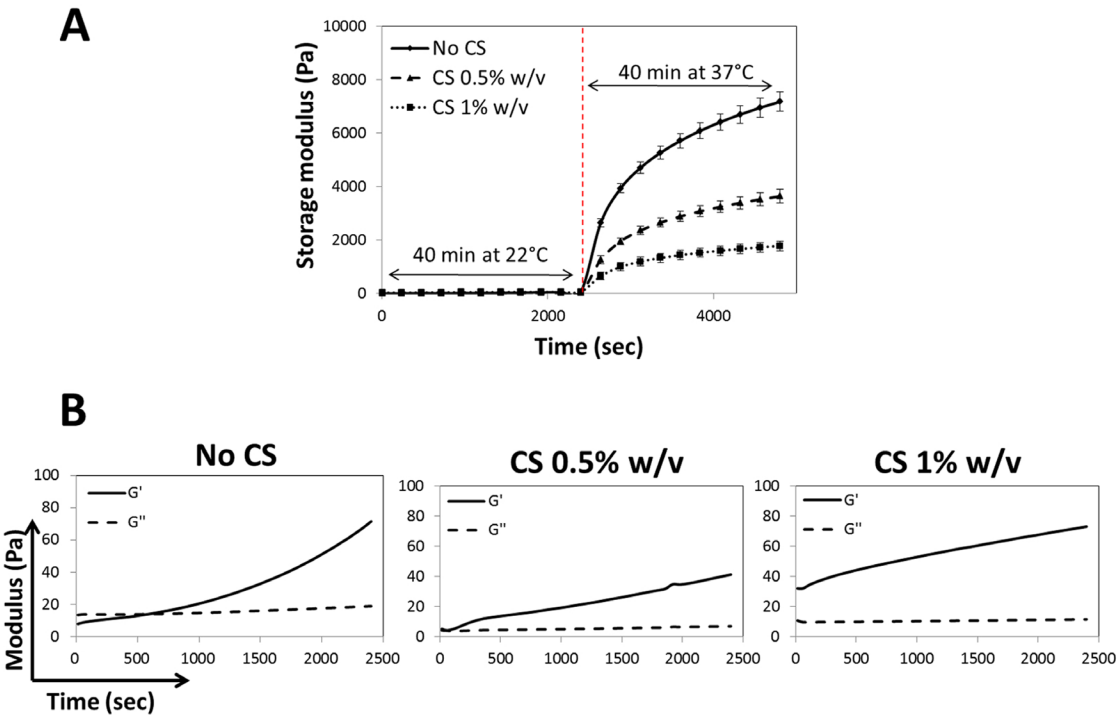

Figure 3. (A) Effect of CS on the thermosensitivity of SHC0.075BGP0.1 hydrogel. Evolution of storage modulus (G') during isotherms at 22°C (40 min) followed by 37°C (40 min) (strain 5%, frequency 1Hz). The dotted line indicates the time of the sudden temperature increase (n=3). (B) Effect of CS on the storage (G') and loss (G'') moduli of SHC0.075BGP0.1 hydrogel at 22°C (n≥3).

3.3.Mechanical properties of the CH-CS hydrogels

Figure 4 presents the shear storage modulus (G') in steady solid state after 24h gelation at 37 °C obtained by ElastoSens^{TM}Bio. BGP0.4 hydrogels presented a very low storage modulus (<1kPa), without significant difference among hydrogels with different concentrations of CS (Figure 4A). In contrast, SHC0.075BGP0.1 hydrogels showed much higher mechanical properties with G' reaching to ~18 kPa in the absence of CS. Addition of CS had a negative and dose-dependent effect on the final mechanical properties of SHC0.075BGP0.1 hydrogels with G' decreasing to ~5 kPa for 1% (w/v) of CS (Figure 4B). This however remains significantly higher than all BGP0.4 hydrogels.

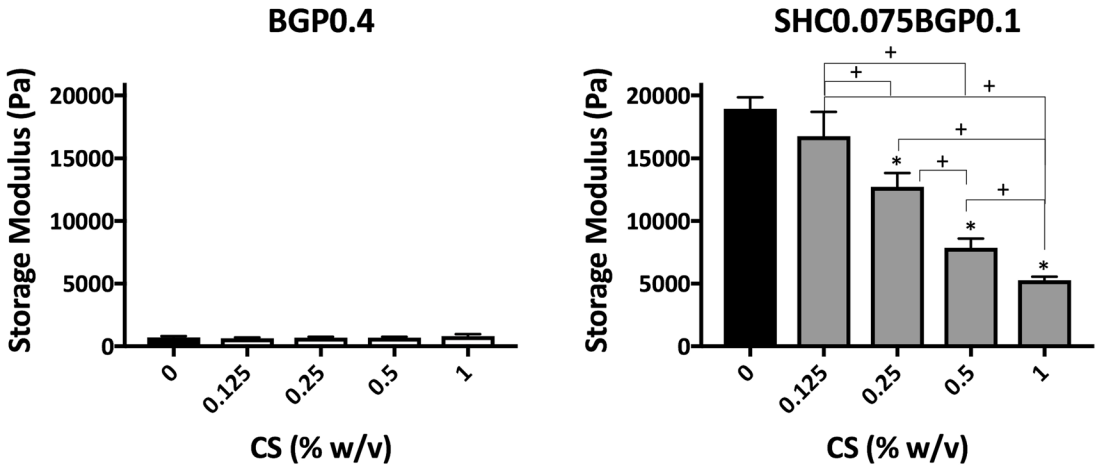

Figure 4. Effect of CS on the shear storage modulus (G') of hydrogels after 24h of gelation at 37°C measured by ElastoSens^{TM}Bio (n=6). * $p < 0.05$ compared to 0% CS; + $p < 0.05$ among different concentrations of CS.

3.4.Injectability of the CH-CS hydrogels

To confirm injectability, the maximal force required to extrude each hydrogel through a microcatheter (inner diameter of 0.54 mm, length=150 cm) immersed in saline at 37 °C was measured. Although all formulations were manually injectable both immediately after introduction to the catheter and after 5 min at 37 °C, the maximum force needed to extrude the hydrogels was increased with time (Figure 5).

Addition of CS (1% w/v) did not have any significant effect on the injectability of BGP0.4 hydrogels while SHC0.075BGP0.1 hydrogels were more easily extruded when containing CS. These results are in direct correlation with rheological properties of SHC0.075BGP0.1 hydrogels during gelation at 37 °C showing a decrease in the storage modulus of the hydrogels containing CS compared to those without CS.

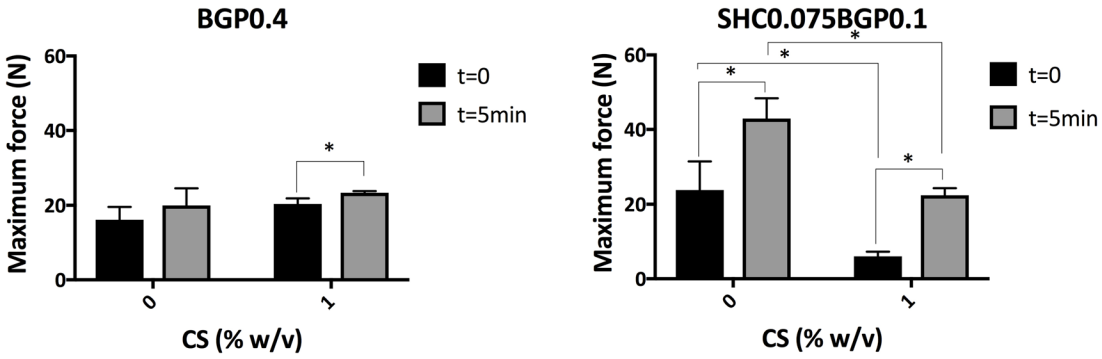

Figure 5. Effect of CS on the injectability of hydrogels immediately after introduction to a catheter at 37 °C (t=0) and after 5 min at 37 °C (t=5 min) (n=3).*$p<0.05$.

3.5.Porous structure of the CH-CS hydrogels

Figure 6 shows the morphology of freeze-dried hydrogels. An open porous structure was seen for all formulations and addition of CS (1% w/v) tend to decrease the pore size (Figure 6). Since lyophilisation alters porosity of the hydrogels by inducing rapid cooling and phase separation followed by sublimation under vacuum which results in void formation in the structure of hydrogels [29], samples were also analyzed in hydrated state, under optical microscope (Figure 6, insets). Non-interconnected bubbles with different sizes could be seen, which may be the result of the mixing process (Luer lock system) [10].

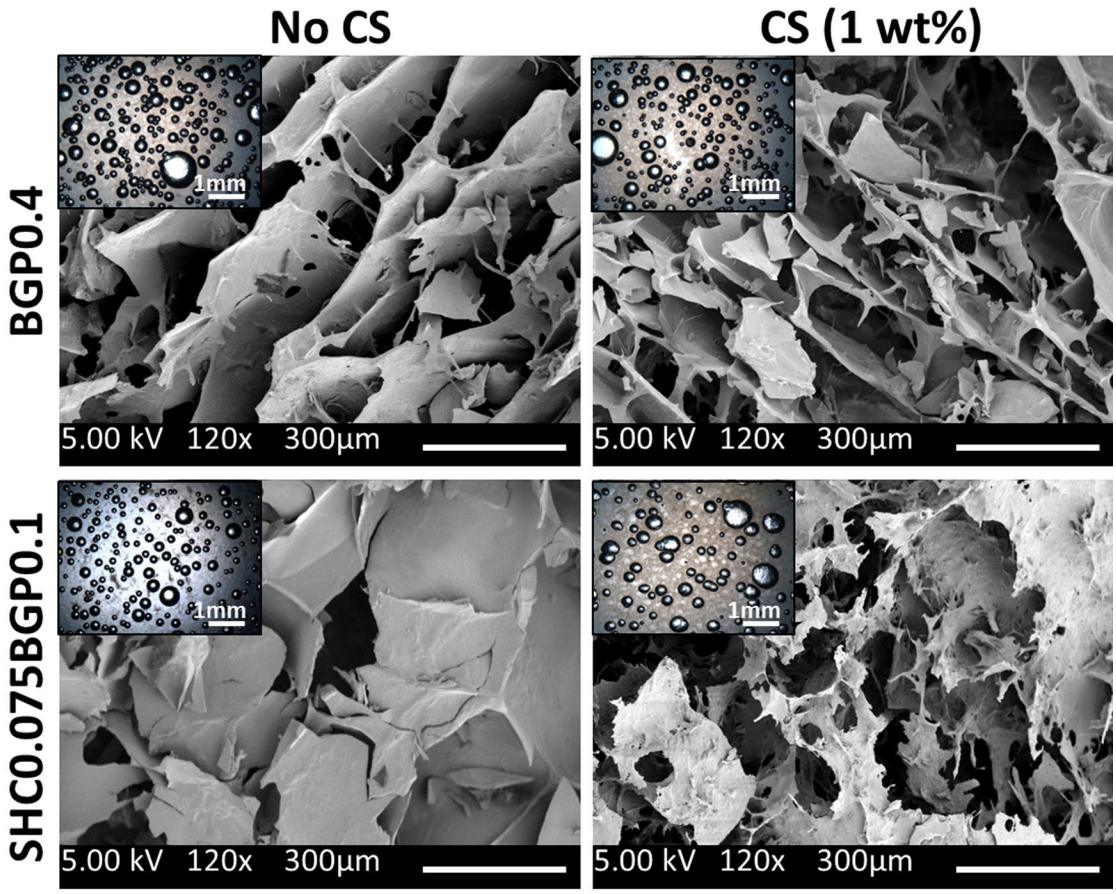

Figure 6. Effect of CS on the porous structure of lyophilized hydrogels visualized under SEM and that of hydrated hydrogels visualized by optical microscopy (insets).

3.6.Swelling properties of the CH-CS hydrogels

Figure 7 presents swelling properties of the hydrogels. Lyophilized BGP0.4 and SHC0.075BGP0.1 hydrogels respectively showed 508 ± 56% and 621 ± 101% increase in weight after 4h immersion in PBS. While addition of CS (1% w/v) had negligible effect on the swelling capacity of BGP0.4 hydrogels , both 0.5 and 1% w/v CS addition resulted in significantly higher swelling of SHC0.075BGP0.1 hydrogels after 4h immersion in PBS. The wet weight of both BGP0.4 and SHC0.075BGP0.1 hydrogels then remained quite stable up to 7 days.

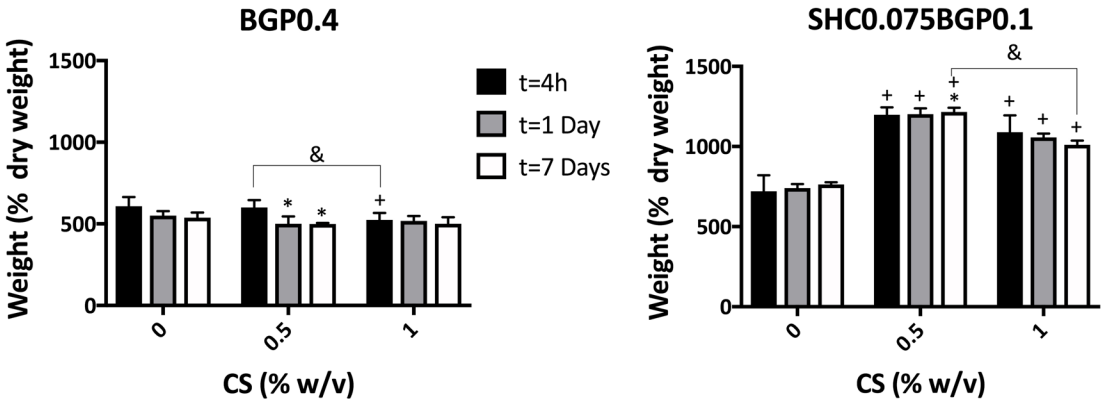

Figure 7. Effect of CS on the swelling properties of hydrogels, expressed as wet weight after 4h, 1 and 7 days immersion in PBS at RT normalized to initial dry weight (n=6).* $p< 0.05$ compared to t=4h for each formulation; + $p< 0.05$ compared to 0% CS for each time point; & $p< 0.05$ between 0.5 and 1% CS.

3.7.CS release from the CH-CS hydrogels

Figure 8 presents the release of CS from SHC0.075BGP0.1 hydrogel containing CS (1% w/v) in PBS, as quantified by DMMB assay. A rapid burst release was observed during the first few hours followed by a plateau after 24h, without further significant release up to 15 days (data not shown). In total, only a small fraction of CS was released from the hydrogels (17% after 15 days) suggesting that most of the CS made complexes with CH. This is in accordance with the changes in the FTIR spectrum for SHC0.075BGP0.1 hydrogel containing CS (1% w/v) compared to that of SHC0.075BGP0.1 hydrogel without CS (Figure 8B). In particular, bands at 2800-3600 cm^{-1} (corresponding to BGP hydroxyl and alkyl groups) and 600-800 cm^{-1} (corresponding to amine groups in CH and sulfur-oxygen compounds in CS) decreased or disappeared after addition of CS indicating importance of these groups in CH-CS combination.

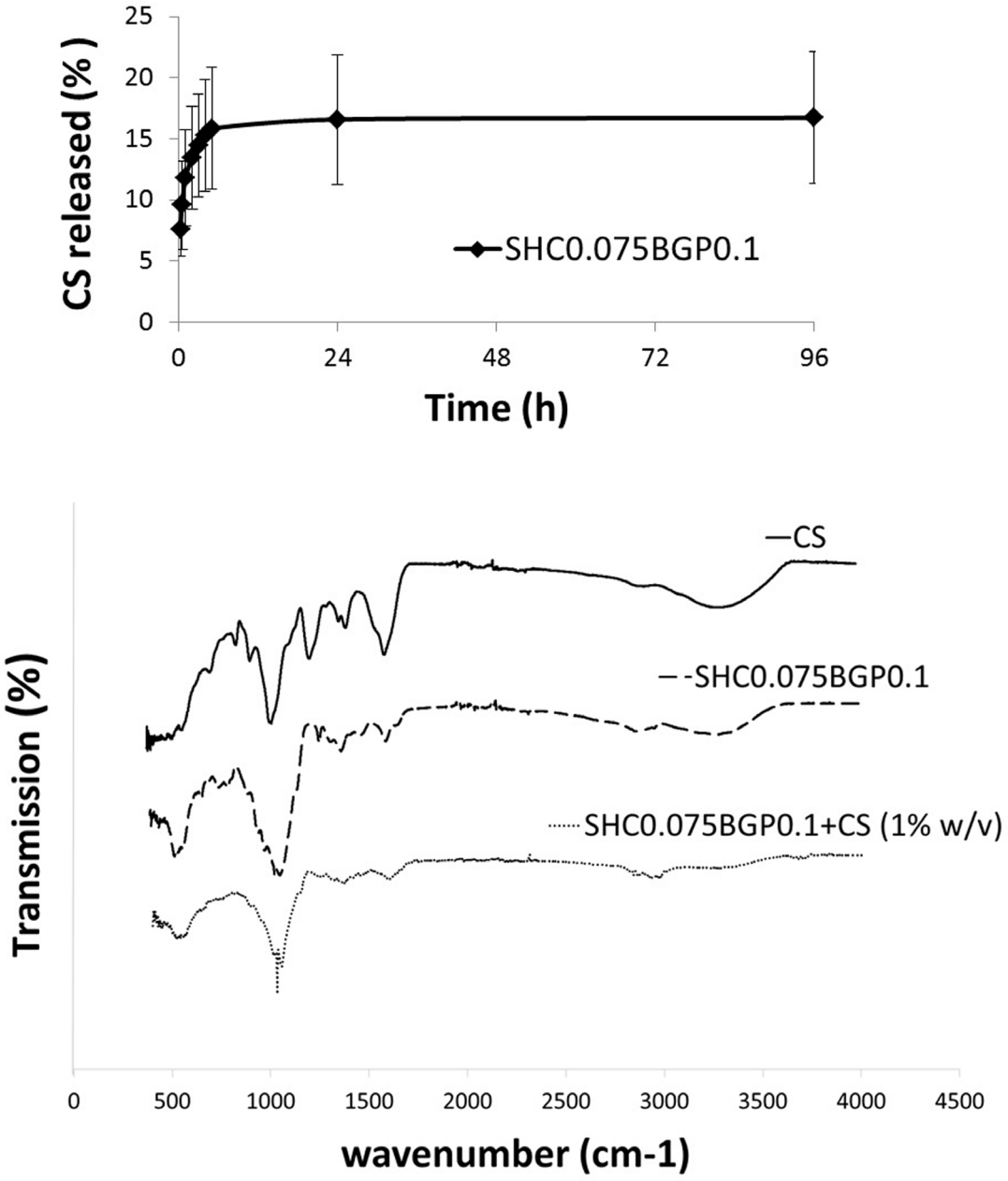

Figure 8. (A) Release of CS from SHC0.075BGP0.1 hydrogel with time measured by DMMB assay (n=6). (B) FTIR spectra of chondroitin sulfate (CS), SHC0.075BGP0.1 hydrogel and SHC0.075BGP0.1 hydrogel containing 1% w/v CS.

3.8.Viability and metabolic activity of the encapsulated cells

Survival and growth of L929 fibroblasts entrapped in CH and CH-CS hydrogels were first studied in normal cell culture media containing serum (10% v/v) (Figure 9 A and B). Cells entrapped in BGP0.4 hydrogels with and without CS showed low initial metabolic activity, which was further reduced over time. After 7 days in culture, no cell viability was detected in BGP0.4 hydrogels as confirmed by LIVE/DEAD assay (Figure 9B).

In contrast, in SHC0.075BGP0.1 hydrogels, the metabolic activity after 24h was at least 3 fold higher than in BGP0.4 gels ($p<0.0001$) and it further increased with time. Moreover, addition of CS (1% w/v) resulted in significantly higher cell viability ($p<0.01$) at each time point. For hydrogels containing 0.5% CS, the difference was only significant at day 1 and 3. These results were confirmed by LIVE/DEAD assay that suggested the presence of fewer dead cells when the hydrogels contained 1% CS (Figure 9B).

In low serum conditions, CS showed an even more pronounced positive effect on the viability of encapsulated cells (Figure 9C); SHC0.075BGP0.1 hydrogels containing 0.5 and 1% w/v CS presenting 2 and 2.8 fold higher signal than hydrogels without CS. Interestingly, cells were able to significantly proliferate until 3 days despite the very low amount of FBS, suggesting that these hydrogels provide an environment that favors cell survival in the absence of serum.

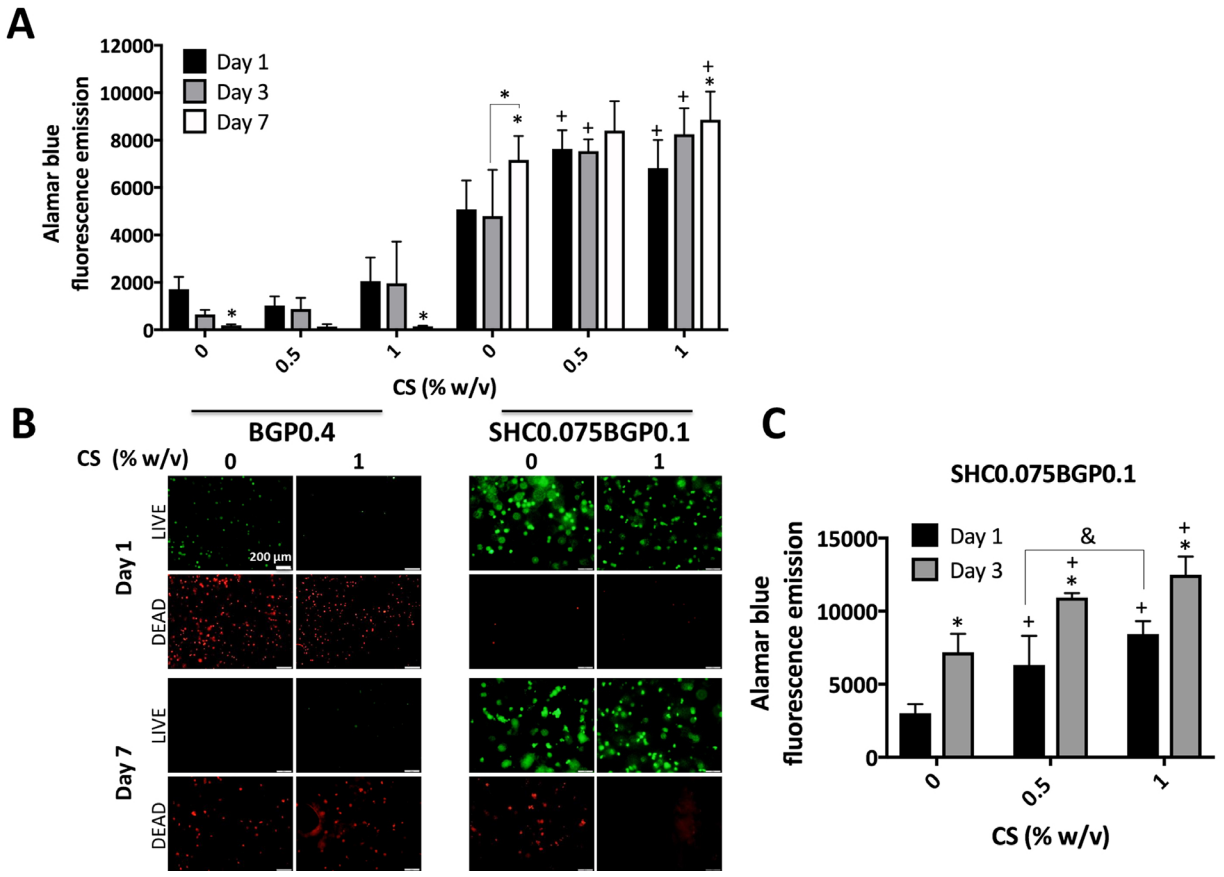

Figure 9. Effect of CS on the cytocompatibility of hydrogels. (A) Metabolic activity of L929 fibroblasts encapsulated in the hydrogels after 1, 3 and 7 days measured by Alamar Blue assay (n=12). Results with BGP0.4 were significantly lower at all time points compared to SHC0.075BGP0.1 hydrogels, thus differences are not indicated in the figure. (B) LIVE/DEAD staining of L929 fibroblasts encapsulated in the hydrogels for 1 and 7 days (bar=200µm). (C) Metabolic activity of encapsulated L929 fibroblasts in SHC0.075BGP0.1 hydrogels after 1 and 3 days in the absence of serum measured by Alamar Blue assay (n=6).* $p<0.05$ compared to Day 1 for the same formulation; + $p<0.05$ compared to 0% CS for the same time point; & $p< 0.05$ between 0.5 and 1% CS.

4. Discussion

The optimal scaffold for tissue engineering and regeneration strategies is injectable and possesses mechanical and physiochemical properties similar to the native ECM. In this study, we proposed to incorporate CS into the physical and thermosensitive CH hydrogels recently developed by our team [10], in order to further mimic the ECM and create a microenvironment that comprises physical, chemical, and mechanical cues closer to native tissue. CH hydrogel synthesized using BGP0.4M as a GA was chosen as a control material in this study since it is known to present an ideal gelation kinetic, which allows injectability through small lumen despite rapid solidification. However, as expected, hydrogels with high concentration of BGP (0.4M) showed cytotoxic effect which is probably due to their hyperosmolarity [30]. Hydrogels made by combining BGP with SHC (SHC0.075BGP0.1) demonstrated a very similar gelation kinetic and thermosensitivity as well as physiological osmolality which at least partly explains the much higher cellular viability observed with these hydrogels. Addition of CS improved in vitro viability and proliferation of encapsulated fibroblasts in the presence of serum and more effectively in serum free conditions. This can be explained by several factors. First, CS anti-apoptotic properties, as CS was shown to increase resistance to apoptosis in the presence of FBS in chondrocytes [16] and in the absence of serum in fibroblasts [17] and vascular smooth muscle cells [14, 15]. Secondly, we showed that addition of CS increased the swelling capacity of SHC0.075BGP0.1 hydrogels due to the hydrophilicity of CS. The higher water content will favor the viability and proliferation of the encapsulated cells by causing lower interfacial tension and increasing the ion, nutrient and metabolite exchange with the surrounding environment [31]. Moreover, CS is known to interact with some growth factors such as FGF and could therefore help retain them inside the hydrogel [19, 21]. Finally, the effect of CS on gel morphology might also have played a role since porosity and pore architecture are known to influence cell survival, proliferation, and migration in hydrogels [29].

The thermosensitive gelation process of these physical hydrogels has several advantages. As shown in Figure 1, hydrogel preparation is simple and involves no chemical reaction. It forms a liquid solution at physiological pH at RT, in which the cells can be suspended and delivered in vivo to the site of interest through a simple injection. This provides not only the elimination of

surgical procedures that are required in the case of implantable matrices, but also minimizes the damage to the surrounding tissue. Upon delivery to the site of interest in the body, the temperature dependent gelation mechanism initiates. It was shown that in CH-BGP hydrogels, the gelation mechanism involves transfer of protons from CH to BGP during heating, resulting in CH neutralization, strengthening of the hydrophobic CH-CH interactions, and physical gel formation [32]. The mechanism implicated is believed to be similar for CH-SHC-BGP hydrogels. At RT, the negatively charged GA components screen the electrostatic repulsions between positively charged CH chains ($–NH_3^+$ groups), preventing precipitation despite physiological pH. Upon raising the temperature to 37°C, proton transfer occurs, CH chains become less soluble and modification of CH molecular confrontation and hydrogen bonding along with increase of hydrophobic interactions result in the formation of a physical hydrogel [9, 33, 34]. The higher mechanical properties of gels formed with SHC compared to BGP only could be explained by more complete neutralization of chitosan chains, the smaller size of SHC molecule and its decomposition into CO_2 which enables stronger interactions between chitosan chains [10].

This thermosensitive hydrogel represents a significant advantage over other systems where cooling down from higher temperatures [35], in situ photo-crosslinking [36, 37] or chemical cross-linking [38] is used because it eliminates safety concerns regarding high temperature, UV or chemical exposure to implanted cells and the tissue itself. In addition, in this study, CS was directly added to the GA (pH~8) to avoid additional chemical modifications such as EDC/NHS [39]. Depending on the hydrogel components and pH of the solutions, CS can either form a polyelectrolyte complex (PEC) with CH [5] or simply be entrapped in the hydrogel network. Theoretically, formation of PEC can only occur at pH values in the vicinity of the pKa interval of the two polymers (i.e. $2.6 \leq pKa \leq 6.5$ since sulfate and carboxyl groups in CS respectively have $pKa \approx 2.6$ and ≈ 4.6 and amino groups in CH have $pka \approx 6.5$) [23, 40]. However, it is shown that when BGP is present, CH is soluble at near neutral pH and apparent proton dissociation constant of CH increases strongly with increased salt [41]. Therefore, the pH interval for the formation of PEC between CH and CS in our system might be larger. In the absence of CS, hydrogel formation is achieved by non-covalent physical associations, such as secondary forces (hydrogen, ionic, or hydrophobic bonding) between CH chains [32]. Introduction of CS into this system may interfere with the arrangement of the network, by spatial occupation and also due to the fact that some of the ionized sites on the CH chain form a complex with CS and therefore less ionic

interaction may occur between the GA and CH during gelation. In our hydrogel system, since some of the CH ionized sites would be occupied with GA, it is plausible that CS could only partly form PEC with CH. The other part was simply entrapped in the network and probably corresponds to the fraction of CS which was found to be rapidly released from the hydrogel (about 17%). The hydrogels were shown to be stable in water. This is in agreement with Piai et al. 2010 who also showed that after an initial burst release of CS, CH/CS hydrogels are stable in buffers with pH of 1 to 12 [42].

This also explains why the addition of CS decreased the gel mechanical properties in a dose-dependent manner. It should be noted, however, that this new chitosan physical hydrogel formulation (prepared with SHC-BGP mixture as gelling agent), even with CS, still presents clearly enhanced mechanical properties compared to conventional CH-BGP hydrogels. Moreover, the dose-dependent diminishing effect of mechanical properties enables to fine tune the mechanical properties of the hydrogel and thus mimic that of tissue of interest. For example, in adipose tissue engineering and especially for applications in plastic and reconstructive surgery, mechanical mismatching between the scaffold and native tissue can result in scar tissue formation that can cause poor implant integration, an unnatural feel and appearance, and possibly implant migration or failure [43]. Therefore, it is of particular importance to be able to tune mechanical properties of the hydrogels to mimic native soft tissues and avoid frictional irritation after implantation. In contrast, in applications where the hydrogel will be under large stresses, such as in situ placement for articular cartilage tissue engineering or intervertebral disc regeneration [44], a hydrogel with high mechanical strength is in demand.

5. Conclusion

In this study CS was added into CH hydrogels with optimized gelling kinetics and high mechanical properties through a simple, non-chemical step. Addition of CS increased the swelling capacity of the hydrogels and favoured viability and proliferation of the fibroblasts encapsulated in the hydrogels, especially in the absence of serum. CS addition did not alter the themosensitive characteristics of the hydrogels and it had a diminishing dose-dependent effect on the final mechanical properties. These results suggest that these CH/CS hydrogels have a potential to be used as vehicles with tunable mechanical properties for delivery of therapeutic cells.

Acknowledgements

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Supplementary data

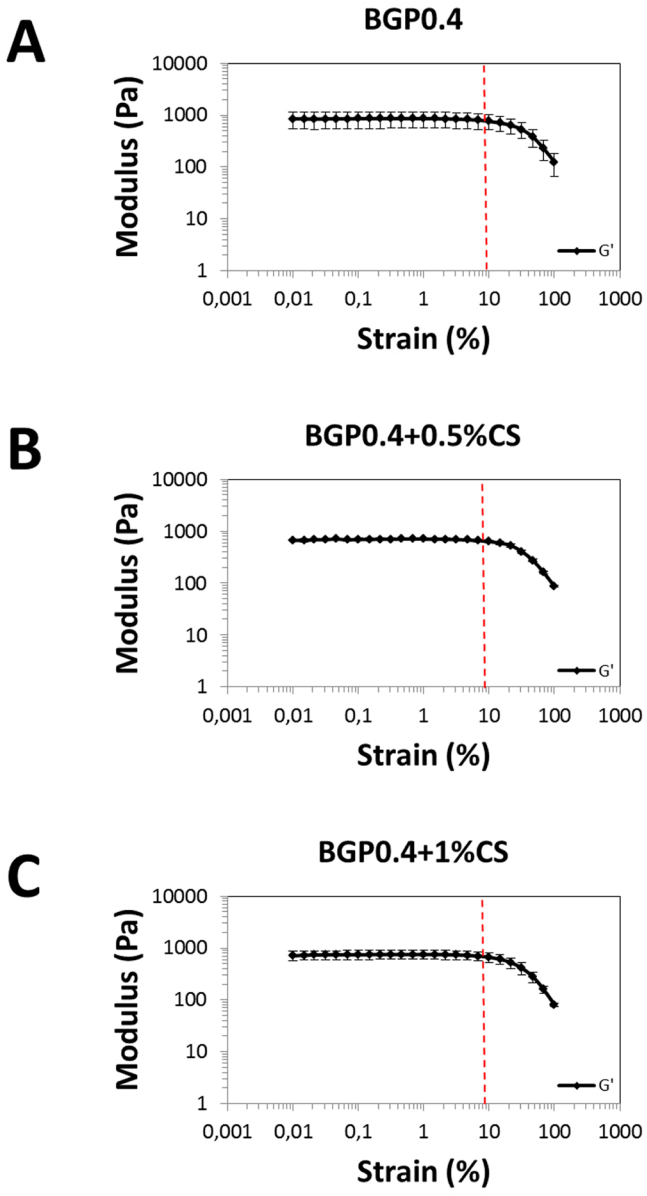

Figure S1. Strain sweeps for BGP0.4 hydrogels (A) without CS and with (B) 0.5% w/v and (C) 1% w/v CS: Storage modulus (G') values for 0.1 to 100% strain. Dotted line shows the limit of the linear viscoelastic region (LVE).

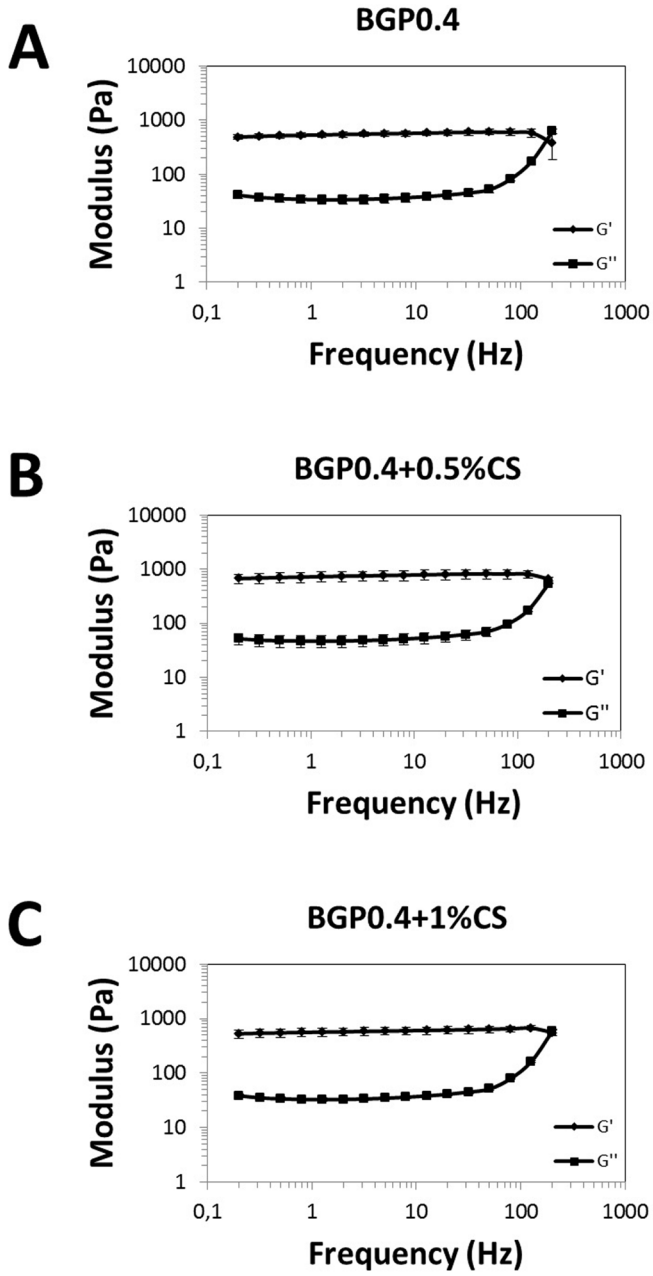

Figure S2. Frequency sweeps for BGP0.4 hydrogels (A) without CS and with (B) 0.5% w/v and (C) 1% w/v CS: Storage (G') and loss (G'') moduli values from 0.01 to 100 HZ (strain 5%).