

1 Biodegradable doubly cross-linked chitosan hydrogels as  
2 sustained drug delivery systems. Influence of chemical cross-  
3 linking and chitosan ratios on rheological properties and  
4 pharmacological performance.

5 *Nieves Iglesias<sup>1</sup>, Elsa Galbis<sup>1</sup>, Concepción Valencia<sup>2,3</sup>, M. Jesús Díaz-Blanco<sup>2,3</sup>, Bertrand*  
6 *Lacroix<sup>4,5</sup>, M.-Violante de-Paz<sup>1,\*</sup>*

7 1. Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia,  
8 Universidad de Sevilla, 41012 Sevilla, Spain

9 2. Departamento de Ingeniería Química, Campus de “El Carmen”, Universidad de  
10 Huelva, 21071 Huelva, Spain

11 3. Pro2TecS—Chemical Process and Product Technology Research Center, Universidad  
12 de Huelva, 21071 Huelva, Spain

13 4. Department of Materials Science and Metallurgical Engineering, and Inorganic  
14 Chemistry, University of Cádiz, Cádiz, Spain.

15 5. IMEYMAT, Institute of Research on Electron Microscopy and Materials of the  
16 University of Cádiz, Cádiz, Spain.

17 \*Correspondence: vdepaz@us.es; Tel.: +34-954-556-740

18

## 19 Abstract

20 This study investigates the impact of dual ionic and covalent cross-links (ion-XrL and  
21 cov-XrL) on the properties of chitosan-based (CTS) hydrogels as eco-friendly drug  
22 delivery systems (DDS) for the model drug diclofenac sodium (DCNa). Citric acid and a  
23 diiodo-trehalose derivative (ITrh) were the chosen ionic and covalent cross-linker,  
24 respectively. The novel hydrogels completely disintegrated within 96 h by means of a  
25 hydrolysis process mediated by the enzyme trehalase. As far as the authors are aware,  
26 this is the first time that a trehalose derivative has been used as a covalent cross-linker  
27 in the formation of biodegradable hydrogels. The impact of CTS concentration and  
28 degree of cov-XrL on rheological parameters were examined by means of an  
29 experimental model design and marked differences were found between the materials.  
30 Hydrogels with maximum elastic properties were achieved at high CTS concentrations  
31 and high degrees of cov-XrL. DCNa-loaded formulations displayed well-controlled drug-  
32 release profiles strongly dependent on formulation composition (from 17% to 40% in 72  
33 h). Surprisingly, higher degrees of covalent cross-linking led to a boost in drug release.  
34 The formulations presented herein provides a simple and straightforward pathway to  
35 design fully biodegradable, tailor-made controlled drug delivery systems with improved  
36 rheological properties.

37 Keywords:

38 Ionic cross-linking; chemical cross-linking; controlled drug release; biodegradable; eco-  
39 friendly formulations; viscoelastic hydrogels.

## 40 1. Introduction

41 Hydrogels constitute a group of polymeric materials that swell and retain a significant  
42 fraction of water within their three-dimensional (3D). They exhibit tunable mechanical  
43 properties, biocompatibility and biodegradability and are therefore widely applied in  
44 biomedical and pharmaceutical fields: cell proliferation and differentiation [1], tissue  
45 engineering and regenerative medicines [2,3], and wound dressing [4,5], among others.  
46 However, one of their most promising uses is as a constituent of matrices for the  
47 controlled release of bioactive molecules, [6,7]. For hydrogel applications as drug  
48 delivery systems (DDS), their original 3D structure must be mechanically strong so that  
49 they do not erode prematurely, and thus early release or diffusion of the drug to non-  
50 target tissues is prevented. This would dramatically improve their therapeutic efficacy  
51 and reduce drug-associated side effects [8]. Moreover, it is necessary their  
52 disintegration under physiological conditions, preferably in harmless products to ensure  
53 a good biocompatibility of the hydrogel [9].

54 Although hydrogels made from natural polymers may not provide sufficient mechanical  
55 properties, they do offer their inherent biocompatibility as an advantageous property  
56 [10]. Therefore, strengthen the natural-based hydrogel system while maintaining its  
57 swelling, flexibility and degradability properties is pursued. Among the natural occurring  
58 polymers, chitosan (CTS) is drawing the attention of the scientific community, in  
59 particular, in biomedical applications because of its interesting biopharmaceutical  
60 characteristics, well documented biocompatibility and low toxicity [11]. CTS is also able  
61 to adhere to the mucosal surfaces within the body [8] and has demonstrated its capacity  
62 to open tight junctions between epithelial cells though well-organized epithelia [12]  
63 acting as a permeation enhancer.

64 An interesting review provides an overview of traditionally used and recently developed  
65 methods for preparation and modification of chitosan-based hydrogels [13]. In general,  
66 crosslinkers serve as a bridge linking different or the same polymer chains, forming a 3D  
67 network, improving the mechanical strength and chemical stability in acidic solutions.  
68 CTS hydrogels have been prepared via ionotropic gelation (ionic cross-linking) [2,14–18]

69 coordination with metal ions [19], and irreversible/covalent cross-linking between CTS  
70 and the cross-linker [7,20–22], among others. Among the covalent stabilization  
71 mechanism, one of the most widely used is the formation of imine bonds between  
72 amino groups from CTS and the aldehyde groups from the cross-linker [8,23,24].

73 Although non-covalent cross-linked hydrogels show unique mechanical properties,  
74 including both stiffness/toughness and elasticity/flexibility to retain the hydrogel  
75 structure, most of them are mechanically weak and prone to fracture, greatly restricting  
76 their applications [25]. Conversely, and regarding drug release, the reversible nature of  
77 ionically cross-linked networks is useful since, once release in the drug in the medium  
78 has been achieved, the formulations can subsequently disintegrate into biocompatible  
79 components that then will be metabolized and eliminated from the body [26].

80 Different approaches can be followed to overcome the instability of ionotropic  
81 hydrogels. Thus, for example; to improve the mechanical properties of ionically cross-  
82 linked calcium-alginate hydrogels under physiological conditions, the formation of  
83 interpenetrating polymer network was successfully conducted by Zhao *et al.* [27]. On  
84 the other hand, covalent cross-linking can improve the mechanical properties of the  
85 hydrogels [28] and consequently, dual covalent and ionic bonds can be considered as an  
86 effective method to enhance the properties of CTS-based materials. However, only few  
87 examples of dual cross-linking methods have been published. Zhuang *et al.* reported the  
88 formation of CTS films through the utilization of citric acid as a dual ionic and covalent  
89 linker [29]. Ionic and covalent cross-linking of CTS with  $\text{CaSO}_4$  and genipin, respectively,  
90 has also been investigated [30]. Additionally, cross-linked iminoboronate-CTS hydrogels  
91 have also been reported in which boronate-based coordination and covalent cross-  
92 linking occur [31]. On the other hand, when chemical cross-linking is involved, a  
93 decrease in degradability is usually observed [28]. Labile bonds need being introduced  
94 in the gels so that the former can be broken under physiological conditions. Thus,  
95 ensuring the presence of labile covalent bonds in the chemical structure of the covalent  
96 cross-linkers is a good strategy to overcome this drawback. Among the breakable  
97 covalent bonds, glycosidic linkages are present in di-, oligo- or polysaccharides. They are  
98 easily hydrolyzed by living systems by means of reactions catalyzed by extremely

99 common non-specific and specific glycosidase enzymes {also called glycoside  
100 hydrolases, [32,33]}, such as cellulase, amylase, mannosidase, lactase and trehalase,  
101 among others.

102 In this work, we aim to manufacture a set of biodegradable and eco-friendly chitosan-  
103 based hydrogels with enhanced rheological properties as DDS by means of a double  
104 cross-linking process. Sodium diclofenac (DCNa), one of the most frequently used non-  
105 steroidal anti-inflammatory drugs (NSAID) was the model drug of choice. The hydrogels  
106 were ionically and covalently cross-linked. The chosen covalent cross-linker was the  
107 2,3,4,2',3',4'-hexa-*O*-acetyl-6,6'-diiodo-6,6'-dideoxy- $\alpha$ -D-Glucopyranosyl- $\alpha$ -D-  
108 glucopyranoside (ITrh), a dielectrophilic derivative from the disaccharide  $\alpha,\alpha'$ -trehalose,  
109 that bears a labile acetal group in its structure. As ionic cross-linker, citric acid (CA) was  
110 the polyprotic acid of choice since it presents excellent antimicrobial and antioxidant  
111 properties as well as excellent biocompatibility [29,34]. The chemical structure,  
112 biodegradability under physiological conditions, thermogravimetric properties and  
113 morphologies of the hydrogels were evaluated. Two parameters, CTS concentration and  
114 degree of covalent cross-linking, were investigated to find the influence they exerted  
115 not only on the physicochemical and rheological properties of the hydrogels prepared  
116 based on CTS, but also on the release profiles of DCNa. Drug release tests under  
117 physiological conditions were conducted to demonstrate the in vitro controlled drug  
118 release of DCNa. The prepared CTS-based hydrogels have demonstrated to be fully  
119 biodegradable and endowed with controlled DCNa release properties under  
120 physiological conditions for advanced therapies in which dual ionic and covalent cross-  
121 links were involved. As far as the authors are aware, this is the first time that a trehalose  
122 derivative has been used as a biodegradable covalent cross-linker in the formation of  
123 structured polymeric materials.

## 124 2. Materials and methods

### 125 2.1. Materials

126 All the chemicals used were purchased from Sigma-Aldrich (Madrid, Spain) and used as  
127 received. The phosphate buffer solution of pH 5.5 (25 °C) used for release assays was

128 freshly prepared when required. Chitosan (CTS) from Sigma-Aldrich, with a  
129 deacetylation degree of 75% was chosen. The molecular weight of the CTS was  
130 determined by viscometric analysis. Its viscosity was measured in a buffered solution of  
131 0.5 M acetic acid - 0.5 M sodium acetate solution at  $25.0 \pm 0.1$  °C using an Anton Paar  
132 AMVn automated microviscometer. The viscometric constants “a” and “K” in the Mark-  
133 Houwink equation were previously determined for this solvent – CTS system and found  
134 to be  $a = 0.59$  and  $K = 0.119 \text{ cm}^3 \text{ g}^{-1}$ . The weight of the CTS used was calculated by means  
135 of the Mark-Houwink equation ( $[\eta] = 3.385 \text{ dL/g}$ ) and its value was 299 kDa. The  
136 di-iodinated trehalose (ITrh) employed for covalent crosslinking (cov-XrL) was prepared  
137 following the recipe described in Section 2.2.2. Trehalase (from porcine kidney, 1UN/0.5  
138 mL) was supplied by Sigma–Aldrich, Spain.

139 Dialysis tubing cellulose membranes avg. flat width 25 mm (Mw 8,000-14,000) were  
140 purchased from Sigma-Aldrich. Before the release, it was necessary to activate the  
141 cellulose membrane of the dialysis tube following this procedure: washing the tubing in  
142 water for 3 h, treating the tubing with a 0.3% (w/v) solution of sodium sulfide at 80 °C  
143 for 1 min. Subsequently it was washed with water at 60 °C for 2 min and with a 0.2%  
144 (v/v) solution of sulfuric acid. Finally, it was washed with hot water the acid.

## 145 2.2. Methods

### 146 2.2.1. General Methods

147 Fourier Transform Infrared (FTIR) spectra were recorded on a Jasco FT/IR 4200  
148 spectrometer (Great Dunmow, Essex, UK) equipped with attenuated total single  
149 reflection (ATR) accessory in the wavenumber range from 4000 to  $400 \text{ cm}^{-1}$ . Nuclear  
150 magnetic resonance (NMR) and mass spectra were recorded at the CITIUS Service  
151 (University of Seville).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 300 K with a Bruker  
152 AMX-500 for solutions in  $\text{CDCl}_3$ . Chemical shifts ( $\delta$ ) are reported as parts per million  
153 downfield from  $\text{Me}_4\text{Si}$  and  $J$  in Hz.  $J$  is assigned and not repeated. All the assignments  
154 were confirmed by COSY and HSQC experiments. Mass spectra were obtained using a  
155 Kratos MS80RFA instrument. High resolution mass spectra were recorded on a Q-  
156 Exactive spectrometer.

157 2.2.2. Synthesis of 2,3,4,2',3',4'-Hexa-*O*-acetyl-6,6'-diiodo-6,6'-dideoxy- $\alpha$ -D-  
158 Glucopyranosyl- $\alpha$ -D-glucopyranoside (ITrh)

159 The title compound was prepared according to a modified method of the procedure  
160 followed by Sizovs *et al.* [35]. A suspension of iodine (22.23 g, 87.6 mmol) in dry  
161 dimethylformamide (DMF, 200 mL) was prepared in round-bottom flask. A solution of  
162 triphenylphosphine (24.20 g, 92.27 mmol) in dry tetrahydrofuran (THF, 38 mL) was  
163 added to the iodine suspension followed by anhydrous trehalose (10 g, 29.2 mmol). The  
164 reaction was allowed to proceed at 80 °C for 12 h and the solvents were removed under  
165 reduced pressure. The resulting syrup was dissolved in methanol (250 mL) and basified  
166 to pH 9 by means of sodium methoxide. The mixture was stirred for 2 h at r.t. and then  
167 neutralized by the addition of the acidic resin DOWEX-2H (H<sup>+</sup> form). Methanol was  
168 removed under reduced pressure to yield a colored oil that was poured into water (150  
169 mL). To facilitate the precipitation of the formed triphenylphosphine oxide, the aqueous  
170 solution was stored at 4 °C for 24 h. A white solid appeared at the bottom of the flask  
171 and the suspension was then filtered and washed with dichloromethane (DCM, 2 x 50  
172 mL). The water from the aqueous solution was mostly removed under reduced pressure,  
173 and the obtained oil was dried at high vacuum for 48 h in the presence of P<sub>2</sub>O<sub>5</sub>. The dry  
174 oil was dissolved in dry pyridine (Py, 200 mL) and the solution was placed in an ice-bath.  
175 Acetic anhydride (38 mL, 403 mmol) was slowly added and the reaction was allowed to  
176 stir at 25 °C for 12 h. The reaction mixture was poured on ice, and then extracted with  
177 dichloromethane (DCM, 4 x 50 mL). The combined organic layers were washed with a  
178 dilute solution of sulfuric acid and dried over Na<sub>2</sub>SO<sub>4</sub>. Na<sub>2</sub>SO<sub>4</sub> was filtered off and the  
179 solvent removed under reduced pressure yielding a yellowish oil. The product was  
180 purified by column chromatography (eluent gradient: from 1:1 *tert*-butyl methyl ether-  
181 hexane to 1:4 *tert*-butyl methyl ether-hexane) to yield the title compound (23.2 g,  
182 25.0%). The ITrh spectra are recorded in Figures S1 to S4 in Supplementary information.

183 <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  ppm: 5.55 – 5.46 (m, 2H, H-3), 5.42 (d, 2H, H-1,  $J_{1,2}$  = 4.0 Hz),  
184 5.19 (dd, 2H, H-2,  $J_{2,3}$  = 10.5 Hz), 4.91 – 4.86 (m, 2H, H-4), 3.95 (dt, 2H, H-5,  $J_{5,6a}$  = 2.5 Hz;  
185  $J_{4,5}$  =  $J_{5,6b}$  = 9.5 Hz), 3.23 (dd, 2H, H-6a,  $J_{6a,6b}$  = 11.0 Hz), 3.06 (dd, 2H, H-6b), 2.14, 2.07,  
186 2.02 (3 s, 19 H, 6 methyl groups). <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  ppm: 169.92, 169.59,

187 169.46 (carbonyl groups from acetyl moieties), 91.78 (C-1), 72.35 (C-4), 69.96, 69.76,  
188 69.31 (C-5, C-3, C-2), 21.17, 20.68, 20.62 (methyl groups), 2.44 (C-6). IR:  $\nu_{\max}$  (cm<sup>-1</sup>) 1742,  
189 1367, 1216, 1028, 656, 593, 566, 558, 528, 513, 493. ESI-MS positive ion mode:  
190 calculated m/z (C<sub>24</sub>H<sub>32</sub>O<sub>15</sub>I<sub>2</sub>Na)<sup>+</sup> ([M+Na]<sup>+</sup>): 836.9723; found m/z: 836.9721;  $\Delta(m/z)$ :-  
191 0.2498 ppm.

192 2.2.3. Preparation of Hydrogels from Cross-linked Chitosan (CTS), Citric Acid (CA)  
193 and Diiodinated Trehalose derivative (CTS<sub>x</sub>-CA<sub>10</sub>-ITrh<sub>y</sub>)

194 Ten systems named CTS<sub>x</sub>-CA<sub>10</sub>-ITrh<sub>y</sub> were prepared according to the procedure  
195 described below (Figure S5). The targeted final CTS concentrations in mass percentages  
196 (weight/weight or w/w) were 3%, 4% or 5% w/w and the degree of ionic cross-linking  
197 (ion-XrL) was fixed by 10% for all the samples. Finally, the degree of cov-XrL was set at  
198 0%, 5% or 10%. In Table 1 and along the text “x” denotes CTS concentration (% w/w),  
199 and “y” denotes the degree of cov-XrL in the hydrogel. The ionic cross-linker was added  
200 dissolved in double-distilled water (10 mg/mL concentration), the covalent cross-linker  
201 was added in ethanol (50 mg/mL concentration) at 40 °C, and the final mass was  
202 adjusted to 25 g with double-distilled water.

203 The general procedure followed for the preparation of aqueous cross-linked CTS-CA-ITrh  
204 conjugates is summarized next (Figure 1, Table S1) and, as an example, the recipe  
205 includes the amounts of the reagents necessary for the preparation of sample  
206 CTS<sub>4</sub>-CA<sub>10</sub>-ITrh<sub>5</sub> (hydrogel with 4% w/w polymer concentration, 10% of degree of ion-XrL  
207 and 5% of degree of cov-XrL): CTS with a deacetylation degree of 75% (CTS, 1 g, 4.36  
208 mmol of free amine groups) was charged in a flask provided with a stirrer bar; then, an  
209 aqueous solution of citric acid (CA, 2.80 mL, 1% w/v, 0.15 mmol), a solution of  
210 di-iodinated trehalose derivative in ethanol (ITrh, 1.78 mL, 5% w/v, 0.11 mmol), a  
211 solution of acetic acid (HAc, 0.25 mL, 52% w/v) and double-distilled water (up to a final  
212 weight of 25 g, and final polymer concentration of 4% w/w) were added in sequence.  
213 Five-minute stirrings were performed between the addition of each reagent. Once all  
214 the reagents were added, the mixture was stirred for another 90 min at 40 °C. The  
215 solution was cooled to room temperature, the stir bar removed, and stirring proceeded

216 overnight on a roller at 25 °C. The gelation of the systems was confirmed when the  
 217 mixtures stopped flowing upon tube inversion for 60 s [23] (Figure S5). Three different  
 218 batches of these conjugates were synthesized for comparative purposes.

219 **Table 1.** CTS concentration and degree of covalent cross-linking of the 10 CTS-based hydrogels  
 220 prepared. Rheological parameters.

Sample	Formulation code	CTS Conc. (%w/w)	Degree cov-Xr (%)	pH	Rheological properties <sup>a</sup>				
					Tan $\delta$ (at 1 rad/s)	G' <sub>1</sub> (Pa)	m	K (Pa·s <sup>n</sup> )	n
1	CTS <sub>3</sub> -CA <sub>10</sub> -ITrh <sub>0</sub>	3%	0	5.6	1.58	10.4	0.70	65.68	0.46
2	CTS <sub>3</sub> -CA <sub>10</sub> -ITrh <sub>5</sub>	3%	5	5.6	1.50	17.2	0.63	43.68	0.50
3	CTS <sub>3</sub> -CA <sub>10</sub> -ITrh <sub>10</sub>	3%	10	5.5	1.14	30.7	0.61	61.67	0.50
4	CTS <sub>4</sub> -CA <sub>10</sub> -ITrh <sub>0</sub>	4%	0	5.8	0.87	73.3	0.46	233.97	0.31
5	CTS <sub>4</sub> -CA <sub>10</sub> -ITrh <sub>5</sub>	4%	5	5.5	0.84	121.5	0.42	237.54	0.30
6	CTS <sub>4</sub> -CA <sub>10</sub> -ITrh <sub>5</sub>	4%	5	5.9	0.77	93.1	0.45	251.33	0.30
7	CTS <sub>4</sub> -CA <sub>10</sub> -ITrh <sub>10</sub>	4%	10	5.3	0.70	146.0	0.39	324.39	0.22
8	CTS <sub>5</sub> -CA <sub>10</sub> -ITrh <sub>0</sub>	5%	0	6.3	0.68	233.9	0.39	475.41	0.18
9	CTS <sub>5</sub> -CA <sub>10</sub> -ITrh <sub>5</sub>	5%	5	6.2	0.63	249.8	0.33	463.41	0.17
10	CTS <sub>5</sub> -CA <sub>10</sub> -ITrh <sub>10</sub>	5%	10	6.3	0.46	653.3	0.29	449.07	0.17

Degree of ionic cross-linking: 10% in every sample;

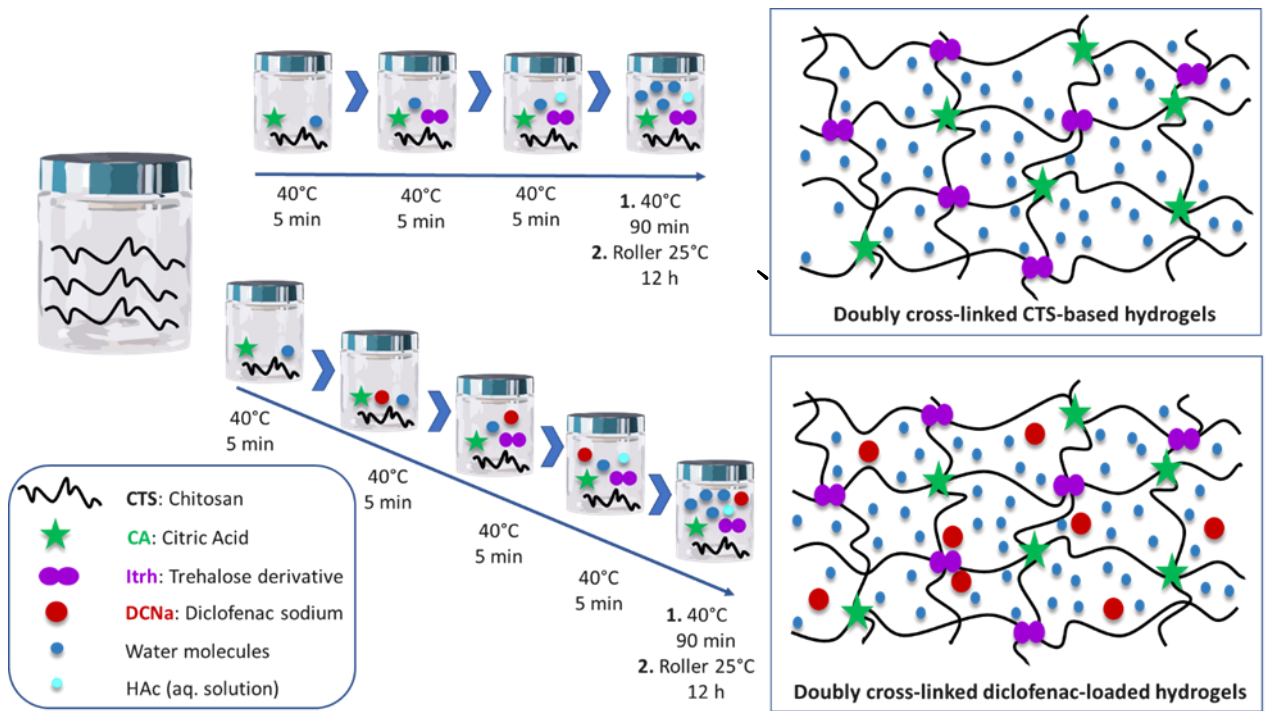
CTS conc = concentration (in w/w percentage) of CTS;

Degree cov-Xr = degree of covalently cross-linked amino groups in CTS material by diiodo trehalose derivative;

<sup>a</sup>Power-law model parameters and loss tangent at 1 rad/s for CTS<sub>x</sub>-CA<sub>10</sub>-ITrh<sub>y</sub> hydrogels studied.

221

222 **Figure 1.** General procedure for the formation of doubly cross-linked CTS-CA-IThr hydrogels.



#### 2.2.4. Preparation of Diclofenac Sodium Loaded Formulations from Cross-linked Chitosan-Conjugates

Ten systems named DCNa-CTS<sub>x</sub>-IThr<sub>y</sub> were prepared. The preparation process was similar to that of non-loaded hydrogels except that a solution of DCNa in ethanol was added into the mixture just after the ionic cross-linker CA (Figure 1). Final CTS concentrations: 3%, 4% or 5% w/w; degree of ion-XrL: 10%; degree of cov-XrL: 0%, 5% or 10%; final DCNa concentration: 1% w/w.

#### 2.2.5. Hydrogel studies

The dried samples were examined by thermogravimetric analysis (TGA), and the decomposition temperatures were observed. The thermogravimetric analyzer was TA Instruments Q-600 SDT (New Castle, DE, USA). Platinum pans containing approximately 5 mg of each sample were used. Trials were conducted under inert atmosphere (nitrogen, flow rate: 100 mL/min, heating rate: 10°C/min), from 0°C to 700°C.

237 To measure the pH of the samples, the selected hydrogel (1 g) was placed in a glass  
238 container and 10 mL of double-distilled water was added. It was shaken gently and the  
239 obtained pHs are shown in Table 1. pH readings of the samples were determined  
240 electrometrically using digital pH-meter (HI98103; Hanna Checker pH-meter, Hanna  
241 Instruments).

#### 242 2.2.6. In vitro degradation of CTS<sub>x</sub>-CA<sub>10</sub>-ITR<sub>h<sub>y</sub></sub> hydrogels

243 The in vitro degradation studies of hydrogels were conducted following the procedure  
244 described by Liu and coworkers for collagen-based hydrogels [36]. Thus, 60-80 mg of  
245 hydrated hydrogels (n = 3 of each formulation) were immersed in vials containing 5 mL  
246 of 0.1 M PBS (pH 5.7), followed by addition of 30 µl trehalase (2UN/mL). The vials were  
247 incubated at 37 °C with shaking at 100 rpm. At different time intervals, the hydrogels  
248 were taken out and rinsed with double-distilled water to remove excess salinity. The  
249 weight of each sample was measured after all surface water was carefully blotted off.  
250 The percent residual mass of hydrogels was calculated according to Equation 1:

$$251 \quad \text{Residual mass (\%)} = \frac{W_t}{W_0} \times 100 \quad (\text{Eq. 1})$$

252 where  $W_0$  is the initial weight of the hydrogel and  $W_t$  is the weight of the hydrogel at  
253 each time point.)

#### 254 2.2.7. Rheological Studies and Experimental

255 CTS<sub>x</sub>-CA<sub>10</sub>-ITR<sub>h<sub>y</sub></sub> hydrogels were rheologically characterized in a controlled-strain (ARES,  
256 Rheometric Scientific, Surrey, UK) rheometer, using a serrated plate-plate (25 mm  
257 diameter, 1 mm gap) geometry. Small amplitude oscillatory shear (SAOS) tests were  
258 carried out inside the linear viscoelastic region in a frequency range of 0.03–100 rad/s  
259 at 25 °C. Strain sweep tests were previously performed to determine the linear  
260 viscoelastic regime. Viscous flow tests were also made by applying a stepped shear rate  
261 ramp in a shear rate range of 0.06–100 s<sup>-1</sup> at 25 °C. Each fresh sample was tested at least  
262 in duplicate. The rheological parameters obtained are recorded in Table 1.

263 2.2.8. Experimental Model Design for the analysis of rheological parameters

264 In order to study the influence of CTS concentration and the degree of cov-XrL in the  
265 rheological properties of the hydrogels, a Box–Behnken experimental design (CSS  
266 Statistica, StatSoft Inc., Tulsa, UK) was used to evaluate the significance of these  
267 independent variables as well as the interactions among them in the rheological  
268 parameters  $G'_1$ ,  $m$ ,  $\tan \delta$ ,  $K$  and  $n$ . The number of experiments ( $N$ ) is defined by the  
269 Equation 2:

270 
$$N = k^2 + k + cp \quad (\text{Eq. 2})$$

271 where  $k$  represents the number of factors (variables) involved in the study and  $cp$  is the  
272 number of replicates of the central point. Box–Behnken could be seen as a cube,  
273 consisting of a central point and the middle points of the edges.

274 The total number of experiments required for our considered independent variables at  
275 three levels was 10. The values of the selected pair of independent variables were  
276 normalized from -1 to +1 by using Equation 3 in order to facilitate direct comparison of  
277 the coefficients and visualization of the effects of the individual independent variables  
278 on the response variable.

279 
$$X_n = \frac{X - \bar{X}}{(X_{max} - X_{min})/2} \quad (\text{Eq. 3})$$

280 where  $X_n$  is the normalized value of independent variables;  $X$  is the absolute  
281 experimental value of the variable concerned;  $\bar{X}$  is the mean of all fixed values for the  
282 variable in question; and  $X_{max}$  and  $X_{min}$  are the maximum and minimum values of the  
283 variable, respectively.

284 2.2.9. Diclofenac Sodium Release Studies

285 The evaluation of drug release was conducted by ultraviolet-visible (UV-vis)  
286 spectroscopy. UV-vis measurements of DCNa-loaded hydrogels were performed with a

287 Shimadzu UV-2102 PC UV–visible spectrophotometer (Kyoto, Japan). The data were the  
288 result of, at least, three measurements.

289 Prior to the release analyses, a calibration curve of DCNa concentration against  
290 absorbance at 280 nm was made with six DCNa standard solutions (buffered solutions  
291 at pH 5.5). The equation obtained (Equation 4) determines the linear relationship  
292 between UV absorbance ( $A$ , at 280 nm) and DCNa concentration ( $C$ , in mg/mL) in the  
293 range of drug concentration studied (from 100  $\mu\text{g/mL}$  to 12.5  $\mu\text{g/mL}$ ).

$$294 \qquad A = 27.525 C + 0.027 \qquad \text{(Eq. 4)}$$

295 The selected hydrogel (1 g) was transferred to a dialysis tubing cellulose membrane  
296 (molecular weight cut-off: 8,000–14,000 Da), then immersed in 200 mL of phosphate  
297 buffer at pH 5.5 in a round-bottom flask provided with a stirrer bar at 37 °C. At pre-  
298 designed time intervals, aliquots were taken from the release medium and the amount  
299 of DCNa released was determined by UV–vis spectroscopy at 280 nm. One mL of pre-  
300 heated buffer solution was added to the release medium to maintain a constant volume.  
301 Experiments were performed in triplicates.

#### 302 2.2.10. Studies of Hydrogels Morphologies by Scanning Electron Microscopy

303 The morphologies of selected samples were examined by Scanning Electron Microscopy  
304 (SEM) using a field emission scanning electron microscope FEI Teneo. Images were  
305 recorded at an accelerating voltage of 5 kV using secondary electrons. Before SEM  
306 observations, the hydrogel scaffold was directly frozen at -20 °C for 3 h, then at -80 °C  
307 for 24 h [37]. After that, the samples were lyophilized by freeze drying for 24 h. Small  
308 pieces of dry hydrogels were cut by razors, then fixed on aluminum stubs using a double-  
309 sided carbon tape and finally sputter-coated with about 20 nm of gold.

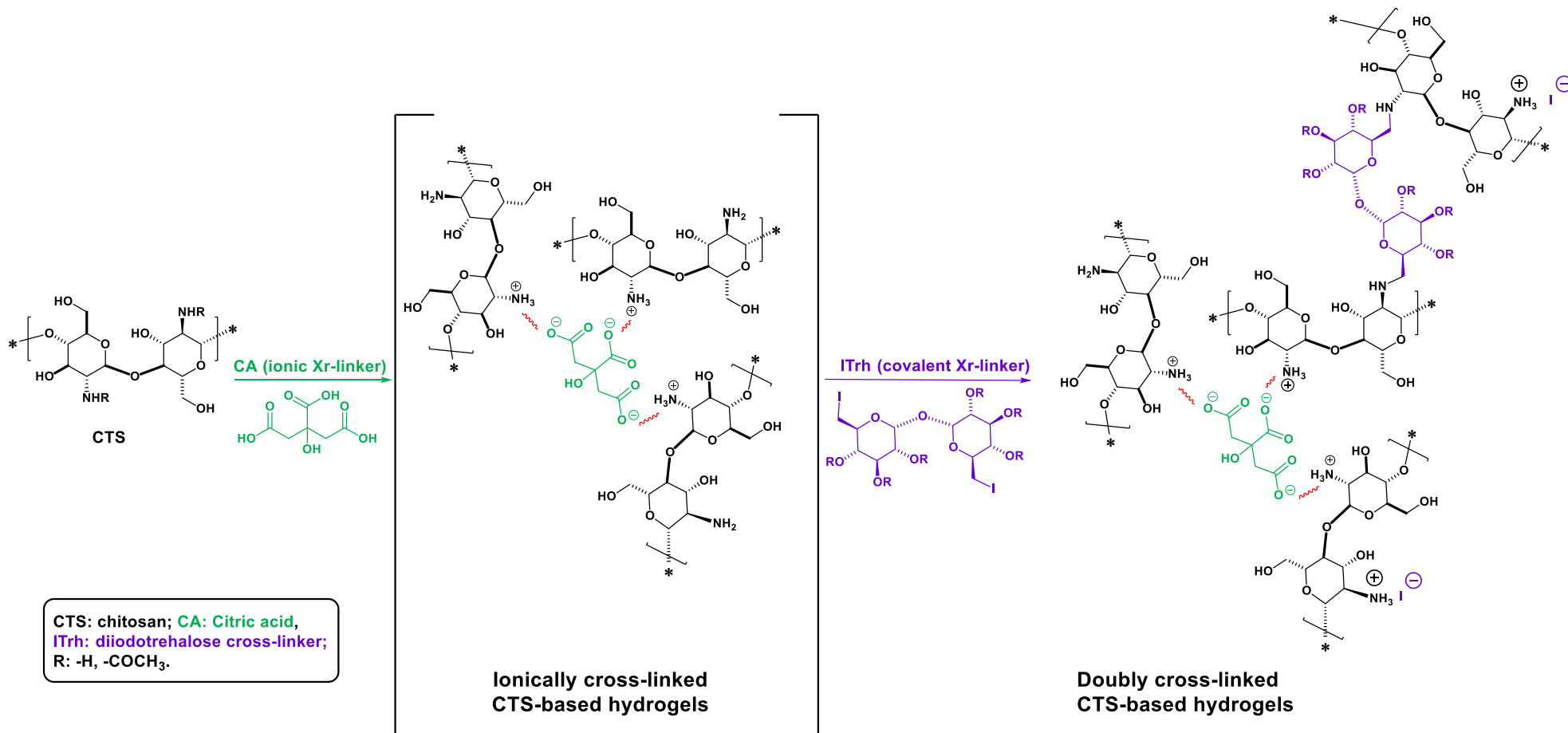
### 310 3. Results and discussion

311 In the present work we focused on the preparation of CTS-based hydrogels by combining  
312 ion-XrL with cov-XrL in order to increase the viscoelastic properties of CTS hydrogels  
313 regarding those recently found for ion-XrL hydrogels [17] and study their impact on the

314 transport of a model anionic API, DCNa. Another parameter to investigate was the effect  
315 of an increment in CTS percentages regarding those tested in the previous study as well  
316 as the use of another ionic cross-linking agent, CA, which could also enhance hydrogen  
317 bonds formation in the hydrogel structure. The impact of this double cross-linking on  
318 the release kinetics of the anionic drug will be addressed.

### 319 3.1. Preparation of Hydrogels from Cross-linked Chitosan (CTS), Citric Acid 320 (CA) and Diiodinated Trehalose (ITrh) (CTS<sub>x</sub>-CA<sub>10</sub>-ITrh<sub>y</sub>)

321 The tricarboxylic ionic cross-linker CA and the electrophile (and covalent cross-linker)  
322 derived from  $\alpha$ -D-trehalose, the 6,6'-dideoxy-2,3,4,2',3',4'-hexa-O-acetyl-6,6'-diiodo- $\alpha$ -  
323 D-glucopyranosyl- $\alpha$ -D-glucopyranoside (ITrh), were used to render a new family of  
324 doubly-cross-linked hydrogels. The ionic cross-linking agent, CA, allowed the  
325 establishment of ionic bonds and hydrogen bonding with the ionized amine groups and  
326 the hydroxyl groups from CTS. The cross-linker ITrh, a disaccharide with hydrolyzable  
327 glycosidic bond in its structure, was used in aqueous media. This cross-linking agent  
328 reacted with the amino groups of the CTS leading to 3D networks, with potential  
329 degradable properties under physiological conditions (Scheme 1).



330

331 **Scheme 1.** Reactions involved in the formation of doubly cross-linked CTS-CA-IThr hydrogels.

332 ITrh was synthesized by a modified procedure from Sizovs *et al.* [35]. Due to the  
333 symmetrical character of the starting disaccharide material  $\alpha,\alpha$ -trehalose, the two  
334 iodine groups at C-6 and C-6' could be considered equivalent, and a double reaction with  
335 amine groups from CTS was likely to occur.

336  $^1\text{H}$  NMR, COSY  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and ESI-MS of the diiodo derivative from  $\alpha$ -D-trehalose  
337 (ITrh) are recorded in Figures S1-S4. Due to the symmetry of the molecule, NMR spectra  
338 —mono and two-dimensional  $^1\text{H},^{13}\text{C}$  and COSY— do not show high complexity. Spectral  
339 analyses confirmed the chemical structure of the cross-linker. Thus, the presence of two  
340 double doublets at *ca.* 3.2 and 3.1 ppm in the  $^1\text{H}$  MNR and COSY spectra, correlated to  
341 the protons from the iodomethylene units of the glucopyranose rings, should be stood  
342 out. It is also noteworthy the presence of a peak at 2.5 ppm in the  $^{13}\text{C}$  NMR spectrum  
343 associated to the mentioned methylene groups. Moreover, the chemical structure of  
344 ITrh was unequivocally confirmed by its Electrospray Ionization (ESI) High Resolution  
345 mass spectrum, with a peak at *m/z* ratio 836.9721, corresponding to  $[\text{M}+\text{Na}]^+$  ion.

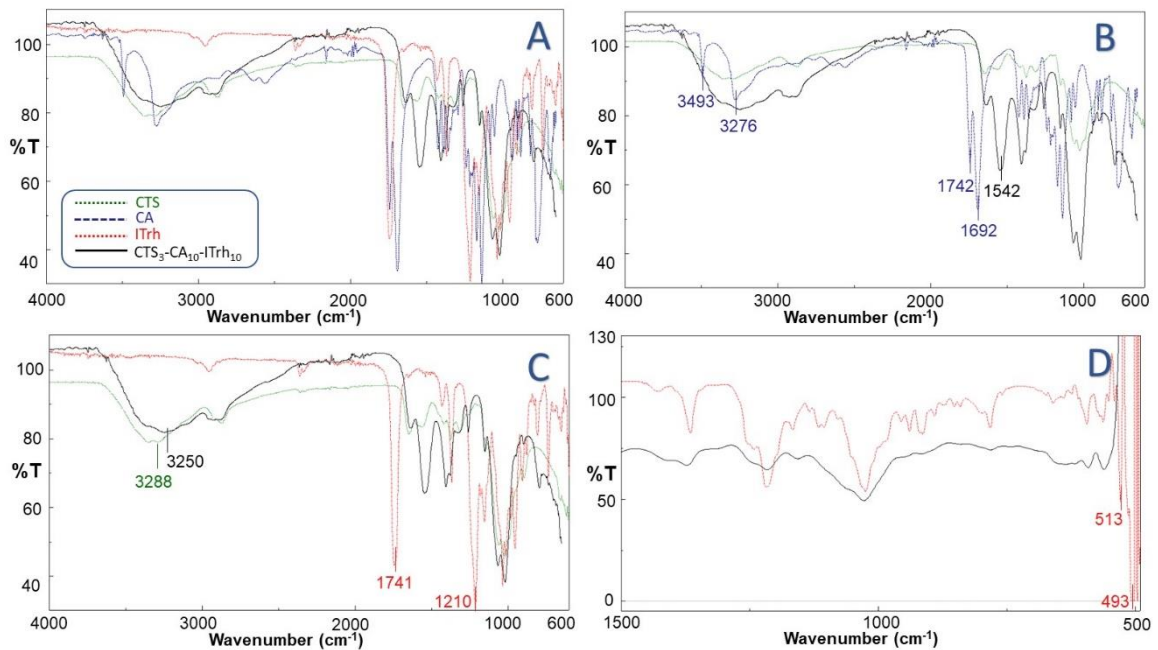
346 To confirm the cross-linking in the hydrogels, FTIR spectra of the starting materials and  
347 the hydrogels were obtained (Figure 2). Figure 2A records the spectra of the polymer  
348 (CTS), the two cross-linkers used (CA and ITrh) and one of the hydrogels formed (CTS<sub>3</sub>-  
349 CA<sub>10</sub>-ITrh<sub>10</sub>). In Figures 2B, 2C and 2D, only selected FTIR spectra and sections were  
350 recorded.

351 The existence of ionic cross-linking interactions in CTS-based hydrogels was firstly  
352 confirmed (Figure 2B). This ionic interaction occurred because of the acid-base reaction  
353 between the carboxylic acid groups from CA and some of the amine groups from CTS. In  
354 the CTS FTIR spectrum, O-H bonds from hydroxyl groups provide a broad band centered  
355 at  $3288\text{ cm}^{-1}$ , which is overlapped with the stretching bands corresponding to the N-H  
356 bonds from amine and amide groups. In addition, several significant bands were of  
357 interest in the FTIR spectrum of CA. The bands at  $3493$  and  $3276\text{ cm}^{-1}$  (sharp and broad  
358 bands, respectively) are mainly due to the stretching of the O-H bonds found in the  
359 carboxylic acid groups of CA. Other two bands ( $1742$  and  $1692\text{ cm}^{-1}$ ), both sharp and  
360 intense, can be correlated to the stretching of C=O bonds present in the two types of

361 carboxylic acid groups of CA. None of these CA bands were present in sample 3  
362 confirming the acid-base reaction mentioned above. In contrast, a new band at 1542  
363  $\text{cm}^{-1}$  (stretching band of C=O in carboxylate ions), has emerged as well as a shift of st  
364 N-H band, (at  $3250 \text{ cm}^{-1}$ ), in this case correlated with the freshly formed ammonium ions  
365 from CTS protonation.

366 From the chemical point of view, the covalent cross-linking took place by a nucleophilic  
367 reaction in which some amine groups of CTS attacked the iodomethylene moieties of  
368 ITrh, giving rise to secondary amino groups and iodide ions (Scheme 1). To detect that  
369 this cross-linking reaction was successfully accomplished, the disappearance of the  
370 stretch band due to the covalent C-I bond in the ITrh cross-linker in the region  
371 comprehended between  $600$  and  $400 \text{ cm}^{-1}$  was investigated. Figure 2D displayed the IR  
372 of sample 3 and ITrh. As can be observed, the bands at  $513$  and  $493 \text{ cm}^{-1}$  associated to  
373 the stretching of C-I bond [38] in unreacted ITrh were not found in the IR spectrum of  
374 sample 3, which is the confirmation that the chemical cross-linking between CTS and  
375 ITrh had taken place. It was also noticed that during the gelling procedure (Figure 2C),  
376 the labile acetate groups from ITrh were hydrolyzed, as confirmed by the disappearance  
377 of the bands at  $1741$  and  $1210 \text{ cm}^{-1}$  (stretching bands of C=O and O-CO bonds,  
378 respectively).

379



380

381 **Figure 2.** FTIR spectra of CTS, the cross-linkers used (CA and ITrh) and one of the prepared  
382 hydrogels freeze-dried (sample 3: CTS<sub>3</sub>-CA<sub>10</sub>-ITrh<sub>10</sub>). The color code for Figures A, B C and D is as  
383 follows: CTS: green; CA: blue; ITrh: red; CTS<sub>3</sub>-CA<sub>10</sub>-ITrh<sub>10</sub> hydrogel: black. Representative bands  
384 have been included.

385 An experimental model design was conducted to study the influence of CTS  
386 concentration and the degree of cov-XrL on the rheological properties of prepared  
387 hydrogels. Ten systems named CTS<sub>x</sub>-CA<sub>10</sub>-ITrh<sub>y</sub>, in which “x” denotes CTS concentration  
388 (% w/w), and “y” denotes the degree of cov-XrL in the hydrogel, were prepared. The  
389 targeted final CTS concentrations ranged from 3% to 5% w/w and the degree of cov-XrL  
390 varied from 0%, to 10%), setting a degree of ion-XrL of 10% for all the trials (Table 1).

### 391 3.2. Thermogravimetric Analyses (TGA)

392 The thermal stability of the freeze-dried hydrogels was evaluated by thermogravimetry  
393 under an inert atmosphere. Characteristic parameters resulting from the experiments  
394 are provided in Table S2. Figure S6 displays the TGA curves of CTS, CA and Sample 3  
395 (CTS<sub>3</sub>-CA<sub>10</sub>-ITrh<sub>10</sub>).

396 The main peak of the thermograms was centered at values close to 300 °C and was  
397 associated with the degradation of the cov-XrL ITrh and CTS backbone due to their

398 structural similarity. The thermo-degradation step centered at 215 °C and characteristic  
399 for the ionic cross-linker CA was not observed in the thermograms of CTS-based  
400 hydrogels. It could be inferred from this fact that CA is structurally integrated into the  
401 hydrogel structures and is exerting its action as cross-linker effectively. This conclusion  
402 is supported by the FTIR results already discussed.

403 On the other hand, and being all the samples highly hydrophilic materials, a weight loss  
404 associated to water content was observed for all the formulations at low and high  
405 temperatures. This was confirmed by the analyses of their thermograms: weight loss vs  
406 temperature and non-reversible heat flow vs temperature plots. For both, CTS starting  
407 material and hydrogel formulations, there was a clear endothermic event with  
408 associated maximum weight loss in the range from 45 °C to 72 °C, probably due to water  
409 molecules slightly attached to hydrogel structures [39]. It is remarkable that, after  
410 experiencing the same freeze-dry procedure, that water content was substantially  
411 higher in the freeze-dried hydrogels than that found in CTS, with increases in *ca.* 67%  
412 (water content in CTS: 9%; water content in freeze-dried hydrogels: from 14 to 17%).

413 Moreover, in the case of hydrogel formulations, a bonus water content (from 7% to 13%)  
414 was sustainably detached when heated to values close to 200 °C with maximum weight  
415 losses at temperatures between 145 °C and 181 °C and depended on the concentration  
416 of CTS: the higher the concentration of CTS, the lower their water content. However,  
417 CTS itself showed no weight loss at temperatures ranging from *ca.* 120 °C to 220 °C. It is  
418 hypothesized that water is firmly retained inside the 3D structure of the cross-linked  
419 materials. Therefore, the new hydrogels possess enhanced water retention capacity  
420 than may be closely related to the presence of more hydrophilic groups, such as -COO<sup>-</sup>,  
421 -COOH, -NH<sub>3</sub><sup>+</sup> and -OH, available as centers to attract water [40].

### 422 3.3. In vitro degradation of CTS<sub>x</sub>-CA<sub>10</sub>-ITRh<sub>y</sub> Hydrogels

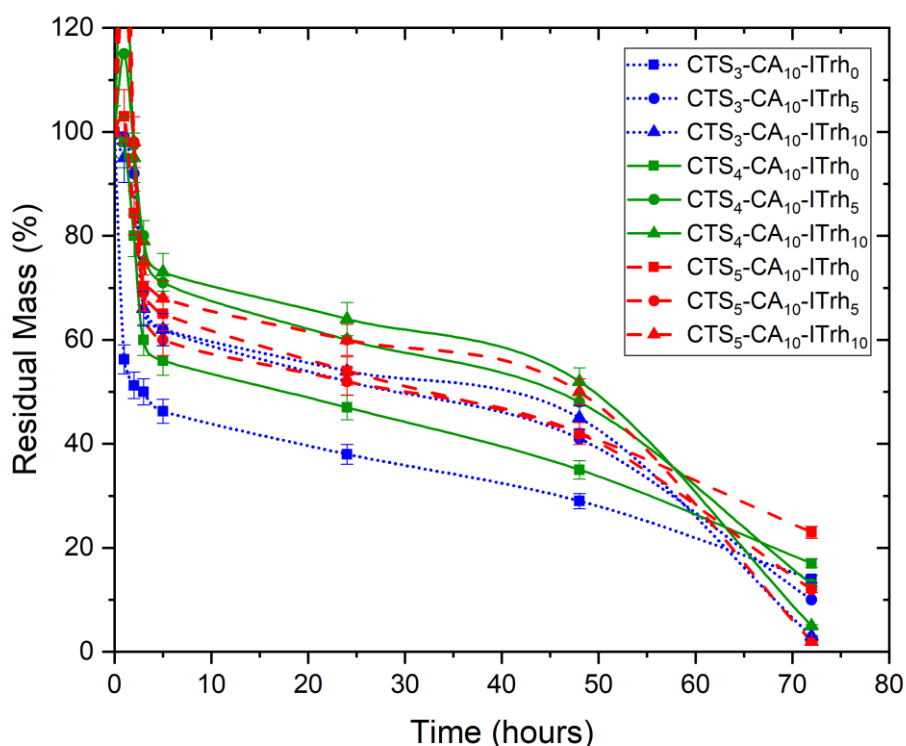
423 In general terms, in vitro degradation of polysaccharide-based hydrogels can occur in  
424 the polymer backbone through a hydrolytic process that usually causes the breakage of  
425 the glycosidic bonds of the polysaccharide chains [41]. In the case of CTS-based  
426 hydrogels, hydrolysis takes place either in an acidic medium [42] or mediated by the  
427 enzyme lysozyme [43]. Other options are breaking the cross-links and thereby releasing

428 the biocompatible polysaccharide. In this work, cross-links are the goal of degradation  
429 experiments.

430 The in vitro degradation of the CTS-CA-ITrh hydrogels focused on the lysis of such  
431 cross-links by hydrolytic procedures —mediated or not by thehalase— in an acidic  
432 microenvironment (pH = 5.7). It was monitored the residual mass percentage of the  
433 matrix as a function of time. The reversibility of ionic cross-linking in aqueous media has  
434 been well established [25] and hence, a straightforward breakdown of the interactions  
435 between CTS and CA was expected. The other cross-linking found in these hydrogels is  
436 the trehalose bridges between the CTS chains that were formed during the gelling. These  
437 bridges are expected to be degraded by the enzyme trehalase, responsible for the  
438 catalytic hydrolysis of the acetal group of trehalose. Under these conditions, certain  
439 degree of hydrolysis of the CTS backbone cannot be ruled out.

440 From the degradation trials, some the samples experienced a water gain in the first few  
441 hours, mainly those with higher CTS content, and then a rapid mass loss was observed  
442 (from 27 to 54%, depending on the system, Figure 3). This effect could be due to the  
443 partial solubilization of the ionic cross-linker in the medium, with the concurrent water  
444 loss, causing a loosening of the hydrogel structure. The slower weight loss rates  
445 observed for higher cross-linked samples were in line with other authors' observations  
446 for covalently cross-linked CTS-based hydrogels [23]. Quasi-plateau regions were  
447 observed in the degradation profiles of covalent-cross-linked hydrogels, suggesting a  
448 slowdown in their degradation patterns. This could be explained by the need for the  
449 enzyme to diffuse into the already eroded hydrogel structure to exert its action.

450 In intermediate stages of the degradation processes, the most cross-linked systems  
451 showed greater hydrolytic stability as demonstrated by Guo and collaborators in the  
452 hydrolysis of injectable hydrogels prepared from chitosan-*graft*-polyalanine and  
453 oxidized dextran as cross-linker [23]. However, as the degradation experiments  
454 progressed, the degradation profiles resembled each other, all being fully disintegrated  
455 within 96 h. Trehalose links have demonstrated to be biodegradable under physiological  
456 conditions.



457

458 **Figure 3.** *In vitro* degradation patterns of CTS-CA-ITrh hydrogels conducted at pH 5.7 and 37 °C  
 459 in the presence of trehalase. The inset shows the successful biodegradation of the samples under  
 460 the experimental conditions. Error bar: standard deviation ( $n = 3$  different samples for each  
 461 formulation)

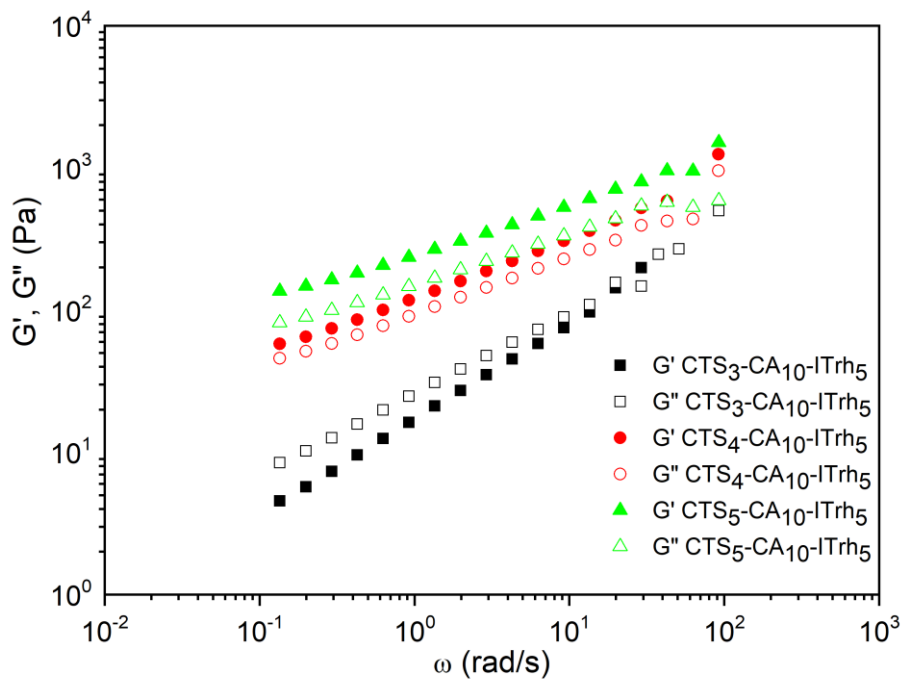
462

463 3.4. Rheological Characterization of CTS<sub>x</sub>-CA<sub>10</sub>-ITRh<sub>y</sub> Hydrogels. Correlations  
 464 of Rheological Parameters with CTS Concentration and Degree of  
 465 Covalent Cross-linking Based on an Experimental Model Design

466 The ten hydrogels prepared were rheologically characterized. The influence of CTS  
 467 concentration and the degree of cov-XrL on significant rheological parameters, such as  
 468 elastic modulus  $G'$ ,  $\tan \delta$ , consistency,  $K$ , and flow,  $n$  indexes were mathematically  
 469 studied by means of Box Behnken experimental designs.

470 Figure 4 shows the evolution of the linear viscoelasticity functions, storage or elastic  
 471 modulus ( $G'$ ) and loss or viscous modulus ( $G''$ ), with frequency, as a function of CTS

472 concentration for samples with ionic degree XrL of 10% and cov-XrL of 5%. As can be  
 473 observed, it was apparent that an increase in CTS concentration yielded larger figures  
 474 for the linear viscoelasticity functions as has been demonstrated for other CTS-based  
 475 hydrogels [20]. The  $G''$  values were higher than those found for  $G'$  at low CTS  
 476 concentration and a tendency to reach a crossover point between these functions was  
 477 obtained at high frequencies. On the other hand,  $G'$  values were higher than  $G''$  ones at  
 478 higher CTS concentrations throughout the studied frequency range.



479  
 480 **Figure 4.** Frequency dependence of the storage,  $G'$ , and loss,  $G''$ , moduli at 25°C, in the linear  
 481 viscoelasticity region, for hydrogels as a function of CTS concentration.

482 Aiming to quantify the dependence on CTS concentration and covalent cross-linked  
 483 degree, a power-law equation (Equation 5) has been used to describe the evolution of  
 484 the storage modulus,  $G'$ , with frequency:

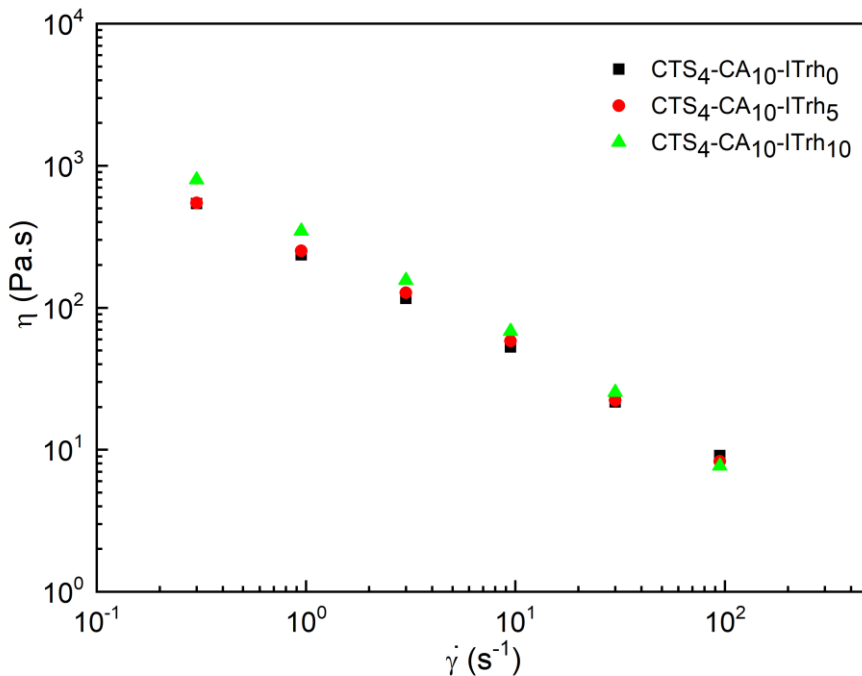
$$485 \quad G' = G'_1 \times \omega^m \quad (\text{Eq. 5})$$

486 where  $G'_1$  and  $m$  are fitting parameters.

487 Figure 5 illustrates the viscous flow behavior exhibited by selected hydrogels as a  
 488 function of the degree of cov-XrL. A shear-thinning behavior was apparent in all samples,  
 489 which could be fitted well to the power-law model (Equation 6):

490 
$$\eta = K \cdot \dot{\gamma}^{n-1} \quad (\text{Eq. 6})$$

491 where  $K$  and  $n$  are the consistency and flow indexes, respectively. The values of fitting  
 492 parameters are shown in Table 1. As can be observed an increase in cov-XrL yielded  
 493 higher viscosity values.



494  
 495 **Figure 5.** Viscous flow curves for hydrogels, at 25°C, as a function of covalent cross-linked  
 496 degree.

497 A Box–Behnken experimental design was used to evaluate the significance of these  
 498 independent variables (CTS concentration and degree of cov-XrL) related to the  
 499 rheological parameters recorded in Table 1. In general, for the dependent parameters  
 500 evaluated in the intervals studied, a greater influence of CTS concentration was  
 501 observed with respect to that found for the degree of cov-XrL.

502 In Table 2 the obtained equations by using polynomial regression and the calculated  
 503 statistics parameters ( $R^2$ ,  $d_f$  and  $F$ ) are shown. In this sense, a suitable ( $>0.97$ )  $R^2$  and  
 504 ( $>35$ )  $F$  values have been found in equations.

505 **Table 2.** Equations yielded for each dependent variable as a function of the independent  
 506 variables (normalized values) for the Box-Behnken experimental design.

Equations	$R^2$	$d_f$	$F$
$\tan \delta = 0.838 - 0.41 [CTS] - 0.146 Xr + 0.218 [CTS]^2 - 0.08 Xr^2 + 0.055 [CTS] Xr$	0.994	5.4	146.32
$G'_1 (Pa) = 110.1 + 179.79 [CTS] + 113.8 Xr + 109.55 Xr^2 + 99.78 [CTS] Xr$	0.972	5.4	35.96
$m = 0.427 - 0.155 [CTS] - 0.0433 Xr + 0.064 [CTS]^2$	0.999	3.6	351.08
$K (Pa s^n) = 251.49 + 202.81 [CTS] + 7.87 Xr^2 - 5.58 [CTS] Xr$	0.999	3.6	712.65
$n = 0.30 - 0.1566 [CTS] + 0.03 [CTS]^2 - 0.0125 [CTS] Xr$	0.990	3.6	423.68

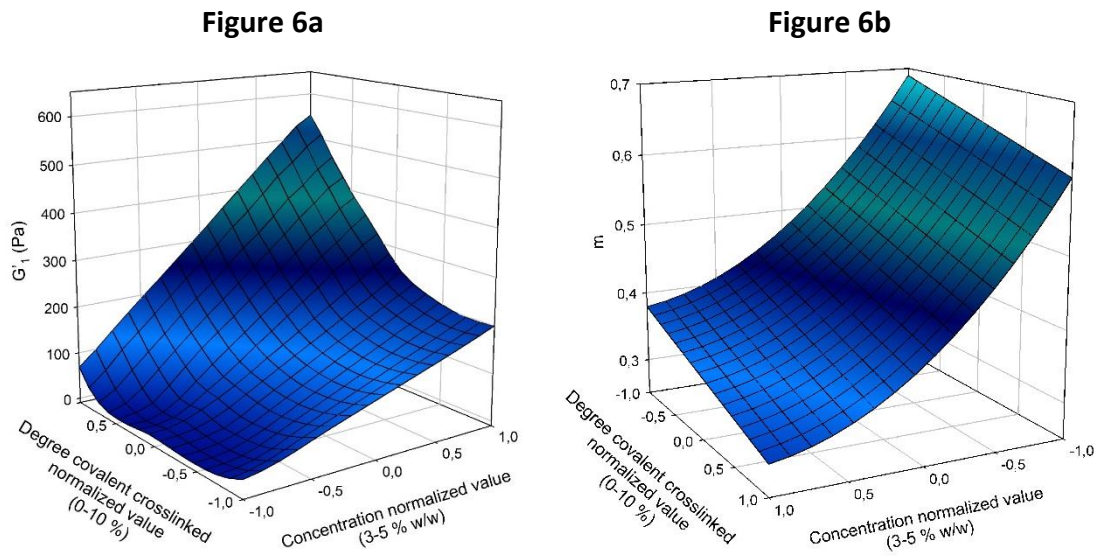
[CTS] = CTS concentration (% w/w), normalized value;  $Xr$  = degree of covalent cross-linking, normalized value;  $\tan \delta$  = loss tangent (at 1 rad/s);  $G'_1$  = storage modulus;  $m$  = power-law index;  $K (Pa s)$  = consistency index;  $n$  = flow index

507 To facilitate the identification, that on the selected dependent variable the independent  
 508 variables are applied, the response surfaces for each dependent variable are shown in  
 509 Figures 6, 7, S7a and S7b. In a set of hydrogels formed from chitosan-*graft*-polyaniline  
 510 copolymer cross-linked with oxidized dextran, an increase in storage modulus was  
 511 observed with the degree of cross-linking [23], similar to what was found for the  
 512 hydrogels from this study, as shown below.

513 Figure 6a allows estimating the variation of  $G'_1$ , with respect to cov-XrL and CTS  
 514 concentration, over the range considered. These variations in  $G'_1$  were more  
 515 pronounced at the highest figures of the independent variables, *i.e.*, at 5% of CTS and  
 516 10% of cov-XrL. Moreover,  $G'_1$  was more sensitive to changes in polymer concentration  
 517 than to the other independent variable. Thus, to obtain hydrogels with high  $G'_1$ , it is  
 518 advisable to operate with high degrees of cov—XrL and high CTS concentrations. On the  
 519 contrary, the lowest values were found at medium-to-low degrees of cov-XrL and low

520 CTS concentrations. No significant  $G'_1$  figures have been found at low concentrations.  
521 These low  $G'_1$  data were independent of the degree of cov-XrL.

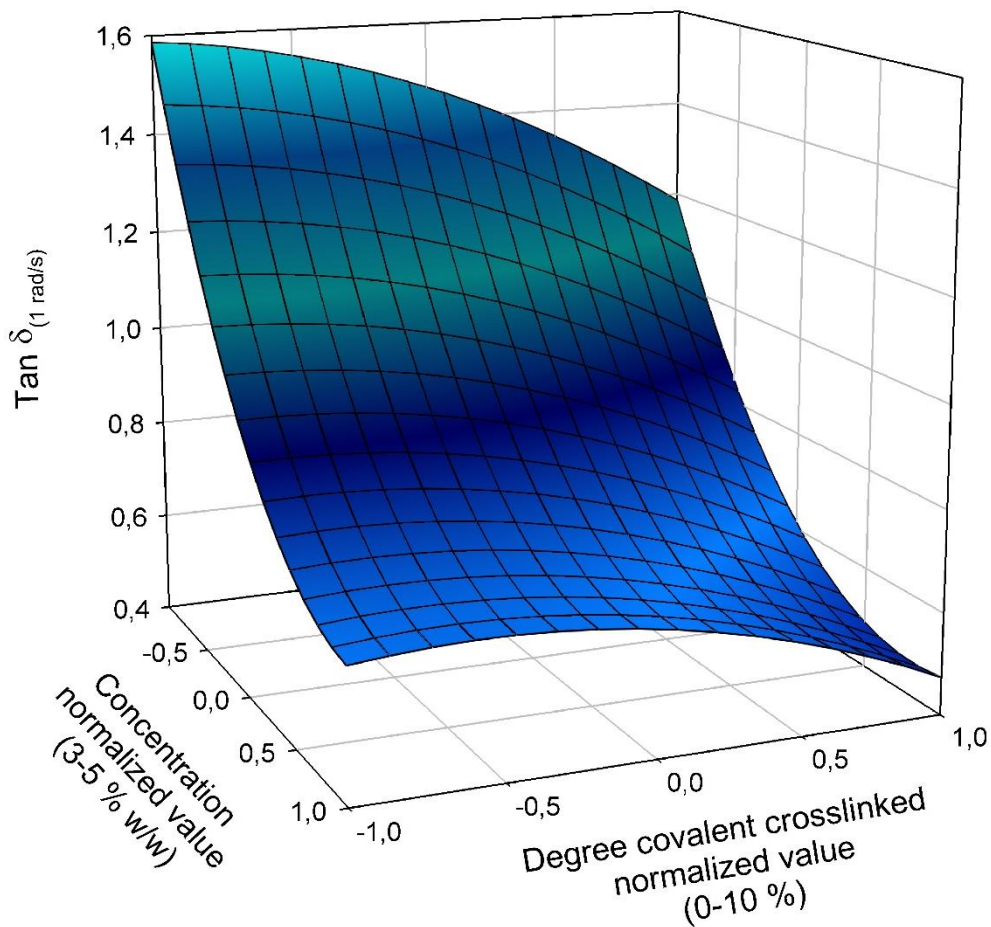
522 Similar to the findings regarding  $G'_1$ , the percentage of cov-XrL displayed lower statistical  
523 influence in the power-law index ( $m$ ) (Figure 6b) than that exerted by CTS rates.  
524 Moreover, a similar negative (lowered values) statistical influence for cov-XrL was found  
525 under high and low CTS concentrations. In this way, the efficient selection of the  
526 parameters studied entails the use of high CTS concentration and a high degree of  
527 cov-XrL to obtain higher  $m$  values, fact that seems to indicate a more developed and  
528 complex hydrogel microstructure.



529 **Figure 6.** Response surface models obtained from the Box–Behnken experimental design to  
530 evaluate the relative influence of the independent variables (CTS concentration and degree of  
531 cov-XrL) on the rheological parameters  $G'_1$  (Fig. 6a) and  $m$  (Fig. 6b).

532 The response surface for the loss tangent ( $\tan \delta = G''/G'$ ) is recorded in Figure 7 to  
533 explore the relationships between the independent variables mentioned above and this  
534 response variable. Thus, the statistical influences exerted by cov-XrL and CTS  
535 concentration followed a similar trend as in the dependent parameters evaluated so far  
536 ( $G'_1$ ,  $m$ ). Thus, in order to achieve the lowest values for  $\tan \delta$ , the use of high CTS

537 concentrations and cov-XrL is advisable, indicating that, under these conditions, the  
538 relative elastic properties of the hydrogels may increase.



539

540 **Figure 7.** Response surface model obtained from the Box–Behnken experimental design to  
541 evaluate the relative influence of the independent variables (CTS concentration and degree of  
542 cov-XrL) on the rheological parameter  $\tan \delta$  at 1 rad/s.

543 The consistency index,  $K$  (Figure S7a) showed a very low statistical dependence on  
544 cov-XrL under the studied conditions. Conversely, and regarding polymer concentration,  
545 a positive (increasing) statistical correlation was observed, with  $K$  growing almost  
546 linearly with this parameter and hence, the highest values of  $K$  were found at high CTS  
547 concentrations. These results may be explained taking into account that, the structural  
548 network of hydrogels became more compact as CTS concentration increased due to the  
549 increase in hydrogen bond formation [17], far exceeding the influence that the other

550 variable can exert. This is also the trend found for flow index ( $n$ ) (Figure S7b). Hence, to  
551 obtain the lowest values for  $n$ , high CTS concentrations should be used.

552 Therefore, it was concluded that, to achieve hydrogels with maximum elastic properties,  
553 the use of high CTS concentrations and high degree of cov-XrL were advisable.

### 554 3.5. Diclofenac Sodium Loaded Formulations from Cross-linked Chitosan- 555 Conjugates and Studies of Drug Release

556 In general terms, and due to the ionizable amino groups present in the polymer  
557 backbone, CTS-based hydrogels behave as pH-sensitive DDS, a fact that has been  
558 demonstrated in numerous examples such as the release of DOX [8,24], amoxicillin and  
559 ibuprofen [23], 5-fluorouracil and diclofenac sodium (DNa) [22] from stabilized CTS-  
560 based hydrogels.

561 Values of pH from 5.5 to 7.5 are within the so-called physiological pH figures in human  
562 beings; thus, slightly acidic microenvironments are found in mucous membranes and  
563 other topical areas. However, maintaining acid-base balance is critical for the survival of  
564 living species since cellular processes are highly sensitive to changes in proton  
565 concentrations. Although in humans, pH varies within a narrow range (in the blood  
566 between pH 7.35 and 7.45), local deviations from the systemic pH are often caused by  
567 pathologies, such as cancer, inflammation, infection, ischemia, renal failure or  
568 pulmonary disease [44]. Therefore, drug delivery systems capable of releasing the active  
569 pharmaceutical ingredient at acidic pH, such as the formulations studied herein, could  
570 find significant applications in a wide range of pathologies and locations.

571 Sodium diclofenac (DCNa) is one of the most frequently used non-steroidal anti-  
572 inflammatory drugs (NSAID) used to treat pain and inflammatory diseases [45]. Because  
573 of the short half-life in plasma (1–2 h) and associated adverse effects [46], it is regarded  
574 as an ideal model drug for controlled delivery system [22]. DCNa-loaded hydrogels may  
575 have potential applications in the treatment of inflammatory bowel disease (IBD),  
576 pathologies of great impact in developed countries [26]. For that use, they may release  
577 the drug at the acidic pH typical of inflamed areas and even the small intestine (pH 5-

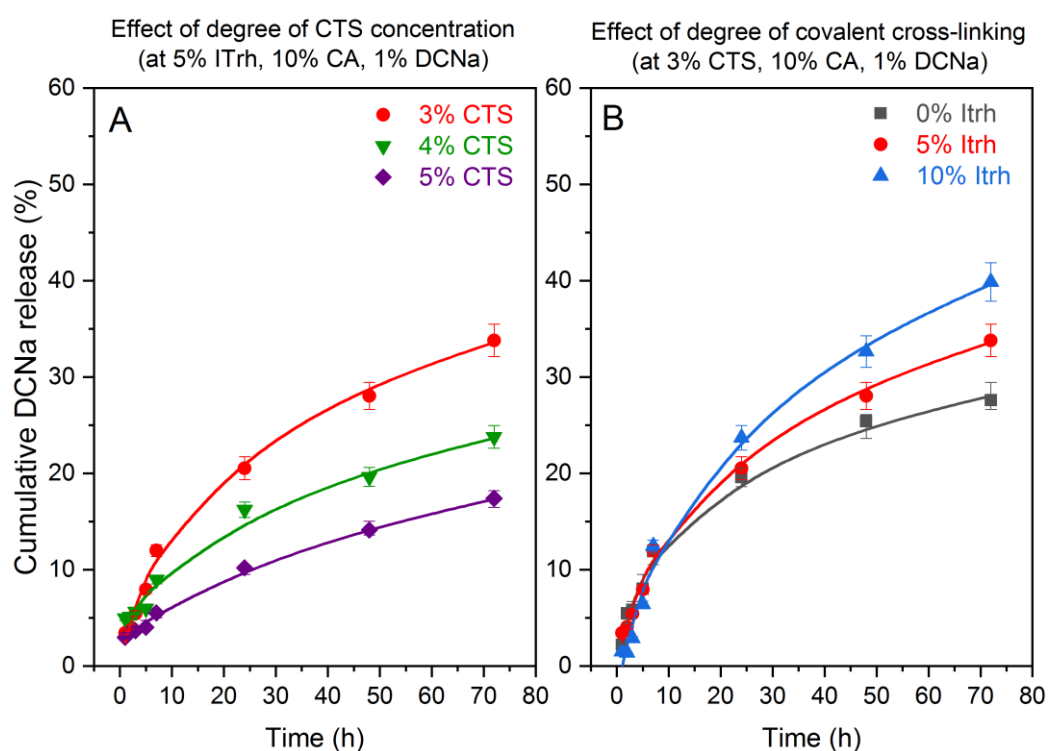
578 7.5) [47–49], and therefore, the release studies of the prepared formulations have been  
579 conducted at pH 5.5.

580 As in the study for the rheological properties, ten drug-loaded hydrogel formulations  
581 named DCNa-CTS<sub>x</sub>-ITrh<sub>y</sub> were prepared and are recorded in Table S3. Several examples  
582 of formulations of DCNa and CTS have been published, mainly beads, microparticles and  
583 hydrogels. For example, the preparation of DCNa-loaded CTS microspheres by double  
584 physical emulsification have been described [45] as well as ionically cross-linked  
585 DCNa-loaded CTS-based beads was prepared using sodium polyphosphate as a cross-  
586 linker. In this case, controlled drug release was confirmed at pH 7.2 and was depended  
587 on several formulation factors, such as CTS concentration, drug-polymer ratio and  
588 percentage of Tween 80 [50]. The role of silica matrices in DDS has also been explored.  
589 DCNa-loaded silica-CTS composites—some of them cross-linked with glutaraldehyde—  
590 have been developed to achieve efficient sustained drug-release systems. In this case,  
591 the protonated chitosan spheres, were the systems that better controlled the release of  
592 DCNa [51]. Regarding gelling systems, Zhang *et al.* prepared DCNa-loaded hydrogels  
593 based on carboxymethyl chitosan-*graft*-poly (*N*-isopropyl acrylamide)-glycidyl  
594 methacrylate by UV cross-linking. Their findings highlighted that the drug release  
595 kinetics depended on pH and temperature. However, the degradability of the networks  
596 was not proved for these systems [22]. We have previously investigated the formation  
597 of DCNa-loaded ionotropic CTS-based hydrogels in which the drug release and the  
598 rheological properties of the systems were strongly dependent on the formulation [17].  
599 Unfortunately, the consistency of the prepared matrices ranged from liquid-like to  
600 viscoelastic gels. With the aim to improve the physical properties of the materials, in the  
601 present study the concentration of CTS has been increased, an additional covalent cross-  
602 linking introduced, and the ionic cross-linker replaced by a more hydrophilic molecule.

603 In order to check the release of the DCNa-loaded hydrogels, a calibration curve of the  
604 drug was made with DCNa standard solutions at 280 nm [17]. From the samples studied,  
605 it was observed that all the hydrogels were able to control the DCNa release for long  
606 periods at 37 °C in phosphate buffer at pH 5.5 (Table S3, Figures 8a and 8b). The drug  
607 was released in a slow and sustained manner, ranging from 17% to 40% after 72 h. These

608 enhanced retention figures found for the described DCNa formulations were in  
609 concordance with the expected results since the anionic drug could be ionically  
610 anchored to the cationic CTS-based hydrogels [7].

611 The influence of two factors, CTS concentration and the degree of cov-XrL, on the  
612 release profiles was studied. When the role played by CTS concentration on DCNa  
613 release was investigated, the other variables, degree of ionic XrL and cov-XrL were set  
614 at 10%, and 5% respectively. The results are recorded in Figure 8a.



615

616 **Figure 8.** *In vitro* release profiles of diclofenac sodium (DCNa) from CTS hydrogels in phosphate  
617 buffer solution at pH 5.5 at 37 °C. Data were obtained from UV-Vis spectroscopy at 280 nm and  
618 reported as mean  $\pm$  S.D from three independent experiments. (Fig. 8A) Effect of CTS  
619 concentration — from 3% to 5% CTS concentration — in drug release (fixed paraments: degree  
620 of covalent cross-linking 5%; degree of ionic cross-linking 10%; DCNa concentration 1%). (Fig. 8B)  
621 Effect of degree of covalent cross-linking — from non-cross-linked samples to 10% of cross-linked  
622 — in drug release (fixed paraments: CTS concentration 3%; ionic cross-linking 10%; DCNa  
623 concentration 1%).

624 It was noted that the greatest changes in DCNa release were found among those  
625 formulations with the largest differences in the starting-hydrogels rheological behaviors.  
626 For example, for the DCNa-CTS<sub>5</sub>-ITrh<sub>5</sub> sample, the DCNa released after 72 h was only the  
627 17% of the drug present in the formulation. This could be due to the more developed  
628 and complex hydrogel microstructures characteristic of the prepared dispersions with  
629 the highest CTS content, as the previous rheological studies had demonstrated. When  
630 comparing these findings with the drug release from the DCNa-CTS<sub>3</sub>-ITrh<sub>5</sub> sample, it was  
631 observed that the percentage of drug delivered by the latter was two-fold the figure of  
632 DCNa released from the DCNa-CTS<sub>5</sub>-ITrh<sub>5</sub> sample. Consequently, the higher the CTS  
633 concentration in the hydrogels, the lower the drug release kinetics. The same trend was  
634 reported for disulfide-cross-linked CTS-based hydrogels [7].

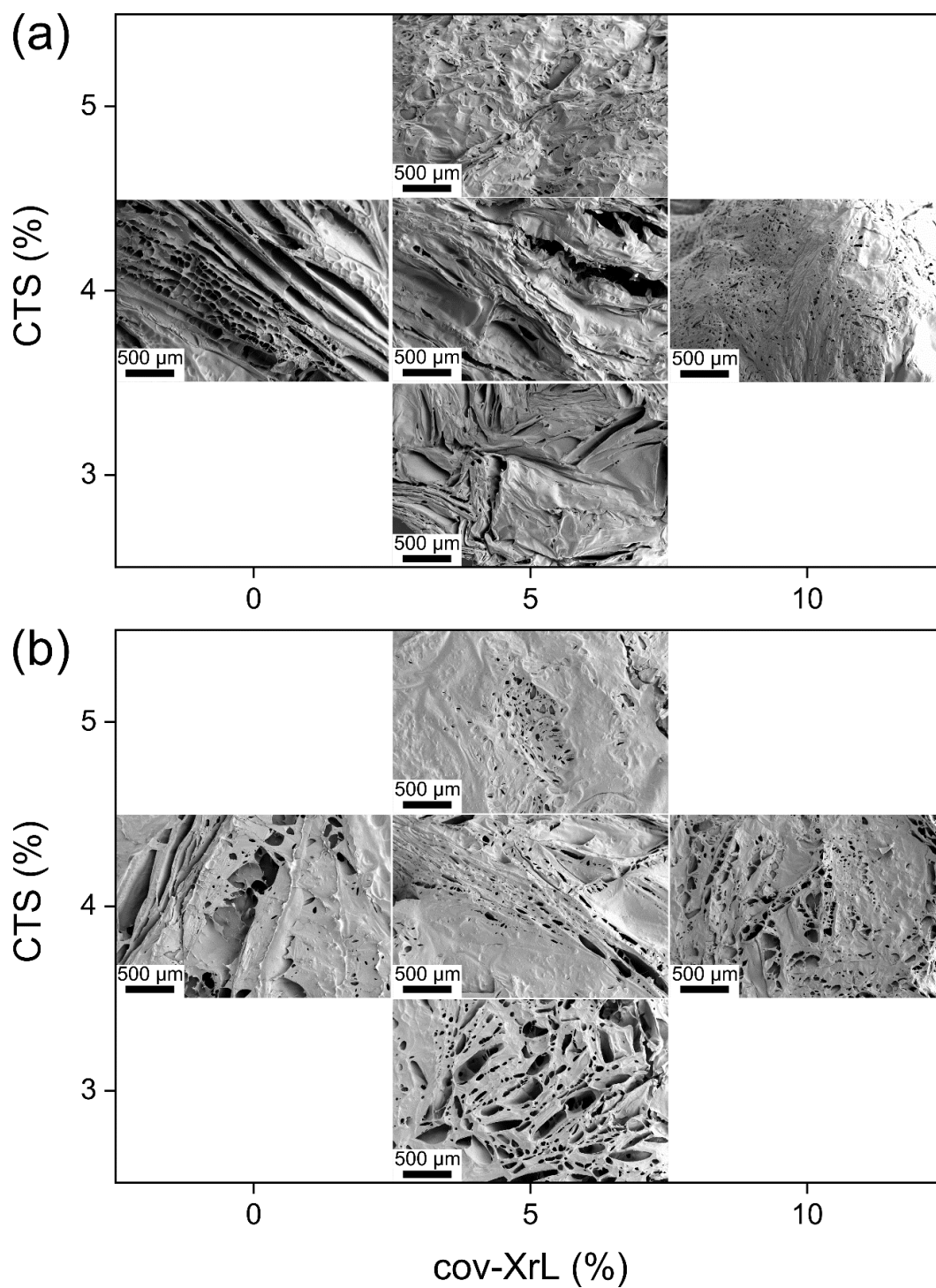
635 Figure 8b shows the release kinetics from selected drug-loaded hydrogels in order to  
636 study the impact of cov-XrL on drug release. On the formulations linked to the displayed  
637 curves, the degree of ionic XrL was set at 10% for all samples, the percentage of CTS at  
638 3% and the concentration of the drug in the formulations at 1%. Although initially  
639 surprising, and being the other parameters constant, drug release was enhanced in the  
640 most cross-linked systems (cov-XrL: 10%) leading to a boost in drug release. For example,  
641 the percentage of DCNa delivered (after 72 h) were 28% and 40% for samples DCNa-  
642 CTS<sub>3</sub>-ITrt<sub>0</sub> and DCNa-CTS<sub>3</sub>-ITrt<sub>10</sub>, respectively. This assessment related to the influence  
643 of cov-XrL in cumulative drug release was accurate for all the formulations studied  
644 regardless CTS percentage (Table S3). This trend has also been observed for the release  
645 of amoxicillin from CTS-oxidized dextrin hydrogels. Other authors reported that when  
646 the cross-linking density of the hydrogels increased, the rate of drug release also  
647 augmented. They explained this observation based on the fact that hydrogels with  
648 higher cross-linking density would have swelled less, thus causing a higher concentration  
649 gradient of the drug. [23] Another hypothesis could be that either the bulky trehalose  
650 units between the CTS chains or the volatile solvent used in the preparation of the  
651 formulations [52] had behaved as a porogen and may have altered, to some extent, the  
652 regular packaging of the CTS in the material. This outcome could have generated  
653 interconnected pores that allowed the penetration of water molecules into the  
654 matrices, modifying the overall drug release rates due to the generation of drug release

655 channels [53]. Therefore, the higher the degree of cov-XrL, the greater the free volume  
656 found in the matrices, and hence, the faster the drug would diffuse into the medium.  
657 This assumption was supported by the SEM images found for DCNa-loaded hydrogels at  
658 4% of CTS.

659 These findings suggested that the prepared hydrogels can not only act as DDS but can  
660 also be used to tune the rate of drug release by changing the degree of cross-linking [8],  
661 and/or by modifying the polymer concentration. It is anticipated that the release of  
662 non-ionic drugs will show a faster kinetics than those exhibited by the anionic drug  
663 DCNa, as it was the case in disulfide-cross-linked CTS-based hydrogels [7].

### 664 3.6. Scanning Electron Microscopy (SEM) Studies

665 The SEM micrographs displayed in Figures 9a and 9b show the scaffolds of CTS-CA-ITrh  
666 and DCNa-CTS-ITrh hydrogels (before and after DCNa loading, respectively) at different  
667 CTS concentrations and degrees of cov-XrL. As can be observed in Figure 9a, CTS-CA-ITrh  
668 hydrogels prepared with  $CTS \leq 4\%$  and  $XrL \leq 5\%$  displayed scaffolds with well-defined  
669 lamellar structures. At higher CTS contents, such structures tended to disappear in favor  
670 of more compact scaffolding. When the degree of cov-XrL reached 10%, matrices  
671 containing small pores were found. Similarly to other findings previously reported by us  
672 [17], the presence of DCNa caused a change in the morphologies of the hydrogels (Figure  
673 9b) and led to more porous microstructures than their non-loaded counterparts. The  
674 role of DCNa as a porogen in the hydrogels is hypothesized. For DCNa-CTS-ITrh  
675 hydrogels, the lower the percentages of CTS, the greater and larger the number of pores  
676 found. These observations are consistent with both the rheological properties of the  
677 precursor hydrogels. Evidence of larger number of interconnected pores at low CTS  
678 percentages also supports the faster DCNa release found for DCNa-CTS<sub>3</sub>-ITrh  
679 formulations. It has been demonstrated that interconnected pores may significantly  
680 reduce the diffusion length for drug release and the volume fraction of polymer [42].  
681 Thus, DCNa-loaded hydrogels prepared with the highest CTS concentration exhibited a  
682 developed and complex hydrogel microstructure, responsible for the retention of the  
683 anionic drug in the gel-like matrices. The release studies confirmed that the increase in  
684 the percentage of CTS decreased the rate of drug release in all the samples studied.



685

686 **Figure 9.** SEM micrographs showing the scaffolds evolution of (a) CTS-CA-ITrh and (b) DCNa-  
 687 CTS-ITrh hydrogels at different CTS concentrations and degrees of cov-XrL. For comparison  
 688 purpose, all the images were recorded at the same magnification.

689 To sum up, all drug-loaded hydrogels displayed a controlled and steadily DCNa release  
 690 at 37 °C with cumulative release figures ranging from 17% to 40% after 72 h. To what

691 extent the drug release occurred was strongly dependent on the composition of the  
692 formulation.

693 In despite of the relevant morphological changes observed by SEM in DCNa-loaded and  
694 non-loaded hydrogels, it was clear that the ionic interactions established between the  
695 anionic drug and the cationic matrices was a relevant parameter regarding drug release.  
696 The high percentage of CTS in the systems contributed to a more densely 3D ionic  
697 anchoring of DCNa, decelerating the drug release. It is anticipated that the release of  
698 non-ionic drugs will show faster kinetics than those exhibited by anionic APIs.

#### 699 4. Conclusions

700 The preparation of a new family of eco-friendly and biodegradable chitosan-based  
701 hydrogels have been successfully achieved by means of ionic and covalent cross-linking  
702 (ion-XrL and cov-XrL). The novel hydrogels completely disintegrated within 96 h by  
703 means of a hydrolysis process mediated by the enzyme trehalase. As far as the authors  
704 are aware, this is the first time that a trehalose derivative has been used as a covalent  
705 cross-linker in the formation of biodegradable hydrogels, converging the improved  
706 physical properties of stable hydrogels with the inherent degradability of ionotropic  
707 hydrogels.

708 Through an experimental model design, the influence of two parameters —polymer  
709 concentration and degree of cov-XrL— on hydrogel rheological properties was disclosed,  
710 being the former the most influential variable. Hydrogels with maximum elastic  
711 properties were achieved at the highest CTS concentrations and degrees of cov-XrL.  
712 These moduli agreed with the scaffold morphologies found by SEM micrographs in  
713 which the hydrogels prepared with lower CTS content and lower degree of cov-XrL  
714 showed well-defined lamellar structures, whereas at higher CTS and cov-XrL, such  
715 structures evolved to more compact scaffolding.

716 Ten DCNa-containing formulations with different polymer concentration, and degree of  
717 cov-XrL were evaluated regarding their release profiles. They displayed well-controlled  
718 drug-release patterns that were strongly dependent on the formulation composition,

719 with cumulative drug release varying from 17% to 40% for 72 h under physiological  
720 conditions. Systems with improved viscoelastic properties exhibited the lowest rates of  
721 drug release. Surprisingly, a boost in drug release was found in those formulations with  
722 the highest levels of covalent cross-linking. This trend could be extrapolated to any CTS  
723 concentration value.

724 In summary, the preparation method of the CTS-based drug formulations presented  
725 herein provides a simple and straightforward pathway to design tailor-made controlled  
726 drug delivery systems with improved rheological properties. Their degradability bears  
727 real relevance to their potential use in biomedical applications. We anticipate that these  
728 systems can be readily adapted to achieve effective encapsulation of other APIs of  
729 interest for sustained release.

## 730 Acknowledgments

731 The authors would like to thank *El Ministerio de Ciencia, Innovación y Universidades*  
732 (MICINN) of Spain (Grant MAT2016-77345-C3-2-P), and *La Junta de Andalucía* (Grant  
733 P12-FPM-1553) for their financial support.

## 734 References

- 735 [1] P.M. Favi, R.S. Benson, N.R. Neilsen, R.L. Hammonds, C.C. Bates, C.P. Stephens,  
736 M.S. Dhar, Cell proliferation, viability, and in vitro differentiation of equine  
737 mesenchymal stem cells seeded on bacterial cellulose hydrogel scaffolds,  
738 *Materials Science and Engineering C*. 33 (2013) 1935–1944.  
739 doi:10.1016/j.msec.2012.12.100.
- 740 [2] L. Liu, Q. Gao, X. Lu, H. Zhou, In situ forming hydrogels based on chitosan for drug  
741 delivery and tissue regeneration, *Asian Journal of Pharmaceutical Sciences*. 11  
742 (2016) 673–683. doi:10.1016/j.ajps.2016.07.001.
- 743 [3] N. Annabi, A. Tamayol, J.A. Uquillas, M. Akbari, L.E. Bertassoni, C. Cha, G. Camci-  
744 Unal, M.R. Dokmeci, N.A. Peppas, A. Khademhosseini, 25th anniversary article:  
745 Rational design and applications of hydrogels in regenerative medicine, *Advanced*

- 746 Materials. 26 (2014) 85–124. doi:10.1002/adma.201303233.
- 747 [4] M.A. Amin, I.T. Abdel-Raheem, Accelerated wound healing and anti-inflammatory  
748 effects of physically cross linked polyvinyl alcohol-chitosan hydrogel containing  
749 honey bee venom in diabetic rats, Archives of Pharmacal Research. 37 (2014)  
750 1016–1031. doi:10.1007/s12272-013-0308-y.
- 751 [5] Y. Liu, S. Hsu, Synthesis and Biomedical Applications of self-healing hydrogels,  
752 Frontiers in Chemistry. 6 (2018) 449(1–10). doi:10.3389/fchem.2018.00449.
- 753 [6] N. Bhattarai, J. Gunn, M. Zhang, Chitosan-based hydrogels for controlled,  
754 localized drug delivery, Advanced Drug Delivery Reviews. 62 (2010) 83–99.  
755 doi:10.1016/j.addr.2009.07.019.
- 756 [7] M.J. Lucero, C. Ferris, C.A. Sanchez-Gutierrez, M.R. Jimenez-Castellanos, M.-V. de-  
757 Paz, Novel aqueous chitosan-based dispersions as efficient drug delivery systems  
758 for topical use. Rheological, textural and release studies., Carbohydrate Polymers.  
759 151 (2016) 692–699. doi:10.1016/j.carbpol.2016.06.006.
- 760 [8] Y. Liang, X. Zhao, P.X. Ma, B. Guo, Y. Du, X. Han, pH-responsive injectable  
761 hydrogels with mucosal adhesiveness based on chitosan-grafted-dihydrocaffeic  
762 acid and oxidized pullulan for localized drug delivery, Journal of Colloid and  
763 Interface Science. 536 (2019) 224–234. doi:10.1016/j.jcis.2018.10.056.
- 764 [9] W.E. Hennink, C.F. van Nostrum, Novel crosslinking methods to design hydrogels,  
765 Advanced Drug Delivery Reviews. 64 (2012) 223–236.  
766 doi:10.1016/j.addr.2012.09.009.
- 767 [10] S.A. Shah, M. Sohail, M.U. Minhas, Nisar-ur-Rehman, S. Khan, Z. Hussain,  
768 Mudassir, A. Mahmood, M. Kousar, A. Mahmood, pH-responsive CAP-co-  
769 poly(methacrylic acid)-based hydrogel as an efficient platform for controlled  
770 gastrointestinal delivery: fabrication, characterization, in vitro and in vivo toxicity  
771 evaluation, Drug Delivery and Translational Research. 9 (2019) 555–577.  
772 doi:10.1007/s13346-018-0486-8.
- 773 [11] S.K. Shukla, A.K. Mishra, O. a. Arotiba, B.B. Mamba, Chitosan-based  
774 nanomaterials: A state-of-the-art review, International Journal of Biological  
775 Macromolecules. 59 (2013) 46–58. doi:10.1016/j.ijbiomac.2013.04.043.

- 776 [12] D. Enescu, C.E. Olteanu, Functionalized Chitosan and Its Use in Pharmaceutical,  
777 Biomedical, and Biotechnological Research, Chemical Engineering  
778 Communications. 195 (2008) 1269–1291. doi:10.1080/00986440801958808.
- 779 [13] B. Qu, Y. Luo, Chitosan-based hydrogel beads: Preparations, modifications and  
780 applications in food and agriculture sectors – A review, International Journal of  
781 Biological Macromolecules. 152 (2020) 437–448.  
782 doi:10.1016/j.ijbiomac.2020.02.240.
- 783 [14] A. Rafique, K.M. Zia, M. Zuber, S. Tabasum, Chitosan functionalized poly (vinyl  
784 alcohol) for prospects biomedical and industrial applications: A review,  
785 International Journal of Biological Macromolecules. 87 (2016) 141–154.  
786 doi:10.1016/j.ijbiomac.2016.02.035.
- 787 [15] R. Vivek, R. Thangam, V. Nipunbabu, T. Ponraj, S. Kannan, Oxaliplatin-chitosan  
788 nanoparticles induced intrinsic apoptotic signaling pathway: A “smart” drug  
789 delivery system to breast cancer cell therapy, International Journal of Biological  
790 Macromolecules. 65 (2014) 289–297. doi:10.1016/j.ijbiomac.2014.01.054.
- 791 [16] Y. Xu, J. Han, H. Lin, Fabrication and characterization of a self-crosslinking chitosan  
792 hydrogel under mild conditions without the use of strong bases, Carbohydrate  
793 Polymers. 156 (2017) 372–379. doi:10.1016/j.carbpol.2016.09.046.
- 794 [17] N. Iglesias, E. Galbis, C. Valencia, M.-V. De-Paz, J.A. Galbis, Reversible pH-sensitive  
795 chitosan-based hydrogels. Influence of dispersion composition on rheological  
796 properties and sustained drug delivery, Polymers. 10 (2018) 1–17.  
797 doi:10.3390/polym10040392.
- 798 [18] A. Chenite, C. Chaput, D. Wang, C. Combes, M.. Buschmann, C.. Hoemann, J..  
799 Leroux, B.. Atkinson, F. Binette, A. Selmani, Novel injectable neutral solutions of  
800 chitosan form biodegradable gels in situ, Biomaterials. 21 (2000) 2155–2161.  
801 doi:10.1016/S0142-9612(00)00116-2.
- 802 [19] Y. Xu, C. Zhan, L. Fan, L. Wang, H. Zheng, Preparation of dual crosslinked alginate-  
803 chitosan blend gel beads and in vitro controlled release in oral site-specific drug  
804 delivery system, International Journal of Pharmaceutics. 336 (2007) 329–337.  
805 doi:10.1016/j.ijpharm.2006.12.019.

- 806 [20] C. Ferris, M. Casas, M.J. Lucero, M. V de Paz, M.R. Jimenez-Castellanos, Synthesis  
807 and characterization of a novel chitosan-N-acetyl-homocysteine thiolactone  
808 polymer using MES buffer., *Carbohydrate Polymers*. 111 (2014) 125–132.  
809 doi:10.1016/j.carbpol.2014.03.078.
- 810 [21] M. Dash, F. Chiellini, R.M. Ottenbrite, E. Chiellini, Chitosan - A versatile semi-  
811 synthetic polymer in biomedical applications, *Progress in Polymer Science*  
812 (Oxford). 36 (2011) 981–1014. doi:10.1016/j.progpolymsci.2011.02.001.
- 813 [22] L. Zhang, L. Wang, B. Guo, P.X. Ma, Cytocompatible injectable carboxymethyl  
814 chitosan/N-isopropylacrylamide hydrogels for localized drug delivery,  
815 *Carbohydrate Polymers*. 103 (2014) 110–118. doi:10.1016/j.carbpol.2013.12.017.
- 816 [23] J. Qu, X. Zhao, P.X. Ma, B. Guo, Injectable antibacterial conductive hydrogels with  
817 dual response to an electric field and pH for localized “smart” drug release, *Acta*  
818 *Biomaterialia*. 72 (2018) 55–69. doi:10.1016/j.actbio.2018.03.018.
- 819 [24] J. Qu, X. Zhao, P.X. Ma, B. Guo, pH-responsive self-healing injectable hydrogel  
820 based on N-carboxyethyl chitosan for hepatocellular carcinoma therapy, *Acta*  
821 *Biomaterialia*. 58 (2017) 168–180. doi:10.1016/j.actbio.2017.06.001.
- 822 [25] R. Dong, Y. Pang, Y. Su, X. Zhu, Supramolecular hydrogels: synthesis, properties  
823 and their biomedical applications, *Biomaterials Science*. 3 (2015) 937–954.  
824 doi:10.1039/c4bm00448e.
- 825 [26] N. Iglesias, E. Galbis, M.J. Díaz-Blanco, R. Lucas, E. Benito, M.V. De-Paz,  
826 Nanostructured Chitosan-based biomaterials for sustained and colon-specific  
827 resveratrol release, *International Journal of Molecular Sciences*. 20 (2019) 398 (1–  
828 16). doi:10.3390/ijms20020398.
- 829 [27] J. Zhao, X. Zhao, B. Guo, P.X. Ma, Multifunctional interpenetrating polymer  
830 network hydrogels based on methacrylated alginate for the delivery of small  
831 molecule drugs and sustained release of protein, *Biomacromolecules*. 15 (2014)  
832 3246–3252. doi:10.1021/bm5006257.
- 833 [28] J. Berger, M. Reist, J.M. Mayer, O. Felt, N.A. Peppas, R. Gurny, Structure and  
834 interactions in covalently and ionically crosslinked chitosan hydrogels for  
835 biomedical applications, *European Journal of Pharmaceutics and*

- 836 Biopharmaceutics. 57 (2004) 19–34. doi:10.1016/S0939-6411(03)00161-9.
- 837 [29] L. Zhuang, X. Zhi, B. Du, S. Yuan, Preparation of Elastic and Antibacterial Chitosan-  
838 Citric Membranes with High Oxygen Barrier Ability by in Situ Cross-Linking, ACS  
839 Omega. 5 (2020) 1086–1097. doi:10.1021/acsomega.9b03206.
- 840 [30] D. Bao, M. Chen, H. Wang, J. Wang, C. Liu, R. Sun, Preparation and  
841 characterization of double crosslinked hydrogel films from  
842 carboxymethylchitosan and carboxymethylcellulose, Carbohydrate Polymers.  
843 110 (2014) 113–120. doi:10.1016/j.carbpol.2014.03.095.
- 844 [31] D. Ailincăi, L. Marin, S. Morariu, M. Mares, A.C. Bostanaru, M. Pinteala, B.C.  
845 Simionescu, M. Barboiu, Dual crosslinked iminoboronate-chitosan hydrogels with  
846 strong antifungal activity against Candida planktonic yeasts and biofilms,  
847 Carbohydrate Polymers. 152 (2016) 306–316. doi:10.1016/j.carbpol.2016.07.007.
- 848 [32] S. Doppalapudi, A. Jain, W. Khan, A.J. Domb, Biodegradable polymers-an  
849 overview, Polymers for Advanced Technologies. 25 (2014) 427–435.  
850 doi:10.1002/pat.3305.
- 851 [33] T. Sun, Y.S. Zhang, B. Pang, D.C. Hyun, M. Yang, Y. Xia, Engineered nanoparticles  
852 for drug delivery in cancer therapy, Angewandte Chemie - International Edition.  
853 53 (2014) 12320–12364. doi:10.1002/anie.201403036.
- 854 [34] N. Zhang, H. Zhang, R. Li, Y. Xing, Preparation and adsorption properties of citrate-  
855 crosslinked chitosan salt microspheres by microwave assisted method,  
856 International Journal of Biological Macromolecules. 152 (2020) 1146–1156.  
857 doi:10.1016/j.ijbiomac.2019.10.203.
- 858 [35] A. Sizovs, L. Xue, Z.P. Tolstyka, N.P. Ingle, Y. Wu, M. Cortez, T.M. Reineke,  
859 Poly(trehalose): Sugar-Coated Nanocomplexes Promote Stabilization and  
860 Effective Polyplex-Mediated siRNA Delivery, Journal of the American Chemical  
861 Society. 135 (2013) 15417–15424. doi:10.1021/ja404941p.
- 862 [36] W. Liu, C. Deng, C.R. McLaughlin, P. Fagerholm, N.S. Lagali, B. Heyne, J.C. Scaiano,  
863 M.A. Watsky, Y. Kato, R. Munger, N. Shinozaki, F. Li, M. Griffith, Collagen-  
864 phosphorylcholine interpenetrating network hydrogels as corneal substitutes,  
865 Biomaterials. 30 (2009) 1551–1559. doi:10.1016/j.biomaterials.2008.11.022.

- 866 [37] B. Palma Santana, F. Nedel, C. Perelló Ferrúa, R. Marques e Silva, A. Fernandes da  
867 Silva, N.L. Fernando Demarco, Flávio Villarreal Carreño, Comparing different  
868 methods to fix and to dehydrate cells on alginate hydrogel scaffolds using  
869 scanning electron microscopy, *Microscopy Research and Technique*. 78 (2015)  
870 553–561. doi:10.1002/jemt.22508.
- 871 [38] R.H. Schuler, The effect of iodine on the infrared spectra of the alkyl iodides, *The*  
872 *Journal of Chemical Physics*. 22 (1954) 947. doi:10.1063/1.1740227.
- 873 [39] S. Iravani, C.S. Fitchett, D.M.R. Georget, Physical characterization of arabinoxylan  
874 powder and its hydrogel containing a methyl xanthine, *Carbohydrate Polymers*.  
875 85 (2011) 201–207. doi:10.1016/j.carbpol.2011.02.017.
- 876 [40] H.A. Essawy, M.B.M. Ghazy, F.A. El-Hai, M.F. Mohamed, Superabsorbent  
877 hydrogels via graft polymerization of acrylic acid from chitosan-cellulose hybrid  
878 and their potential in controlled release of soil nutrients, *International Journal of*  
879 *Biological Macromolecules*. 89 (2016) 144–151.  
880 doi:10.1016/j.ijbiomac.2016.04.071.
- 881 [41] M. Naveed, L. Phil, M. Sohail, M. Hasnat, M.M.F.A. Baig, A.U. Ihsan, M. Shumzaid,  
882 M.U. Kakar, T. Mehmood Khan, M.D. Akabar, M.I. Hussain, Q.G. Zhou, Chitosan  
883 oligosaccharide (COS): An overview, *International Journal of Biological*  
884 *Macromolecules*. 129 (2019) 827–843. doi:10.1016/j.ijbiomac.2019.01.192.
- 885 [42] J. Li, D.J. Mooney, Designing hydrogels for controlled drug delivery, *Nature*  
886 *Reviews Materials*. 1 (2016) 16071 (1–38). doi:10.1038/natrevmats.2016.71.
- 887 [43] Y. Hong, H. Song, Y. Gong, Z. Mao, C. Gao, J. Shen, Covalently crosslinked chitosan  
888 hydrogel: Properties of in vitro degradation and chondrocyte encapsulation, *Acta*  
889 *Biomaterialia*. 3 (2007) 23–31. doi:10.1016/j.actbio.2006.06.007.
- 890 [44] S. Düwel, C. Hundshammer, M. Gersch, B. Feuerecker, K. Steiger, A. Buck, A.  
891 Walch, A. Haase, S.J. Glaser, M. Schwaiger, F. Schilling, Imaging of pH in vivo using  
892 hyperpolarized <sup>13</sup>C-labelled zymonic acid, *Nature Communications*. 8 (2017)  
893 15126. doi:10.1038/ncomms15126.
- 894 [45] S. Dreve, I. Kacso, A. Popa, O. Raita, F. Dragan, A. Bende, G. Borodi, I. Bratu,  
895 Structural investigation of chitosan-based microspheres with some anti-

- 896 inflammatory drugs, *Journal of Molecular Structure*. 997 (2011) 78–86.  
897 doi:10.1016/j.molstruc.2011.05.001.
- 898 [46] J. Schwaiger, H. Ferling, U. Mallow, H. Wintermayr, R.D. Negele, Toxic effects of  
899 the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological  
900 alterations and bioaccumulation in rainbow trout, *Aquatic Toxicology*. 68 (2004)  
901 141–150. doi:https://doi.org/10.1016/j.aquatox.2004.03.014.
- 902 [47] E. Gökbulut, İ. Vural, M. Aşıkoğlu, N. Özdemir, Floating drug delivery system of  
903 itraconazole: Formulation, in vitro and in vivo studies, *Journal of Drug Delivery  
904 Science and Technology*. 49 (2019) 491–501. doi:10.1016/j.jddst.2018.12.019.
- 905 [48] M. Goud, V. Pandey, Gastroretentive drug delivery system, *International Journal  
906 of Pharma and Bio Sciences*. 6 (2016) 158–165.
- 907 [49] R.J. Xavier, D.K. Podolsky, Unravelling the pathogenesis of inflammatory bowel  
908 disease, *Nature*. 448 (2007) 427–434. doi:10.1038/nature06005.
- 909 [50] B.S.M. Zadeh, F. Moshtaghi, F. Rahim, A. Akhgari, Preparation and evaluation of  
910 sodium diclofenac loaded chitosan controlled release microparticles using  
911 factorial design, *International Journal of Drug Development and Research*. 2  
912 (2010) 468–475.
- 913 [51] R.B. Kozakevych, Y.M. Bolbukh, V.A. Tertykh, Controlled Release of Diclofenac  
914 Sodium from Silica-Chitosan Composites, *World Journal of Nano Science and  
915 Engineering*. 3 (2013) 69–78. doi:10.4236/wjnse.2013.33010.
- 916 [52] L. Xinming, C. Yingde, A.W. Lloyd, S. V. Mikhalovsky, S.R. Sandeman, C.A. Howel,  
917 L. Liewen, Polymeric hydrogels for novel contact lens-based ophthalmic drug  
918 delivery systems: A review, *Contact Lens and Anterior Eye*. 31 (2008) 57–64.  
919 doi:10.1016/j.clae.2007.09.002.
- 920 [53] Y. Zhang, X.T. Zhang, Q. Zhang, B. Wang, T. Zhang, Formulation development and  
921 evaluation of gastroretentive floating beads with *Brucea javanica* oil using  
922 ionotropic gelation technology, *Chinese Journal of Natural Medicines*. 16 (2018)  
923 293–301. doi:10.1016/S1875-5364(18)30059-1.

924