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Optimization of Injectable Thermosensitive Scaffolds With Enhanced Mechanical Properties For Cell Therapy

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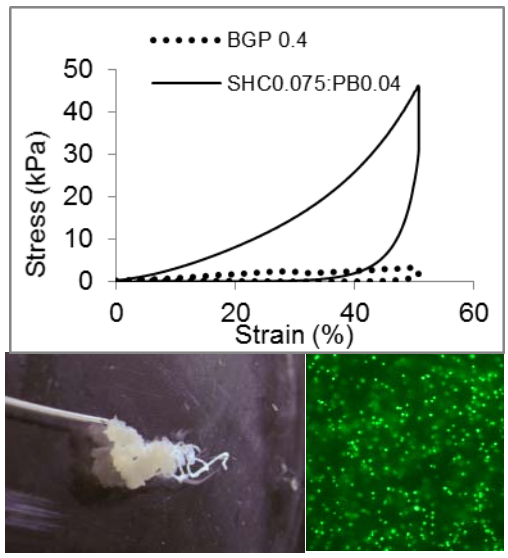
Abstract: Strong injectable chitosan thermosensitive hydrogels can be created, without chemical modification, by combining sodium hydrogen carbonate (SHC) with another weak base, namely beta-glycerophosphate (BGP) or phosphate buffer. Here we studied the influence of gelation agent concentration on the mechanical properties, gelation kinetics, osmolality, swelling and compatibility for cell encapulation, in order to find

the most optimal formulations and demonstrate their potential for cell therapy and tissue engineering.

The new formulations present up to a 50 fold increase of the Young's modulus after gelation compared with conventional chitosan-BGP hydrogels, while reducing the ionic strength to the level of iso-osmolality. Increasing PB concentration accelerates gelation but reduces the mechanical properties. Increasing BGP also has this effect, but to a lesser extent. Cells can be easily encapsulated by mixing the cell suspension within the hydrogel solution at room temperature, prior to rapid gelation at body temperature. After encapsulation, L929 mouse fibroblasts were homogeneously distributed within scaffolds and presented a strongly increased viability and growth compared with conventional chitosan-BGP hydrogels. Two particularly promising formulations were evaluated with human mesenchymal stem cells. Their viability and metabolic activity were maintained over 7 days in vitro.

Key Words : chitosan, thermogels, cell therapy, stem cells, mechanical properties



1. Introduction

Injectable hydrogels are increasingly used in biomedical applications, because they provide an excellent platform for minimally-invasive local delivery of cells, drugs and/or other bioactive products. They look especially interesting for cell therapy, a young sector that promises profound change to medical practices in the near future. However, developing a highly resistant scaffold that is compatible with cell seeding and injectable by catheter or small needle is still a challenge. Most hydrogels, including those presently used as cell delivery systems to enhance tissue and organ regeneration (based on collagen, gelatin, hyaluronic acid, cellulose derivatives, alginate, chitosan (CH) or a combination of these), are mechanically weak.[1-3] Increased strength can be achieved by triggering in situ crosslinking using photochemistry or chemical crosslinkers but these methods are generally limited either by toxicity issues, lack of uniform crosslinking or slow, the rate of reaction. Click chemistry can solve some of these issues but requires previous chemical modification of the biological product.[3-6] Materials can also be made to respond to environmental changes, such as temperature, pH or the ionic strength of the surrounding medium.[7]

Among these, thermosensitive chitosan hydrogels are particularly interesting due to their ability to form solutions with neutral pH at room temperature which gel in situ at body temperature. This can be achieved by mixing chitosan acidic solution with a weak base such as beta-glycerophosphate (BGP).[8] Their temperature dependant gelation mechanism was found to involve the heat-induced transfer of protons from CH to glycerol phosphate, which reduces the repulsive forces among positively charged ammonium groups and allows interaction of CH chains.[9] While CH-BGP gels have been used in several applications,[10, 11] the slowness of gelation and the weakness of mechanical properties after gelation limit their performance. Gelation can

be accelerated by increasing BGP concentration,[BGP], but this significantly decreases the cytocompatibility of the resulting hydrogel, and does not improve final mechanical properties.[12-14] This strongly limits their potential as a cell delivery vehicle. While several thermogelling chitosan derivatives were investigated, such as chitosan–g-PEG, chitosan–g-NIPPAm, hydroxy butyl chitosan, chitosan–g-(polyalanine–poly(ethylene glycol)) (CS–g-(PA–PEG)),[7] none of them combine rapid gelation, high mechanical strength, macroporosity and low ionic strength required for cell encapsulation.

Our group has recently developed new formulations which enable remarkable increase of mechanical properties of chitosan thermogels and rapid gelation, while reducing the concentration of BGP or eliminating it.[15] This was achieved by combining sodium hydrogen carbonate (SHC) with another weak base, such as BGP or phosphate buffer (PB). These hydrogels may thus represent excellent candidates for minimally-invasive therapeutic cell delivery. In this paper, we studied the influence of these gelling agents (GA) in order to optimize the hydrogels with respect to their use as injectable scaffolds for cell therapy, namely their osmolality, pH, mechanical properties, gelation kinetic and thermosensitive properties. The best formulations have been further characterized and evaluated as 3D scaffolds for cells, by testing survival and growth of encapsulated L929 mouse fibroblasts and human mesenchymal stem cells (MSCs) over 7 days of culture.

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 $C_3H_7Na_2O_6P \cdot 5H_2O$ (BGP), sodium phosphate monobasic NaH_2PO_4 (SPM) and sodium phosphate dibasic Na_2HPO_4 (SPD) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Sodium hydrogen carbonate $NaHCO_3$ (sodium bicarbonate, hereafter SHC) was purchased from MP Biomedicals (Solon, OH, USA). The other chemicals were of reagent grade, and were used without further purification.

2.2. Preparation of CH Hydrogels

2.2.1. Chitosan Solution: Chitosan (CH) was first purified using sodium dodecyl sulfate, an anionic surfactant, following the method described in detail by Assaad et al.[15] Chitosan powder was then solubilized in HCl (0.1 M) at 3.33% (w/v) overnight with a magnetic stirrer. The solution was sterilized by autoclaving (20 min, 121 °C) and stored at 4 °C.

2.2.2. Gelling agent (GA) Solutions: Three different GAs were used in this study, namely BGP, SHC and PB at pH = 8, prepared with a mixture of sodium phosphate dibasic and sodium phosphate monobasic at a ratio of 0.932/0.068 in Milli-Q water. SHC was combined with either PB or BGP. BGP alone, at 0.2 and 0.4 M, served as reference, because it was used to create CH-BGP hydrogels in previously published work.[12, 16] Gelling agents were sterilized by filtration through 0.2 µm filters and stored at 4 °C.

2.2.3. Preparation of Hydrogels for Physico-chemical Characterization: Chitosan hydrogels were prepared by mixing CH solution with one of the GA solutions at a volume ratio of 3:2 respectively. The two solutions were introduced in separate syringes, which were joined by a Luer lock connector. The content of the GA syringe

was pushed into the CH syringe and the mixture was pushed from side to side for 15 repeats immediately prior to use.

2.2.4. Preparation of Hydrogels for Cell Encapsulation: hydrogels of similar composition were prepared, but in two consecutive steps: CH was first mixed with 2X concentrated GA solution (at a volume ratio of 3:1) to create a solution at physiological pH at room temperature. The cell suspension (in culture media) was then mixed using a third syringe at a volume ratio of 4:1.

All hydrogels had a final CH concentration of 2% (w/v) and only the GA concentration was changed. Throughout the balance of the paper, each hydrogel is identified by its final molar concentration in GA. For instance, BGP0.1:SHC0.075 corresponds to a gel containing 2% (w/v) of CH, 0.1M BGP and 0.075M SHC.

2.3. Characterization of Hydrogels

2.3.1. pH and Osmolality: After 24 hours of gelation at 37 °C, each hydrogel was pressed and filtered in order to recover entrapped solution. The pH (UltraBasic pH-meter, Denver Instrument) and osmolality (Advanced® Micro Osmometer, Model 3300, Advanced Instruments) of the extracted solution were measured.

2.3.2. Rheological Tests: Rheological properties were investigated using an Anton Paar instrument (Physica MCR 301, Germany) with coaxial cylinder geometry (CC10/T200). The evolution of the storage modulus (G'), loss modulus (G") and complex viscosity were measured in the linear viscoelastic range, at a constant shear stress (1 Pa) and constant frequency (1 Hz), immediately following the preparation of each hydrogel. The gelation kinetic was thusfollowed during isotherm at 37 °C for 1 hour. In addition, to investigate the thermosensitive properties of the hydrogels,

rheological parameters were recorded during temperature ramps between 4 °C and 65 °C (1 °C /min) and during isotherms at 22 °C.

2.3.3. Injectability: Injectability of thermogels through small diameter catheters (length = 150 cm and inner diameter = 0.53 mm, Fas Tracker™-18 MX Microcatheter, Boston Scientific) was first verified manually, and then by measuring the maximal force required to extrude hydrogels using the ElectroForce® 3200 instrument (Bose Corporation, USA) equipped with a 220 N load cell. Immediately after mixing CH and GA, the hydrogel was placed in a 1 mL syringe connected to the catheter previously filled with NaCl 0.9% at 37 °C. The catheter was filled with gel and left for 5 minutes at 37° C. The force required to extrude the gel was then monitored, and the maximum force recorded for the various formulations was compared. The morphology of the extruded material (continuous thread, segments, droplets, etc.) was also observed.

2.3.4. Mechanical Properties: To evaluate mechanical properties after complete gelation, unconfined compression tests were performed using a Bose ElectroForce® 3200 instrument equipped with a 22 N load cell. Two (2) mL of hydrogel was poured into cylindrical containers (14 mm diameter) and incubated at 37 °C for 24 hours for gelation. Samples were gently removed from the container and a compression was applied at a constant rate of 0.5mm/s until reaching 50% deformation. The secant Young's moduli were calculated as the slope of a line connecting the point of zero strain to a point at a specified deformation. Tensile tests were also performed on some formulations.

2.3.5. Swelling Behavior**:** Swelling behavior and stability of the hydrogels was studied using gravimetric method. After 24h gelation, 2 mL samples were immersed in water at room temperature. Sample weight was measured at predetermined time points after removing the excess of water using an analytical microbalance.

2.3.6. Gel Morphology After 24h gelation, samples were frozen at -20 °C overnight, and freeze-dried under vacuum for 24 hours. They were carefully cut in the thickness using a surgical blade, coated with a gold layer and observed by Scanning Electron Microscopy SEM (Hitachi S-3600). In addition, to observe the morphology of hydrated gels, a thin layer of hydrogel was deposited on a glass slide and left to gel for 24h before visualization by optical microscopy.

2.4. Survival and Growth of Encapsulated Cells

2.4.1. Cell Encapsulation: L929 mouse fibroblast cells (ATCC, Manassas, VA, USA) cultured in normal Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Medicor, Montreal, QC, Canada) and 1% glutamine (PS, Gibco BRL, Invitrogen) and used at passages 10 to 15. Human MSCs (hMSCs, StemCell Technology, Canada) were expanded in supplemented serum-free MSC NutriStem XF medium (Biological industry, Israel) and used at passages 4 to 6. Cells were mixed to gel solution as described in section 2.2.4. A volume of 0.5 mL gel solution containing cells (at 1M cells/mL) was left to gel in 48 wells for 5 min. at 37 °C, before adding culture medium on the top and incubating further up to one week. Culture media was changed twice a week.

2.4.2. Live-Dead: After 24 hours of encapsulation, cell viability and repartition in hydrogels were evaluated by Live-Dead assay with a viability/cytotoxicity kit (FluoProbes®, Interchim). Hydrogels were washed once with DMEM without FCS and immersed (45 min, 37 °C) in the presence of 2 µM ethidium homodimer-3 and 1 µM calcein AM to stain dead cells in red and live cells in green. Hydrogels were then washed with DMEM without FCS and observed in inverted fluorescent microscopy (Leica DM IRB).

2.4.3. Alamar Blue Assay: the metabolic activity of encapsulated cells was evaluated by Alamar Blue (AB) VR (10%v/v Cedarlane Corp., Burlington, ON, Canada) at 1, 3 and 7 days. To optimize the access of AB to entrapped cells, it was mixed with the hydrogel by gently pipetting up and down with a 1000 µl pipette (P1000, Gilson, Middleton, WI). After 3 hours of incubation at 37 °C, the coloration was homogeneously distributed inside hydrogels samples and diffused in supernatant. 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).

2.5. Statistics

All experiments were repeated at least in triplicate. Results are expressed as mean ± SD. Statistical comparison of the data was performed using GraphPad Prism software with a two-way ANOVA and post-Bonferroni's test for comparison of more than two groups. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Influence of GA Concentration on Gel Properties

3.1.1. Effect of GA on Osmolality

To adapt chitosan thermogels for cell compatibility and encapsulation, we first determined the impact of each gelling agent concentration on osmolality. As expected, the osmolality increased linearly, but with different slopes for BGP, SHC, and PB. A theoretical equation of the osmolality as a function of the hydrogel formulation was derived from these data:

(Osmolality (mOsmol/L) = 158 + 1475 [SHC] + 1153 [PB] + 1789 [BGP],

where [SHC], [PB] and [BGP] are the molar concentrations (M) of SHC, PB and BGP respectively, and 158 is the osmolality due to HCl (chitosan solvent) and culture medium.

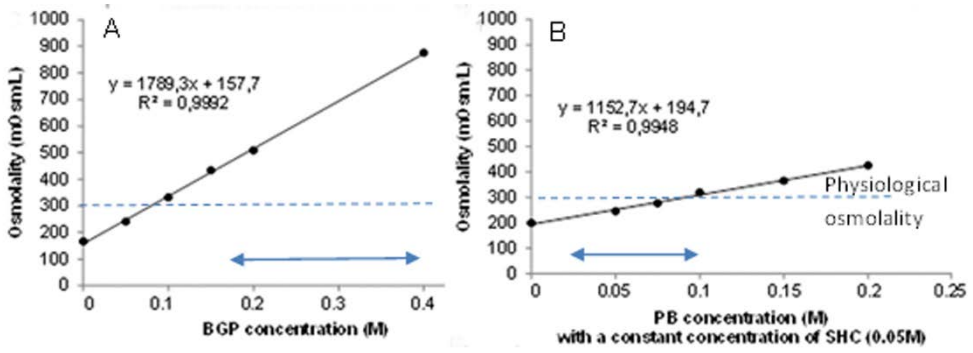


Figure 1*: Osmolality of hydrogels as a function of the final gelling agent concentration, prepared with culture medium as described in 2.2.4; A) conventional chitosan-BGP hydrogels ; B) Gels with [SHC]= 0.05M and increasing [PB]. The arrow indicates the range of concentrations needed to get rapid gelation.*

As shown in Figure 1A, at the range of BGP concentrations used in conventional CH-BGP hydrogels (0.2-0.4M) are hypertonic, which at least partly explains the extent of cell death obtained with these gels in previous publications.[12, 15] PB and SHC had a slightly lower impact on the final ionic strength (slopes of 1153 and 1475 mOsmol/L.M, respectively). More importantly, at the range of concentrations used in

the new formulations, the hydrogels are close to the physiological level of osmolality (as shown with SHC-PB gel on Figure 1B).

3.1.2. Effect of GA Concentration on Mechanical Properties after Gelation

Similarly, the influence of hydrogel formulation on the mechanical properties was evaluated after 24 hrs of gelation. Figure 2 presents the typical stress-deformation curves in compression of conventional chitosan-BGP hydrogel and of the new formulations after 24 hours of gelation at 37 °C. Hydrogels prepared with BGP were difficult to handle, broke at approximately 30% deformation, and their average secant modulus was only of 3.0±0.5 kPa. In contrast, after 24 hour gelation, all BGP:SHC and PB:SHC hydrogels were cohesive, easy to handle and sustained 50% deformation. All presented significantly higher mechanical properties than BGP hydrogels, but to an extent that varies depending on the GA concentration.

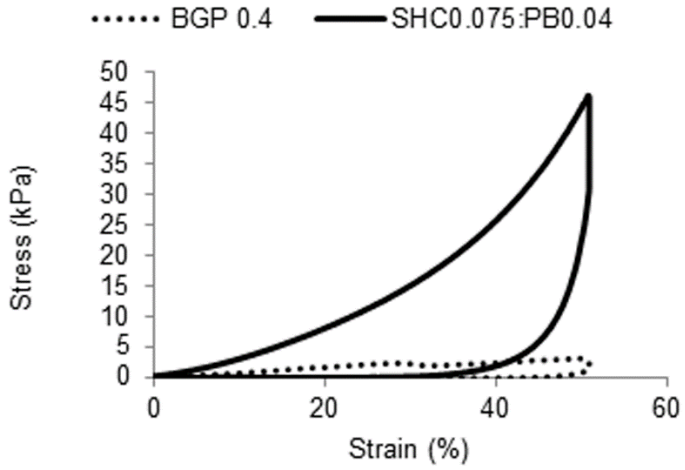


Figure 2: *Typical stress-deformation curves in unconfined compression of PB0.04:SHC0.075 versus BGP0.4 hydrogels after 24h gelation. Hydrogels present a non-linear behavior, therefore further results are expressed as secant modulus;*

Previous work from our laboratory had shown that [SHC] has a strong impact on the final mechanical properties, with a narrow window of appropriate concentration.[15]

Based on these results, the effect of [PB] and [BGP] was studied for hydrogels with [SHC] kept constant at 0.075M (Figure 3). Maximal properties were obtained with pure SHC hydrogels, with secant modulus reaching up to 165 kPa for SHC0.075 hydrogel, i.e. about 50 fold increase compared with CH-BGP gels. Increasing [PB] led to reduced secant modulus from 165 kPa for SHC0.075 down to 19 kPa for PB0.08:SHC0.075. Addition of BGP to SHC had less impact, with less than 25% variation between samples prepared with 0 to 0.2M BGP (Figure 3B).

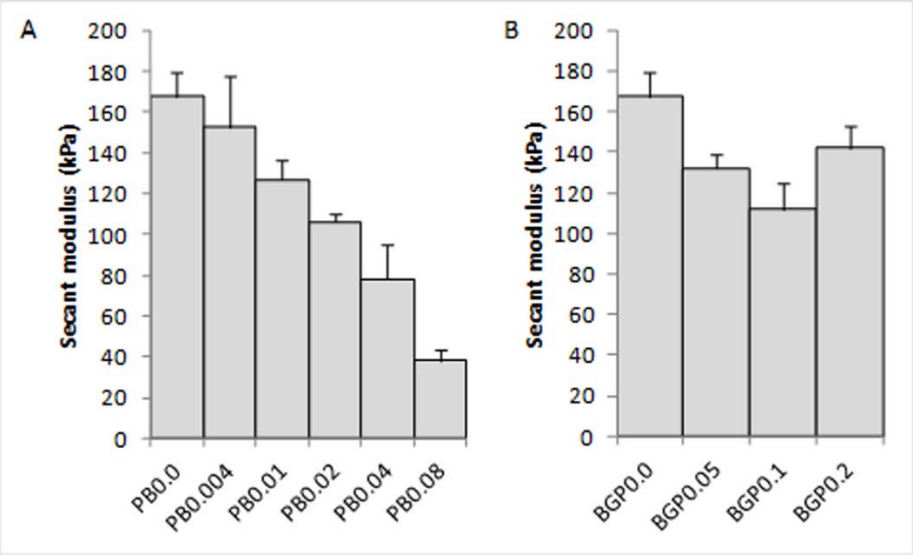


Figure 3*: Influence of [PB] and [BGP] on mechanical properties of CH hydrogels containing SHC0.075M. Secant modulus measured at 50% of deformation after 24h gelation, as a function of A) [PB] and B) [BGP] (mean + SD; n = 3).*

3.1.3. Effect of GA Concentration on the Kinetic of Gelation

In addition to the mechanical properties after complete gelation, the kinetic of gelation is also important to consider for in vivo applications since it describes the rate of solidification of the product after injection. Rheological study showed that SHC hydrogel presents a very slow evolution of the storage modulus (G') at 37°C, but increasing [PB] strongly accelerates gelation (Figure 4A). BGP had a similar, yet more moderate effect (Figure 4B).

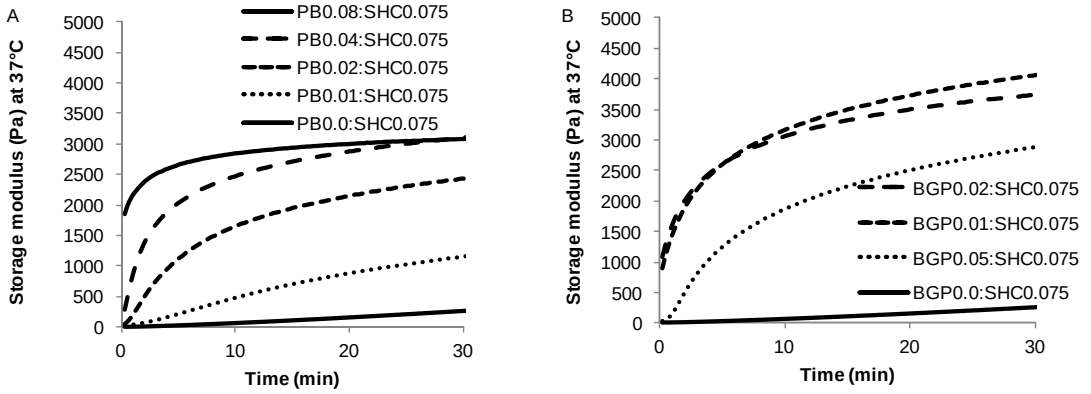


Figure 4: Evolution of storage modulus, G', at 37°C as a function of A) [PB] and B) [BGP] ([SHC]=0.075M)

Therefore, increasing [PB] enables to accelerate gelation but reduces final mechanical properties, as determined by compression testing. Thus, hydrogels prepared with PB at 0.04M gel more slowly than PB at 0.08M but achieved higher Young modulus after 24 h of gelation (88 versus 26 kPa). Increasing [BGP] also accelerates gelation, with less effect on gel final mechanical properties. Altogether, these results help tailoring hydrogels as a function of the targeted application.

3.2. Evaluation of Specific Formulations

Based on these data, six different SHC:PB and SHC:BGP formulations (see Table 1), selected for their adequate osmolality, rapid gelation and good mechanical properties were further evaluated in this study. The chitosan-BGP hydrogels (with [BGP]=0.2 M and 0.4 M) that are usually described in the literature[12, 16] were kept as controls for comparison.

Hydrogel name	Final %CH	Final GA concentration (M)	pH	Osmolality (mOsmol/L)	$E_{50\%}$ (Pa)

	w/v	PB	BGP	SHC			
BGP0.2	2	-	0.20	-	6.83	516	< 3
BGP0.4	2	-	0.40	-	7.01	874	6 ± 1
BGP0.1:SHC0.05	2	-	0.10	0.05	7.27	250	134 ± 10
BGP0.1:SHC0.075	2	-	0.10	0.075	7.39	287	146 ±14
PB0.04:SHC0.05	2	0.04	-	0.05	7.1	278	103 ± 8
PB0.04:SHC0.075	2	0.04	-	0.075	7.31	314	88 ± 3
PB0.08:SHC0.05	2	0.08		0.075	7.15	324	43 ± 15
PB0.08:SHC0.075	2	0.08	-	0.075	7.33	361	26 ± 7

Table 1 : *Composition, pH, osmolality and secant Young modulus of chitosan hydrogels.*

3.2.1. Thermosensitive Properties

Sudden increase of G' between 30 and 40 °C during temperature ramps (Figure 5A) confirmed the thermosensitive properties of each formulation and give an indication of their temperature of incipient gelation.[16] Interestingly, the slope of G' increase was much more pronounced with BGP0.1:SHC (0.05 M and 0.075 M) hydrogels compared to BGP hydrogels. PB:SHC formulations presented intermediate values.

During isotherms at 37°C (Figure 5B), the gelation time (time where G' = G'' according to the approach from Winter and Chambon[17]) was less than 15s for all hydrogels except for BGP0.2. G' of the new formulations rapidly rise, suggesting rapid increase of their elastic properties. In contrast, isotherms at 22 °C (figure 5C) confirmed that, except BGP0.4, G' values increased very slowly and remained below 1000 Pa within 1 hour, thus demonstrating their relative stability at room temperature. This allows to easily and homogenously mix the cell suspension in the hydrogel solution, and is a first indication of their injectability at room temperature.

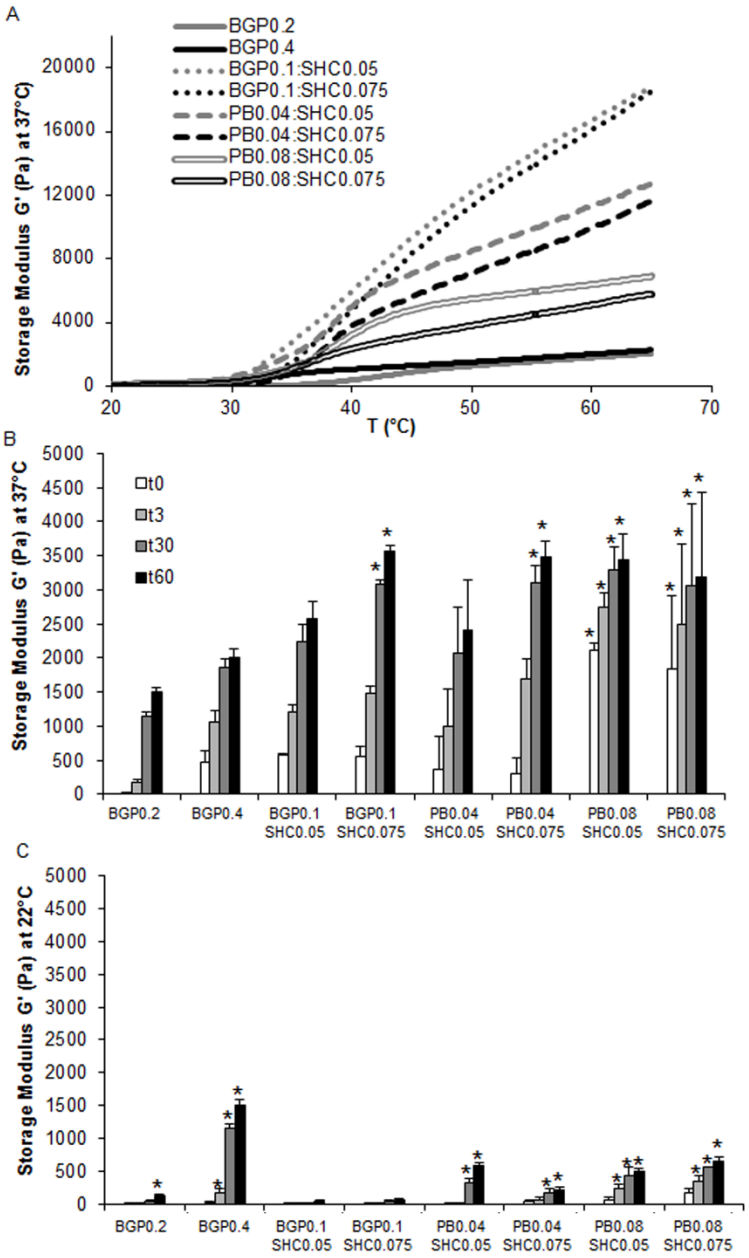


Figure 5. *Thermosensitive properties of hydrogels. A) Evolution of the storage modulus G' during 4-65°C temperature ramps (only data from 20 to 65°C are presented here); B) Evolution of G' during isotherm at 37 °C (* p< 0.05 compared with BGP04 at the same time point); C) Evolution of G' during isotherm at 22 °C (* p< 0.05 compared to their own initial G' value).*

3.2.2. Injectability

To confirm injectability, the maximal force required to extrude each hydrogel from a catheter (inner diameter of 0.53 mm), immersed in saline at 37 °C for 5 min was measured (Figure 6A). All formulations were manually injectable. The maximal force

required varied between 17 and 43 N, with only weak correlation with rheological properties of the hydrogels measured at small amplitude of oscillation, probably due to shear thinning effect. Extruded hydrogels were cohesive, and generally formed a linear and continuous shape after injection in a saline bath at 37 °C (as represented in Figure 6B for PB0.04:SHC0.075). In the case of PB0.08 formulations, however, discontinuous segments were obtained after extrusion (Figure 6B, arrows), probably due to some chitosan precipitation leading to lower gel homogeneity.

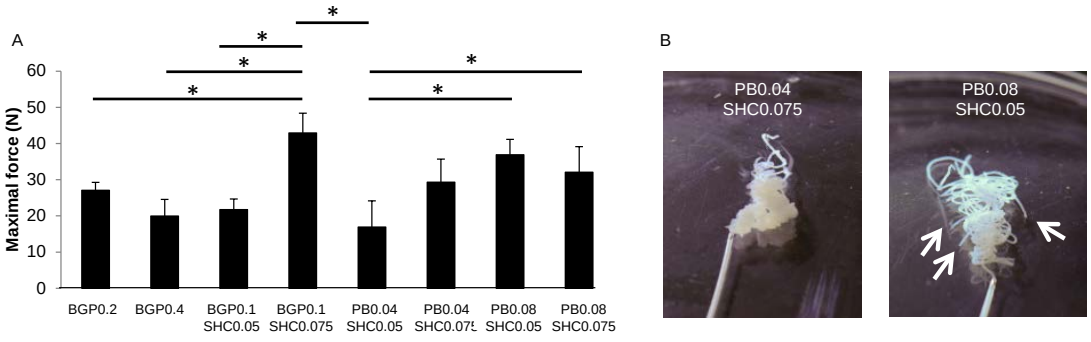


***Figure 6**: Injectability of chitosan thermogels. A) Maximal force needed to extrude hydrogels from the catheter (Φ_{intern}=0.53 mm) immersed for 5 min. in saline at 37 °C (* p <0.05); B) Aspect of extruded PB0.04:SHC0.075 and PB0.08:SHC0.05 (arrows point discontinuities in the PB0.08:SHC0.05 hydrogels after extrusion)*

3.2.3. Swelling properties

Swelling behavior is a key parameter for the design of implantable hydrogels, as it is an indication of hydrophicility and their short term stability after in vivo implantation. Figure 7 reports the evolution of sample weight during immersion in water for up to 160 minutes. During the first minutes, most hydrogels decrease in weight due to water loss, the only exception being BGP0.2 which slightly swelled during this period. Weight loss was about 5 to 25% depending on the formulation, and was more pronounced for BGP0.4 and hydrogels containing PB at 0.08M. After a couple of

hours, hydrogel weight remained quite constant over a 10 day period (data not shown), showing good stability in water.

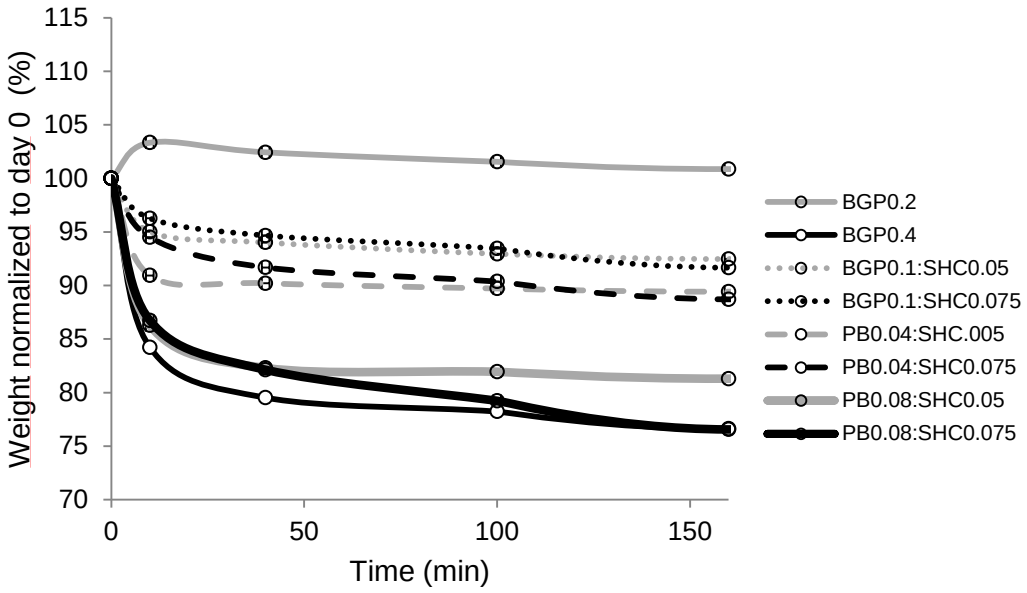


Figure 7. *Swelling properties of chitosan thermogels after immersion in water at 22°C during the firsts 160 minutes*

3.2.4. Gel Morphology

The morphology of freeze dried hydrogels, as observed by SEM (Figure 8a-h), showed porous structures of variable size and homogeneity. Although the freeze-dried porosity does not represent the hydrated porosity, because the freeze-drying process creates artifacts, these data suggest that changing the type and concentration of GA allow for the creation of different morphologies that could have an impact on the access to O_2 and nutrients, and therefore on cell viability. In addition, interestingly, numerous bubbles are visible within the hydrated gels, creating a non-interconnected porous structure (Figure 8i,j). These bubbles can be explained by the preparation process (mixing the two components using the Luer lock system), as well as by the generation of CO_2 when SHC is mixed with the acidic solution of CH.[15]

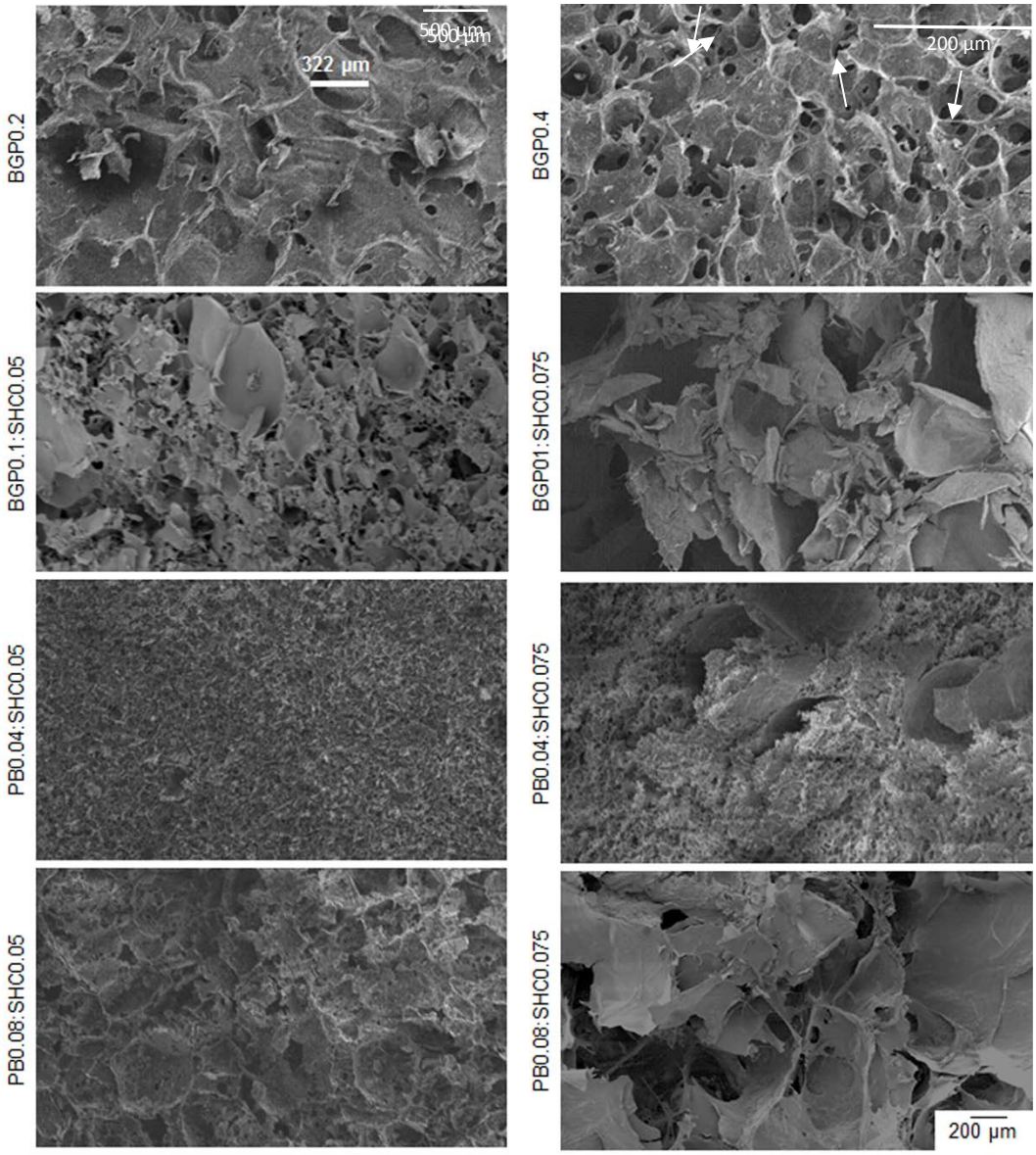


Figure 8. *Gel morphology : a-h) Scanning electron microscopy (SEM) images of cross section of freeze-dried hydrogels (scale bar = 200 µm); i) Optical microscopy of hydrated SHC-PB004 gel after 24h gelation, showing numerous pores up to 300 µm ; j) Confocal microscopy images of L929 fibroblasts (in green) within the hydrogel, showing some pores (arrows) lined with live cells (Live/dead staining).*

3.3. Potential of Hydrogels for Cell Encapsulation

3.3.1. In Vitro Cell Cytocompatibility

L929 were encapsulated in each formulation of hydrogels. Cell metabolic activity was followed over 7 days (Figure 9A), and live/dead staining was performed after 24 hours of culture (Figure 9B).

As expected, encapsulation in BGP0.4 led to significant cell death, as shown by numerous red cells in live/dead at day 1 and a decrease in cell metabolic activity over time. The initial viability was better with BGP0.2, but the cell number also decreased over time. The metabolic activity of entrapped fibroblasts was significantly improved in the new hydrogels, with marked improvement at day 7 for all formulations. The variation of measured metabolic activity over time tends to differ among the formulations. A clear increase in metabolic activity was observed for BGP0.01:SHC0.05, PB0.04:SHC0.075, PB0.08:SHC0.05 and PB0.08:SHC0.075 ($p<0.05$ compared to day 1), which suggests that they were able to sustain significant cell proliferation.

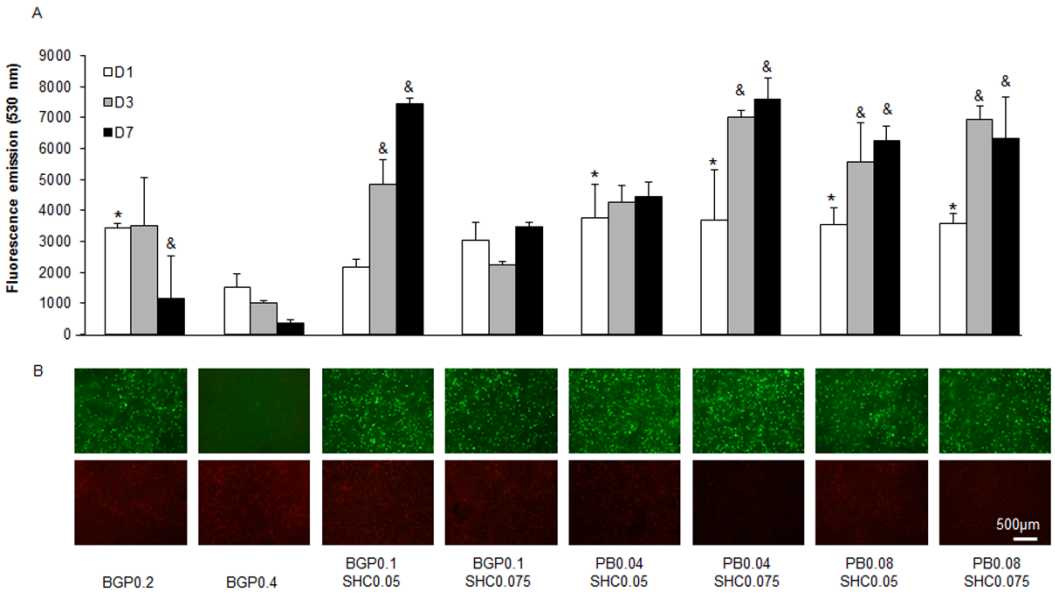


Figure 9. *Cytocompatibility of chitosan hydrogels. A) Metabolic activity of encapsulated cells for 1 week. ANOVA analysis was performed to compare* **) at day 1, metabolic activity of new formulations with BGP0.4M hydrogels and* $^{\&}$*) for each formulation, day 3 and day 7 to day 1(**, $^{\&}$ $p<0.05$*) ; B) Viability of L929 cells after 24 hours in hydrogels in 48 wells, evaluated by fluorescent microscopy after Live/dead staining (hydrogel dimensions: s=1.1 cm*^{2}*; h=4.5mm).*

3.3.2. Viability of Mesenchymal Stem Cells in Hydrogels

The two best formulations obtained previously (BGP0.1:SHC0.05 or PB0.04:SHC0.075) were tested for their ability to encapsulate human MSCs, in comparison with BGP0.2. Once again, live/dead staining was performed at 24 hours, and metabolic activity was tested by AB for 7 days (Figure 10).

The initial metabolic activity and Live/Dead staining showed excellent cell survival and no difference between the formulations tested after 24 hours encapsulation. However, after 7 days of culture, significant cell mortality was observed with the BGP0.2M control, whereas cell metabolic activity remained constant in the new formulations.

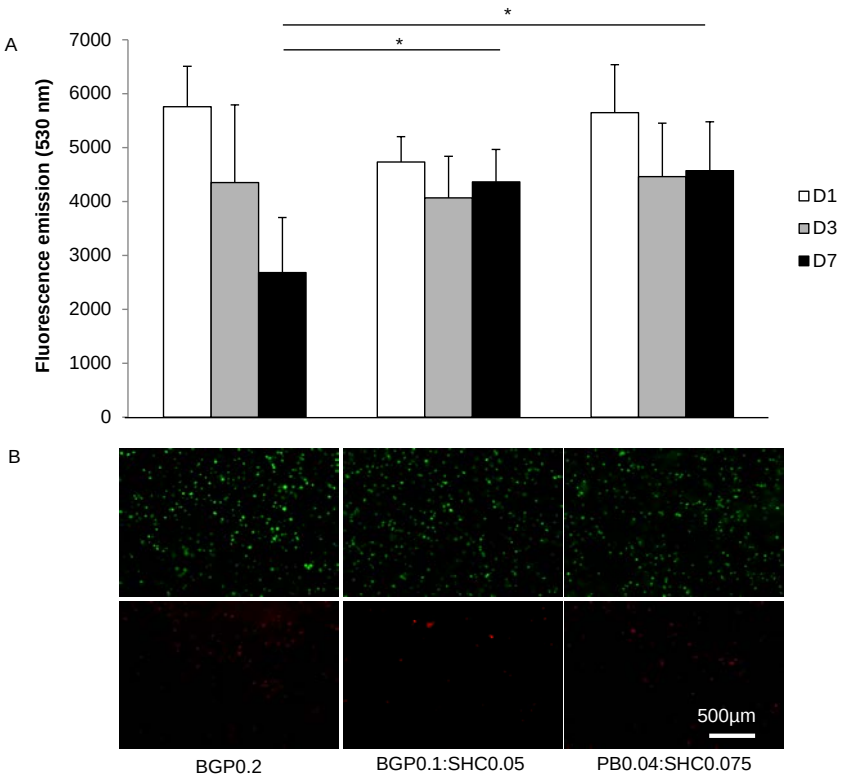


Figure 10. *Compatibility of chitosan thermogels with human MSCs 3D culture: A) Metabolic activity (AlamarBlue fluorescence) of MSCs entrapped for 1 week in BGP0.2, BGP0.1:SHC0.05 and PB004:SHC0.075 and in as control (*p< 0.05 compared with BGP0.2); B) Live/Dead staining of human MSCs after 24 hours in hydrogels.*

4. Discussion

Injectable hydrogels that rapidly solidify at body temperature have raised a great deal of interest in terms of cell therapy or local drug delivery. The new chitosan thermogels reported here (particularly, BGP0.01:SHC0.05 and PB0.04:SHC0.075) hold great promise as vehicles for cell therapy, because they combine the required properties, namely a relatively low viscosity before and during injection, rapid in situ gelling and strong final mechanical strength to ensure matrix cohesion and resistance to in vivo stresses, as well as cytocompatibility and biodegradability.

In particular, these hydrogels overcome the well-known limitations of chitosan-BGP hydrogels, which were demonstrated here again, namely a) the difficulty in producing an adequate combination of gelation kinetic and cytocompatibility[12] and b) their very poor mechanical properties after gelation.[15] BGP0.2 presented very slow gelation kinetic and poor cell survival and proliferation. This was in accordance with previous studies reporting that BGP becomes cytotoxic at concentrations above 0.12 M.[12, 14] The gelation rate was increased with BGP0.4, but the gel was limited by clear cytotoxicity. Some authors replaced BGP with other weak bases, such as dibasic ammonium hydrogen phosphate,[18] dibasic hydrogen phosphate[19] or SHC[13] to reduce ionic strength and increase the gelation rate (from 30 min to 4-5 min). However, none of them reported the rapid gelation (less than one min) and drastic increase in mechanical properties as observed here with BGP01:SHC and PB004:SHC combinations. Used alone, SHC, at specific concentration, can also enhance the mechanical properties of chitosan-based hydrogels.[15] However, its gelation kinetic at body temperature is very slow, which increases the risk of gel migration and makes it

difficult to get a cohesive scaffold in vivo. The present results shows how changing the ratio and combination of SHC with either PB or BGP allows for the osmolality, gelation kinetic and Young modulus to be adjusted in order to optimize the hydrogel as a function of the need for a particular application.

However, the precise role of PB and BGP when added to SHC gel remains to be clarified. As discussed in our previous paper,[15] the gelation agents do not act as crosslinkers but instead influence the quality of CH chain interactions and entanglements through the extent and rate of deprotonation of amino groups during gelation. Thus PB is able to accelerate gelation and help stabilize the pH at a physiological range but with a decrease in mechanical properties, probably since it enters into competition with carbonate ions from SHC. Interestingly, BGP is able to accelerate gelation with less of a compromise in terms of lower mechanical properties.

Strong mechanical properties of injectable scaffolds are important in order to ensure their cohesion and resistance to in vivo stresses, and to increase cell retention, which play a key role in the efficiency of cell therapies. In addition, matching the mechanical properties of tissues is important in terms of promoting adequate stem cell differentiation and inducing tissue neoformation.[20, 21] While further study would be required in order to evaluate hydrogel behavior under complex or cyclic loading, the present mechanical data suggest that these hydrogels may be used for the regeneration of soft tissues and load-bearing structures, such as cartilage and intervertebral disk.
This desirable elasticity and resistance in compression was achieved with a biodegradable and biocompatible hydrogel, with no crosslinking agent or chemical modification of chitosan, an important advantage in terms of regulatory approval

compared to smart polymers such as Poly(N-isopropylacrylamide) (PNIPAAm)[22] and its copolymers, or poly(N,N-diethylacrylamide) and poly(N-vinlycaprolactam)[23] to name just a few. Our hydrogels remain liquid at room temperature, gel rapidly when increasing the temperature to 37°C and do not shrink extensively during gelation, which enables to adequately cast a tissue defect. It is yet important to note that, in contrast to PNIPAAMs, they are not thermoreversible, i.e. hydrogels formed at 37°C remain solid when decreasing temperature back to ambient.

In addition, excellent survival of encapsulated cells was observed thanks to the physiological pH and osmolality of the gels. How the pores present within the gel influence survival of encapsulated cells remains unclear and further investigation would be necessary to explain why some formulations are more favorable to cell growth, as observed here with fibroblasts and previously with entrapped T Lymphocytes.[24] Future in vivo studies will also investigate the biocompatibility of the hydrogels, as well as the survival and the phenotype of encapsulated cells, as insufficient diffusion of oxygen and nutrients within the scaffold in vivo could lead to cell death.

5. Conclusion

In this paper we optimized and evaluated new in situ gelling chitosan hydrogels for cell therapy and tissue engineering purposes, based on the combination of SHC and PB or BGP. After studying the impact of each gelation agent on osmolality, mechanical properties and gelation kinetic, 6 promising formulations that presented iso-osmolality as well as good mechanical properties and rapid gelation were selected. Thanks to their sol state at room temperature, they present sufficiently low viscosity to homogeneously

mix cells and to be injected by catheter and they quickly gel at 37 °C. These new formulations lead to excellent cell survival, as demonstrated with fibroblasts and human mesenchymal stem cells. These results demonstrate the high potential of these injectable scaffolds as platforms for cell therapy and tissue engineering.

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