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1 Rational formulation design of injectable  
2 thermosensitive chitosan-based hydrogels  
3 for cell encapsulation and delivery

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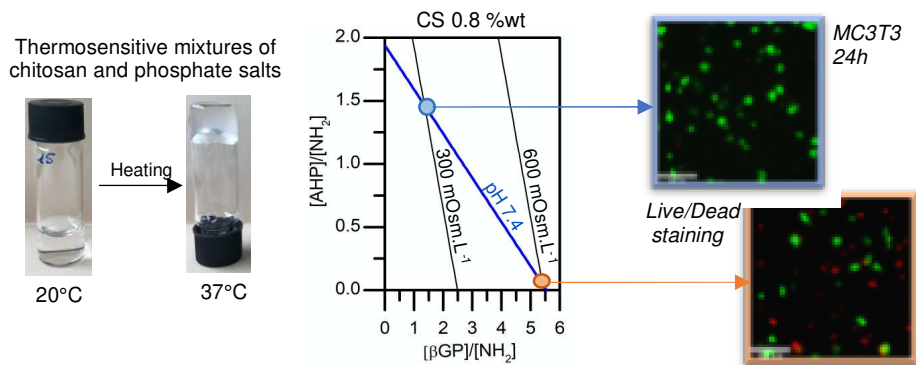
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13 **ABSTRACT** (150 words)

14

15 This work reports a rational design of injectable thermosensitive chitosan systems for cell  
16 encapsulation and delivery. Using mixtures of two phosphate salts, beta-glycerophosphate and  
17 ammonium hydrogen phosphate, we demonstrate that the pH and the osmolarity can be adjusted  
18 separately by varying the molar ratios between the salts and the D-glucosamine monomers. We  
19 found the existence of a critical temperature above which gelation time decays following a  
20 power-law. This gelation kinetics can be finely tuned through the pH and salt-glucosamine ratios.  
21 Formulations having physiological pH and osmolarity were produced for chitosan concentrations  
22 ranging from 0.4 to 0.9 wt%. They remain liquid for more than 2 h at 20°C and form a  
23 macroporous gel within 2 min at 37°C. *In vitro* encapsulation of pre-osteoblastic cells and  
24 gingival fibroblasts showed homogeneous cell distribution and good cell viability up to 24 h.  
25 Such an approach provides a valuable platform to design thermosensitive cell-laden systems.

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29 **KEYWORDS:** thermosensitive hydrogel, chitosan, phosphate salt, injectable vehicle, cell

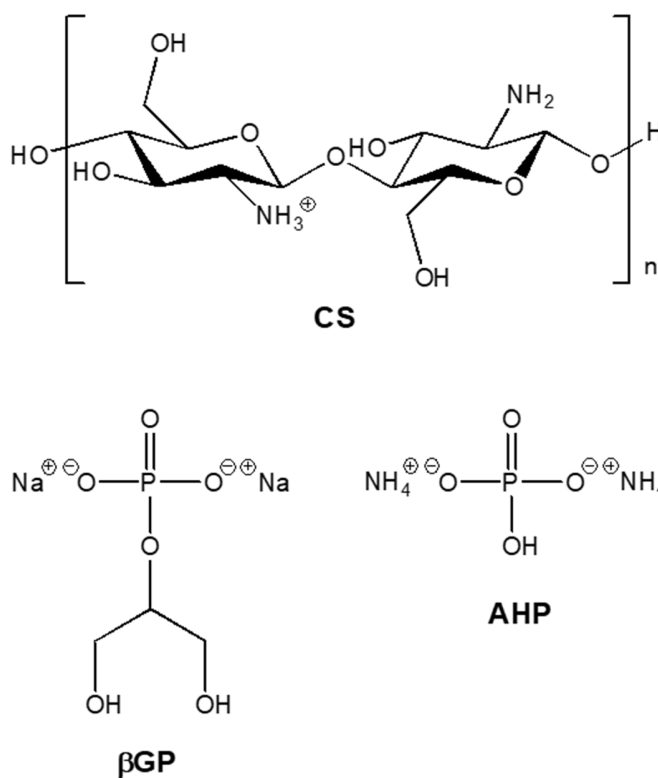
30 encapsulation, cell delivery

## 31 INTRODUCTION

32 Cell-laden injectable hydrogels have raised a great interest during the last decades as valuable  
33 delivery vehicles or matrices for cell therapies (Li, Rodrigues, & Tomás, 2012). Administrated in  
34 a minimally invasive manner, these gels fill-in the defects in hardly accessible and fragile tissues,  
35 and might provide a temporary scaffold to injected cells to ensure their viability and stimulate  
36 their activity. For effective clinical applications, such systems should stay liquid before and  
37 during injection, then gel rapidly in physiological conditions for temperature, pH and osmolarity.  
38 In the liquid state, pH and osmolarity should be finely controlled for the survival of the  
39 encapsulated cells. After gelation, the gel scaffold must provide appropriate mechanical and  
40 chemical properties as well as a macroporosity enabling cell invasion and growth. Different  
41 stimuli-responsive systems for *in situ* gelling have been investigated, triggered by temperature  
42 (Yap & Yang, 2020), pH (Z. Li et al., 2016), or light (Neves et al., 2017). Among those,  
43 thermogelling hydrogels based on biocompatible chitosan have been extensively studied  
44 (Mekhail & Tabrizian, 2014).

45 Chitosan (CS) is a linear copolymer composed of D-glucosamine and N-acetyl-D-glucosamine  
46 monomers (**Figure 1**). CS is obtained by alkaline deacetylation of parent chitin, a primary  
47 component of cell walls in fungi and crustacean shells. As one of the few representatives of  
48 biodegradable and biocompatible polymers approved for clinical use (Berger et al., 2004; Riva et  
49 al., 2011; Saravanan, Vimalraj, Thanikaivelan, Banudevi, & Manivasagam, 2019) CS has long  
50 been a popular biomaterial for developing cell delivery matrices (Di Martino, Sittinger, &  
51 Risbud, 2005; Zhou, Jiang, Cao, Li, & Chen, 2015). Amino groups of chitosan present an  
52 apparent  $pK_{a_{app}}$  in the vicinity of 6.2. At  $pH < pK_{a_{app}}$ , CS behaves as a cationic polyelectrolyte  
53 bearing protonated amino groups, soluble in aqueous media. Near neutral pHs where CS is

54 mostly deprotonated, the strong tendency of the carbohydrate polymer to self-associate through  
55 H-bonding and hydrophobic interactions dominates, giving rise to the formation of a hydrated  
56 gel-like precipitate (Rinaudo, 2006). This poor solubility of CS at physiological pHs has long  
57 been a major limitation for biomedical uses, until the pioneering work by Chenite et al., who  
58 demonstrated that in presence of beta-glycerophosphate ( $\beta$ GP, **Figure 1**), cationic CS solutions  
59 turned into thermally sensitive solutions, remaining liquid at room temperature even at neutral  
60 pHs, and forming hydrogels around 37°C (Chenite et al., 2000, 2001).



61  
62 **Figure 1.** Structures of chitosan CS,  $\beta$ -glycerophosphate  $\beta$ GP and ammonium hydrogen phosphate AHP.  
63  
64 The mechanisms responsible for the thermoresponsivity of CS- $\beta$ GP mixtures are still debated.  
65 Cho et al. associated the gelling mechanism with enhanced hydrophobic interactions with  
66 increasing temperatures (Cho, Heuzey, Bégin, & Carreau, 2005, 2006). Filion et al. underlined

67 that the  $pK_{a,app}$  of CS decreases by heating, producing a release of protons that may be accepted  
68 by the negatively charged  $\beta$ GP, a weak base having a  $pK_{a,2}$  of 6.65 at 25°C. The CS  
69 neutralization induced by the temperature-dependent proton transfer would result in a phase  
70 separation of the chitosan solution (Filion, Lavertu, & Buschmann, 2013; Lavertu, Filion, &  
71 Buschmann, 2008). Thermodynamic insights into the mechanism of the phase transition of CS  
72 induced by  $\beta$ GP have been recently related by Grinberg et al. (2020). From calorimetric analyses,  
73 they concluded that the  $\beta$ GP binding induces the formation of a highly ordered hydrate structure  
74 of the polysaccharide, causing a large entropic stress despite the stability brought by stabilization  
75 of the complexes. Heating thus leads to dehydration and release of  $\beta$ GP ligands, accompanied by  
76 a transfer of protons from CS to  $\beta$ GP due to the simultaneous decrease in the ionization constant  
77 of CS as already suggested by Lavertu, Filion, & Buschmann (2008). By losing their charges and  
78 hydrated structures, CS chains eventually tend to self-associate and form a gel.

79 These CS- $\beta$ GP mixtures appeared clearly as a promising thermo-gelling system for cell injection,  
80 remaining liquid at physiological pH and turning rapidly into a gel at body temperature.  
81 However, increasing salt concentrations to reach appropriate values of pH also increases the  
82 osmolarity of the mixture up to cytotoxic values (Ahmadi & De Bruijn, 2008). Hence, strategies  
83 were implemented to improve the standard system, mostly by replacing partially or totally  $\beta$ GP  
84 by other salts. Using ammonium hydrogen phosphate (AHP, **Figure 1**), a lower salt concentration  
85 was required to reach neutral pHs (7.0 – 7.2) resulting in physiological osmolarity ( $300 \pm 30$   
86  $mOsm.L^{-1}$ ) (Nair, Starnes, Ko, & Laurencin, 2007). Other inorganic phosphate salts without  
87 polyol moieties, like dipotassium hydrogen phosphate  $K_2HPO_4$  (Ta, Han, Larson, Dass, &  
88 Dunstan, 2009), basic sodium phosphates as  $NaH_2PO_4$ ,  $Na_2HPO_4$ , or even  $Na_3PO_4$  (X. Y. Li et al.,  
89 2010; Casettari, Cespi, Palmieri, & Bonacucina, 2013), have also been studied to produce

90 thermo-sensitive CS formulations. Sodium hydrogen carbonate (SHC) has been studied as a  
91 gelling agent, alone (Huang, Yu, Feng, & Li, 2011; Liu, Tang, Wang, & Guo, 2011) or in  
92 combination with  $\beta$ GP (Assaad, Maire, & Lerouge, 2015; Ceccaldi et al., 2017; Deng, Kang,  
93 Zhang, Yang, & Yang, 2017; Alinejad, Adoungotchodo, Hui, Zehtabi, & Lerouge, 2018).  
94 Mixtures of CS/AHP (Nair, Starnes, Ko, & Laurencin, 2007) or CS/ $\beta$ GP/SHC (Ceccaldi et al.,  
95 2017 ; Alinejad, Adoungotchodo, Hui, Zehtabi, & Lerouge, 2018) have been evaluated *in vitro* as  
96 3D scaffolds for cells. Encapsulation of mouse preosteoblastic cells and fibroblasts as well as  
97 human mesenchymal stem cells confirmed the cytocompatibility for days for some formulations.  
98 The feasibility of using these chitosan-based hydrogels as a cell carrier vehicle has therefore been  
99 demonstrated. Nevertheless, the numerous variables, pH, osmolarity, salt and chitosan  
100 concentrations leading to injectable cytocompatible solutions are all interdependent. A general  
101 platform is still lacking, enabling to control independently pH and osmolarity, and therefore  
102 gelation kinetics and cytocompatibility, whatever the chitosan concentration. Yet, controlling  
103 chitosan concentration is a key to adjust the viscoelastic and microstructural properties of the  
104 injected hydrogels, which are of the utmost importance to favor cell anchorage and growth.

105 In this study we present a rational formulation design to make thermosensitive CS solutions, by  
106 partially replacing  $\beta$ GP by AHP in the mixture. With this third component, AHP, we can  
107 decouple the control on pH and osmolarity, whatever the CS concentration. We show that these  
108 two parameters can be adjusted independently by the simple ratios  $[\text{salt}]/[\text{NH}_2]$ , where  $[\text{NH}_2]$   
109 denotes the concentration in D-glucosamine monomer in the solution, *i.e.* the concentration in  
110 deacetylated chitin monomers bearing  $\text{NH}_2$  functions. This approach gives rise to a robust  
111 platform, to easily formulate physiological solutions able to form a gel within a couple of minutes  
112 at body temperature. We investigated in depth the gelation kinetics for physiological formulations

113 and determined a critical temperature of gelation, under which the system remains liquid  
114 overtime, for different CS concentrations. We checked the cytocompatibility of our controlled  
115 CS/ $\beta$ GP/AHP formulations and explored the effect of osmolarity on cell activity by  
116 encapsulating preosteoblastic mouse cells (MC3T3-E1) and human gingival fibroblasts (HGF).  
117 By means of confocal microscopy and rheology, we showed that hydrogel microstructure and  
118 mechanical properties can be varied by changing CS concentration while maintaining fixed pH  
119 and osmolarity.



## 120 **EXPERIMENTAL METHODS**

### 121 **1. Materials & Cells**

122 Three grades of chitosan (CS) having high purity (impurity level < 1.0%) were purchased from  
123 Glentham Life Sciences Limited. All the study was performed with chitosan low molar mass  
124 (viscosity 30-100 cps) having the following characteristics given by the supplier: deacetylation  
125 degree DD = 91%, as measured by potentiometric titration, average molar mass  $M_w = 250$   
126  $\text{kg}\cdot\text{mol}^{-1}$ , as measured by viscosimetry. For pH titration experiments, two other grades with DD  
127 of 95 % were also used: chitosan very low molar mass (10 cps) with  $M_w = 30 \text{ kg}\cdot\text{mol}^{-1}$ , and  
128 chitosan (100-300 cps) with  $M_w = 890 \text{ kg}\cdot\text{mol}^{-1}$ .

129  $\beta$ -glycerophosphate disodium salt hydrate BioUltra ( $\beta$ GP) and ammonium hydrogen phosphate  
130 BioUltra (AHP) were purchased from Sigma-Aldrich. Glacial acetic acid (AcOH, 99%) was  
131 purchased from Alfa Aesar. Alexa Fluor 488 was purchased from Abcam. The Live/Dead<sup>TM</sup> kit  
132 and AlamarBlue<sup>TM</sup> reagent were purchased from Invitrogen. MC3T3-E1 pre-osteoblastic cells  
133 were procured from ATCC<sup>®</sup>, and human gingival fibroblasts were isolated from healthy donors  
134 and kindly donated by Laboratory BioS, Reims University.  $\alpha$ -MEM, DMEM, phosphate buffer  
135 saline without calcium and magnesium (PBS) were purchased from Gibco. Deionized distilled  
136 water from a Milli-Q system was used to prepare all aqueous solutions.

137

### 138 **2. Methods**

#### 139 *2.1. Preparation and sterilization of reactants*

140 Aqueous suspensions of CS were sterilized in autoclave at 121°C for 10 min using standard  
141 liquid cycle according to San Juan et al. (2012). After autoclaving, acetic acid (AcOH) was added

142 to the suspension with molar ratio  $[\text{AcOH}]/[\text{NH}_2] = 1$ , which was stirred at room temperature  
143 ( $21 \pm 1^\circ\text{C}$ ) for 24 h. The concentration of the CS stock solution was 1.33 wt%. CS stock solutions  
144 were stored at  $5^\circ\text{C}$  until further use.  $\beta\text{GP}$ , AHP and AcOH solutions at  $1 \text{ mol.L}^{-1}$  were prepared  
145 in deionized water and sterilized by filtration using syringe filters having pore size of  $0.2 \mu\text{m}$ .

#### 146 *2.2. pH titration of CS/ $\beta\text{GP}$ solution with AHP solution*

147 Stock solutions of  $\beta\text{GP}$  and AHP as well as CS solution at 0.8 wt% were chilled in an ice bath  
148 prior to mixture. All pH titrations were performed at  $4 \pm 1^\circ\text{C}$ . The  $\beta\text{GP}$  solution was added  
149 dropwise to the CS solution under vigorous stirring. Solutions with molar ratio  $[\beta\text{GP}]/[\text{NH}_2]$   
150 ranging from 0 to 4 were prepared. For each  $[\beta\text{GP}]/[\text{NH}_2]$  ratio, titration was carried out with  
151 AHP solution until the pH reached 7.4.

#### 152 *2.3. Osmolarity measurement*

153 Osmolality of different solutions was measured with a cryoscopic osmometer (Osmomat 30,  
154 Gonotec). The apparatus measures the osmolality ( $\text{mOsm.kg}^{-1}$ ), which is proportional through  
155 the density to the osmolarity ( $\text{mOsm.L}^{-1}$ ). In this study, since all solutions are diluted, the  
156 difference between osmolarity and osmolality is insignificant.

#### 157 *2.4. Gelation time measurement*

158 The gelation times were determined by the test tube inversion method. The sol phase was defined  
159 as the flowing phase and the gel phase as the non-flowing phase when the test tube was inverted.  
160 1 mL of CS/ $\beta\text{GP}$  solutions was introduced into 1.5 mL tubes that were incubated in a thermostat  
161 at fixed temperature ( $15\text{-}37^\circ\text{C}$ ). Time between inversions was as follows: 30 s from 0 to 10 min,  
162 1 min from 10 to 30 min, 5 min from 30 to 60 min, 10 min from 60 to 120 min, 15 min from 2 h

163 to 5 h, 30 min from 5 h to 10 h and 3 h after 10 h. The time at which the gel did not flow anymore  
164 was recorded as the gelation time and the time interval between two inversions gave the  
165 experimental uncertainty. Each measure was duplicated. The shortest measurable gelation time is  
166 given by the duration of thermal equilibration, which in these tubes was measured to be 1 min for  
167 a solution at 4°C immersed in a thermostat at 37°C.

#### 168 *2.5. Microstructure study of CS/βGP/AHP hydrogels*

169 CS was labelled with Alexa Fluor 488 (Alexa Fluor monomer units:NH<sub>2</sub> = 1:150) following a  
170 protocol adapted from Zhang et al. (2011). The fluorescently labelled CS was then added to  
171 regular CS suspensions before the autoclave process. Labelled CS chains represented only 0.5  
172 wt% of the total amount of CS in each formulation. The amount of grafted fluorophore was three  
173 orders of magnitude smaller than in Zhang et al. (AlexaFluor/NH<sub>2</sub> units 1:30000). Hence, the  
174 perturbation caused by the labelling was considered to be negligible. Accordingly, there was no  
175 difference in gelation time at 37°C between regular CS and fluorescently labelled CS.

176 A sample holder was filled with a few hundreds of microliters of fluorescently labelled  
177 CS/βGP/AHP solution, sealed to prevent evaporation and incubated in a regular oven at 37°C for  
178 30 min. Hydrogels at CS concentrations between 0.5 and 0.8 wt% were observed at room  
179 temperature using Laser Confocal Scanning Microscopy (LCSM, Zeiss Axiovert 200M). For  
180 each sample, stacks of 10 images of (225 μm x 225 μm) with an interval of 1 μm were recorded  
181 in eight random positions at 100 μm depth.

182 Deconvolution of the image stacks was performed using the Iterative Deconvolve 3D plugin with  
183 a theoretical point spread function PSF calculated by the Diffraction PSF 3D plugin (ImageJ).  
184 Deconvoluted images were binarized using Li's method (C. H. Li & Tam, 1998). The polymer-

185 rich zones are presented in white and the polymer-poor zones (pores) are presented in black. The  
186 porosity was determined as the percentage of black pixels over the total number of pixels. The  
187 pore size distribution was characterized quantitatively using a customized algorithm (Python)  
188 based on the morphological sieve technique (Wu, van Vliet, Frijlink, & van der Voort  
189 Maarschalk, 2007). This technique calculates on each image the fraction of porosity that is able to  
190 fit a structuring element having a given shape and size, here a disk having a given diameter. For  
191 each formulation, the average porosity and average pore size distribution were calculated over 8  
192 stacks of 10 images.

### 193 *2.6. Rheological characterization*

194 Rheological properties of physiological formulations prepared with 0.5 wt% or 0.8 wt% of CS  
195 were studied using rheometer MCR502 (Anton Paar) with a cone-and-plate geometry (CP50-  
196 2TG). The temperature was managed by a Peltier controller and the geometry was previously  
197 stabilized at 18°C. Formulations were loaded immediately after preparation and silicone oil was  
198 added to the interface between the sample and the air to prevent evaporation.

199 For isothermal measurement, the temperature was rapidly increased 37°C at a rate of 20°C.min<sup>-1</sup>.  
200 The values of storage and loss moduli ( $G'$ ,  $G''$ ) were measured during 6 h, at a constant  
201 oscillatory strain of 0.1% and an angular frequency of 1 rad.s<sup>-1</sup>. For temperature sweep  
202 measurement, the temperature was increased from 4°C to 60°C then decreased from 60°C to 4°C,  
203 at a rate of 5°C/min. The values of storage and loss moduli ( $G'$ ,  $G''$ ) were measured during one  
204 heating/cooling cycle, at a constant oscillatory strain of 0.1% and an angular frequency of 1 rad.s<sup>-1</sup>.  
205 <sup>1</sup>.

### 206 *2.7. Injectability evaluation*

207 Injectability test was performed using the protocol reported by Kondziolka, Gobbel, Fellows-  
208 Mayle, Chang, & Uram (2011). For visualization, a small amount of rose Bengal dye was added  
209 to CS/βGP/AHP mixture. Immediately following the preparation, the mixture was loaded into a 5  
210 mL syringe equipped with a 21G needle and placed on a pump syringe. A volume of 1.5 mL was  
211 then injected at a rate of 250 μL.min<sup>-1</sup> into a PBS solution at pH 7.4 and 37°C.

## 212 2.8. Cell encapsulation in thermosensitive chitosan hydrogels

213 We compared two formulations of hydrogels for cell encapsulation: CS/βGP (600 mOsm.L<sup>-1</sup>) and  
214 CS/βGP/AHP (300 mOsm.L<sup>-1</sup>) at fixed CS concentration (0.8 wt%) and at pH 7.4 on two cell  
215 models: primary human gingival fibroblast (HGF) and pre-osteoblast immortalized murine cell  
216 line (MC3T3-E1). HGF were cultured in DMEM and MC3T3-E1 cells were cultured in  
217 α-MEM, both cell culture medium supplemented with 10%v/v fetal calf serum and 1%v/v  
218 penicillin-streptomycin. For both cell types, the encapsulation protocol was as follows: (1) cells  
219 were trypsinized and mixed gently with CS/βGP or CS/βGP/AHP solutions at a final  
220 concentration of 10<sup>6</sup> cells.mL<sup>-1</sup>; (2) 300 μL of the mixture containing cells were dispensed into a  
221 24-well culture plate (Nunc™ F96 MicroWell™) and incubated for 1 h at 37°C and 5% CO<sub>2</sub> to  
222 induce gelation; (3) 1 mL of culture medium supplemented with 10%v/v fetal calf serum and  
223 1%v/v penicillin-streptomycin was added into each well. Hydrogels without cells were also  
224 prepared by replacing the cell suspension by culture medium.

## 225 2.9. Live/Dead assay

226 After 1 h or 24 h, the encapsulated cells were stained using calcein AM/ethidium homodimer  
227 (CalAM/EthD-1) mixture at optimized concentrations ([CalAM]/[EthD-1]: 2 μM/2 μM and 1

228  $\mu\text{M}/0.5 \mu\text{M}$  for MC3T3-E1 cells and HGF, respectively). For positive control, cells were seeded  
229 directly into the well at a density of  $10^4 \text{ cells.cm}^{-2}$ . For negative control, cells were killed using  
230 methanol solution at 70 wt%. 1 mL of the staining mixture was added into each well and the  
231 plates were incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 45 min then the mixture was removed and the  
232 hydrogels or cells were rinsed with PBS. 500  $\mu\text{L}$  of PBS solution were added into each well to  
233 prevent drying. The distribution and the state of the cells were visualized using a laser scanning  
234 confocal microscope (LSM 710, Zeiss) using lasers at 488 nm and 561 nm. Each hydrogel sample  
235 was scanned from bottom to 300  $\mu\text{m}$  depth at 8 random positions. Stacks were taken at 20x  
236 magnification and a 512x512 pixel resolution with the pinhole at 1 Airy unit and interval of 4  
237  $\mu\text{m}$ . Recorded images were then analyzed with ImageJ using 3D Object counter plugin (Bolte &  
238 Cordelières, 2006). The cell viability (%) was determined as the number of living cells over the  
239 total number of cells. Experiments were carried out with  $n = 2$  for MC3T3 and  $n = 1$  for HGF and  
240 all samples were prepared in triplicate.

#### 241 *2.10. AlamarBlue assay*

242 The cell metabolism was evaluated using the AlamarBlue assay at 1 h and 24 h following  
243 encapsulation. After incubation, a solution of 10 %v/v AlamarBlue in culture medium (1  
244 mL/well) was added directly on each hydrogel and incubated for 3 h at  $37^\circ\text{C}$  and 5 % of  $\text{CO}_2$ .  
245 Then, the supernatant was homogenized by pipetting and 150  $\mu\text{L}$  were transferred into a 96-well  
246 plate (Nunc™ F96 MicroWell™). The intensity of fluorescence was determined by using Xenius  
247 XM spectrofluorometer (SAFAS Monaco) at 560 nm excitation and 590 nm emission. A value of  
248 the metabolic activity per cell was estimated by normalizing the measured intensity by the  
249 number of living cells obtained from the Live/Dead assay.

250      *2.11. Statistical analysis*

251      Statistical analysis was performed using a t-test with normality assumption. Statistical  
252      significance was accepted at the level of  $p < 0.05$ .

## 253 RESULTS & DISCUSSION

254 In all the following,  $[\text{NH}_2]$  denotes the concentration in D-glucosamine monomer in the solution.

### 255 1. Controlling the pH using CS/ $\beta$ GP/AHP formulations

256 Control of the pH of thermogelling CS/ $\beta$ GP solutions is determinant for the viability and the  
257 injectability of encapsulated cells. Cell viability requires that the pH be maintained close to 7.4  
258 during cell suspension and storage until injection and immersion in other buffering media. The  
259 gelation time of CS/ $\beta$ GP solutions was also shown to depend greatly on pH (Chenite et al., 2001).

260 Accordingly, we measured that the gelation time of CS/ $\beta$ GP solutions increases from 1 min to  
261 10 h when pH decreases from 7.5 to 7.1 (**Figure S1**). In the present study, CS was dissolved in  
262 acidic aqueous solutions with a fixed molar ratio  $[\text{AcOH}]/[\text{NH}_2] = 1$ . This ratio was chosen as the  
263 minimum amount of acid required to ensure the solubilization of chitosan chains (Rinaudo,  
264 2006). In CS/ $\beta$ GP/AHP formulations,  $\beta$ GP and AHP are both weak bases that raise the pH of the  
265 solution. As demonstrated thereafter, the pH can be finely controlled by the molar ratios between  
266 each phosphate salt and D-glucosamine monomer,  $[\beta\text{GP}]/[\text{NH}_2]$  and  $[\text{AHP}]/[\text{NH}_2]$ .

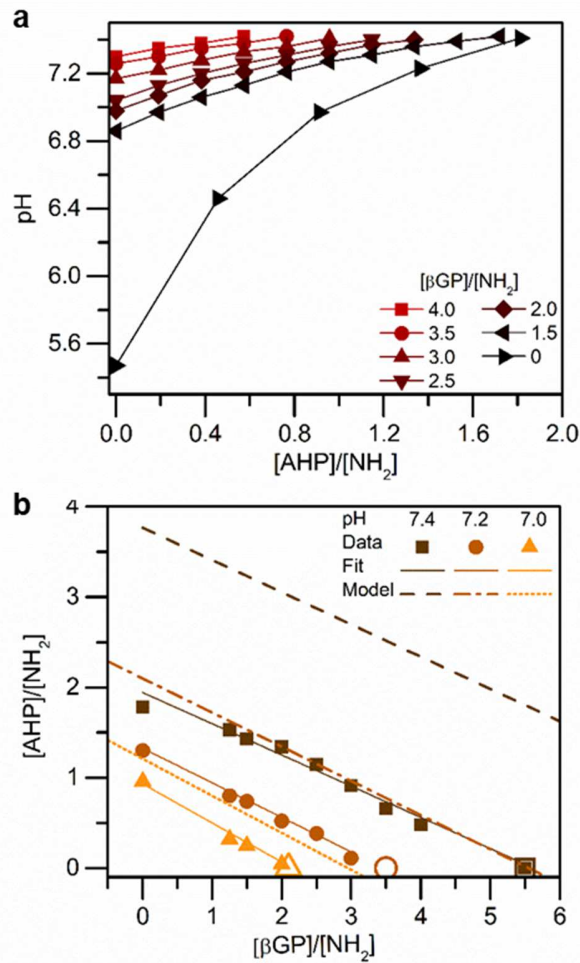
267 We carried out pH titration at 4°C by adding AHP (1 mol.L<sup>-1</sup>) to solutions having an initial CS  
268 concentration of 0.8 wt% and different ratios of  $[\beta\text{GP}]/[\text{NH}_2]$ . The evolution of pH as a function  
269 of  $[\text{AHP}]/[\text{NH}_2]$  is reported in **Figure 2a**. For a given  $[\beta\text{GP}]/[\text{NH}_2]$ , all titration curves show an  
270 increase in pH with increasing  $[\text{AHP}]/[\text{NH}_2]$ . Similarly, for a given  $[\text{AHP}]/[\text{NH}_2]$ , pH increases  
271 with increasing  $[\beta\text{GP}]/[\text{NH}_2]$ . From those curves, we extracted the pairs of ratios  $[\beta\text{GP}]/[\text{NH}_2]$   
272 and  $[\text{AHP}]/[\text{NH}_2]$  producing a given pH and built the iso-pH curves represented in **Figure 2b**.  
273 Those iso-pH curves are linear in the studied range of pH from 7.0 to 7.4. Formulations using the



274 ratios of the iso-pH curve at 7.4 but different initial CS concentrations (0.5 and 0.6 wt%) exhibit  
275 a pH 7.4 as well, which confirms that the pH is fully determined by the ratios  $[\beta\text{GP}]/[\text{NH}_2]$  and  
276  $[\text{AHP}]/[\text{NH}_2]$ , irrespectively of the CS concentrations.

277 The pH of CS and CS/ $\beta$ GP solutions is known to decrease substantially with increasing  
278 temperature (Lavertu et al., 2008; Supper et al., 2013). A similar effect was observed in the  
279 CS/ $\beta$ GP/AHP formulations. For instance, the pH of a solution at CS 0.8 wt% decreases from 7.40  
280  $\pm 0.05$  at 4°C to 6.95  $\pm 0.05$  at 37°C (**Figure S2**). Therefore, it seems more appropriate to target  
281 formulations having a pH 7.4 at 4°C to ensure that the pH of cell-laden solutions remains within a  
282 physiological range (typically 7.0-7.4) during cell dispersion and encapsulation.

283

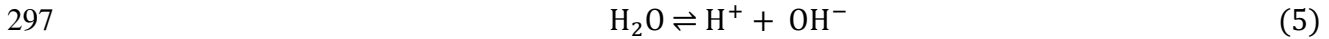
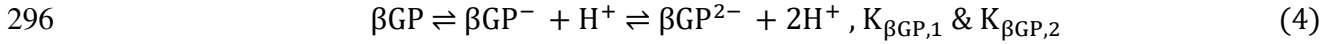
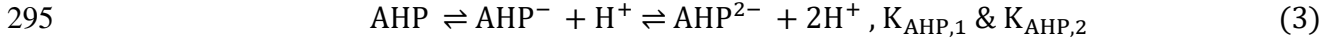
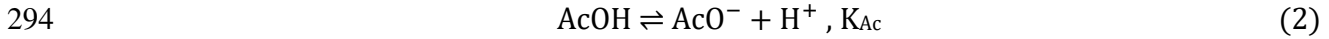


284

285 **Figure 2.** (a) Evolution of the pH as a function of [AHP]/[NH<sub>2</sub>] for formulations with initial CS  
 286 concentration of 0.8 wt% and [βGP]/[NH<sub>2</sub>] ranging from 0 to 4. (b) Iso-pH curves at 7.0, 7.2 and 7.4 as a  
 287 function of [AHP]/[NH<sub>2</sub>] and [βGP]/[NH<sub>2</sub>]. Experimental data are shown as filled symbols. Linear fitting  
 288 curves are shown as continuous lines and model predictions as interrupted lines. Predictions accounting  
 289 for the polyelectrolyte effect based on Filion et al., 2007 are shown as open symbols for CS/βGP mixtures  
 290 ([AHP]/[NH<sub>2</sub>] = 0).

291 The linear relationship observed in **Figure 2b** can be rationalized by considering the dissociation  
 292 of the different species present in the solution, as follows:





298 The values of the dissociation constants of AHP and  $\beta\text{GP}$  were measured by pH titration at 4°C  
 299 and 25°C (**Figure S3**). Only the second equilibrium is observed for pH ranging from 2 to 8:

300  $K_{\text{AHP},2} = 10^{-6.7}$  and  $K_{\beta\text{GP},2} = 10^{-6.2}$ . For CS and AcOH, values are chosen from the literature:  $K_{\text{CS}}$   
 301  $= 10^{-7.1}$  (4°C) (Filion et al., 2013) and  $K_{\text{Ac}} = 10^{-4.8}$  (Kortum, Vogel, & Andrussow, 1960).

302 To a first approximation, we assume here that CS behaves like a small molecule and we do not  
 303 take into account its polyelectrolyte nature. Writing electroneutrality, conservation of matter and  
 304 acid-base equilibria, we find that for the pH range of interest (7.0-7.4), the ratios  $[\text{AHP}]/[\text{NH}_2]$   
 305 and  $[\beta\text{GP}]/[\text{NH}_2]$  satisfy the following relation (see detailed calculation in SI):

306 
$$\frac{[\text{AHP}]}{[\text{NH}_2]} = -\frac{C_{\beta\text{GP}}}{C_{\text{AHP}}} \cdot \frac{[\beta\text{GP}]}{[\text{NH}_2]} + \frac{C_{\text{Ac}}}{C_{\text{AHP}}} \cdot \frac{[\text{AcOH}]}{[\text{NH}_2]} - \frac{C_{\text{CS}}}{C_{\text{AHP}} \cdot \text{DD}} \quad (6)$$

307 where  $[\text{AcOH}]$ ,  $[\beta\text{GP}]$ ,  $[\text{AHP}]$ ,  $[\text{NH}_2]$  are respectively the initial concentrations in AcOH,  $\beta\text{GP}$ ,  
 308 AHP and D-glucosamine monomer, DD is the degree of deacetylation of CS. Factors  $C_{\text{Ac}}$ ,  $C_{\beta\text{GP}}$ ,

309  $C_{\text{AHP}}$  and  $C_{\text{CS}}$  are all functions of the concentration in protons  $h$  as follows:  $C_{\text{Ac}} = \left(1 + \frac{h}{K_{\text{Ac}}}\right)^{-1}$ ,

310  $C_{\beta\text{GP}} = \left(1 + \frac{K_{\beta\text{GP},2}}{h}\right)^{-1}$ ,  $C_{\text{AHP}} = \left(1 + \frac{K_{\text{AHP},2}}{h}\right)^{-1}$  and  $C_{\text{CS}} = \left(1 + \frac{K_{\text{CS}}}{h}\right)^{-1}$ .

311 For a fixed ratio  $[\text{AcOH}]/[\text{NH}_2]$ , here equal to 1, equation (6) captures that for a given pH, the  
 312 relationship between  $[\text{AHP}]/[\text{NH}_2]$  and  $[\beta\text{GP}]/[\text{NH}_2]$  is linear. The negative slope  $-\frac{C_{\beta\text{GP}}}{C_{\text{AHP}}}$  depends

313 weakly on the pH of the mixture and the dissociation constants of the two salts  $\beta$ GP and AHP.  
314 The model predicts the experimental values of the slopes with a good precision including the  
315 slight increase when the pH decreases from 7.4 to 7.0, as shown in **Figure 2b** and **Table S1**.  
316 However, it fails to predict the intercept. We attribute this discrepancy to the polyelectrolyte  
317 nature of CS. In particular, the protonation of  $\text{NH}_2$  along the CS chains is very sensitive to  
318 entropic effects and  $\text{pK}_{\text{CS}}$  increases with increasing salt concentration (Filion et al., 2007).  
319 Accordingly, predictions of pH for CS/ $\beta$ GP accounting for these polyelectrolyte effects using a  
320 model by Filion et al. (**Figure S4**) are in perfect agreement with our experimental measurements,  
321 as indicated by the open symbols in **Figure 2b**. The simple small molecule model also confirms  
322 that the linear relationship between  $[\text{AHP}]/[\text{NH}_2]$  and  $[\beta\text{GP}]/[\text{NH}_2]$  is independent on the CS  
323 concentration as long as the ratio  $[\text{AcOH}]/[\text{NH}_2]$  is fixed. Therefore, such iso-pH curves may  
324 serve as an efficient platform to prepare CS/ $\beta$ GP/AHP formulations having a controlled pH.

## 325 **2. CS/ $\beta$ GP/AHP formulations with adjustable pH and osmolarity**

326 The contribution of  $\beta$ GP, AHP and AcOH to the osmolarity of the formulations was determined  
327 by measuring independently their osmolarity at different concentrations. We verified that  
328 osmolarity was proportional to the molar concentration for all three components (**Figure S5**). As  
329 expected from the dissociation constants, the osmolarity induced by  $\beta$ GP is higher than the one  
330 induced by AHP. The osmolarity of a CS solution at 0.8 wt% is negligible. The total osmolarity  
331 (expressed in  $\text{Osm}\cdot\text{L}^{-1}$ ) was calculated as the linear combination of the three contributions:

$$332 \quad \text{Osmolarity} = 1.03\cdot[\text{AcOH}] + 2.35\cdot[\beta\text{GP}] + 1.82\cdot[\text{AHP}] \quad (7)$$

333 Normalizing Equation (7) by  $[\text{NH}_2]$  gives a relationship between  $[\text{AHP}]/[\text{NH}_2]$  and  $[\beta\text{GP}]/[\text{NH}_2]$ :

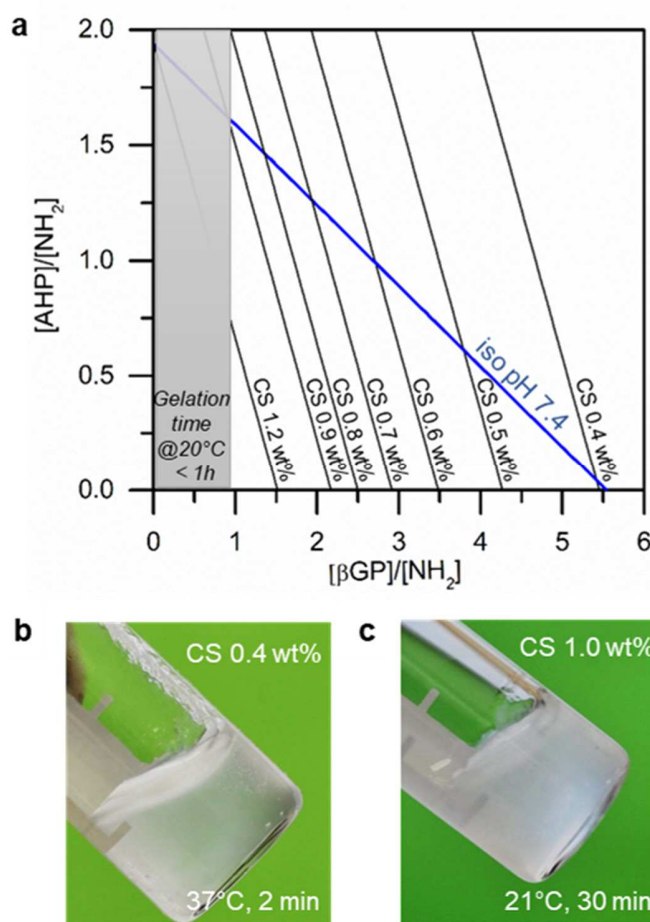
334 
$$\frac{[\text{AHP}]}{[\text{NH}_2]} = -A_1 \cdot \frac{[\beta\text{GP}]}{[\text{NH}_2]} - A_2 \cdot \frac{[\text{AcOH}]}{[\text{NH}_2]} + A_3 \cdot \frac{\text{Osmolarity}}{[\text{NH}_2]} \quad (8)$$

335 where  $A_1 = 1.29$ ,  $A_2 = 0.57$  and  $A_3 = 0.55$  are dimensionless constants.

336 For a fixed ratio  $[\text{AcOH}]/[\text{NH}_2]$ , we obtain linear iso-osmolarity lines between  $[\text{AHP}]/[\text{NH}_2]$  and  
 337  $[\beta\text{GP}]/[\text{NH}_2]$ . Unlike the iso-pH curves above, the intercept of these curves depends on the CS  
 338 concentration. The curves obtained for a physiological osmolarity of  $300 \text{ mOsm.L}^{-1}$  are presented  
 339 in **Figure 3a** for CS concentrations ranging from 0.4 to 1.2 wt%. For a given CS concentration,  
 340 the intersection between the iso-pH ( $\text{pH} = 7.4$ ) and iso-osmolarity lines give the unique  
 341 formulation satisfying both conditions on targeted pH and osmolarity.

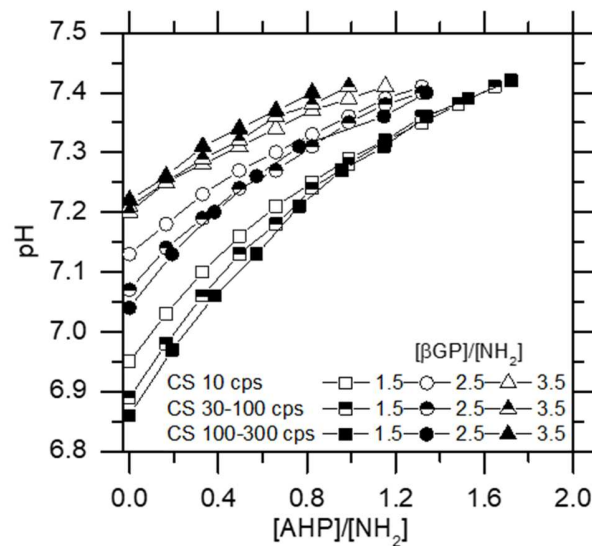
342 The representation in **Figure 3a** provides a way to determine the formulations of interest for  
 343 thermosensitive cell-laden hydrogels. In particular, it shows that the double constraint on pH and  
 344 osmolarity restricts the range of pertinent CS concentrations. As shown in **Figure 3a**, CS  
 345 concentration of 0.4 wt% is the lowest concentration allowing to achieve physiological pH (7.4)  
 346 and osmolarity ( $300 \text{ mOsm.L}^{-1}$ ). CS concentrations lower than 0.4 wt% would require either a  
 347 hypotonic mixture to satisfy  $\text{pH} = 7.4$  or a basic pH ( $>7.4$ ) to satisfy an osmolarity of 300  
 348  $\text{mOsm.L}^{-1}$ . We verified that the physiological formulation at 0.4 wt% corresponding to  
 349  $[\beta\text{GP}]/[\text{NH}_2] = 5.5$  and  $[\text{AHP}]/[\text{NH}_2] = 0$  forms a macroscopically homogeneous gel after 2 min  
 350 at  $37^\circ\text{C}$  (**Figure 3b**). In **Figure 3a**, the highest CS concentration allowing to achieve  
 351 physiological pH and osmolarity is 1.2 wt%. However, this concentration (corresponding to  
 352  $[\beta\text{GP}]/[\text{NH}_2] = 0$ ) is not pertinent for practical applications, because the gelation time at room  
 353 temperature (i.e. before injection) decreases when decreasing  $[\beta\text{GP}]/[\text{NH}_2]$ , as will be presented  
 354 in section 3. We found that a minimum ratio  $[\beta\text{GP}]/[\text{NH}_2] = 1.0$ , corresponding to a CS  
 355 concentration of 0.9 wt%, is required to maintain the thermosensitive solution in a liquid state for

356 more than 1 h at  $21\pm 1^\circ\text{C}$ . A duration of 1 h corresponds to the time needed to comfortably carry  
 357 out cell trypsination, dispersion in CS/salt solution and injection. Accordingly, solutions prepared  
 358 at a higher concentration than 0.9 wt% form a gel in less than 1 h at room temperature, as shown  
 359 for example for a 1 wt% solution ( $[\beta\text{GP}]/[\text{NH}_2] = 0.7$ ,  $[\text{AHP}]/[\text{NH}_2] = 1.7$ ) which formed a gel  
 360 within 30 min (**Figure 3c**).



361  
 362 **Figure 3.** (a) Iso-osmolarity curves at  $300\text{ mOsm}\cdot\text{L}^{-1}$  (black) and iso-pH curve at 7.4 (blue) as a function  
 363 of  $[\text{AHP}]/[\text{NH}_2]$  and  $[\beta\text{GP}]/[\text{NH}_2]$  for CS concentration ranging from 0.4 to 1.2 wt%. Shaded area  
 364 indicates formulations for which gelation time at  $21^\circ\text{C}$  is less than 1 h. (b) Hydrogel ( $300\text{ mOsm}\cdot\text{L}^{-1}$ ; pH =  
 365 7.4) obtained with 0.4 wt% of CS after 2 min of incubation at  $37^\circ\text{C}$ , (c) Hydrogel ( $300\text{ mOsm}\cdot\text{L}^{-1}$ ; pH =  
 366 7.4) obtained with 1.0 wt% of CS after 30 min at  $21\pm 1^\circ\text{C}$ .

367 These results and methodology show how the pH and osmolarity of CS/ $\beta$ GP/AHP mixtures can  
 368 be independently adjusted for different CS concentrations. It is worth to note that the  
 369 concentration range for cytocompatible values of pH and osmolarity has been measured here for  
 370 one type of CS having a given degree of deacetylation (DD = 91 %) and a given molar mass  
 371 distribution (viscosity 30-100 cps,  $M_w = 250 \text{ kg}\cdot\text{mol}^{-1}$ ). It has been reported that the gelation rate  
 372 of CS/ $\beta$ GP solutions at given pH increases with CS of higher DD (Ruel-Gariépy, Chenite,  
 373 Chaput, Guirguis, & Leroux, 2000). Therefore, the upper bound of the accessible concentration  
 374 range is expected to decrease with increasing DD. As regards the molar mass, we verified that the  
 375 pH can be adjusted by the salts/ $\text{NH}_2$  ratios whatever the chitosan chain length is as long as the  
 376 deacetylation degree is known, as shown for CS with DD = 95 % for two different molar masses  
 377 (**Figure 4**). Hence, this rationalized formulation is applicable to other types of CS.

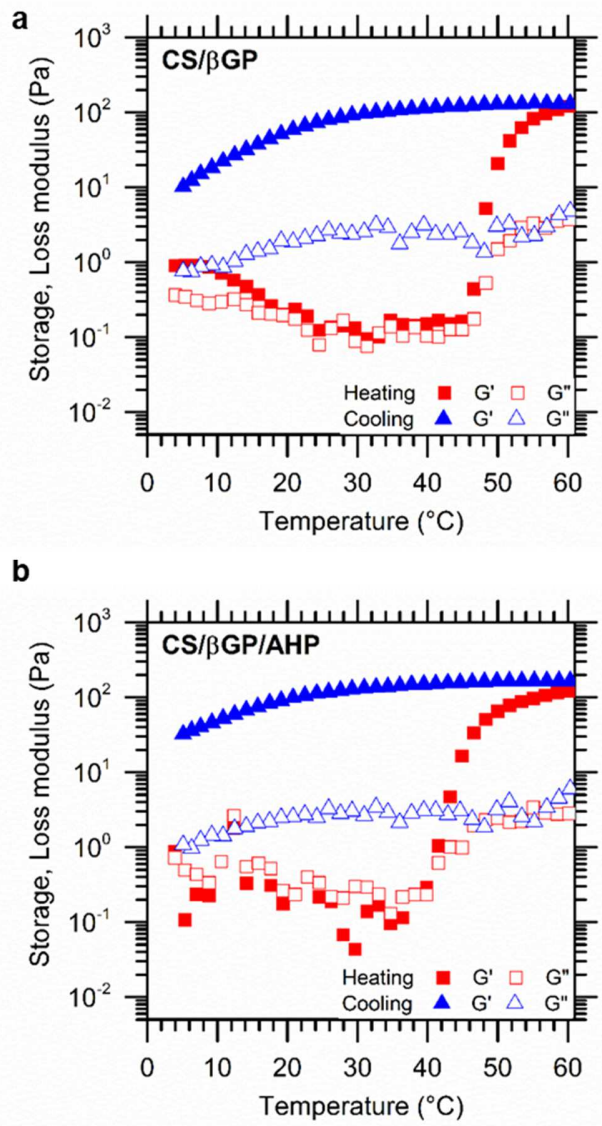


378  
 379 **Figure 4.** Evolution of the pH as a function of  $[\text{AHP}]/[\text{NH}_2]$  in formulations with initial CS concentration  
 380 of 0.8 wt% and fixed  $[\beta\text{GP}]/[\text{NH}_2] = 1.5, 2.5$  and  $3.5$  for three different chitosan grades (10 cps, 30-100  
 381 cps and 100-300 cps) having DD = 95%, 91% and 95%, respectively.

382        **3. Gelation kinetics of CS/βGP/AHP formulations**

383    It is important to notice that the gelation time of CS/βGP systems depends greatly on the pH  
384    (Chenite et al., 2001). As a result, we focus here on the gelation kinetics at a fixed pH = 7.4.  
385    **Figure 5** shows the evolution of storage,  $G'$ , and loss modulus,  $G''$ , during a heating-cooling cycle  
386    between 4 and 60°C for CS/βGP and CS/βGP/AHP, both at pH 7.4. While heating, both moduli  
387    exhibit a sharp increase and the gel point ( $G' = G''$ ) is reached near 45°C for CS/βGP and 40°C  
388    for CS/βGP/AHP. Upon cooling, a large hysteresis is observed and both moduli decrease slowly  
389    below 30°C. This weak thermoreversibility is very similar to what has been reported earlier by  
390    Chenite et al. (2001).





391

392 **Figure 5.** Evolution of the storage and loss modulus during a heating/cooling cycle for CS/ $\beta$ GP mixture

393 (a) and CS/ $\beta$ GP/AHP mixture (b), both at fixed pH of 7.4, CS concentration of 0.8 wt%.

394 A more systematic characterization of the thermosensitivity of CS/ $\beta$ GP/AHP mixtures was

395 performed using the test tube inversion method (**Figure S6a**). We verified that the time measured

396 by this method is consistent with the time to reach the gel point as determined by rheology

397 (**Figure S6b-c**). In a first series of experiments, we studied the effect of the relative salt ratio

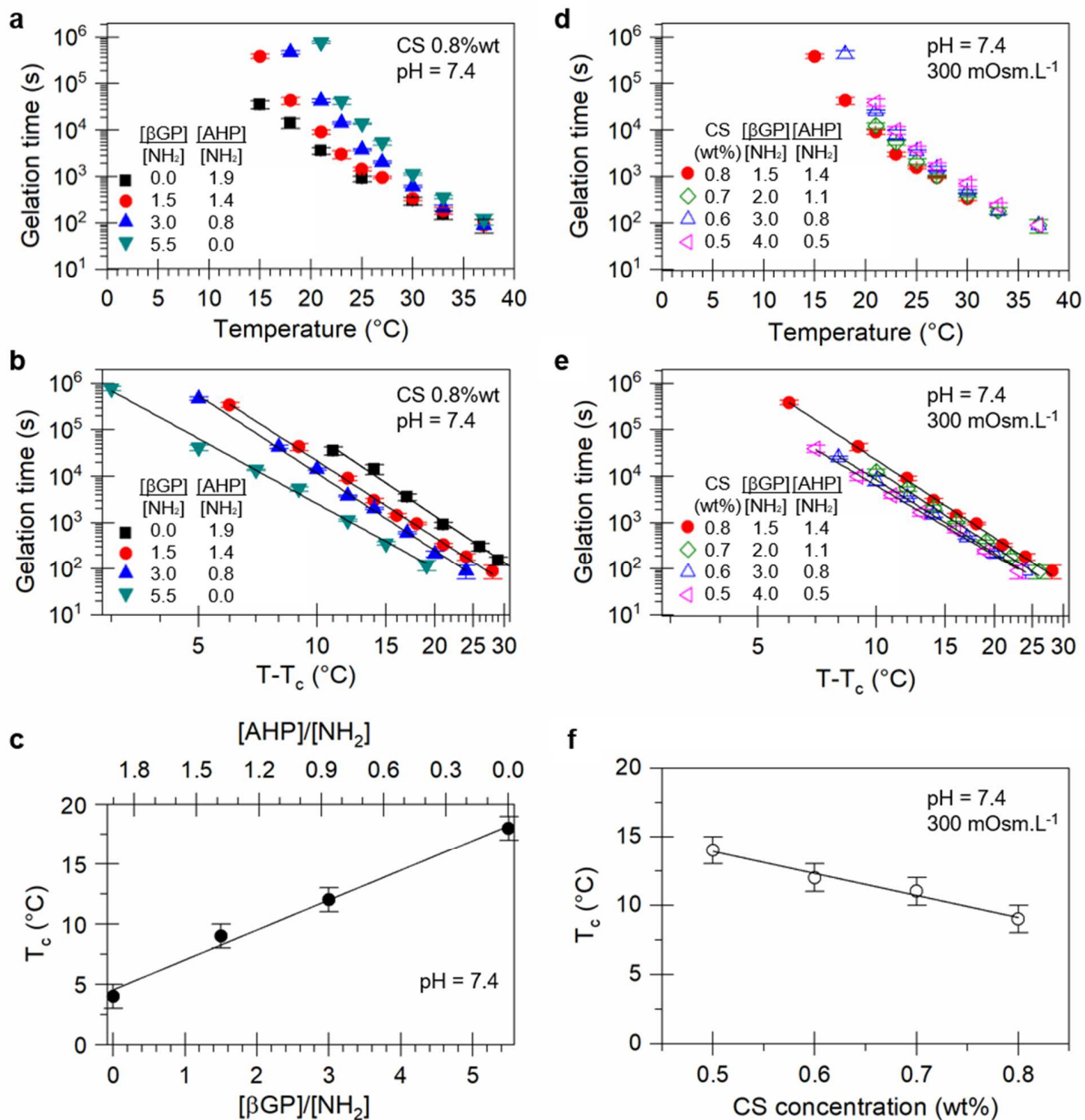
398  $[\text{AHP}]/[\beta\text{GP}]$  on the gelation time. For that, a fixed CS concentration of 0.8 wt% was chosen.

399 Four ratios  $[\beta\text{GP}]/[\text{NH}_2]$  were studied (0, 1.5, 3 and 5.5) and the corresponding  $[\text{AHP}]/[\text{NH}_2]$   
400 ratios were adjusted to maintain  $\text{pH} = 7.4$  (1.9, 1.4, 0.8 and 0, respectively). In those solutions,  
401 the osmolarity varied from 300 to 600  $\text{mOsm}\cdot\text{L}^{-1}$ . **Figure 6a** shows that the gelation time  
402 decreases by at least four orders of magnitude when temperature increases from  $15^\circ\text{C}$  to  $37^\circ\text{C}$  for  
403 all four formulations. At a given temperature, the gelation time decreases with decreasing  
404  $[\beta\text{GP}]/[\text{NH}_2]$  ratio or increasing  $[\text{AHP}]/[\text{NH}_2]$  ratio. This sensitivity to  $[\text{AHP}]/[\beta\text{GP}]$  becomes  
405 increasingly pronounced with decreasing temperature. At  $20^\circ\text{C}$ , the formulation with only  $\beta\text{GP}$   
406 become a gel more than 100 times slower than the one with AHP only.

407 The temperature dependence of the gelation time of CS/ $\beta\text{GP}$  formulations was presented in an  
408 earlier study by Chenite et al. (2001) who concluded that the gelation time displayed an  
409 exponential decay with temperature. Our data which cover a larger range of times and  
410 temperatures do not fit with a simple exponential decay, but rather with a model of critical  
411 transition, as already observed in thermosensitive gelatin gels (Te Nijenhuis, 1979). Our  
412 experimental data were successfully fitted with the critical-like model  $t_{\text{gel}} = k(T-T_c)^\gamma$  where  $T_c$  is  
413 a critical temperature above which the sol-gel transition occurs and  $\gamma$  is a critical exponent  
414 (**Figure 6b**). The exponent  $\gamma$  and the prefactor  $k$  decrease with increasing  $[\beta\text{GP}]/[\text{NH}_2]$  (**Figure**  
415 **S7a-b**). Conversely,  $T_c$  increases from  $4$  to  $18^\circ\text{C}$  when going from CS/AHP to CS/ $\beta\text{GP}$  as shown  
416 in **Figure 6c**. Overall these results show that the thermal stability of CS/ $\beta\text{GP}$ /AHP solutions at a  
417 fixed  $\text{pH}$  increases with  $[\beta\text{GP}]/[\text{NH}_2]$ . This observation together with the rheological results in  
418 **Figure 5** supports the hypothesis that the polyol part of  $\beta\text{GP}$  hinders the interactions between CS  
419 chains as suggested by Supper et al. (2013).

420 Similar experiments were performed in a second series with formulations defined by the iso-pH  
421 (7.4) and iso-osmolarity (300 mOsm.L<sup>-1</sup>) curves for different CS concentrations ranging from 0.5  
422 to 0.8 wt%. The gelation time decayed strongly with increasing temperature in a similar way to  
423 the first series, as shown in **Figure 6d**. For a given temperature, gelation occurred faster with  
424 increasing CS concentration (together with decreasing [βGP]/[NH<sub>2</sub>] and increasing [AHP]/[NH<sub>2</sub>]  
425 to maintain physiological conditions). Again, a critical power law behavior was observed as  
426 shown in **Figure 6e** (and **Figure S7c-d**). The critical temperature, and therefore the thermal  
427 stability of the solution, decreased with increasing CS concentration.

428 From a practical point of view, these results show that all the formulations having physiological  
429 pH and osmolarity present an adequate gelation kinetics for use as cell delivery vehicles. At  
430 21°C, they remain liquid for more than 2 h, which is appropriate for cell dispersion and  
431 manipulation. At body temperature, they form a gel within less than 1 min, therefore preventing  
432 cells from being dispersed away. The injectability of the formulation having a CS concentration  
433 of 0.8 wt% was verified using standard injection parameters into a PBS solution at 37°C. A  
434 continuous injection could be performed with the quasi-instantaneous formation of a macroscopic  
435 hydrogel (**Video S1**).



436

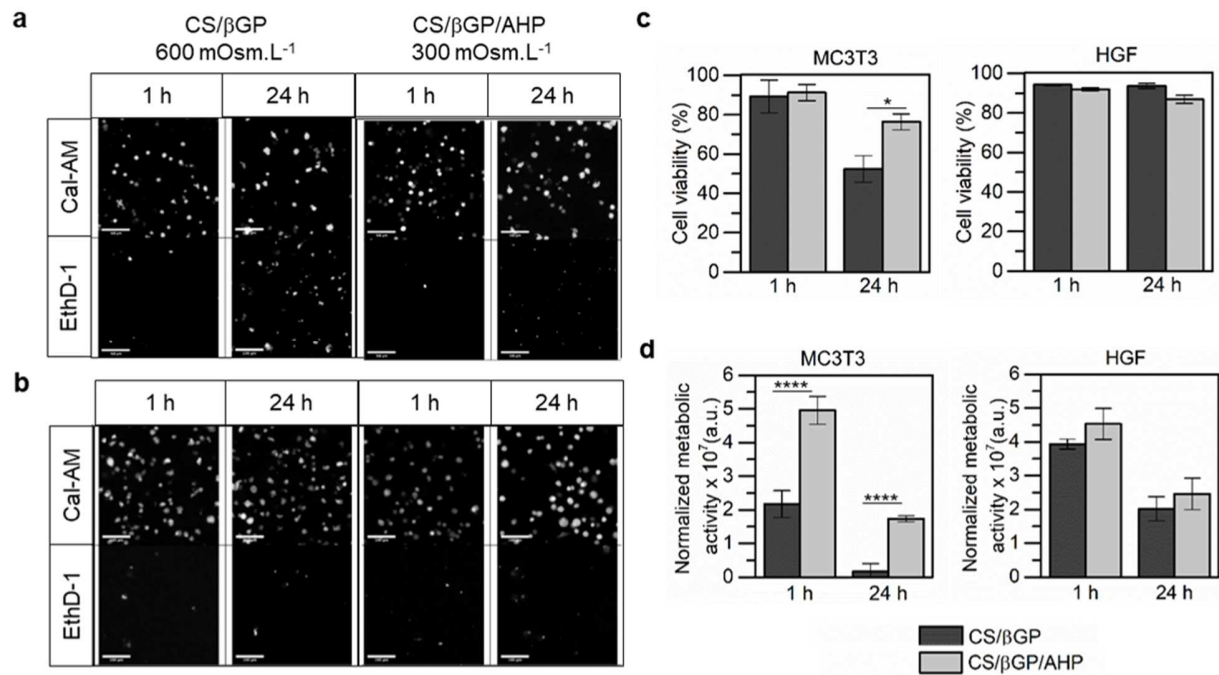
437 **Figure 6.** (a-c) Gelation kinetics of formulations having fixed CS 0.8 wt% and pH 7.4 and various  
 438 [βGP]/[NH<sub>2</sub>] and [AHP]/[NH<sub>2</sub>] ratios. Gelation time as a function of temperature (a) and (T-T<sub>c</sub>) (b). Full  
 439 lines show fits by a power law. (c) Critical temperature as a function of [βGP]/[NH<sub>2</sub>] and [AHP]/[NH<sub>2</sub>].  
 440 (d-f) Gelation kinetics of physiological formulations (pH = 7.4 and 300 mOsm.L<sup>-1</sup>) for CS concentration  
 441 ranging from 0.5 to 0.8 wt%. Gelation time as a function of temperature (d) and (T-T<sub>c</sub>) (e). Full lines show  
 442 fits by a power law. (f) Critical temperature as a function of CS concentration.

#### 443        **4. Cell encapsulation in CS/βGP/AHP hydrogels**

444        The viability and the metabolism of encapsulated cells was studied using Live/Dead and  
445        AlamarBlue assays with formulations having a CS concentration of 0.8 wt% and pH 7.4. Two  
446        formulations were compared to assess the effect of osmolarity: an isotonic formulation denoted  
447        CS/βGP/AHP (300 mOsm.L<sup>1</sup>) produced with ratios [βGP]/[NH<sub>2</sub>] = 1.5 and [AHP]/[NH<sub>2</sub>] = 1.4,  
448        and a hypertonic formulation denoted CS/βGP (600 mOsm.L<sup>-1</sup>) obtained with ratios [βGP]/[NH<sub>2</sub>]  
449        = 5.5 and [AHP]/[NH<sub>2</sub>] = 0. Two cell types were studied: pre-osteoblast immortalized murine cell  
450        line (MC3T3-E1) and primary human gingival fibroblasts (HGF). Cells were dispersed in the  
451        liquid formulations at 20°C at a concentration of 10<sup>6</sup> cells. mL<sup>-1</sup>. Encapsulation was achieved by  
452        gelation at 37°C for 1 h in an incubator prior to the addition of culture medium. Cells were  
453        stained and observed 1 h and 24 h after encapsulation. The staining efficiency was verified on  
454        positive and negative controls (**Figure S8**). The homogeneity of the cell dispersion was verified  
455        by cell counting in multiple areas of the gels (**Figure S9**) and a local cell concentration of 1.2 x  
456        10<sup>6</sup> ± 0.2 cells per cm<sup>3</sup> was found, which is in agreement with the density of seeded cells.

457        Typical images obtained with Live/Dead staining are shown in **Figure 7a-b** for MC3T3-E1 and  
458        HGF, respectively. At 1 h, the viability for both cell types is high (90%) for the two studied  
459        formulations (**Figure 7c**). At 24 h, differences are observed: for MC3T3-E1 cells, a significantly  
460        better cell viability is observed in CS/βGP/AHP hydrogel (78 ± 6 %) than in CS/βGP hydrogel  
461        (52 ± 5 %); for HGF, cell viability remains high in both hydrogels. The metabolic activity of the  
462        encapsulated cells was assessed with an AlamarBlue assay (**Figure 7d**). A value of the metabolic  
463        activity per cell was estimated by normalizing the measured intensity by the number of living  
464        cells. At 1 h, a clear difference can be seen between the two cell types: MC3T3 have a much

465 higher metabolic activity in the isotonic formulation CS/βGP/AHP than in CS/βGP while HGF  
 466 have a similar metabolic activity in both formulations. At 24 h, the same trend is observed but the  
 467 metabolic activity drops significantly for both cell types.



468  
 469 **Figure 7.** Cell encapsulation in CS/βGP/AHP and CS/βGP hydrogels for 1 h and 24 h. (a-b) Confocal  
 470 images of Live/Dead assays for MC3T3 (a) and HGF (b) cells. Images are obtained from the projection of  
 471 76 slices taken at a random position in the hydrogel with a field of 425 x 425 x 300 μm. (c) Cell viability.  
 472 (d) Normalized metabolic activity. Statistically significant at  $p < 0.05$  (\*) or  $p < 0.0001$  (\*\*\*\*).

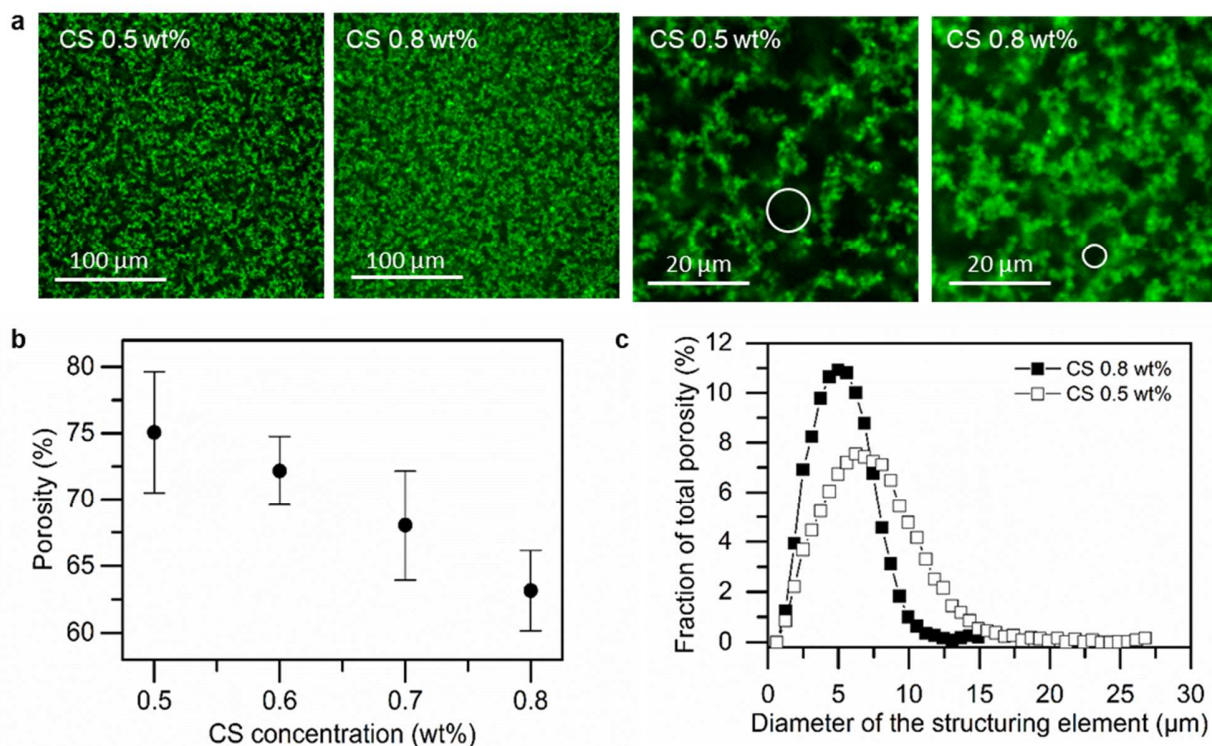
473  
 474 These differences in viability and activity may be explained by the different ability of the cell  
 475 types to adapt to hypertonic conditions (Dmitrieva & Burg, 2005). To our knowledge, no study  
 476 has been reported on MC3T3-E1 whereas it has been shown that HGF are resistant to osmotic  
 477 shocks (Huynh, Everts, Leethanakul, Pavasant, & Ampornaramveth, 2016). In our study, the  
 478 encapsulated cells exhibit rounded phenotypes after 24 h, which reflects a poor adhesion to the  
 479 chitosan matrix. This observation is in agreement with a previous study on encapsulated MC3T3-

480 E1 in CS/AHP hydrogels (Nair et al., 2007). Therefore, the proposed approach could be  
481 advantageously combined with bio-functionalization strategies providing adhesion sites to the  
482 encapsulated cells. For example, covalent grafting of peptides or proteins responsible for cell  
483 adhesion on chitosan chains have shown promising results (Custodio, Alves, Reis, & Mano,  
484 2010; Tsai, Chen, & Liu, 2013).

## 485 **5. Microstructure and mechanics of CS/ $\beta$ GP/AHP hydrogels**

486 The possibility to vary the CS concentration while maintaining physiological conditions, provides  
487 a way to adjust the microstructure and the mechanical properties of cytocompatible formulations.  
488 The microstructure of hydrogels (pH = 7.4 and 300 mOsm.L<sup>-1</sup>) was characterized by LSCM using  
489 fluorescently labelled formulations. After 30 min at 37 °C, heterogeneous structures were  
490 observed composed of interconnected polymer-rich and polymer-poor phases, the latter being  
491 qualified as pores (**a**). We measured that the porosity (%) decreases linearly with increasing CS  
492 concentration as shown in **Figure 8b**, from  $75 \pm 5$  % for CS 0.5 wt% to  $63 \pm 7$  % for CS 0.8 wt%.  
493 Such a linear relationship was reported earlier for CS/ $\beta$ GP (Crompton et al., 2006). Besides  
494 porosity, the pore size is a crucial parameter governing cell-cell interactions and vascularization  
495 (Kaivosoja et al., 2012). Targeted pore sizes vary from tens to hundreds of micrometers  
496 depending on the cell type and on the application (Kaivosoja et al., 2012; O'Brien, Harley,  
497 Yannas, & Gibson, 2005). With the present systems, we observed large interconnected elongated  
498 macropores. The longest dimension of the largest pores was found to reach 15  $\mu$ m for CS 0.8  
499 wt% and up to 30  $\mu$ m for CS 0.5 wt%. Pore size distribution was analyzed quantitatively by  
500 image analysis using a morphological sieve algorithm. The distributions in **Figure 8c** show the  
501 fraction of porosity able to fit a circular structuring element of a given diameter. This analysis

502 clearly reveals that the characteristic size of the macropores increases with decreasing CS  
503 concentration. Indeed, 98% of the porosity can contain structuring elements having diameters  
504 ranging from 1 to 9  $\mu\text{m}$  for CS 0.8 wt% and from 1 to 14  $\mu\text{m}$  for CS 0.5 wt%.

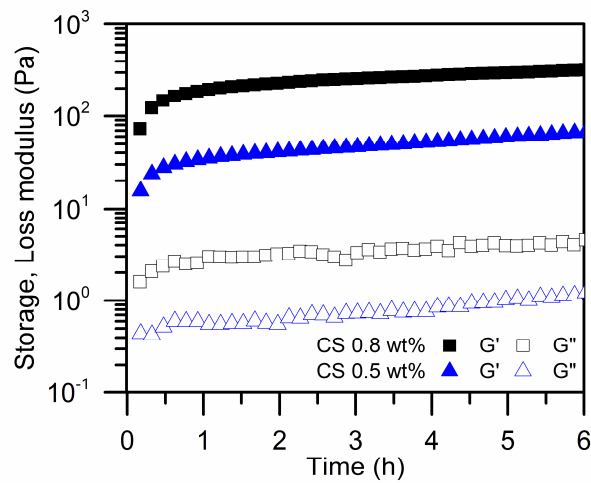


505  
506 **Figure 8.** (a) Confocal images of fluorescently labelled CS/ $\beta$ GP/AHP hydrogels prepared with 0.5 or 0.8  
507 wt% after 30 min at 37°C, white circles show circular structuring elements having the mean diameter  
508 determined by a morphological sieve algorithm. (b) Hydrogel porosity as a function of CS concentration.  
509 (c) Pore size distributions determined by morphological sieve technique for CS/ $\beta$ GP/AHP hydrogels  
510 prepared with 0.5 and 0.8 wt%.

511 The mechanical properties of hydrogels prepared with 0.5 and 0.8 wt% at 4°C were characterized  
512 by oscillatory rheology during an isotherm at 37°C as shown in **Figure 9**. Gelation occurs within  
513 one minute for both formulations with a rapid increase in the storage modulus ( $G'$ ) reaching  
514 approximately 30 Pa for 0.5 wt% and 180 Pa for 0.8 wt% at 1 h of incubation. After 6 h,  $G'$



515 reaches 60 Pa for 0.5 wt% and 300 Pa for 0.8 wt%. These values of  $G'$  are low as compared to  
516 the ones of most biological tissues. For this reason, these hydrogels might be more particularly  
517 appropriate for very soft tissues, such as brain tissue which  $G'$  is in the range 100-300 Pa (Tabet  
518 et al., 2019). Accordingly, CS gels were shown to be suitable matrices for cells of the nervous  
519 system (Kornev, Grebenik, Solovieva, Dmitriev, & Timashev, 2018).



520  
521 **Figure 9.** Storage modulus ( $G'$ ) and loss modulus ( $G''$ ) as function of time at 37°C for physiological  
522 formulations prepared with a CS concentration of 0.5 wt% ( $[\beta\text{GP}]/[\text{NH}_2] = 4.0$  and  $[\text{AHP}]/[\text{NH}_2] = 0.5$ )  
523 and 0.8 wt% ( $[\beta\text{GP}]/[\text{NH}_2] = 1.5$  and  $[\text{AHP}]/[\text{NH}_2] = 1.4$ ).

## 524 CONCLUSION

525 In this study, we report a rational formulation design to prepare thermo-gelling systems  
526 composed of chitosan CS and two phosphate salts,  $\beta$ GP and AHP. We show how the pH and the  
527 osmolarity can be adjusted independently by the ratios [salts]/[NH<sub>2</sub>], arising possibility to obtain  
528 physiological formulations having tunable CS concentration. Moreover, a systematic study of the  
529 gelation kinetics revealed that the gelation time follows a power law that diverges at a critical  
530 temperature. With this approach, injectable solutions having a pH of 7.4 and a physiological  
531 osmolarity of 300 mOsm.L<sup>-1</sup> can be produced for CS concentrations ranging from 0.4 to 0.9 wt%.  
532 The cytocompatibility of the subsequent gels was demonstrated by *in vitro* encapsulation of  
533 MC3T3-E1 and HGF cells. Elastic moduli of the gels can reach up to 300 Pa, a value found in  
534 brain tissues. The proposed methodology could be applied to solutions of chitosan or  
535 functionalized chitosan having various molar mass and deacetylation degrees, therefore providing  
536 a general platform to prepare valuable thermosensitive hydrogels for cell encapsulation and  
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538

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