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Bioinspired pH-sensitive riboflavin controlled-release alkaline hydrogels based on blue crab chitosan: Study of the effect of polymer characteristics

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4 1 **Bioinspired pH-sensitive Riboflavin controlled-release alkaline**
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6 2 **hydrogels based on blue crab chitosan: Study of the effect of wall**
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9 3 **polymer characteristics.**

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62 14 **Abstract**

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65 15 Recently, the application of natural biocompatible polymeric hydrogels for the
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67 16 conception of drug delivery matrices has attracted widespread interest. Thus, in the present
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69 17 study, riboflavin pH-sensitive drug delivery hydrogels were developed based on blue crab
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71 18 chitosan (Cs), via direct dissolution in alkali/urea aqueous solution at low temperatures. First,
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73 19 the effect of Cs characteristics in terms of acetylation degree (AD) and molecular weight (Mw)
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75
76 20 on the structural, mechanical, thermal, swelling and *in vitro* biodegradation of Cs-based
77
78 21 hydrogels were studied. Data from overall analysis revealed that Cs with low AD and high Mw
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80 22 exhibited improved mechanical properties, as evidenced by the compressive and rheological
81
82 23 behaviors tests, thermal resistance, swelling behavior and *in vitro* degradation kinetics.
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84 24 However, hydrogels pore sizes were reduced with the AD decrease and Mw increase.
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86 25 Additionally, hydrogels in PBS (pH 5.5) underwent quicker degradation, compared to those
87
88 26 immersed in PBS (pH 7.4). In the drug delivery model, the kinetics of Riboflavin release,
89
90 27 through the Cs-based hydrogels were monitored. The Riboflavin release exhibited a typical tri-
91
92
93 28 phasic deliverance pattern, with significantly higher released amounts in more acidic systems.
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95 29 Therefore, drug encapsulation within the conceived pH-sensitive Cs-based hydrogels could
96
97 30 provide suitable and promoting microenvironment for drugs delivery.

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105 33 **Keywords:** Hydrogels; Acetylation degree and Molecular weight; Drug controlled-
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107 34 release.

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121 **37 1. Introduction**

122
123 38 Hydrogels are three-dimensional hydrophilic polymeric networks with the ability to
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125 39 absorb large amounts of water or biological fluids [1-2]. Because of their high-water content,
126
127 40 porosity and soft consistency, they closely simulate natural living tissues, more than any other
128
129 41 class of synthetic biomaterials and thus open up many possibilities for applications in
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131 42 biomedical fields [3-5]. Physical hydrogels are distinguished from chemical hydrogels. The
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133 43 network of physical hydrogels is maintained through weak bonds (hydrophobic, hydrogen,
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135 44 ionic) that are not permanent because they are disconnected continuously depending on the
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137 45 medium (pH, temperature, ionic strength). The chemical hydrogels, however, have a network
138
139 46 which is maintained by covalent crosslinks providing them a permanent character [6-7].

140
141
142 47 The high porosity that characterizes hydrogels can easily be adjusted by controlling the
143
144 48 density of the crosslinks in their matrix and their affinity to water. Moreover, their porous
145
146 49 structure allows to controllably loaded and released drugs [8-9]. The benefits of hydrogels for
147
148 50 drug delivery applications include the possibility of controlled and sustained release, which
149
150 51 permits a high local concentration of an active pharmaceutical ingredient (drug) to be
151
152 52 maintained over a long period of time. The drug can be loaded into a hydrogel, and then released
153
154 53 by several mechanisms: controlled release, controlled swelling, chemically controlled release
155
156 54 and environmental release [10].

157
158
159 55 The rate of release can be managed by modifying some factors such as polymer
160
161 56 concentration, crosslinking density, and water content. Some «smart» hydrogels have the ability
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163 57 to respond to external stimuli such as pH, temperature, ionic strength, etc., making them
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165 58 excellent site-specific active ingredient in delivery matrices for diseases prevention and
166
167 59 treatment [11-12].

170
171 60 Hydrogels are of great interest for other biomedical applications because of the ability to
172
173 61 control their swelling, mechanical properties, chemical and physical structures, crosslinking

178
179
180 62 density and porosity. Therefore, hydrogels are frequently used in tissue engineering for cell
181
182 63 encapsulation or drug delivery, but as well as wound dressings, bioadhesives and biosensors
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185 64 [13-14]. In fact, hydrogels can serve as templates for directing cell behavior and promoting cell
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187 65 organization. In addition, the biocompatibility of hydrogels has generated a lot of interest in
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189 66 hygiene products, implants and soft contact lenses [15-16].

190
191 67 Advantageously, chitosan can be used in the preparation of hydrogels which serve as a
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193 68 matrix for the incorporation of active agents [17-19]. As part of this research, chitosan obtained
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195 69 by partial deacetylation of chitin was chosen. Chitosan-based hydrogels have shown important
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197 70 advantages in terms of drug delivery, as they allow site-specific and / or time-controlled
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199 71 administration for small and large drugs [17,20]. They offer, furthermore, many benefits, such
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201
202 72 as improving biosecurity and drug efficacy. Chitosan hydrogels can provide targeted delivery
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204 73 and improved stability of therapeutic agents against physiological degradation [17].

205
206 74 To the best of our knowledge, there is a lack of information in literature regarding the
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208 75 effect of acetylation degree and molecular weight on chitosan-based hydrogels, although
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210 76 several reports describe their developpement and application in particularly the biomedical
211
212 77 field. Therefore, the objective of this work was the conception of high strength hydrogels based
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214 78 on chitosans with different acetylation degrees and molecular weights, to assess the effects of
215
216 79 these two structural parameters on the properties of the resulting hydrogels. Subsequently, the
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218
219 80 selected hydrogel was applied for controlled release of Riboflavin with very interesting
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221 81 biological potential, as drug model.

222 223 82 **2. Materials and methods**

224 225 226 83 **2.1. Materials**

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228 84 Riboflavin was purchased from LOBA CHEMIE (India) and the other used chemical
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230 85 reagents from commercial sources were of analytical grade and employed without further
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232 86 purifications.
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2.2. Chitosans preparation and purification

Chitosans (Cs) from blue crab *Portunus segnis* shells were prepared in our laboratory, as described in our previous study [21]. Briefly, Cs with different AD were obtained through chitin N-deacetylation with NaOH 12.5 M at a w/v ratio of 1/10 at 140 °C, for 2, 3 and 5 h and produced Cs were named Cs I, Cs II and Cs III. After filtration, Cs was washed to neutrality and then dried for 12 h at 50 °C. Based on the nuclear magnetic resonance (¹³C NMR) analysis, ADs of 17%, 13% and 8% were reached for Cs I, Cs II and Cs III, respectively. Further, Cs were characterized by size exclusion chromatography (SEC-HPLC) and average molecular weights (Mw) of 125 600, 118 900 and 115 000 g mol⁻¹ were obtained for CsI, CsII and CsIII, respectively.

To generate Cs with different Mw, Cs, at different ADs, were hydrolyzed with Cellulase (10 U/g chitosan) in 0.5 N acetate-bicarbonate buffer (pH 5.2) at 55 °C, for 1 and 3 h, as described by Chang *et al.* [22] with slide modifications. The Cs obtained are lyophilized and analyzed to study the evolution of their molecular mass. The respective Mw were reported in

Table S1.

Subsequently, Cs were purified according to the method described by Qian and Glanville [23]. Thus, crude Cs (6 g) was dissolved in 600 ml of HCl 0.1 M under stirring overnight at a temperature of 40 °C. The acidic solution was vacuum filtered to remove insoluble particles. Cs was then precipitated with NaOH 0.5 M under continuous stirring until approximately pH 8.5. Thereafter, 6 ml of 10% (w/v) sodium dodecyl sulfate (SDS) was added to the suspension and the mixture was heated at 95 °C for 5 min. After cooling at room temperature, the pH was adjusted to 10.0 with 0.5 M NaOH. The mixture was vacuum filtered and the hydrated Cs was washed 5 times with 600 ml of deionized water at 40 °C. A solution of barium chloride was used to confirm the absence of residual SDS in the filtrate. Finally, the obtained purified Cs were lyophilized, milled to powder and then sieved.

2.3. Conception of blue crab chitosan-based hydrogels

Hydrogels were prepared based on the freezing/thawing approach described by Duan *et al.* [5]. Briefly, Cs, derived from the action of Cellulase, were dissolved in an alkaline solution, widely used for the dissolution of cellulose and chitin, consisting of 4.5 wt. % LiOH, 7.5 wt. % KOH and 8.5 wt. % urea. Then, the reaction mixtures were maintained at -30 °C until complete freezing, followed by a thawing step at 20 °C under vigorous agitation, until a clear and transparent solution of Cs was obtained. After removal of air bubbles by centrifugation at 5000 ×g for 15 min at 4 °C, the prepared solutions were maintained at 60 °C for 1 h (solvent evaporation technique), promoting the formation of Cs physical gels. After exhaustive washing with Milli-Q water, to remove the residual alkali/urea solution, prepared hydrogels were immersed in an ethanol solution (100%) for 3 days to improve the resistance of the gels [24].

Foremost, Cs-based hydrogels with different AD and Mw were prepared at a concentration of 3% (w/v) [24], to study the effect of these two parameters on the structural, mechanical and rheological features of elaborated hydrogels. The corresponding code to each hydrogel was recorded in **Table S2**. Subsequently, an optimization of the ideal concentration for the formation of Cs-based hydrogels was performed. Accordingly, different concentrations were used, namely 1%, 2%, 3%, 4% and 5% of Cs, and the obtained hydrogels were characterized.

2.4. Blue crab chitosan rheological behavior in the alkali/urea aqueous solution

To study the stability of the Cs in alkali/urea system, hydrogels (15 mm of diameter × 1 mm of thickness) rheological and gelation behaviors were investigated with dynamic viscoelastic measurements. For all the experiments, a rheometer apparatus (Physica MCR, Anton Paar, GmbH, France) equipped with a plate-plate measuring geometry (25 mm diameter, 0.1 mm gap) was used. Oscillatory measurements of the storage modulus (G') and loss modulus (G'') were carried out under a strain sweep from 0.1% to 1000% at 37 °C with a frequency of 1

355
356
357 137 Hz. Thermo-viscoelasticity properties in a ramp temperature from 20 to 80 °C was investigated,
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359 138 under constant frequency (1 Hz) and strain (1%), at a heating rate of 2 °C/min. A solvent trap
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361
362 139 was applied to prevent water evaporation when heating. The data were analyzed with Rheoplus
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364 140 software from Anton Paar.

366 141 **2.5. Analytical methods**

368 142 **2.5.1. Hydrogels microstructure**

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370
371 143 The cross-section of Cs-based hydrogels was studied using scanning electron microscopy
372
373 144 SEM (Hitachi S4800), at an angle of 90° to the surface, at different magnifications. Prior to
374
375 145 imaging their cross-section, hydrogel samples were lyophilized, sectioned and fixed on the
376
377 146 SEM support using double side adhesive tape, and observed up to a 2000 x magnification, under
378
379 147 an accelerating voltage of 2.0 kV and an absolute pressure of 60 Pa, after being sputter coated
380
381 148 with a 5 nm thick gold.

384 149 **2.5.2. Moisture content of Cs-based hydrogels**

385
386 150 The water content was determined according to the methods described by AOAC (2000)
387
388 151 [25]. The water content of the elaborated hydrogels was measured, in triplicate, by drying about
389
390 152 100 mg of each sample in an oven at 105 °C until the dry weight of the sample was reached
391
392
393 153 (constant weight). Weights before and after drying were measured. The moisture content of
394
395 154 hydrogels was determined by measuring the mass loss of each film in triplicate and expressed
396
397 155 as follows:

$$400 \quad 156 \quad MC (\%) = \frac{W_0 - W_1}{W_0} \times 100$$

403 157 where W_0 and W_1 are the respective masses (g) of hydrogels before and after drying at 105 °C.

405 158 **2.5.3. Swelling rate of hydrogels**

406
407 159 The swelling test was performed on pieces of hydrogels with masses of 20-30 mg. The
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410 160 samples were immersed in phosphate-buffered saline (PBS) at 37 °C and after 24 h of

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416 161 incubation, the samples were removed, oven-dried and the masses were measured again [26].

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418 162 The swelling rate (SR), repeated three times, was calculated as follows:

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$$SR (\%) = \frac{M_s - M_d}{M_d} \times 100$$

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423
424 164 where SR is the swelling rate (%), M_d is the mass (g) of the oven-dried hydrogel and M_s is the
425
426 165 mass (g) of the swollen hydrogel.

427 428 429 166 **2.5.4. Infrared spectroscopy analyses**

430
431 167 The prepared Cs-based hydrogels FT-IR analysis was performed by means of a
432
433 168 spectrometer (Agilent Technologies, Carry630 series) with an attenuated reflection accessory
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435 169 (ATR) containing a diamond/ZnSe crystal, at room temperature (25 °C). Spectra were recorded
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437
438 170 in the spectral range frequencies of 650-4000 cm^{-1} , with 32 scans of interferograms and a
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440 171 resolution of 4 cm^{-1} . Prior to analysis, FT-IR spectrometer was calibrated via a background
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442 172 spectrum recorded from the clean and empty diamond for each spectrum. Data analysis and
443
444 173 treatment were carried out using the OMNIC Spectra software (ThermoFisher Scientific).

445 446 447 174 **2.5.5. X-ray diffraction studies**

448
449 175 To further investigate the structural characteristics of the prepared hydrogels, XRD
450
451 176 patterns were recorded using an X-ray diffractometer (D8, Advance Bruker XRD
452
453 177 diffractometer, Germany). Ni-filtered Cu $K\alpha$ radiation ($k = 1.5406 \text{ \AA}$) was used to record the
454
455 178 X-ray patterns. The relative intensity was recorded in the scattering range 2θ of 5–50° with a
456
457 179 step size of 0.02° and a counting time of 5 s/step, with an error of $\pm 1^\circ$.

458 459 460 180 **2.5.6. Thermal properties of blue crab chitosan-based hydrogels**

461
462 181 Thermogravimetric analysis (TGA Q500 High Resolution, TA Instruments), operating
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464 182 under nitrogen flow, was used to study the thermal stability of Cs-based hydrogels. The mass
465
466 183 change of a sample as a function of temperature augmentation is the basis of TGA, and the
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468
469 184 progressive change in mass (%) as function of temperature, is recorded. Cs-based hydrogels,

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474
475 185 initially about 4 mg, were heated from 25 to 700 °C at a heating rate of 20 °C/min and constantly
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477 186 measured with an accuracy of 0.01 mg. Cs-based hydrogel thermograms were subsequently
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479
480 187 analyzed using TA Universal V4.5A software.

481 482 188 **2.5.7. Evaluation of hydrogels mechanical properties**

483
484 189 Hydrogel compression tests were carried out using the DMA50 (Dynamic Mechanical
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486
487 190 Analyzer) universal testing machine (Metravib, Brand of ACOEM, France) at a temperature of
488
489 191 25 °C and a compression speed of 1 mm/min. Samples were compressed at 10%, 20%, 30%,
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491 192 40%, 50% and 60%, and then reverted at the same speed of 1 mm/min, to obtain the stress-
492
493 193 strain curves for gels' compression-recovery. The dimensions of the hydrogel specimens
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495 194 (parallelepiped) for compression tests were 10 mm × 5 mm × 5 mm (based on the apparatus
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497 195 requirements). The stress-strain curve hysteresis was recorded and treated by the instrument
498
499 196 software.

500 501 502 197 **2.5.8. Hydrogels *in vitro* degradation test**

503
504 198 Cs-based hydrogels *in vitro* biodegradation study was monitored through the gravimetric
505
506 199 method described by Qu *et al.* [27]. Briefly, hydrogels (approximately 100 mg) were
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508 200 immersed in 10 ml of phosphate buffer saline (PBS) at pH 7.4 (physiological microenvironment
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510
511 201 simulation) and pH 5.5 (acidic microenvironment), at 37 °C and under gentle shaking
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513 202 (approximately 100 rpm). Thereafter, hydrogel samples were removed, at each desired
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515 203 interval time, washed with Milli-Q water to remove the excess of salinity, oven-dried for 48 h
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517 204 at 60 °C and then weighed. The remaining weight of hydrogels (%) was calculated based on the
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519 205 following equation:

$$520$$
$$521$$
$$522$$
$$523 \text{ 206 } \text{Remaining weight ratio (\%)} = \frac{M_t}{M_i} \times 100$$
$$524$$

525 207 where M_t is the remaining hydrogels dry weight after degradation at each selected time interval
526
527 208 and M_i is the initial hydrogels dry weight.

2.6. *In vitro* riboflavin loading and release kinetics

The amount of riboflavin incorporated in hydrogels was studied. Briefly, wet Cs-based hydrogel (30 mg) were suspended in 10 ml of riboflavin solution (1-5 g/l) in dark at 5 °C for 48 h. The riboflavin entrapment efficiency and loading capacity by the hydrogels were determined considering the derivative thermogravimetric (DTG) thermograms [28], by subtracting the amount of riboflavin in the supernatant from the total amount applied [24]:

$$\text{Riboflavin loading capacity (\%)} = \frac{\text{Mass of loaded riboflavin}}{\text{Mass of hydrogel samples}} \times 100$$

$$\text{Riboflavin entrapment efficiency (\%)} = \frac{\text{Mass of loaded riboflavin}}{\text{Mass of initial riboflavin}} \times 100$$

Regarding the riboflavin release studies, loaded hydrogel samples (30 mg) were subsequently incubated in 10 ml of aqueous HCl and NaCl (0.1 M) with different pH values (pH 2.0, 4.5 and 7.4) at 37 °C, with stirring. At each time interval, an aliquot of the supernatant (2.5 ml) was withdrawn and replaced by fresh medium at the same volume. The amount of released riboflavin was determined spectrophotometrically, considering the cumulative amount of riboflavin in each of the release system. The amount of riboflavin was estimated using a UV-visible spectrometer (Agilent Technologies, Carry 630 series) at 450 nm on the basis of a riboflavin calibration curve (**Data not shown**). All studies were performed in duplicate and the average values were reported.

2.7. Statistical analysis

Statistical analyses were performed with SPSS ver. 17.0, professional edition (SPSS, Inc., Chicago, IL, USA) using ANOVA analysis at a p-value < 0.05. A standard deviation at the 95% confidence level was used to compare all parameters analyzed for the different hydrogels. All assessments were repeated three times and average values with standard deviation errors were reported.

3. Results and Discussion

3.1. Microstructure analysis of Cs-based hydrogels

Since understanding biomaterials functional properties is based on their structure knowledge, the examination of Cs-based hydrogels microstructure, reflecting polymer and molecules interactions, is required [29].

SEM images showing the pore microstructure (cross section) of Cs-based hydrogels, with different AD and Mw, are displayed in **Fig. 1**. The pore size of the prepared hydrogels changed in the range of 1 ~ 6 μm and became bigger and bigger as the Cs AD increased, with more compact distribution. For example, pore size values of ~ 1 μm for CsIII-0 based hydrogel (**Fig. 1G**), 2 μm for CsII-0 based hydrogel (**Fig. 1D**) and 3 μm for CsI-0 based hydrogel (**Fig. 1A**) were reached, suggesting that lower AD allowed the preparation of a more well-organized network structure, which could contribute to mechanical support [30].

It was found likewise that the hydrogel pore size had the tendency to decrease with the increase of Cs Mw. Indeed, the pore size (approximately 4 μm ; **Fig. 1I**) of CsIII-3 based hydrogel, showing a microstructure, filled with larger interconnected pores, was about twice of that for CsIII-1 based hydrogel (approximately 2 μm ; **Fig. 1H**), which could lead to modulations in Cs-based hydrogels swelling and drug release behaviors [17].

In another aspect, overall Cs-based hydrogels, regardless Cs AD and Mw, as shown in **Fig. 1**, the formed hydrogels network revealed a uniformly distributed porous three-dimensional architecture, with, to variable extend, a roughness matrix surface. The pores interconnected in a recurrent style inside the hydrogels network affords a suitable medium flow and drug transport channels, being therefore appropriate for drug delivery [26,31].

3.2. Hydrogels moisture content determination

Moisture content (MC) of Cs-based hydrogels, reported in **Table 1**, revealed that values decreased with the decrease of the AD, reaching 82%, 81% and 79%, for CsI-0 (AD=17%),

650
651
652 257 CsII-0 (AD=13%) and CsIII-0 (AD=8%) based hydrogels, respectively ($p<0.05$). however,
653
654 258 considering Cs Mw, MC values were found to be strengthened with the decrease of Cs Mw.
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656 259 Indeed, regarding an AD of 8% (CsIII), hydrogels MC rates of 79.90%, 80.26% and 81.83%
657
658 260 were reached with respective Mws of 115 kDa (CsIII-0), 78.43 kDa (CsIII-1) and 16.04 kDa
659
660 261 (CsIII-3), probably due a decrease of crosslinking density [5]. In fact, hydrogels are systems
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662 262 known for their remarkable water-holding capacity during their preparation, and the water
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664 263 content is one of the most features that distinguishes hydrogels from other biomaterials. Their
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666 264 water-rich structure facilitates, indeed, the transport of nutrients and molecules between the
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668 265 external environment and the hydrogel, which allows to mimic the function of cells in the body
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670 266 [9].
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674 267 Consequently, it could be proposed that the increase of CS MW, besides the decrease of
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676 268 its AD, as well as Cs-based hydrogels soaking in ethanol solution (100%), allowed the decrease
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678 269 in Cs-based hydrogels water content, and thereby, reducing pore sizes [24], which was
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680 270 consistent with SEM data. This finding seems to be beneficial for Cs-based hydrogels
681
682 271 mechanical properties enhancement.
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685 272 **3.3. Evaluation of Cs-based hydrogels swelling properties**

687 273 As an absorbent matrix, the degree of swelling of a hydrogel is a key parameter that is
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689 274 closely related to the ability of hydrogels to release active ingredients [32]. Swelling is defined
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691 275 as a continuous transition process from the solvent-free glassy state or partially rubbery state to
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693 276 a relaxed rubbery solvent containing state. The solvent infiltration and the elastic contraction
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695 277 from the network strain, as two opposite forces, create a skirmish in the swelling process, which
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697 278 reaches the equilibrium, when they reach a dynamic balance [33]. In this context, the effect of
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699 279 Cs AD and Mw on Cs-based hydrogels swelling ratio (SR) was studied and results are recorded
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701 280 in **Table 1**.
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710
711 281 Results display that Cs-based hydrogels water absorption capacity was found to be
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713 282 dependent to the Cs AD and Mw. Indeed, SR values increased with the decrease of Cs AD as
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715 well as the decrease in its Mw. For example, hydrogels developed with CsI (CsI-0; AD=17%)
716 283 exhibited SR value of 13.59 ± 0.45 g/g, compared to CsIII-0 based hydrogel, prepared with lower
717
718 284 AD Cs (CsIII-0; AD=8%), with SR exceeding 18 g/g (**Table 1**). Moreover, regarding CsIII-
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720 285 based hydrogel, respective SR values of more than 18 g/g, 22 g/g and 26 g/g were reached with
721
722 286 hydrogels prepared with GCsIII-0 (115 kDa), GCsIII-1 (78.43 kDa) and CsIII-3 (16.04 kDa).
723
724 287
725

726 288 It is well known that the swelling properties of hydrogels depend on the hydrophilic
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728 289 nature of polymeric chains and the nature of bonds inside the matrix structure. Thus, it is
729
730 290 possible to deduce that the decrease of Cs AD, and thereby, the increase of the $-NH_2$ groups
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732 291 number, allowed the improvement of the hydrophilicity of the elaborated hydrogels, favoring
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734 292 their interaction with water molecules [34,35]. In addition to the polarity of the hydrogels, the
735
736 293 degree of crosslinking and hydrogels porosity were found to well correlate with the ability of
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738 294 Cs-based hydrogels to absorb water [12,27].
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742 295 **3.4. Hydrogels spectroscopic characterizations**

743 744 296 **3.4.1. FT-IR analysis**

745
746 297 The FT-IR spectra of Cs-based hydrogels were shown in **Fig. 2**. Compared to the polymer
747
748 298 powder [21], characteristic Cs absorption bands at 3417 cm^{-1} , 1627 cm^{-1} , 1544 cm^{-1} , 1407 cm^{-1}
749
750 299 and $1020\text{--}1097\text{ cm}^{-1}$ linked to the $-OH$, amide I groups ($-C=O$), amide II ($-NH_2$), $-CH$ and
751
752 300 glycoside rings, respectively, were noted to be rearranged. Indeed, the $N-H$ peak in the FT-IR
753
754 301 spectra of the Cs-based hydrogels was found to be shifted significantly to higher wavenumbers
755
756 302 overlapping with the peak of the $O-H$ stretching vibrations. The peak at $3500\text{--}3200\text{ cm}^{-1}$
757
758 303 straitened, and revealed the tendency to break into several small peaks, demonstrating the
759
760 304 weakening of the inter- and intra-molecular hydrogen bonds and the occurrence of some
761
762 305 reactions on the two groups [34-39].
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769
770 306 The peak of the amide I group weakened remarkably and almost disappeared, indicating
771
772 307 that concentrated alkali has reacted with the acetyl amino group of Cs [38,39]. Moreover, new
773
774 308 stretch vibration absorption bonds appeared at about 3264 cm^{-1} , 2500 cm^{-1} and 785 cm^{-1} ,
775
776 309 indicating that the alkali/urea aqueous solvent affected the structure of Cs to some extent.
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779 310 Indeed, active hydroxyl group of Cs reacted with the concentrated alkali, leading thereby to the
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781 311 destruction of the native hydrogen bonds of Cs effectively and making Cs highly swell or even
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783 312 dissolve in the alkali solution.
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785 313 These findings confirmed further during the dissolution process, alkali not only reacts
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787 314 with the hydroxyl group, but also with the acetyl amino group of Cs, leading to the weakening
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789 315 of the amide I peak ascribed to acetyl amino group [40,41]. Moreover, The FT-IR spectra of all
790
791 316 Cs-based hydrogels are quite similar, regardless Cs AD and Mw, accounting for the stability of
792
793 317 Cs in the alkali/urea aqueous solution system [24, 42,43].
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795

796 318 **3.4.2. XRD patterns study**

799 319 To further clarify the structural changes in the Cs matrix, during dissolution in the
800
801 320 alkali/urea aqueous system and gelling, and regarding the effects of Cs Mw and AD, XRD
802
803 321 patterns of Cs-based hydrogels were studied and compared to the polymer powder profiles (**Fig.**
804
805 322 **3**). X-diffractograms of Cs-based hydrogels revealed marked differences in the molecular state.
806
807 323 Indeed, diffraction peaks nearby 13.1° and 21.3° , attributed to (020) and (110) planes of Cs,
808
809 324 respectively, were detected, reflecting the semi-crystalline structure of Cs [21,44]. The major
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811 325 peaks at $2\theta = 37.7^\circ$, 34.32° , 32.4° and 28.9° observed in the X-diffractograms could be
812
813 326 attributed to the alkali (LiOH) used for the dissolution of Cs [45].
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816 327 As shown in **Fig. 3**, the Cs-based hydrogels displayed the characteristic diffraction
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818 328 patterns of both Cs and alkali at the same time. However, the crystallinity of the physical Cs-
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820 329 based hydrogels clearly diminished in comparison with that of Cs powder, where the above
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822 330 mentioned initial characteristic peaks became broader and weaker, depending on Cs AD and
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829 331 Mw. In fact, convenient with the SEM images, the crystallinity of Cs-based hydrogels decreased
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831 332 with the increase of Cs AD (CsI-0 based hydrogel) and the decrease in its Mw (Cs-1 and Cs-3
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833 based hydrogels), suggesting a transition from a crystalline structure to an amorphous state
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835 during the dissolution and the gelling process [46,47]. This result strongly confirmed that Cs
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837 solubility was related to its crystallinity.
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840 336 With the aid of alkali solution and the freezing to -30 °C, water molecules diffuse in the
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842 337 Cs macromolecular chain. The subsequent thawing and stirring steps during hydrogels
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844 338 preparation process are beneficial for the dissolution of Cs, as the intra- and inter-molecular
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846 339 hydrogen bonds of Cs would be broken during dissolution, leading to the loss of crystallinity
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848 340 [48,49].
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850

851 341 The dissolution of Cs in the alkali aqueous system begins at the amorphous side with a
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853 342 loose structure at first, and afterwards reaches the crystal zone with a rather thicker construction
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855 343 and low temperature. This exhibits a crucial role in the Cs crystalline configuration destruction
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857 344 [26,50]. Meanwhile, overall findings proposed that the chemical structure of Cs was relatively
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859 345 stable in the alkali/urea aqueous solution [5,24].
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862 346 **3.5. Cs AD and Mw affected based hydrogels thermal properties**

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864 347 Thermal stability/degradation behavior of Cs-based hydrogels, with respect to their AD
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866 348 and Mw, was studied, and results in terms of TGA and derivate (DTG) thermograms, are shown
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868 349 in **Supplementary data Fig. S1**. The thermal decomposition data in terms of the corresponding
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870 350 degradation temperatures, the weight loss (Δw) and the residue (**R**), were estimated (**Table 2**).
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872 351 Based on data from the obtained TGA thermograms, the thermal decomposition profiles
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874 of the overall Cs-based hydrogels exhibited a similar weight loss process in the temperature
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876 range of 20–800 °C, indicating the polymer pyrolysis, and characterized by three major phases
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878 (**Supplementary data Fig. S1**), typical fingerprint of Cs thermal decomposition [32].
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888 355 The first phase corresponded to a weight loss of 6% (CsIII-0 based hydrogel) to 19%
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890 356 (CsI-3 based hydrogel), apparently resulted from evaporation of adsorbed water by Cs at a
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893 357 temperature (Td1) range from 28-46 °C to 129-158 °C, reaching its maximum mainly below
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895 358 130 °C, expect for the CsIII-0 based hydrogel (133.54 °C). The different content of bound and
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897 359 unbound water in the hydrogels could explain the observed difference in onset temperature [51].
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899 360 Indeed, in line with data from the SEM analysis and results above mentioned, CsIII-0 based
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901 361 hydrogel exhibited higher onset temperature as a result of more strongly bound water, related
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903 362 to a more homogeneous network structure. However, less uniform network with macro-phase
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905 363 separation like structure, observed in hydrogels based on CsI-3, was found to responsible of
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907 364 more unbound water in the based hydrogel structure [28,48].
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910 365 Considering the DTG curves, the temperature, at which the decomposition process was
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912 366 the shrillest, was revealed by the weight loss peak (**Supplementary data Fig. S1**). In the range
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914 367 the third phase of Cs-based hydrogels pyrolysis process (200-550 °C), where higher ΔW were
915
916 368 reached, peaks located at temperatures of 268.81, 266.09 and 261.55 °C, were found for the
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918 369 CsI-0, CsI-1 and CsI-3 based hydrogels, respectively, whereas those found at 279.71, 274.26
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920 370 and 273.35 °C were related to the CsIII-0, CsIII-1 and CsIII-3 based hydrogels, respectively
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922 371 (**Table 2**). Thus, polymers pyrolysis temperature differences are mainly assigned to the
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924 372 macromolecular interaction, crystallinity index, or orientation. The TGA results proved that
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926 373 CsIII-0 based hydrogel was reorganized in more well-ordered network structure, during the
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928 374 dissolution in the alkali/urea system at low temperatures and the regeneration process in
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930 375 ethanol, ensuing a rather high crystallinity and more homogeneous architecture. Finally,
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932 376 residual decomposition reactions leading to the total degradation of the Cs ring in the hydrogels
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934 377 was found to be around 550 °C [52].
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937 378 Regarding the residual mass (R) of the prepared hydrogels, values were found to decrease
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939 379 with the decrease of the Cs Mw and the increase of its AD, with values of 33.70% (CsI-0 based
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947 380 hydrogel), 30.59% (CsI-1 based hydrogel) and 29.88% (CsI-3 based hydrogel). Considering
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949 381 CsII-based hydrogels, R values of 34.34%, 33.05% and 29.58% were reached for the CsII-0,
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951 382 CsII-1 and CsII-3 based hydrogels, respectively. R values of 47.69%, 40.53% and 38.68% were
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953
954 383 noted for CsIII-0, CsIII-1 and CsIII-3 based hydrogels, respectively (**Table 2**). Accordingly, it
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956 384 could be concluded that the thermal stability of the Cs-based hydrogels is positively correlated with
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958 385 its AD and disproportionate to its Mw. Moreover, as discussed earlier, TGA findings confirmed
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960 386 that the increased stability of the Cs-based hydrogels was due to increasing macromolecular
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962 387 chains crosslinking [28,32].

963 964 965 388 **3.6. Hydrogels mechanical properties as affected by Cs structural parameters**

966 967 389 **3.6.1. Rheological behavior**

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969 390 The rheological properties of Cs-based hydrogels, storage modulus (G') and loss modulus
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971 391 (G''), were shown as a function of strain at 37 °C (**Fig. 4A-C**). Independently of Cs AD or Mw,
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973 392 Cs-based hydrogels displayed higher elastic behavior ($G' > G''$) than the viscous behavior
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975 393 ($G' < G''$), suggesting a distinctive feature of a strong hydrogel.

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978 394 Since the elasticity of the sample, defined as the stored energy due to the elastic
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980 395 deformation, is reflected by the storage modulus G' , the higher the G' value is, the tougher
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982 396 against distortion the hydrogel is [33]. **Fig. 4A-C** shows that for all the Cs-based hydrogel
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984 397 samples, the moduli (G' and G'') fluctuated slightly with deformation in the test strain range of
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986 398 500%. Comparing the hydrogel samples, for CsIII-0 based hydrogel, the G' (more than 130
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988 399 kPa) was found to be significantly higher than that of CsII-0 (more than 82 kPa) and CsI-0
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990 400 (more than 35 kPa) based hydrogels. Additionally, for CsIII-1 (G' about 30 kPa) and CsIII-3
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992 401 (G' more than 17 kPa) based hydrogels, the G' values were significantly lower than that of
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994 402 CsIII-0 based hydrogel ($p < 0.05$). The same tendency was detected with the other hydrogels
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996 403 based on CsII (AD of 13%) and GCsI (AD of 17%).
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1006 404 Before hydrolysis, G' of Cs-based hydrogels, independently of Cs AD, was four times
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1008 405 more than the value of Cs-1 and Cs-3 based hydrogels, thus, the creation of a more stable
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1010 406 network, leading to a greater maximum storage modulus G' .

1012 407 This finding could be mainly assigned to the stronger interactions of Cs macromolecular
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1014 408 chains, with high average molecular weight, than medium and lower average molecular weights
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1016 409 Cs (Cs-1 and Cs-3, respectively). The formation of covalent bonds between the Cs and the
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1018 410 alkali/urea system, allowed thereby remarkably the enlargement of the strength of hydrogels
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1020 411 and the enhancement of their mechanical properties [5,27,33].

1023 412 Data of gradually decreasing storage modulus G' of the hydrogels, with the decrease of
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1025 413 Cs AD and the increase of the Mw, were consistent with the gradually increasing SR results
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1027 414 above described (**Table 1**), further, principally ascribed to the progressively hydrogels
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1029 415 crosslinking density diminishing [24,53].

1031 416 As displayed in **Fig. 4A-C**, it is likewise found that an increase in the deformation, more
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1033 417 than 500%, was associated with loss/viscous modulus G'' enlargement and storage/elastic
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1035 418 modulus G' decline, as the network movement increased [54], demonstrating that at higher
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1037 419 strains elastic flow of the gel network decreased. However, unlike the G' profile, the gel-sol
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1039 420 transition strain value was found not significantly influenced with the Cs AD or Mw.

1041 421 In another aspect, there was found no remarkable difference of Cs-based hydrogels
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1043 422 rheological properties as function of the temperature (**Supplementary data Fig. S2**). Indeed,
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1045 423 interestingly, as the temperature increased from 10 to 60 °C, there was no change of G' and G''
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1047 424 of the hydrogel samples. Therefore, physical Cs-based hydrogels, based on the dissolution in
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1049 425 the alkali/urea aqueous system at low temperatures, exhibited stable rheological properties
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1051 426 against temperature, regardless the Cs AD or Mw.

1055 427 Such rheological behaviors of Cs-based hydrogels, with interesting elastic and solid
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1057 428 characteristics, showed the attendance of a promising material for tissue engineering application
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1065 429 [55]. Moreover, rapid drug loss prevention and better sustained release properties could be
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1067 430 potentially provided by high gel strength and short gelation time of the hydrogel, as obtained
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1069 431 with the Cs-based hydrogels [56].
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1072 **432 3.6.2. Compressive study**

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1074 433 Considering the bone tissue engineering, the mechanical properties of biomaterials like
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1076 434 hydrogels are design features of priority [57,58]. To have additional insight into the mechanical
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1079 435 behavior of Cs-based hydrogels, compressive properties were evaluated by exposing wet gels
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1081 436 to compression testing and the stress vs. strain compressive curves, with different Cs ADs and
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1083 437 Mws, are shown in **Fig. 4D-F**. Mechanical experimental outcomes display that under
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1085 438 compression, the compressive strength of the Cs-based hydrogel samples increased with the
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1087 439 increase in compressive strain for all hydrogel formulations. More particularly, the compressive
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1089 440 stiffness of Cs-based hydrogels increased concomitantly with the increase of the applied
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1091 441 compressive strain in the range of 5-7% deformation, and then stuck to rise but in a slower
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1093 442 manner, indicating that the Cs-based hydrogels were sturdy and ductile.

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1096 443 The order of the mechanical properties was found as: CsIII>CsII>CsI. Indeed, when the
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1098 444 Cs AD increased from 8% (CsIII based hydrogel) to 17% (CsI based hydrogel), the fracture
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1100 445 stress values of the Cs-based hydrogels decreased to 0.35 MPa for CsI-0 based hydrogel, while
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1102 446 values of 0.49 MPa and 0.71 MPa were reached with CsII-0 and CsIII-0 based hydrogels,
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1104 447 respectively ($p<0.05$). CsIII-3 based hydrogel was found to endure 40% deformation, whereas
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1106 448 CsII-3 and CsI-3 based hydrogels were capable to bear only 35% and 30% deformation,
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1108 449 respectively, because of the decrease in the physical crosslink density of the Cs network (**Fig.**
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1110 450 **4D-F**), suggesting a relatively high strength for CsIII based hydrogel, with the highest
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1112 451 macromolecular interactions, the most stable network and the smallest pore size [39,59].

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1115 452 In another side, the increase in Cs Mw was found to result in a significant enhancement
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1117 453 in ultimate compressive stress and strain of fracture ($p<0.05$). In fact, hydrogels developed
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1124 454 based on low Mw Cs (CsIII-3, CsII-3 and CsI-3) exhibited mechanical stiffness of more than
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1126 455 0.29 and 0.27 and 0.11 MPa, respectively. However, ultimate stress modulus increased by 2
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1128 456 and more than 3-folds for medium (CsIII-2, CsII-2 and CsI-2) and high (CsIII-0, CsII-0 and
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1131 457 CsI-0) Mw Cs, respectively (**Fig. 4D-F**). Interestingly, with ADs less than 13% (CsII and CsIII),
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1133 458 even with medium Mw, based hydrogels kept the structural integrity and stability without
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1135 459 evidences of any sign of fracture and could recover by themselves subsequently to external
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1137 460 pressure removal. This finding strongly demonstrated that the synthesized hydrogel exhibited
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1139 461 interesting mechanical properties, thanks to stiffer chains resulting in stronger pore wall,
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1141 462 allowing gels thereby to undergo more intense external forces and conditions [33,51,57].
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1143 463 These mechanical data were further consistent with the crystallinity results (**Fig. 3**) and
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1145 464 the microstructure of the Cs-based hydrogels (**Fig. 1**).
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1148 465 **3.7. Hydrogels *in vitro* degradation behavior**

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1150 466 The degradation behavior, based on the weight loss system, was monitored in PBS at pH
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1152 467 values of 7.4 and 5.5 to simulate the physiological and acidic microenvironments, respectively.
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1155 468 Significant differences ($p < 0.05$) were observed, in terms of Cs AD and Mw, considering
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1157 469 degradation kinetics over 7 days of incubation time (**Fig. 5**). Additionally, the degradation
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1159 470 behavior patterns were peculiar after immersion under simulated physiological (pH 7.4) and
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1161 471 acidic (pH 5.5) conditions at 37 °C. However, independently of the swelling conditions and Cs
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1163 472 characteristics (AD and Mw), more than 75% of the initial mass was preserved (**Fig. 5**).
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1165 473 Independently of Cs characteristics, hydrogels, in PBS of pH 5.5, underwent most quicker
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1167 474 degradation, compared to samples immersed in PBS of pH 7.4. For example, CsIII-0 based
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1169 475 hydrogel retained about 91% mass in acidic microenvironment (**Fig. 5A**), and more than 95%
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1171 476 mass in PBS pH 7.4 (**Fig. 5B**), within 4 days. Qu *et al.* [27], based on the morphology of the
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1173 477 hydrogels observed under SEM images, proved that differences in degradation behavior could
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1175 478 be ascribed to an increase in pore sizes, which increased significantly after immersion in PBS.
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1183 479 This increase was more pronounced for hydrogels swollen in acidic conditions (PBS of pH 5.5),
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1185 480 that in the long term, could quick the degradation of hydrogels, due to protonation of chitosan
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1187 481 amino groups. Indeed, pKa value of D-glucosamine residue is about 6.2~7.0. Subsequently,
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1190 482 macromolecular chains bonding become brittle, leading to the hydrogel's networks destruction
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1192 483 and decomposition.

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1194 484 As expected, Cs-based hydrogels with higher AD and lower Mw exhibited a faster weight
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1196 485 loss after immersion in both media (PBS of pH 5.5 and pH 7.4). After incubation for 7 days,
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1198 486 the attained mass losses were about 20%, 22% and 25% for CsI-0, CsI-1 and CsI-3 based
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1200 487 hydrogels, respectively, while, CsIII based hydrogels still retained more than 80% (CsIII-0),
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1202 488 78% (CsIII-1) and 75% (CsIII-3) of their original weight, under swelling in pH 5.5 PBS, after
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1204 489 7 days of incubation time (**Fig. 5A**). These findings corroborate well and directly with the pore
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1207 490 diameters distribution based on the SEM images, the swelling and mechanical behaviors of
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1209 491 prepared hydrogels and the observed crystallinity data. These results proposed that Cs-based
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1211 492 hydrogels, with good biodegradability and interesting stability in PBS, could exhibit potential
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1213 493 and promising application in tissue engineering [12,55,59].
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1215 494 During the last decades, smart biomaterials as oral administrative drug carriers attracted
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1217 495 day-by-day the attention of researchers in the biomedical field [60]. Therefore, Cs-based
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1219 496 hydrogels developed in the present study could be considered as porous promising pH-sensitive
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1221 497 biomaterial, with sufficient space for the diffusion of small molecules and drugs, and exercising
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1223 498 drug release management capability.
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1226 499 **3.8. Optimization of blue crab chitosan concentration for hydrogels construction**

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1228 500 An optimization of the ideal concentration of Cs for the formation of hydrogels was
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1230 501 performed on the basis of the compressive property test and storage modulus determination.
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1232 502 CsIII-0 based hydrogel was selected for the optimization of Cs concentration study, considering
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1235 503 its appropriate structural architecture, swelling behavior and mechanical strength.
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1242 504 Mechanical properties of the different Cs-based physical hydrogels, considering the
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1244 505 compression stress-strain diagrams are illustrated in **Fig. 6A**. The application of a compressive
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1246 506 force on the hydrogels made it possible to obtain two phases. At low deformation values (around
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1248 507 5%), an elastic (reversible) deformation was observed as a straight line where the deformation
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1250 508 was proportional to the stress. Beyond 5%, the stress increased slowly, giving evidence of a
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1252 509 plastic deformation (irreversible) occurrence, due to the breaking of the bonds or rearrangement
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1254 510 of the structure [12].
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1257 511 As displayed in **Fig. 6A**, hydrogel compressive strength was improved with the increase
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1259 512 of Cs concentration from 0.24 MPa for 1% of Cs to more than 0.48 MPa for hydrogel at 4% of
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1261 513 Cs. The Cs solution at 1% concentration was found to be not able to form solid physical
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1263 514 hydrogel and the hydrogel prepared was too weak to be handled and analyzed, mainly due to
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1265 515 the rather low polymer amount. Indeed, the formed hydrogel was fractured under compressive
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1267 516 deformation less than 30%. However, hydrogels prepared with more than 2% Cs were already
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1269 517 relatively rigid and resistant. Further, data revealed that fracture resistance of all hydrogel
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1271 518 samples was found to be Cs concentration dependent, where 2% Cs-based hydrogel tolerated
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1273 519 more than 45% of compressive deformation to be fractured, whereas, 50% of compressive
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1275 520 deformation was not sufficient to induce hydrogels fracture at more than 3% Cs (**Fig. 6A**).
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1277 521 However, 5% Cs-based hydrogel exhibited a significant decrease in the compressive stress to
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1279 522 around 0.4 MPa ($p < 0.05$), compared to 3% and 4% Cs-based hydrogels, with an average value
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1281 523 of 50 MPa.
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1284 524 In order to further study the influence of Cs concentration on the resulting hydrogels
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1286 525 structure, their rheological behavior was investigated. The shear (G') and loss (G'') moduli
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1288 526 curves of the Cs-based hydrogels as a function of strain were considered in the rheometer
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1290 527 dynamic stress environment (**Fig. 6B**).
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1301 528 Profiles displayed that G' values of Cs-based hydrogels increased with the increase of Cs
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1303 529 concentration, with an increasing strain from 0.1% to 1000%. In fact, G' of Cs 1%-based
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1305 530 hydrogel ($15.9 \cdot 10^3$ Pa) at 100% strain was 11.3 times lower than that of Cs 4%-based hydrogel
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1308 531 ($181 \cdot 10^3$ Pa). Although, in all cases, the storage modulus G' was below the loss modulus G'' ,
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1310 532 indicating a predominantly gel-like behavior, the storage modulus G' decreased significantly
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1312 533 ($p < 0.05$), reaching $63.2 \cdot 10^3$ Pa, for higher concentration of Cs (5%). In another aspect, 1% Cs-
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1314 534 based hydrogel was found to be able to support only around 123% deformation and maintain
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1316 535 the gel-like behavior (the point at which $G'' > G'$), while 3% and 4% Cs-based hydrogels were
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1318 536 capable to bear more than 574% and 491% deformation, respectively. However, at higher Cs
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1320 537 content, the gel structure was kept only at strains below 185% (**Fig. 6B**).

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1323 538 Consequently, from the mechanical and rheological testing data, 3% Cs-based hydrogel
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1325 539 exhibited better rheological properties than those of 2% Cs, and the fracture deformation was
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1327 540 greater than that of 5% Cs. Similarly, excessive Cs concentration led to thicker and too rigid
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1329 541 hydrogel, which could be attributed to chains entanglements excess, leading to hydrogels
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1331 542 brittleness [61]. However, due to the gradually improved number of the hydrogen-bonded
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1333 543 crosslink regions between the Cs chains, in a relatively concentrated solution, aggregation and
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1335 544 entanglement amongst the Cs macromolecular chains will significantly took place.

1337 545 Therefore, 3% concentration of chitosan solution was carefully chosen to elaborate Cs-
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1339 546 based hydrogels in the following section, for the Riboflavin *in vitro* release study.

1342 547 **3.9. Encapsulation of riboflavin, *in vitro* loading and release profiles**

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1344 548 Hydrogels structure is characterized by three major parameters, namely the volume
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1346 549 fraction of polymer in the inflated state, the average molecular weight of based polymers as
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1349 550 well as the pore size distribution of the network [9-10]. This architecture of hydrogels allows
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1351 551 the diffusion of molecules of different sizes in the network, which makes these biomaterials
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1353 552 interesting for drug release applications [7,12].

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1360 553 As the model drug, the kinetics of riboflavin release, through the Cs-based hydrogels were
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1362 554 monitored based on the cumulative amounts of released riboflavin as a function of time.
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1364 555 Different concentrations of riboflavin (1-5 g/l) were used to investigate the influence of drug
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1366 concentration on EE and release profiles.
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1369 557 The EE and LC of Cs-hydrogels for riboflavin, as reported in **Table 3**, were found to be
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1371 558 drug concentration-dependent ($p < 0.05$). Indeed, the EE values increased from more than 75%
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1373 559 for 1 g/l of riboflavin to about 85% for 3 g/l of riboflavin. However, above 3 g/l of riboflavin,
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1375 560 the EE dropped dramatically to 68% and 56%, for 4 g/l and 5 g/l of riboflavin, respectively.
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1377 561 Regarding the LC of riboflavin, values increased concomitantly with the increase of drug
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1379 562 concentration from 24% for 1 g/l of riboflavin to the saturated capacity of 37% at 3 g/l of
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1381 563 riboflavin ($p < 0.05$). No significant differences in the riboflavin LC values was noted beyond 3
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1384 564 g/l of riboflavin ($p \geq 0.05$). The decrease in the amount of encapsulated riboflavin at high
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1386 565 concentration could be related to the saturation of hydrogel (limitation of riboflavin loading
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1388 566 into Cs-based hydrogel), since the encapsulation of riboflavin was monitored through its
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1390 567 diffusion in the hydrogel network.
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1392 568 Riboflavin release profiles from Cs-based hydrogels, at 37 °C, in HCl-NaOH (0.1 M), pH
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1394 569 5.5, exhibited deliverance patterns, characterized by an initial short-time rapid release, during
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1396 570 the first 8 h, followed by low riboflavin release to 72 h. Beyond 72 h, the rate of released
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1398 riboflavin tended to stabilize (**Fig. 7**). Data reveal that hydrogel with lower riboflavin charge
1399 571 showed high initial release in terms of percentage. Indeed, at a riboflavin concentration of 1 g/l,
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1401 572 the initial release rate (after 4 h) was 11% relative to the amount of initial riboflavin loaded in
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1403 573 the hydrogel. It was of 46% and 79%, after 24 h and at the end of the study (96 h). Regarding
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1405 574 a concentration of 5 g/l, the release of riboflavin was 5%, 18% and 36%, after 4 h, 24 h and 96
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1407 575 h, respectively. In terms of the total mass of riboflavin released, the hydrogel group with higher
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1409 576 charge released more riboflavin. For example, at a riboflavin concentration of 1 g/l, the amount
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1419 578 released riboflavin was 790 mg after 96 h, while for the 5 g/l riboflavin hydrogel group, about
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1421 579 2 g of riboflavin were released (**Fig. 7A**). This finding could be assigned to the concentration
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1424 580 gradient phenomenon as diffusion management force. The more Riboflavin loading increased,
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1426 581 the higher concentration gradient increased [27].
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1428 582 The riboflavin (3 g/l) release patterns were further investigated in HCl-NaCl (0.1 M)
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1430 583 under different pH conditions (pH 2.0, pH 4.5 and pH 7.4). Deliverance curves, as reported in
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1432 584 **Fig. 7B**, showed that riboflavin was barely released from the Cs-based hydrogels, at pH 7.4, of
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1434 585 only 13% after 4 days of incubation. However, high amounts of riboflavin were released in
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1436 586 acidic pH ($p < 0.05$), further, the amount of released riboflavin from Cs-based hydrogels was
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1438 587 higher in pH than pH 4.5 in pH 2.0. Indeed, 16% and 38% of riboflavin were released from Cs-
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1440
1441 588 based hydrogel, after 8 h, at pH 4.5 and pH 2.0, respectively. Therefore, Cs-hydrogels were
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1443 589 found to release significantly more riboflavin in acidic microenvironments, probably due to
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1445 590 higher swelling rates or exhaustive hydrogels structure destruction and thereby faster
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1447 591 degradation and release of riboflavin [26,59,60].
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1450 592 4. Conclusion

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1452 593 Different Cs-based hydrogels were successfully engineered, considering Cs AD and Mw,
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1454 594 based on the alkali/urea aqueous system following the freezing/thawing/solvent evaporation
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1456 595 approach. As expected, hydrogels pore size distribution, mechanical strength, swelling and
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1458 596 thermal resistance behaviors besides the *in vitro* biodegradation patterns, were extremely
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1460 597 depending on Cs structural characteristics. Low AD coupled with high Mw seemed to be very
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1462 598 interesting for the development of promoting biomaterials with stable and appropriate features.

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1464 599 Moreover, Cs-based hydrogels were monitored to study the *in vitro* release of riboflavin
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1466 600 selected as the model drug. The obtained release patterns displayed that Cs-based hydrogels
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1469 601 could be applied as smart pH-sensitive carrier for drug-controlled release for further biomedical
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1471 602 applications (antitumor, protein and peptide, gene and antibiotic drug delivery). Additionally,
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603 due to its suitable structural architecture, swelling behavior and mechanical strength, the
604 application of Cs-based hydrogels seems to be a very interesting alternative in the tissue
605 engineering field.

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Figure captions

Figure 1: SEM images of Cs-based hydrogels (GCs) cross sections: GCsI-0 with AD of 17% and Mw of 125.6 kDa (**A**), GCsI-1 with AD of 17% and Mw of 17.8 kDa, (**B**) GCsI-3 with AD of 17% and Mw of 10.44 kDa (**C**), GCsII-0 with AD of 13% and Mw of 118.9 kDa (**D**), GCsII-1 with AD of 13% and Mw of 59.27 kDa (**E**), GCsII-3 with AD of 13% and Mw of 18.54 kDa (**F**), GCsIII-0 with AD of 8% and Mw of 115 kDa (**G**), GCsIII-1 with AD of 8% and Mw of 78.43 kDa (**H**) and GCsIII-3 with AD of 8% and Mw of 16.04 kDa (**I**).

Figure 2: ATR-FTIR profiles of Cs-based hydrogels (GCs) with different AD and Mw, (**A**) GCsI, (**B**) GCsII and (**C**) GCsIII, compared to Cs powders spectra.

Figure 3: XRD patterns of Cs-based hydrogels (GCs) with different AD and Mw, (**A**) GCsI, (**B**) GCsII and (**C**) GCsIII, compared to Cs powders spectra.

Figure 4: Mechanical features of Cs-based hydrogels (GCs) as function of Cs AD and Mw. Rheological behavior (**A**) GCsI, (**B**) GCsII, (**C**) GCsIII, $f=1$ Hz, $T=37$ °C. Compressive properties, at a temperature of 25 °C and a compression speed of 1 mm/min, (**D**) GCsI, (**E**) GCsII, (**F**) GCsIII.

Figure 5: *In vitro* biodegradation Cs-based hydrogels (GCs) as function of Cs AD and Mw, in PBS at (**A**) pH 5.5 (acidic microenvironment) and (**B**) pH 7.4 (physiological microenvironment simulation), at 37 °C.

Figure 6: Mechanical behavior of Cs-based hydrogels as function of Cs concentration. (**A**) Compressive stress vs. compressive strain profiles. (**B**) Viscoelastic properties patterns as function of strain (%), $f=1$ Hz, $T=37$ °C.

Figure 7: Riboflavin, as the model drug, *in vitro* release profile and kinetics from Cs-based hydrogels. Riboflavin incorporation was monitored by immersion in riboflavin solution (0-5 g/l) in dark at 5 °C for 48 h. The release tests were performed in HCl and NaCl (0.1 M) with different pH values (pH 2.0, pH 4.5 and pH 7.4) at 37 °C. (**A**) Riboflavin release kinetics at different concentrations (0-5 g/l of riboflavin) in pH 5.5. (**B**) Riboflavin (3 g/l) release kinetics at different pH values (pH 2.0, pH 4.5 and pH 7.4).

Fig. 1

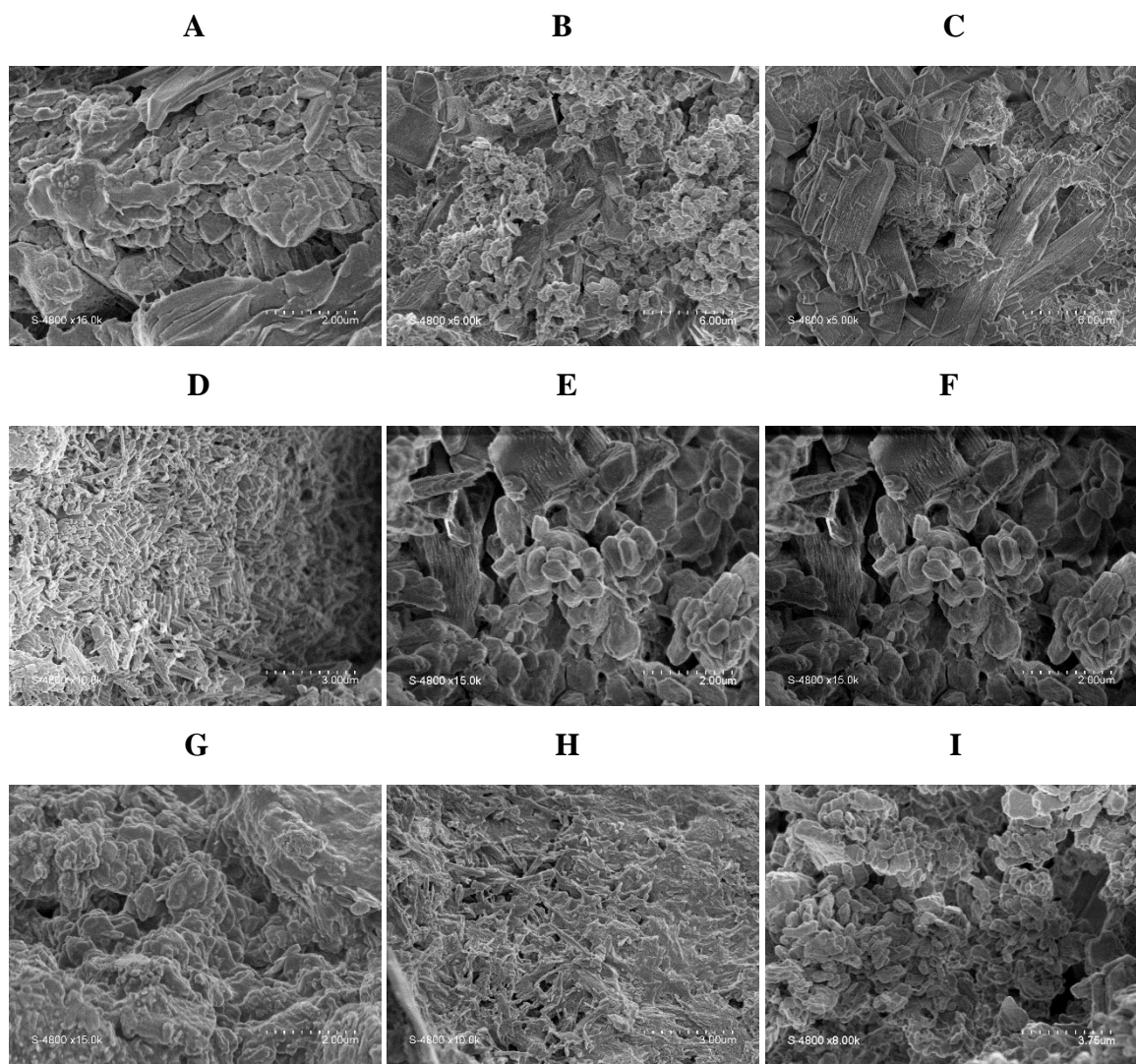


Fig. 2

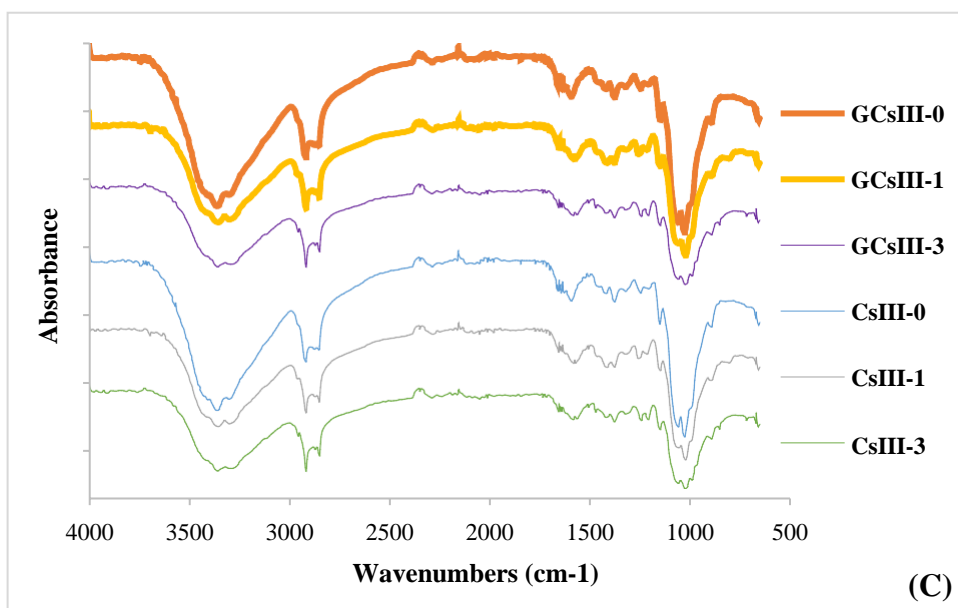
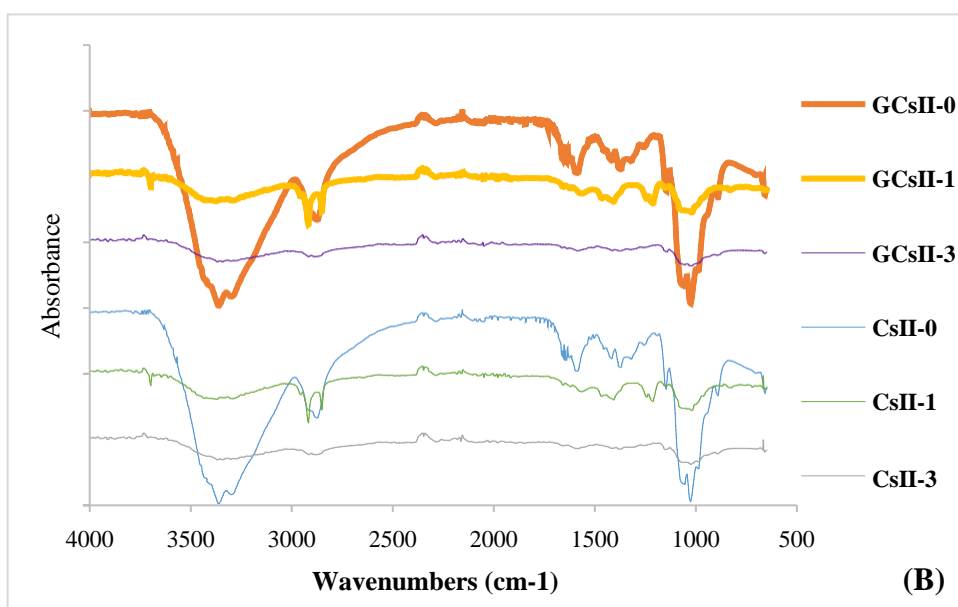
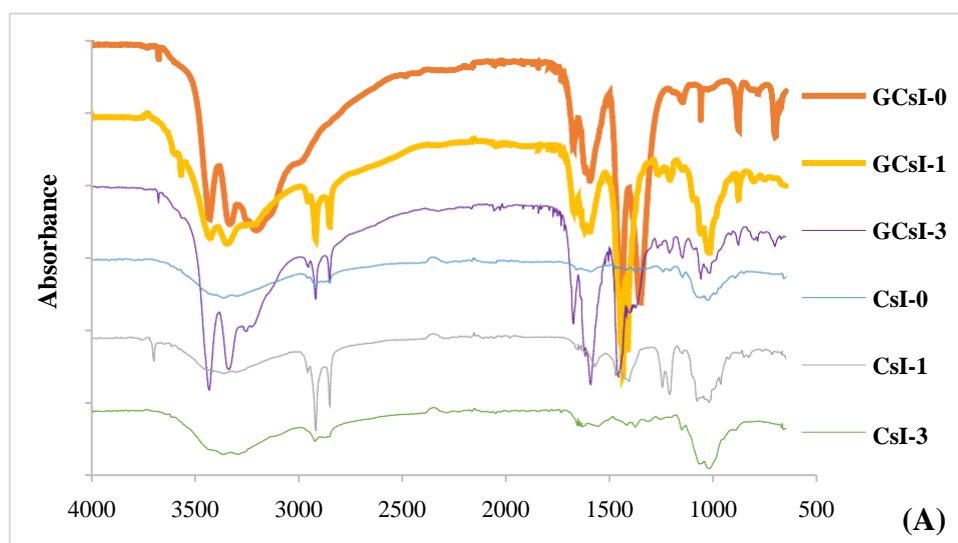


Fig. 3

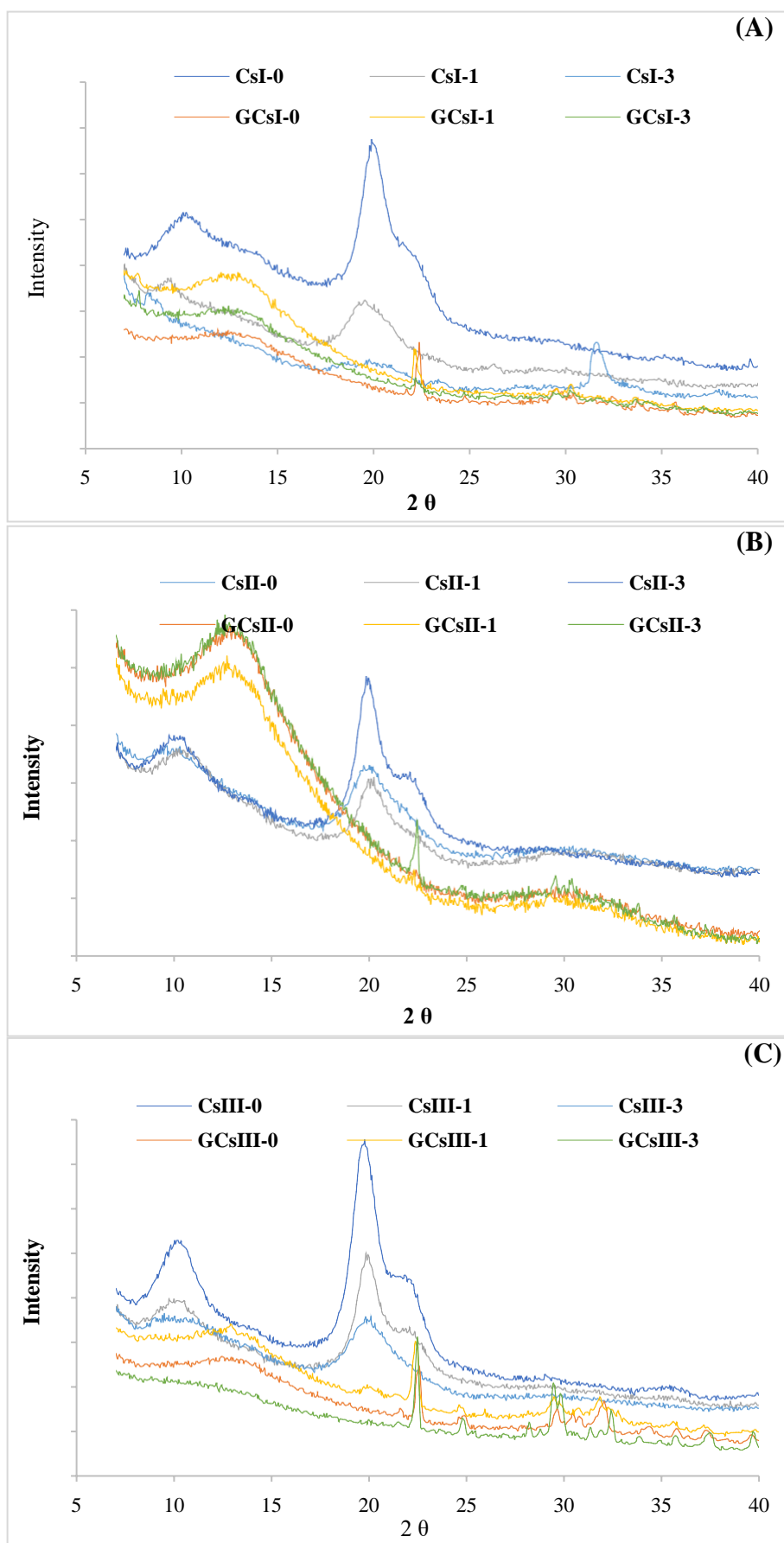


Fig. 4

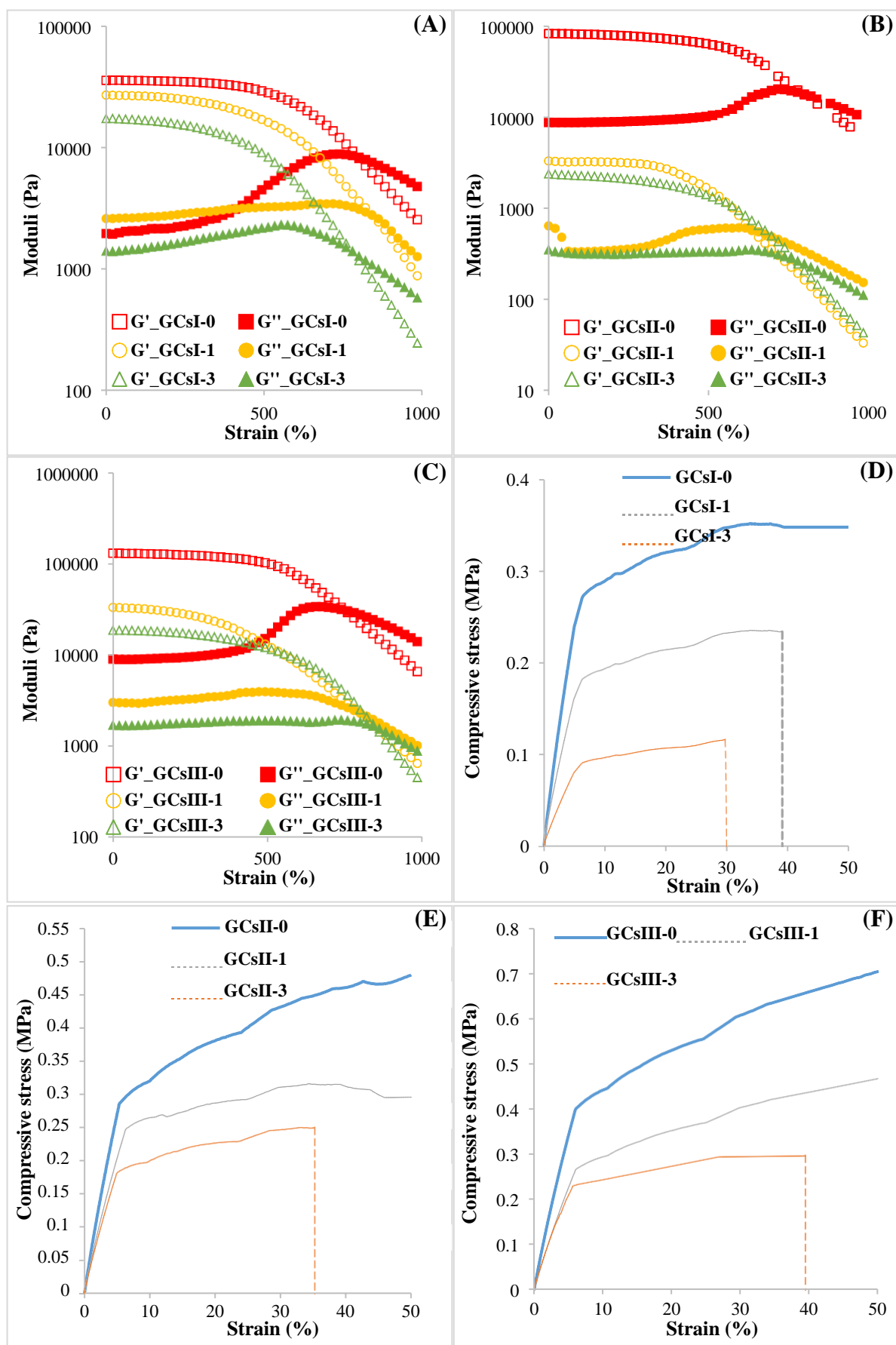


Fig. 5

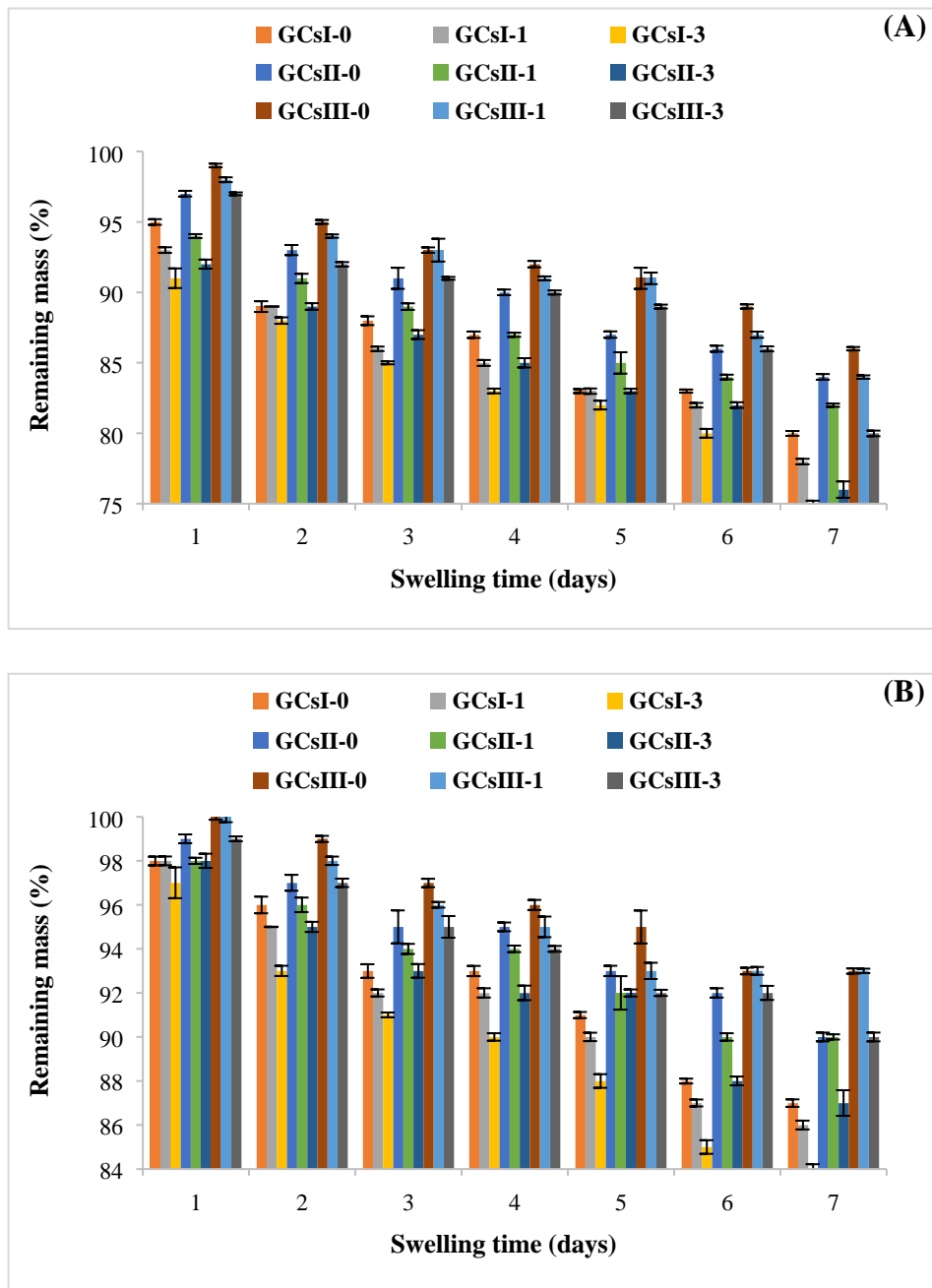


Fig. 6

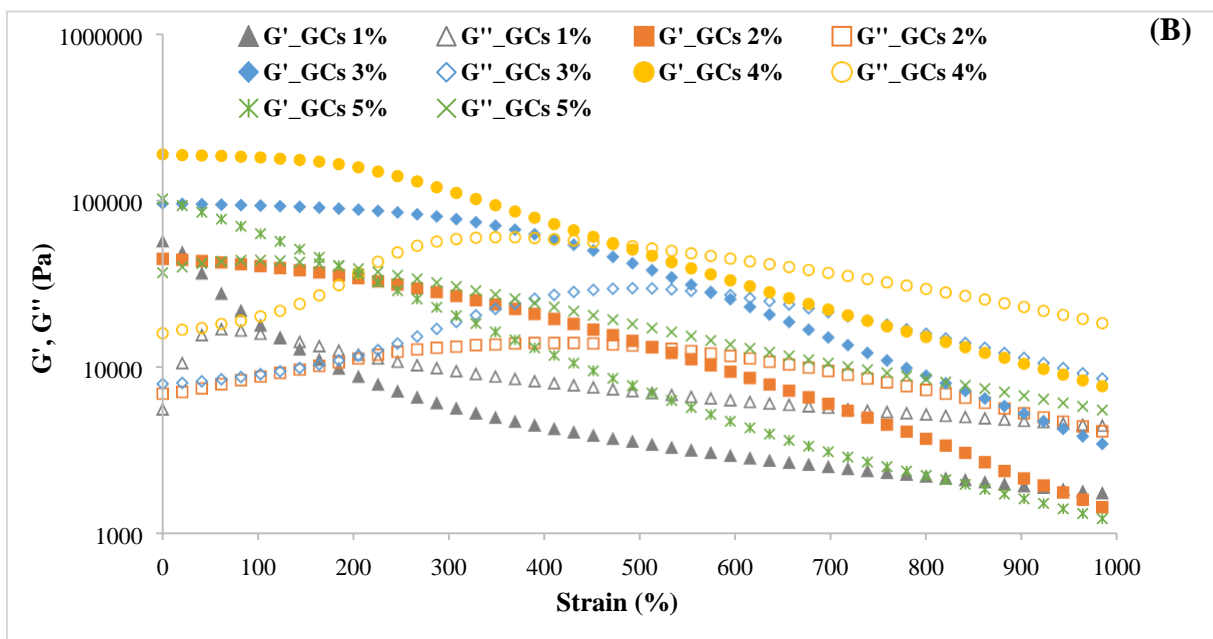
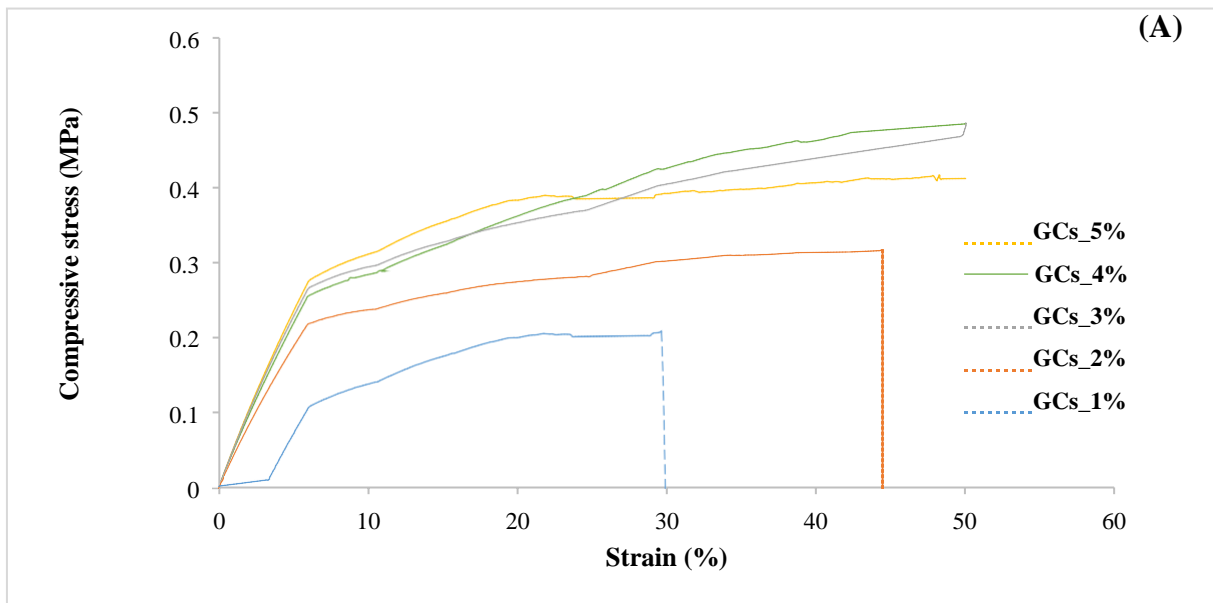


Fig. 7

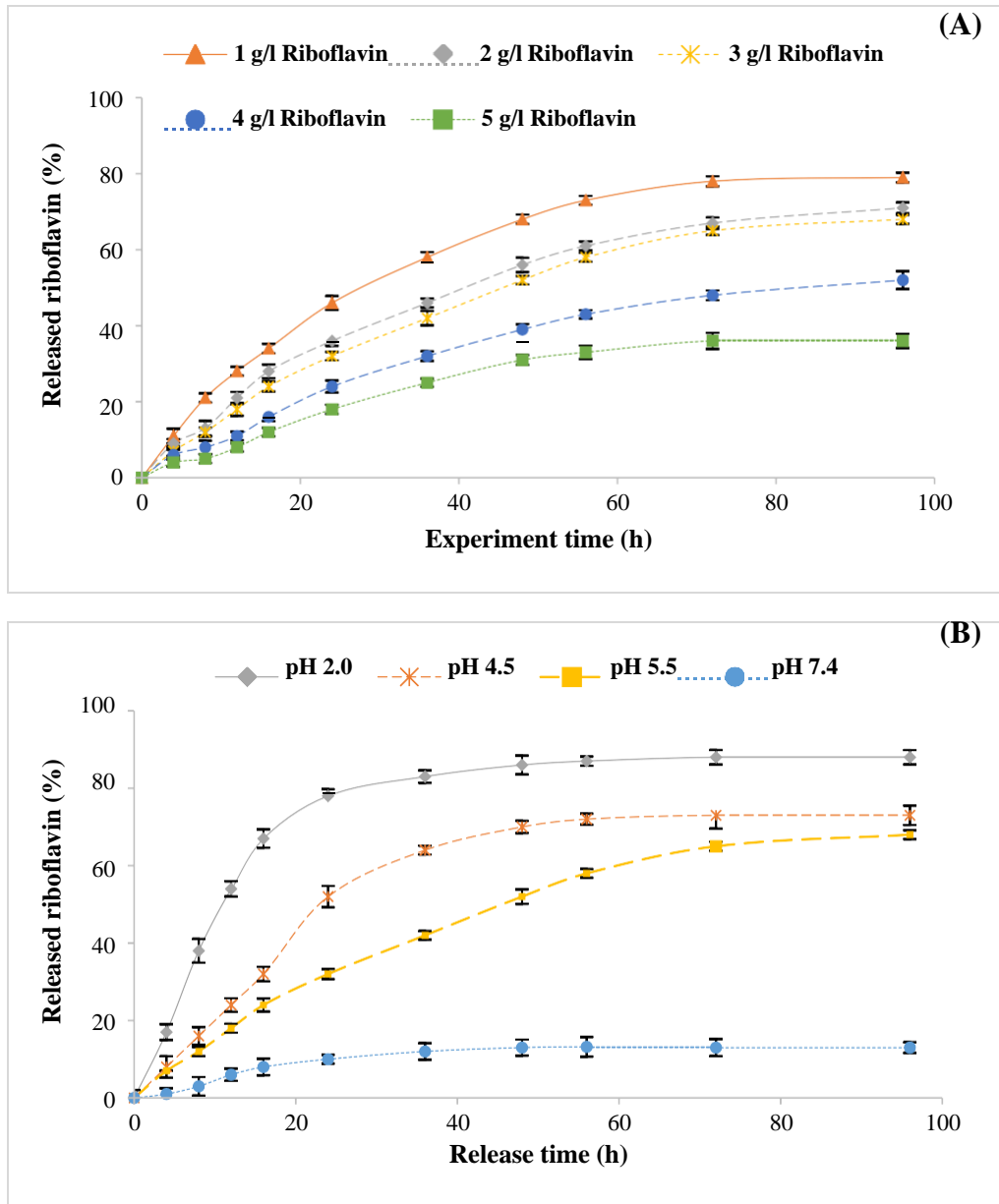


Fig. S1

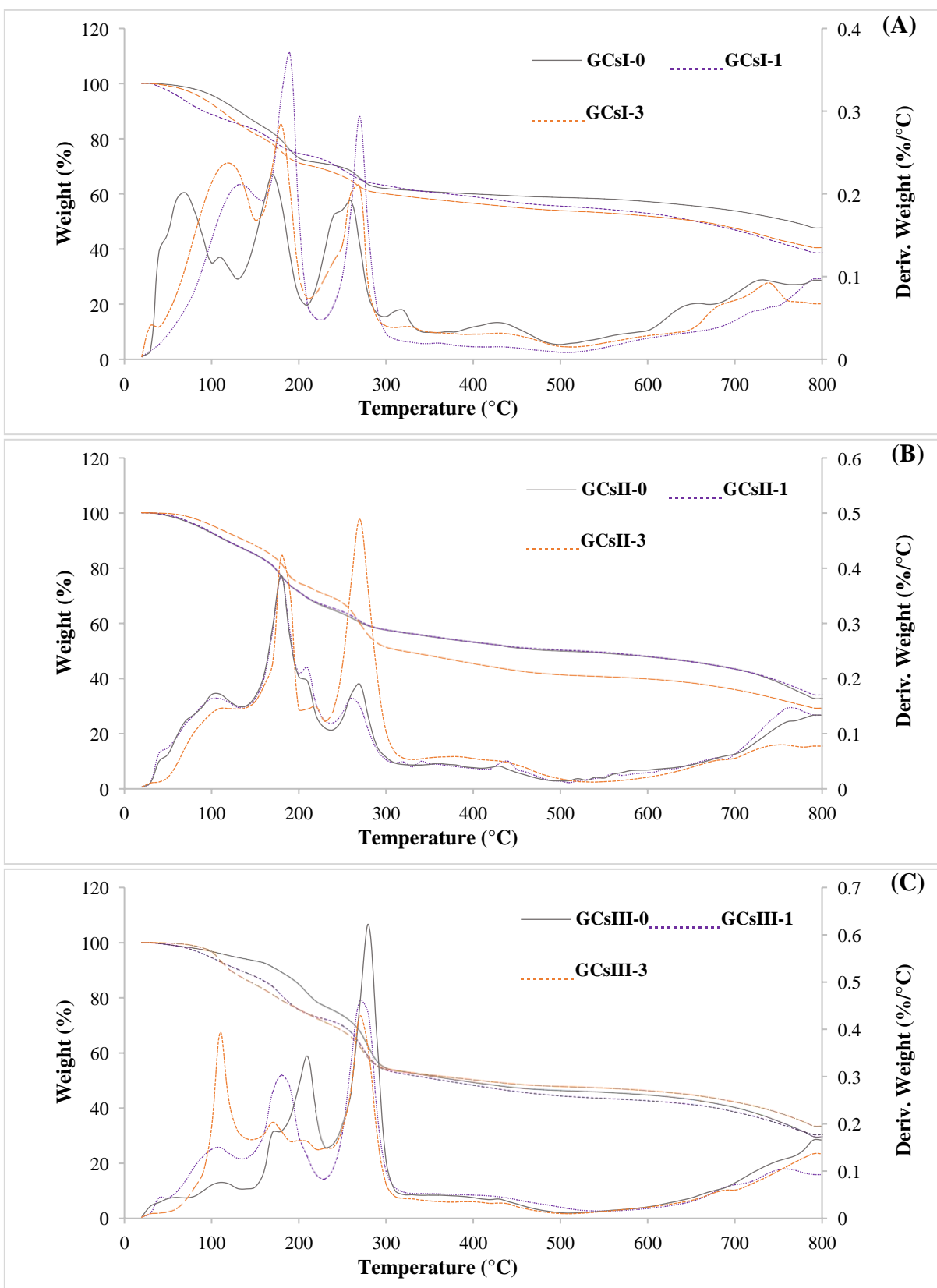


Fig. S2

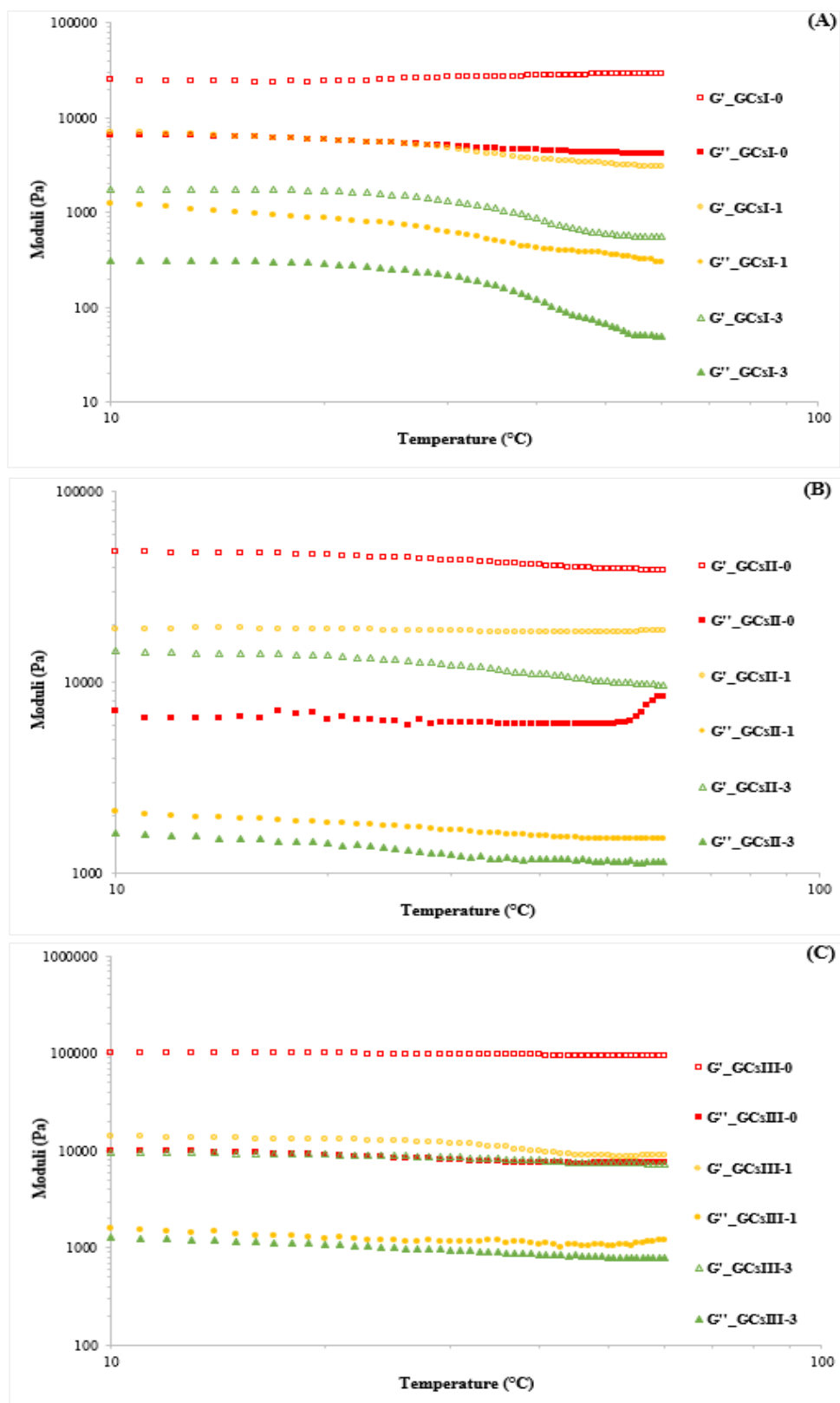


Table 1: Moisture content (MC) and swelling ratio (SR) of different prepared Cs-based hydrogels.

| Cs-based hydrogels | 3% (w/v) Cs content | | | | | |
|--------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | GCsI | | GCsII | | GCsIII | |
| | MC (%) | SR (g/g) | MC (%) | SR (g/g) | MC (%) | SR (g/g) |
| GCs-0 | 82.36±0.72 ^{aB} | 13.59±0.45 ^{aA} | 81.34±0.22 ^{aB} | 16.57±1.04 ^{aB} | 79.90±0.09 ^{aA} | 18.10±0.47 ^{aC} |
| GCs-1 | 86.52±1.35 ^{bC} | 14.92±0.32 ^{bA} | 81.78±0.11 ^{aB} | 18.93±0.32 ^{bB} | 80.26±0.04 ^{bA} | 22.21±0.53 ^{bC} |
| GCs-3 | 89.08±0.69 ^{cC} | 16.38±0.27 ^{cA} | 83.20±0.06 ^{bB} | 22.86±0.78 ^{cB} | 81.83±0.08 ^{cA} | 26.24±0.93 ^{cC} |

Different letters (a-c) in the same column are significantly different as determined by ANOVA test (p<0.05).

Different letters (A-C) in the same line indicated significant differences within hydrogels based on Cs with different AD (p<0.05).

Table 2: Cs-based hydrogels degradation temperatures (**Td**: onset temperature of degradation, **Tmax**: maximum degradation temperature and **Tf**: temperature of the end of degradation), the weight loss (Δw) and the residue (**R**).

| Parameters | GCsI | | | GCsII | | | GCsIII | | | |
|------------------|------------------|--------|--------|---------|---------|---------|----------|----------|----------|--------|
| | GCsI-0 | GCsI-1 | GCsI-3 | GCsII-0 | GCsII-1 | GCsII-3 | GCsIII-0 | GCsIII-1 | GCsIII-3 | |
| rnase I | ΔW (%) | 11.46 | 15.69 | 18.93 | 8.66 | 12.14 | 12.73 | 5.65 | 10.30 | 14.75 |
| | Td (°C) | 27.32 | 33.30 | 40.93 | 30.03 | 37.75 | 42.75 | 36.39 | 41.71 | 45.47 |
| | Tmax (°C) | 114.47 | 104.49 | 66.35 | 118.10 | 109.02 | 104.49 | 133.54 | 109.93 | 106.35 |
| | Tf (°C) | 147.16 | 133.54 | 129.00 | 153.51 | 134.45 | 129.91 | 158.96 | 135.35 | 130.81 |
| rnase II | ΔW (%) | 13.30 | 14.43 | 10.72 | 20.95 | 21.24 | 22.50 | 17.18 | 18.19 | 14.04 |
| | Td (°C) | 147.16 | 133.54 | 129.00 | 153.51 | 134.45 | 129.91 | 158.96 | 135.35 | 130.81 |
| | Tmax (°C) | 184.38 | 176.21 | 169.85 | 188.01 | 179.84 | 172.58 | 208.89 | 179.84 | 175.30 |
| | Tf (°C) | 227.05 | 210.71 | 207.08 | 235.22 | 229.77 | 226.13 | 235.22 | 231.59 | 227.05 |
| Phase III | ΔW (%) | 12.42 | 16.59 | 18.63 | 29.34 | 15.52 | 15.29 | 28.77 | 29.72 | 23.29 |
| | Td (°C) | 227.05 | 210.71 | 207.08 | 235.22 | 229.77 | 226.13 | 235.22 | 231.59 | 227.05 |
| | Tmax (°C) | 268.81 | 266.09 | 261.55 | 269.72 | 267.00 | 262.46 | 279.71 | 274.26 | 273.35 |
| | Tf (°C) | 511.22 | 507.58 | 502.14 | 537.54 | 508.49 | 502.14 | 547.53 | 510.31 | 509.40 |
| R (%) | 38.68 | 40.53 | 47.69 | 29.58 | 33.05 | 34.34 | 29.88 | 30.59 | 33.70 | |

Table 3: Riboflavin entrapment efficiency (EE) and loading capacity (LC) of CsIII-0 based hydrogel, at a concentration of 3% (w/v).

| Riboflavin concentration (g/l) | EE (%) | LC (%) |
|--------------------------------|---------------------------|---------------------------|
| 1 | 75.58 ± 0.61 ^c | 23.51 ± 1.26 ^a |
| 2 | 80.64 ± 1.59 ^d | 31.14 ± 0.94 ^b |
| 3 | 84.54 ± 1.35 ^e | 36.57 ± 1.45 ^c |
| 4 | 66.28 ± 0.52 ^b | 37.19 ± 0.62 ^c |
| 5 | 53.27 ± 1.72 ^a | 37.81 ± 1.32 ^c |

Different letters (a-e) in the same column are significantly different as determined by ANOVA test ($p < 0.05$).

Table S1: Blue crab chitosan (Cs) nomenclature and respective acetylation degrees (AD) and average molecular weights (g mol^{-1}).

| average molecular weights (g mol^{-1}). | | | | |
|--|--------|---------|---------|---------|
| Cs | AD (%) | | | |
| | — | 17 | 13 | 8 |
| Cellulase digestion reaction-time (h) | 0 | CsI-0 | CsII-0 | CsIII-0 |
| | 1 | CsI-1 | CsII-1 | CsIII-1 |
| | 3 | CsI-3 | CsII-3 | CsIII-3 |
| Cs | AD (%) | | | |
| | — | 17 | 13 | 8 |
| Digestion reaction-time (h) | 0 | 125 600 | 118 900 | 115 000 |
| | 1 | 17 800 | 59 270 | 78 430 |
| | 3 | 10 440 | 18 540 | 16 040 |

Table S2: Different blue crab chitosan-based hydrogels (GCs) feed compositions and respective nomenclature.

| Cs-based hydrogels | 3% (w/v) Cs content | | |
|-------------------------------|----------------------------|-------------|--------------|
| | CsI | CsII | CsIII |
| Cs-0 | GCsI-0 | GCsII-0 | GCsIII-0 |
| Cs-1 | GCsI-1 | GCsII-1 | GCsIII-1 |
| Cs-3 | GCsI-3 | GCsII-3 | GCsIII-3 |