Macromolecular Materials and Engineering
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Influence of Natural Crosslinkers on Chitosan Hydrogels for Potential Biomedical Applications

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Chitosan (CH) is a very well-known biopolymer that has been widely used for the development of biomaterials with a wide range of applications in the biomedical field, such as the preparation of hydrogels, owing to its outstanding anti-inflammatory, antibacterial and antifungal properties, biocompatibility and biodegradability, although they present limited mechanical properties. Chemical crosslinking is one of the most recurrent strategies for the reinforcement of these structures and, above all, crosslinking with natural-origin compounds that do not compromise their biocompatibility is considered a hot topic in this research field. D-fructose (F), obtained from the hydrolyzation and further isomerization of starch, an abundant raw material and genipin (G), which is extracted from the fruits of *Gardenia jasminoides Ellis* are used as natural crosslinkers. Chitosan-based hydrogels crosslinked with each crosslinking agent are prepared and characterized through Fourier transform infrared (FTIR) spectroscopy, crosslinking and swelling degree determination, rheological, microstructural, and biological studies. The results demonstrate that crosslinking with G is more beneficial for chitosan-based hydrogels since these samples showed more compact structures and better rheological performance. Additionally, excellent biological in vitro behavior due to the crosslinking with G, unlike that of F.

1. Introduction

In recent decades, humanity has witnessed significant advances within the field of biomedicine, where multiple investigations have promoted the development of materials and treatments that continuously improve previous alternatives and solve some of the problems raised in research areas, such as controlled wound healing, drug delivery, and tissue engineering, among others.[1] For the satisfactory fulfillment of the requirements of these applications, hydrogels have attained striking attention due to their semisolid phase and inherent flexibility, as well as their great absorption capacity, which makes them capable of absorbing water over thousands of times their dry weight of water without losing their structural integrity.[2,3] Due to this excellent water content, hydrogels are quite friendly to water-rich biological environments, especially in human tissues.[4] These hydrogels also excel among other alternatives due to many interesting properties, such as their mechanical performance, excellent biocompatibility, adhesion, degradation, self-healing, and environment responsivity, which can be adjusted and controlled by their composition and/or preparation method.[5,6] In fact, chitosan (CH) gels at a pH over 6.5, alginate can form gels with calcium ions and gelatin can be dissolved at high temperatures (>50 °C), subsequently gelling after cooling to temperatures close to 0 °C.[7–9]

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mame.202300195

DOI: 10.1002/mame.202300195

Regarding the materials used, it is expected that, in the near future, research in the field of hydrogels for biomedical applications will be focused on the exploitation of biopolymers obtained through environmentally friendly methodologies that minimize the waste of resources.[10] Among them, CH is one of the best-known biopolymers used for the development of hydrogels, mainly due to its structure and versatility. It is composed of repetitive units of D-glucosamine and *N*-acetyl-D-glucosamine connected by β-(1,4) linkages.[11] CH is obtained from the partial deacetylation of chitin, which is the most abundant natural amino polysaccharide in the world, by strong alkali treatment at high temperatures.[11–13] These features grant CH remarkable properties, such as biocompatibility, biodegradability, mucoadhesiveness, as well as its anti-inflammatory, antibacterial, and antifungal properties.[14,15] However, while it is true that CH is one of the best candidates for hydrogel formation, its mechanical properties may not be sufficient for applications that require higher tensile strength or better heat resistance.[15,16]

To overcome these limitations, one of the most recurrent strategies is the crosslinking of hydrogels.[17,18] Hydrogels can be classified as chemically or physically crosslinked, depending on their intermolecular interactions. Therefore, the so-called "chemical" or "permanent" are those that make up covalently crosslinked networks. On the other hand "physical" or "reversible" hydrogels are those whose crosslinked structures are based on hydrogen bonds, hydrophobic, or ionic interactions.[1,19,20] In this sense, selecting appropriate crosslinking strategies and crosslinkers is very important to obtain chitosan-based hydrogels with enhanced mechanical properties and expand their application in the biomedical field.[21–23] Therefore, the chemical crosslinking method can be carried out using either naturally or synthetically derived crosslinkers to form strong covalent bonds. Synthetically derived chemical crosslinkers have the limitation of the possibility of inducing cytotoxicity. In addition, there is a considerable risk involving the unreacted crosslinkers, which might remain inside the scaffolds, causing biocompatibility issues.[24,25] For this reason, the employment of natural crosslinkers is an increasing trend. The main advantages of natural crosslinkers are their low cost in most cases and their lack of cytotoxicity, although most crosslinkers are not as efficient as most synthetic crosslinkers. The most frequently used natural crosslinkers are genipin, enzymes, citric and tannic acid, and some sugars such as glucose, sucrose, and fructose.[24,26,27]

Genipin (G) is the most frequently used natural crosslinker, despite its high cost, for the development of crosslinked biomaterials, due to its outstanding biocompatibility, biodegradability, and stability of the resulting crosslinked products.[26,28] Hydrolysis with β-glucosidase of geniposide isolated from the fruits of *Gardenia jasminoides Ellis* produces the monoterpenoid genipin (methyl 1-hydroxy-7-(hydroxymethyl)−1,4a,5,7a-tetrahydrocyclopenta [c]pyran-4-carboxylate). Compared to other chemical crosslinkers, G is significantly less toxic.[29,30] The resulting crosslinked complexes are not cytotoxic for the animal and human cells so far examined. The safety and beneficial actions of genipin emerge from a number of research projects in the areas of therapies for diabetes, periodontitis, cataract, hepatic dysfunction, as well as in wound repair and nerve regeneration.[31] It is widely accepted that G is characterized by its high selectivity, as it can only react with primary amine groups rather than secondary or tertiary ones,[28] making it interesting for crosslinking reactions with proteins or CH. Particularly, G can form bifunctional crosslinks with CH molecules, obtaining blue-colored, fluorescent hydrogels.[32] Thus, two separate reactions lead to the formation of crosslinks with the primary amine groups of CH, as shown in **Figure 1**. The fastest and, thereby, first reaction to occur is the nucleophilic attack of the G C3 carbon atom from a primary amine group that leads to the formation of a heterocyclic compound of G linked to the glucosamine residue of CH. The second reaction is the nucleophilic substitution of the ester group of G to release methanol and form a secondary amide link with CH. Acid catalysis is necessary for the reactions to occur and it has been proved to take place in the same way with gelatin and bovine serum albumin.[32,33] In addition, G exhibits biocompatibility, biodegradability, and low cytotoxicity that is ≈10 000 times lower than glutaraldehyde. Besides, the ability of the cells to multiply after contact with genipin is 5000 times higher than glutaraldehyde. G also gives rise to materials with increased mechanical properties and a better swelling capacity, while the degree of crosslinking of the hydrogels can be regulated by changing the pH value of the reaction medium.[34]

On the other hand, fructose is a well-known carbohydrate present in different vegetables, fruits, and honey. It is an isomer of glucose, which can be obtained through hydrolyzation of the starch extracted from different cereals using microbial enzymes on glucose. Through an isomerization process, it becomes fructose, specifically D-fructose (F).[35–37] Reducing sugars can undergo chemical crosslinking and D-fructose was selected on the basis that among the natural crosslinking agents based on sugar molecules, it presents a higher degree of crosslinking due to a higher degree in the Maillard reaction that takes place.[35,38,39] The Maillard reaction, also called nonenzymatic browning, is a mild reaction of amino groups in the presence of the carbonyl groups of sugars.[40,41] Therefore, nucleophilic amino groups of CH react with carbonyl groups of the reducing saccharides, forming a Schiff base.[42,43] After Schiff base formation, two possible rearrangements can occur depending on the reducing sugar. The Amadori rearrangement engages a reaction between an aldehyde group, such as glucose and the amino group of CH. In contrast, the Heyns rearrangement follows the same pattern but engages a ketose, such as fructose (Figure 1), while generating two possible epimers.[43] Thus, CH can be modified by the Maillard reaction with sugars, thereby changing its physical and biological properties.[40]

The objective of this work was to comparatively evaluate the effect of two well-known natural crosslinkers, namely G and F, on the structure and both the rheological and biological properties of chitosan. To this end, different amounts of both crosslinkers were added to assess the effect of this variable on the crosslinking degree, which is a fundamental property that has a great impact on the target properties. Therefore, each resulting hydrogel was characterized to determine its microstructure, as well as its rheological and biological performance. The comparison of the obtained results of the crosslinked hydrogels with each other and between each crosslinker represents the novelty of this article.

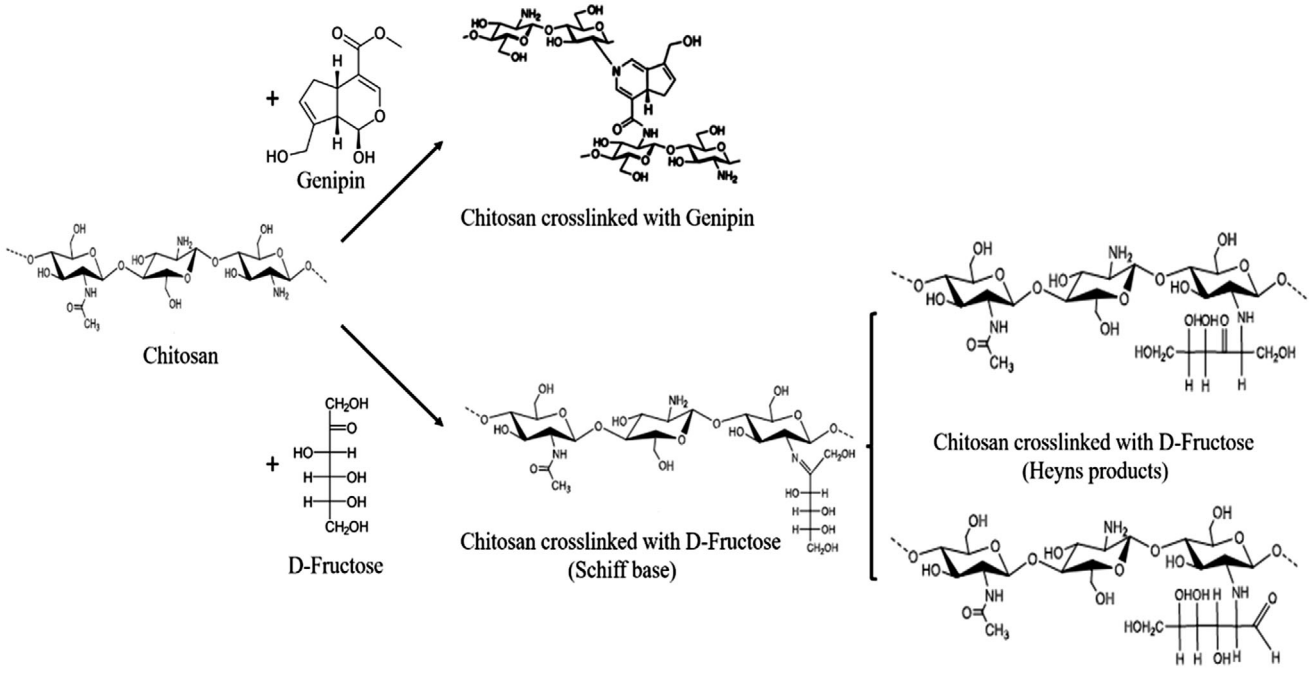

Figure 1. Schematic representation of the reactions between genipin and fructose with chitosan.

2. Experimental Section

2.1. Materials

CH (M_W = 130 000 g mol^{-1}; deacetylation degree = 75–85%) was provided by Sigma-Aldrich (Darmstadt, Germany). Acetic acid was employed as a solvent in a 0.05 M solution (pH = 3.2). Additionally, 4 M sodium hydroxide (NaOH) spray solution was used to increase the pH of the solutions during the preparation process. These reagents were provided from Panreac Química S.A. (Barcelona, Spain). F (≥99%) and G (>98%, extracted from *Gardenia jasminoides*) were used as crosslinkers; they were supplied by Sigma-Aldrich S.A. (Darmstadt, Germany) and Guangxi Shanyun Biochemical Science and Technology Co. (Liuzhou, China), respectively.

2.2. Hydrogels Preparation

The different hydrogels were prepared following the protocol indicated in previous work,[44] with the addition of the two compounds that performed as crosslinking agents. Specifically, in this process, the biopolymer and the crosslinkers were primarily dissolved with agitation at 50 °C to improve chain mobility and favour the interconnection of the biopolymeric chains,[45] as well as to guarantee a better interaction with the crosslinking agents, consequently attaining a better polymerization [46] with a theoretical improvement of the final properties.[1,2] In brief, 20 mL of 1.5 wt% CH solutions were prepared using 0.05 M acetic acid. The crosslinking agents (F and G) were also added in different concentrations (0.5, 1, and 2 wt% with respect to the total amount of biopolymer) to study the influence of the crosslinker amount on the crosslinking degree and, consequently, on the properties of the resulting hydrogels. Subsequently, the solutions were magnetically stirred at 50 °C for 1 h. Afterward, the solutions were subjected to a neutralization stage, increasing pH from 3.2 to 7 by adding 4 M NaOH with a sprayer. Finally, the samples were kept in a refrigerator at 4 °C for 24 h to enhance gelation. To facilitate the reading of the codes of each hydrogel, **Table 1** lists all those used throughout this study.

2.3. Hydrogels Characterization

2.3.1. Chemical Characterization

The chemical bonds formed in each crosslinking reaction were analysed by Fourier-transform infrared spectroscopy (FTIR) using a Hyperion 1000 spectrophotometer (Bruker, Santa Clara, CA). For this analysis, hydrogels were freeze-dried (<15 Pa for 24 h, LyoQuest, TELSTAR, Barcelona, Spain). Afterward,

Table 1. Codification of the crosslinked chitosan hydrogels with 0.5, 1, and 2 wt% of D-fructose and genipin.

Crosslinker	Crosslinker amount [wt%]	Codification
None	0	CH
Fructose	0.5	CH F 0.5
	1.0	CH F 1
	2.0	CH F 2
Genipin	0.5	CH G 0.5
	1.0	CH G 1
	2.0	CH G 2

samples were introduced in an ATR diamond sensor to obtain their corresponding infrared profile, between 4500 and 600 cm^{-1} with an opening of 4 cm^{-1} and an acquisition of 200 scans. Baseline correction was performed by measuring without the sample.

2.3.2. Crosslinking Degree

The degree of crosslinking was assessed slightly modifying the procedure of Huber et al.[47] Thus, 1 mL of ninhydrin reagent (consisting of 75 mL of dimethyl sulfoxide, 300 mg of hydrindantine, 2 g of ninhydrin, and 25 mL of a 4 M sodium acetate solution) was added to 1 mg of the freeze-dried hydrogels. The reaction mixture was incubated for 20 min in boiling water and subsequently cooled down on ice bath to room temperature. Before the measurement, 500 µL of the reaction mixtures were stabilized with 2500 µL of 50% v/v of 2-propanol, and the absorbance was measured at 570 nm with a spectrophotometer Genesys-20 Thermo Spectronic (Thermo Scientific, Waltham, MA). CH without crosslinker (hence no possible crosslinking) was used as blank. The optical absorbance of the solution is proportional to the number of free amino groups in the test sample. The degree of crosslinking was calculated according to Equation (1)

$$\text{CD } (\%) = \frac{\text{CH}_{\text{blank}} - \text{CH}_{\text{crosslinked}}}{\text{CH}_{\text{blank}}} \times 100 \quad (1)$$

where CH_{blank} represents the mole fraction of free NH_2 groups of CH without crosslinking agent and $CH_{crosslinked}$ is the mole fraction of free NH_2 groups of CH with crosslinking agent.

2.3.3. Swelling Properties

For the determination of the swelling degree, the tea-bag method was used, as described by Zhang et al.,[48] with slight modifications. An initial mass of hydrogel (W_0) between 0.4 and 0.6 g was employed. Subsequently, the hydrogel was placed in a tea bag and the bag was immersed in an excessive amount of distilled water (100 mL) for 24 h, taking measurements at 0.5, 1, 4, and 24 h. For each measurement, the tea bag was placed on a dry cloth and gently rubbed with another dry cloth to remove excess liquid. Next, the bag was weighed (W_2). W_1 was determined following the same procedure with an empty bag. The swelling capacity at time t was calculated using Equation (2)

$$S_t = \frac{(W_2 - W_1 - W_0)}{W_0} \times 100 \quad (2)$$

2.3.4. Rheological Characterization

To determine the rheological features of each hydrogel, three different shear tests were performed using a AR 2000 oscillatory rheometer (TA Instruments, New Castle, DE) with parallel serrated plate-plate geometry (diameter: 40 mm).

- Strain sweep tests: The strain range analyzed was established between 0.1% and 100% strain at a constant frequency of 1 Hz and 20 °C. The main objective of this test is the determination of the linear viscoelastic range (LVR) and the critical strain (maximum strain that the hydrogel can bear within the LVR).
- Frequency sweep tests: The selected frequency range for these tests started from 0.02 to 20 Hz at a constant strain within the LVR (2%) at 20 °C. Elastic and viscous moduli (G'' and G', respectively) were obtained, along with the loss tangent ($\tan\delta = G''/G'$). Furthermore, for a better comparison of the results, the values for G' and $\tan\delta$ at 1 Hz (G'_1 and $\tan(\delta)_1$) were tabulated as representative ones.
- Temperature ramp tests: The temperature ramp was fixed from 10 to 40 °C at increasing temperature by 5 °C min^{-1} at constant frequency (1 Hz) and strain (2%). These tests were conducted to assess the properties and stability of the hydrogels both at suitable storage temperatures and at body temperature (even in feverish conditions).

2.3.5. Microstructural Characterization

Prior to the analysis, hydrogels were freeze-dried (<15 Pa for 24 h, LyoQuest, TELSTAR, Barcelona, Spain). In addition, the samples were coated with a thin layer of palladium–gold using a Leica AC600 metallizer (Leica Microsystems, Wetzlar, Germany) and subsequently observed in a Zeiss EVO microscope (Pleasanton, CA) at an acceleration voltage of 10 kV. A digital processing free software (FIJI Image-J, National Institutes of Health, Bethesda, MD) was used to calculate the mean pore size and the pore size distribution of the selected hydrogels.

2.3.6. Biological Characterization

In vitro CyQUANT LDH cytotoxicity assay was used to estimate the cytotoxicity of hydrogels at 36 h according to the manufacturer's instructions (Invitrogen from Thermo Fisher Scientific, USA). U937 (human leukemia monocytic cells), Vero E6 (normal monkey kidney epithelial cells), Jurkat (human T leukemia cells), U2OS (human osteosarcoma epithelial cells), and HeLa (human cervical carcinoma epithelial cells) were used as cell lines (ATCC, USA). All of them were seeded at 10^5 cells per well in Nunc flat-bottomed 96-well plates (ThermoFisher Scientific, USA) following the protocol described in the previous studies.[49–51] Cytotoxicity was determined by fluorescence in a CLARIOstar (BMG LABTECH, Germany). Each hydrogel concentration (wt%) was measured in triplicate and the tests were repeated thrice independently. Cell viability was calculated using the following Equation (3)

$$\%\ \text{Cell viability} = 100 - \left(\left[\frac{\text{Compound} - \text{treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100 \right) \quad (3)$$

Cell viability was also checked using the trypan blue method.[52]

The hemocompatibility assay of the hydrogels at 4 h was examined in Red Blood Cells (RBCs), obtained at the Regional Center for Blood Transfusion and Tissue Bank Sevilla-Huelva (Seville, Spain). They were isolated in vacutainer tubes containing EDTA from 3 healthy human donors (BD, Franklin Lakes, NJ). RBCs were isolated by centrifugation at 1800 rpm for 5 min and the stock solution was prepared according to the protocol mentioned in the previous study.[51] The hydrogels were evaluated at the same concentration (wt%) values selected for in vitro cytotoxicity assays. The positive control was ACK Lysing Buffer (Gibco from Thermo Fisher Scientific, USA) and 1X PBS (Gibco from Thermo Fisher Scientific, USA) was used as negative control. The in vitro hemolytic effect of hydrogels was examined following the instructions described in the previous work.[51] Each hydrogel concentration (wt%) was measured in duplicate and the tests were repeated thrice independently. Finally, absorbance was read at 540 nm, and the hemolysis percentage was calculated following Equation (4)

$$\%\ \text{Hemolysis} = \frac{\text{Compound} - \text{treated Hemoglobin release} - \text{Spontaneous Hemoglobin release}}{\text{Maximum Hemoglobin release} - \text{Spontaneous Hemoglobin release}} \times 100 \quad (4)$$

2.4. Statistical Analyses

For each measurement, at least three replicates were performed. *t*-tests and one-way analysis of variance ($p < 0.05$) where used for statistical analyses, using PASW Statistics for Windows (Version18: SPSS Inc., Endecott, NY). In addition, standard deviations and significant differences were calculated for selected parameters with a confidence level of 95% ($p < 0.05$), indicating them using different letters and symbols in the tables when necessary.

3. Results and Discussion

3.1. FTIR Characterization

First, it was essential to verify that the crosslinking reactions between F and G with CH occurred, as proposed in Figure 1. For this purpose, FTIR measurements of the raw materials and selected hydrogels (1 wt% of crosslinking agent as representative) were performed. **Figure 2**A shows the spectra obtained for CH, F, and CH F 1. The resulting profiles for CH and F were very similar to those observed in works published by other authors.[36,53]

The spectrum obtained for the CH F 1 hydrogel shows numerous differences compared to the CH and F profiles, which could indicate that a reaction took place, specifically the one proposed above. The most determining fact to demonstrate that the reaction has taken place is the appearance of two characteristic bands of C═N bonds (R-CH═N-R′ at 1678 cm^{-1} and δ C═N at 792 cm^{-1}).[54] The formation of the imine bond would mean that this crosslinking reaction follows the mechanism of the Maillard reaction, leading to the Schiff base formation as previously proposed (Figure 1) for the reaction between sugars and amino groups of CH.[27] It is worth mentioning that there are several peaks in the F spectrum, such as ΔCCH + δOCH (1330 cm^{-1}), νCO + νCC + δCCC (1148 cm^{-1}), νOH + νCH_2 associated with sugars (1052 cm^{-1}), νCO + δCCO (974 cm^{-1}), and δCCO + δCCH (781 cm^{-1}), among others, which do not disappear in the CH F 1 spectrum. Due to the low amount of crosslinker, the signal of the latter bands ends up being included in the CH bands. This is another fact that justifies the crosslinking reaction, as this inclusion leads to an increment of some bands, such as the νOH and νNH band (3600–3000 cm^{-1}), δNH (1549 cm^{-1}), and δCH_2OH (1407 cm^{-1}), due to the consequent increase in alcohol groups in the crosslinked structure of the resulting hydrogel.[27,36,54,55]

On the other hand, Figure 2B depicts the spectra obtained for the different proposed crosslinking reactions with G, thus representing the CH, G, and CH G 1 profiles. The G profile was also very similar to those observed in the literature.[32] A clear and consistent fact proves that the proposed crosslinking reaction in Figure 1 took place. First, and most importantly, the disappearance of the signal attributed to the stretching of the C═C bond of the carboxymethyl group (1679 cm^{-1}), and the consequent increase in the intensity of the amide bands I and II (1646 and 1549 cm^{-1}, respectively), demonstrate that the amino groups of CH reacted with the carboxymethyl groups of G, forming secondary amides. In addition, the overlap between the C═O stretching band in secondary amides (1646 cm^{-1}) with the C═C stretching of the olefin ring in G (1621 cm^{-1}) caused the amide band I to become slightly broader in curve, as reported by Reay et al.[32] Second, the formation of these secondary amides is also demonstrated by the absence of the signals attributed to the asymmetric stretching of C—O—C of methyl ester (1297 and 1104 cm^{-1}). This group would disappear after the amidation reaction.[32,54]

3.2. Crosslinking and Swelling Degree

After verifying that the proposed crosslinking reactions took place, the next logical step was determining the extent of these reactions. For this purpose, crosslinking assays were carried out as described in the Experimental Section and the results are gathered in **Table 2**.

As can be observed, there was a similar trend between both crosslinkers, where only 0.5 wt% of added crosslinker led to around 20% of crosslinking degree. Subsequently, by increasing the amount of crosslinker up to 1 wt%, the highest crosslinking values were achieved, namely 32.5 and 31.1 for F and G, respectively. However, contrary to what can be expected, a further increase in the amount of crosslinker up to 2 wt% did not imply greater crosslinking, but rather the opposite, with CD decreasing to values similar to those obtained for systems with 0.5 wt% of crosslinker in the case of F. Although, in G did not show significant differences between 1 and 2 wt%. Nevertheless, this decay effect when increasing the amount of crosslinking agent has also been observed in previous studies with different

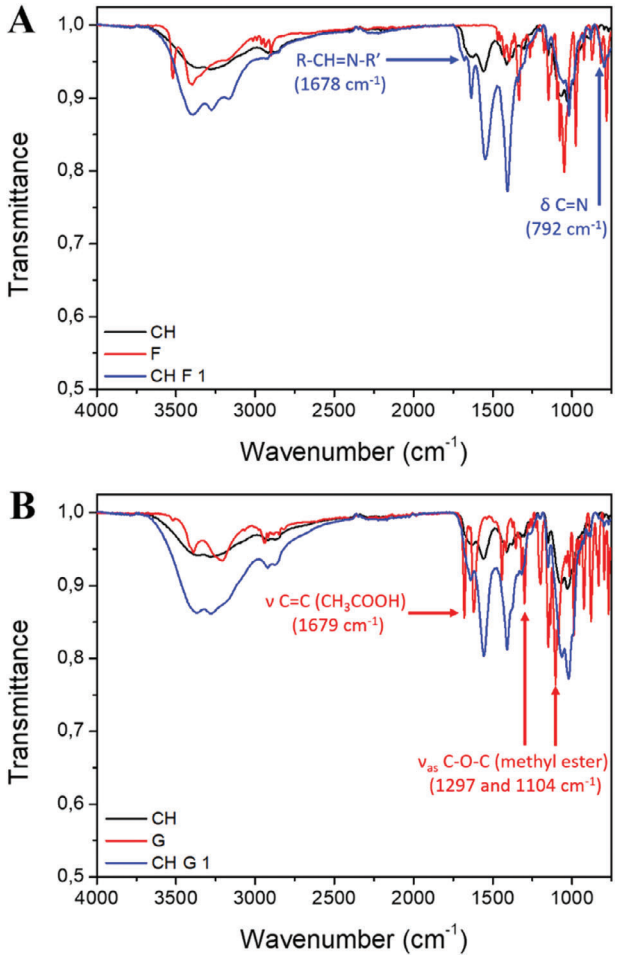

Figure 2. FTIR spectra of chitosan with D-fructose and the hydrogel of chitosan crosslinked with 1 wt% of fructose A) and with genipin and the corresponding hydrogel B).

crosslinking agents, where results showed that, depending on the crosslinking agent and biopolymer, an increase in the amount of crosslinking agent can cause saturation during the formation of the network and, consequently, less interconnection between polymer chains.[15,56–58] In addition, the bioavailability of active amino groups to enable crosslinking may also be hampered due to steric hindrance between those residues or the degree of ionization. The latter has an important contribution to swelling and mechanical properties, as it would have a direct effect in the equilibrium between the degree of crosslinking and the mixing potential, which at the same time is directly affected by temperature, polymer volume fraction and the interaction between the polymer and the solvent, as reported by Jahren et al. (2010).[59]

It is well-known that CD has an important influence on the structure and, thus, on the properties of hydrogels.[60] In the case of SD, whose values are collected in Table 2 from 0.5 to 24 h, it can be observed that both crosslinkers exert a different effect on this property when crosslinked with CH. Regarding the systems with G, it can be observed that the results present a trend similar to that shown for CD; that is, the highest swelling degrees throughout the test were those obtained for the hydrogels with 1 wt% (CH G 1) G. Additionally, doubling the amount of crosslinker up to 2 wt% (CH G 2) led to a slight decrease in SD, similar to what was observed by Dimida et al., who reported that increasing the number of fixed charges on the polymer network increases the hydrogel swelling capacity, which, on the other hand, decreases by increasing the ionic strength of the external solution.[61] This fact was mainly due to the neutralization of the fixed charges by the free charges present in the outer solution, thus reducing both the repulsion effect between polymer chains and the osmotic contribution to the swelling due to the Donnan contribution, which explains why CH G 1 displays a greater swelling capacity than CH G 2.[61] In addition, these two systems presented a better SD throughout the whole test compared to the bare CH hydrogel, except for the measurement of 24 h for the CH G 2 system, which lost the ability to take up water after the first hour. No fructose-crosslinked chitosan hydrogel improved the degree of swelling of bare chitosan. The system with the highest SD was 0.5 wt% F and, the results obtained with G, a decreasing tendency was observed in this property as the amount of F was increased.

3.3. Rheological Evaluation

Rheological tests were carried out for each system as indicated in the Experimental Section. First, it was necessary to determine the critical strain (**Table 3**) in order to determine the linear viscoelastic range to select a suitable strain for the subsequent frequency sweep tests, aiming to obtain information on the stability of the hydrogel network.[62] The results obtained from these frequency tests are shown in **Figure 3**, along with the temperature ramp tests, which were performed to explore variations when the temperature was increased from a suitable storage temperature to an estimated range for body temperature (37 °C).

Table 2. Crosslinking Degree (CD) and Swelling Degree (SD) progression in time of the crosslinked chitosan hydrogels with D-fructose and genipin.

Crosslinker	Crosslinker amount [wt%]	CD [%]	SD 0.5 h [%]	SD 1 h [%]	SD 4 h [%]	SD 24 h [%]
None	0	0	42.1 ± 0.6	99.9 ± 0.3	92.3 ± 0.3	108 ± 0.3
Fructose	0.5	20.8 ± 0.7	18.1 ± 0.8	81.7 ± 0.3	92.1 ± 0.2	87.6 ± 0.7
	1.0	32.5 ± 0.6	27.9 ± 0.4	50.1 ± 0.3	60.9 ± 0.3	71.9 ± 0.4
	2.0	20.3 ± 0.6	7.72 ± 0.2	52.8 ± 0.5	48.9 ± 0.2	64.9 ± 0.4
Genipin	0.5	19.3 ± 0.6	1.04 ± 0.2	47.4 ± 0.3	38.6 ± 0.3	42.6 ± 0.2
	1.0	31.1 ± 0.6	95.8 ± 0.3	149 ± 0.2	159 ± 0.2	169 ± 0.2
	2.0	30.5 ± 0.5	87.0 ± 0.6	124 ± 0.6	110 ± 0.5	99.6 ± ± 0.5

Table 3. Critical strain, elastic modulus (G'_1), and loss tangent (tan δ_1) measurements at 1 Hz of chitosan-based hydrogels crosslinked with different amounts of D-fructose and genipin. Different letters (a–d; A–E; I–III) as superscripts were included to denote significant differences in the values shown in each column ($p < 0.05$).

Crosslinker	Crosslinker amount [wt%]	Critical strain [%]	G'_1 [Pa]	tan δ_1 [-]
None	0	1.018^{a}	1122^{A}	0.050 ± 0.020^{I}
Fructose	0.5	$5.144^{b,c}$	1697^{B}	0.092 ± 0.002^{II}
	1.0	8.193^{b}	2901^{C}	0.076 ± 0.004^{III}
	2.0	3.245^{c}	490.2^{D}	$0.075 \pm 0.008^{I,III}$
Genipin	0.5	$10.34^{b,d}$	2906^{C}	0.075 ± 0.003^{III}
	1.0	12.39^{d}	3568^{E}	$0.075 \pm 0.005^{I,III}$
	2.0	13.72^{d}	2685^{C}	0.078 ± 0.003^{III}

Figure 3A shows the results obtained for the CH hydrogels crosslinked with F, while Figure 3B represents the results obtained for G. In all cases, G' values were significantly higher than the G'' values, which indicated a predominantly elastic response of the systems, and thus, hydrogels with a very slight dependence on frequency were obtained since both G' and G'' values slightly increased when submitted to increasing frequency.[63,64] Delving into the obtained results, a similar trend can be observed in Figure 3A,B, in which the minimum concentration of crosslinker (0.5 wt%) is sufficient to improve, even slightly in the case of F, both moduli compared to the bare chitosan hydrogels.[44] Furthermore, by increasing the amount of crosslinkers up to 1 wt%, this improvement was greater and more noticeable, while further increases in the amount of crosslinking agent (2 wt%) caused both moduli to decrease, obtaining lower values than those of bare CH in the case of F. This tendency in the results is consistent with that observed in the results of the crosslinking tests; thus, it can be asserted that the rheological properties are strongly related to the degree of crosslinking.[65,66] For a better comparison of the effect that each crosslinking agent exerted on the CH hydrogels, the values of critical strain and both elastic modulus and loss tangent at 1 Hz (G'_1 and tan$(\delta)_1$) are shown in Table 3.

From the results in Table 3, it can be observed that both crosslinkers, regardless of their concentration, improved both the critical strain and G' of bare CH, although G produced a more significant improvement. As was previously stated, the results of the rheological tests are consistent with the crosslinking degree results, although it should be noted that no significant differences were observed for the critical strain values obtained with G, which are higher than those obtained for hydrogels with F. The same occurred with G', whose results follow the same trend observed, and more resistant and stable hydrogels were obtained when CH crosslinked with G, obtaining higher G' values compared to F. In fact, the highest value obtained with F (CH F 1) was very similar to those obtained with the lowest and highest concentration of G. Regarding the loss tangent results, minimal differences were found between the systems in all cases, being under 0.1, which means that strong and consistent hydrogels were obtained.[67]

Therefore, it was verified that F had a beneficial effect on the rheological properties of CH, as has been in other biopolymer-based hydrogels based on biopolymers, such as collagen and

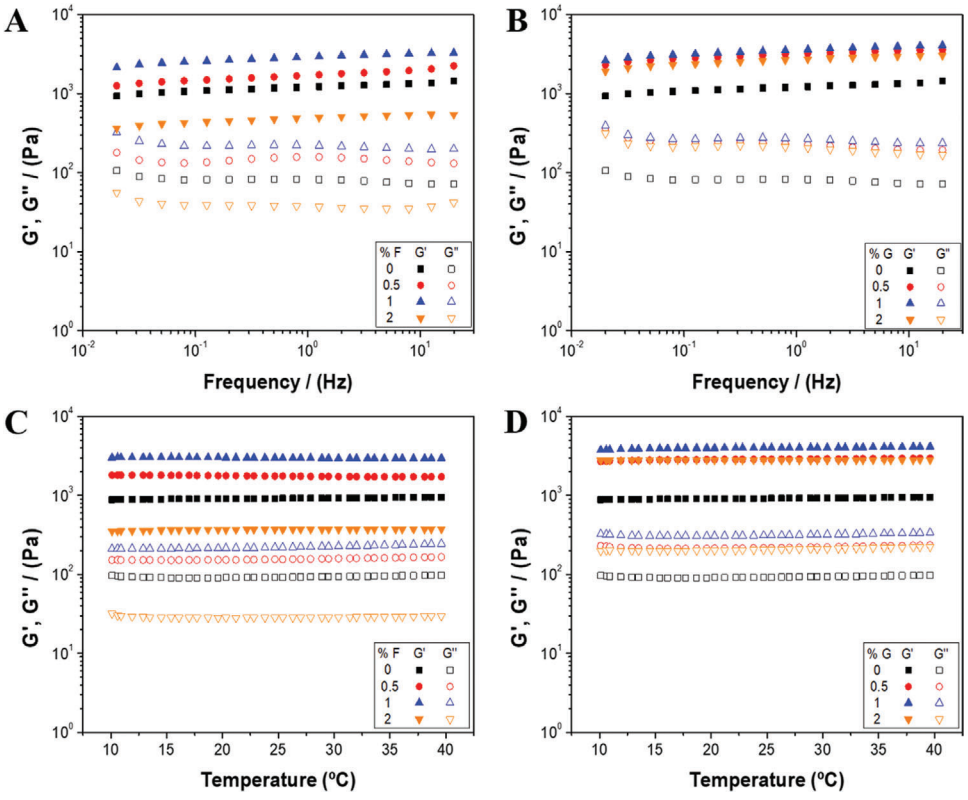

Figure 3. Frequency sweep tests and temperature ramps of CH hydrogels crosslinked with different amounts of fructose A,C) and genipin B,D).

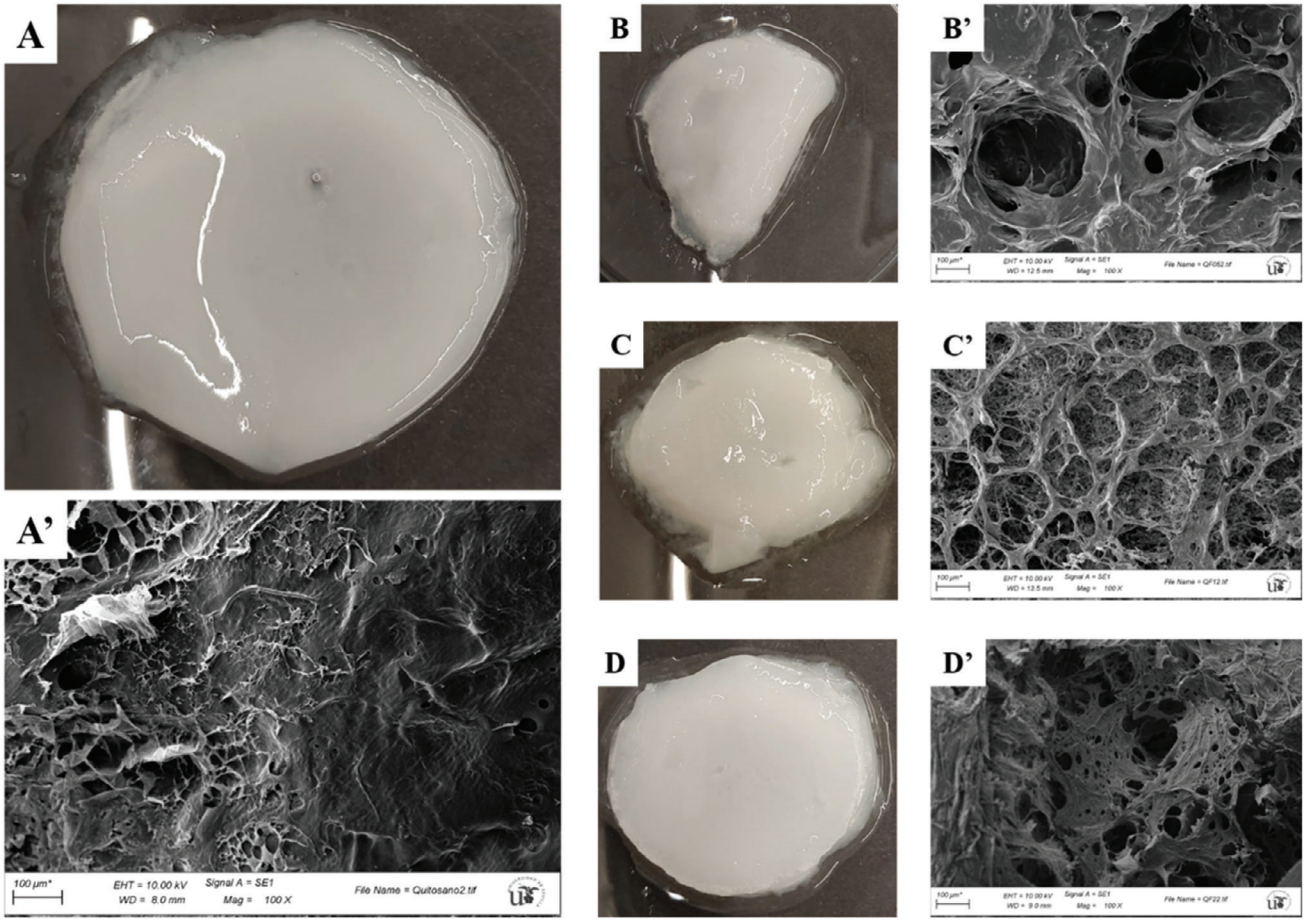

Figure 4. Macroscopic and SEM images of the reference chitosan hydrogel A, A', respectively) and the chitosan-based hydrogels crosslinked with 0.5, 1, and 2 wt% of D- fructose B–D and B'–D', respectively).

hyaluronic acid.[35,68] However, the results obtained and the comparison with those of other authors demonstrates that crosslinking with G offered a remarkable improvement in the rheological performance,[69–71] being better when compared to F or other crosslinkers such as phenolic compounds,[47] dually crosslinked with citric acid and diiodo-trehalose derivative chitosan-based hydrogels,[72] tannic acid,[73] or when crosslinked via photoinitiated click polymer interpenetrated polymer network (IPN) reaction.[15]

Additionally, as was previously indicated, temperature ramp tests were carried out to study the rheological stability of the hydrogels against temperature (Figure 3C,D). The hydrogels maintained excellent stability for the studied temperature range in all cases since the storage and loss moduli were not altered during the test. This fact demonstrates that these hydrogels can maintain their properties at low temperatures, suitable for storage, and at body temperatures once implanted.

3.4. Microstructural Evaluation

Figure 4 shows a macroscopic and microscopic view of bare CH hydrogel (Figure 4A and 4A', respectively) and the ones crosslinked with F (Figure 4B–D and 4B'–D', respectively). From the macroscopic point of view, hardly any differences were observed between the systems, except that, in the case of 1 wt%, a slightly yellowish hue was observed, which became apparent after the freeze-drying process. This is the characteristic effect that the Maillard reaction exerts on the coloration of hydrogels, although a much more apparent change can be observed when heating at temperatures near 100 °C.[74] Apart from this, hydrogels were obtained with an appearance similar to what was expected and observed in other studies.[44,75] Regarding the results obtained from the microscopic characterization, more differences were detected. Figure 4A' shows that CH hydrogels had a characteristic microstructure similar to that observed in other studies,[44,76] where a fairly solid and consistent structure was observed, although with considerable porosity (72.5%), with small-sized pores (51.29 µm).

After the crosslinking reaction by incorporating F, the structure was remarkably transformed, as has been previously reported with collagen[35] and by adding fructose compounds,[77] with increasing amounts of the crosslinking agent. In this way, 0.5 wt% content of F (Figure 4B') changed the structure from a heterogeneous structure with small pores to a less porous structure (59.2%), although with larger pores (129.4 µm) with much

Table 4. Porosity and mean pore size of chitosan-based hydrogels crosslinked with different amounts of D-fructose and genipin.

Crosslinker	Crosslinker amount [wt%]	Porosity [%]	Mean pore size μm]
None	0	72.5 ± 5.1	51.29 ± 19.05
Fructose	0.5	59.2 ± 3.2	129.4 ± 90.48
	1.0	54.4 ± 2.4	76.57 ± 45.36
	2.0	57.8 ± 1.9	82.59 ± 54.06
Genipin	0.5	37.7 ± 4.2	91.83 ± 44.78
	1.0	45.3 ± 2.4	69.55 ± 30.77
	2.0	37.3 ± 4.9	101.5 ± 93.99

more heterogeneity in pore size (not in porosity). Data for a better comparison are included in **Table 4**. Increasing F concentration to 1 wt%, as was previously proved, led to a more crosslinked structure and, as can be observed in Figure 4C', to a more homogeneous one, both in pore size and distribution, with smaller pores (76.57 μm). This fact is consistent with the rheological results, as the more homogeneous and less porous the structure, the better the rheological performance.[15,78] However, further increasing the amount of crosslinker caused a deleterious effect, as was already stated, due to a structural regression, returning to a more heterogeneous structure, both in pore size and distribution, as the structure loses crosslinking, which is also consistent with the results obtained and proves the rheological behavior.

On the other hand, **Figure 5** also shows the macroscopic and microscopic representation of the same hydrogels but crosslinked with G (Figure 5B–D and B'–D'). In the case of these hydrogels, the macroscopic change was much more noticeable since, even with the smallest amount of genipin, the hydrogel acquired a brownish-green coloration, which was more accentuated when the concentration of the crosslinker was increased. G usually gives rise to products with a marked and characteristic blue coloration.[69,78] However, these hydrogels may turn into a brown hue when changing pH from ≈3.2 to 5, as reported by Yang et al.[79]

From the results obtained from the micrographs (Table 4), it can be concluded that, compared to the hydrogels with F, those crosslinked with genipin would have a more consistent and solid structure since the porosity and mean pore size, in most cases, present lower values, which explains the improvement in their rheological properties. Moreover, these results also explain that even having no significant differences with CH G 1 in the CD,

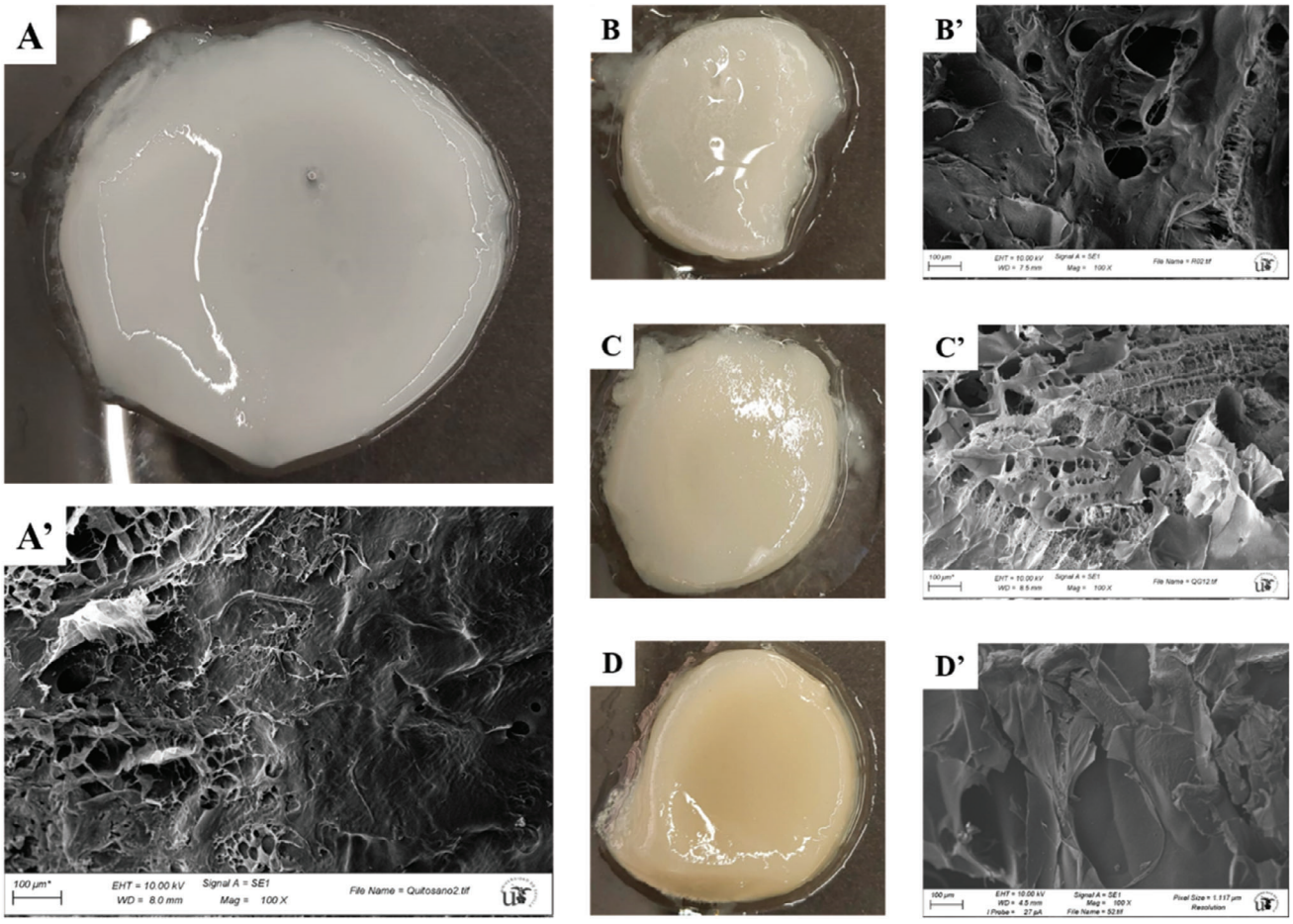

Figure 5. Macroscopic and SEM images of the reference chitosan hydrogel A, A', respectively) and the chitosan-based hydrogels crosslinked with 0.5, 1, and 2 wt.% of genipin B–D and B'–D', respectively).

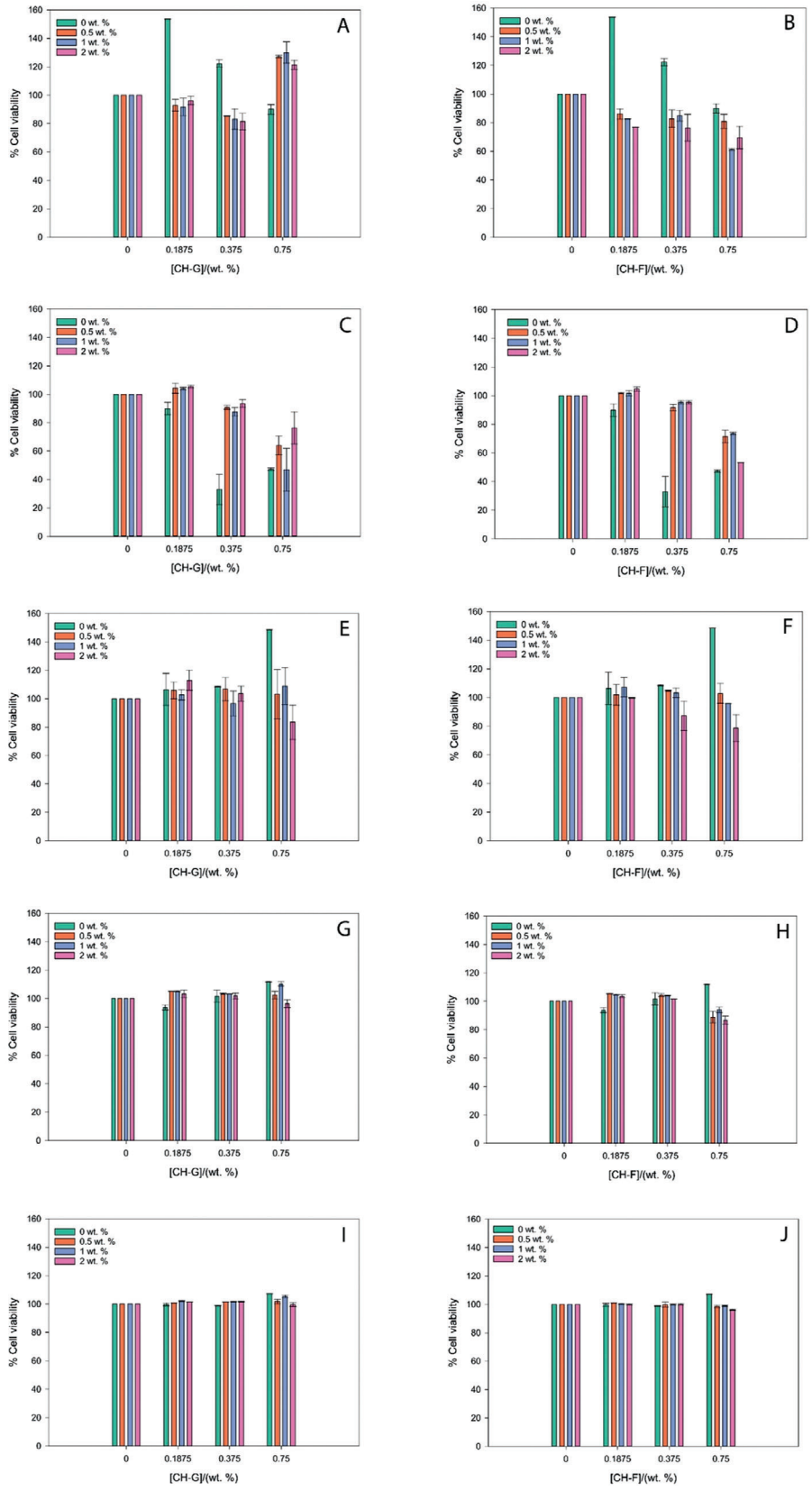

Figure 6. In vitro cytotoxicity results obtained in the chitosan hydrogels crosslinked by genipin or D-fructose: Vero E6 A,B); HeLa C,D); U2OS E,F); U937 G,H); and Jurkat I,J) cell lines evaluated.

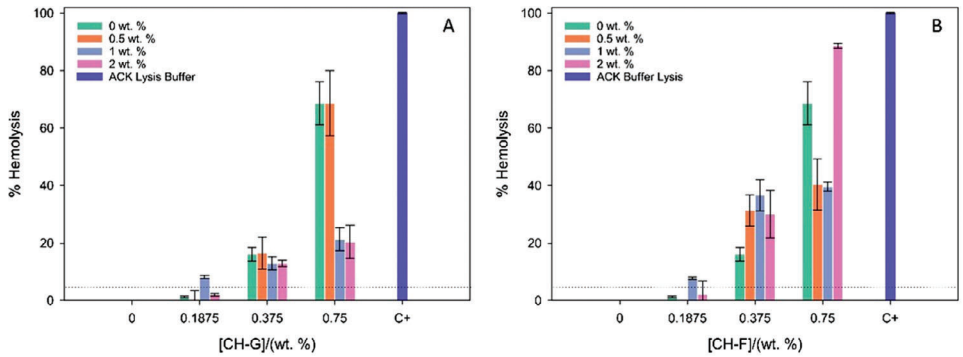

Figure 7. In vitro hemolysis results obtained in the chitosan hydrogels crosslinked with: genipin A); D-fructose B).

the hydrogel with 2 wt% of G had poorer rheological performance since the mean pore size was higher and, consequently, the structure would not be as resistant as the one with 1 wt%.

3.5. Biological Evaluation

In vitro cytotoxicity assays were carried out for easily screening the biocompatibility of the hydrogels formulated as potential scaffolds in tissue engineering and regenerative medicine. **Figure 6** shows the results obtained for each of the systems studied at 48 h. No significant cytotoxic effects were found for any of the two crosslinkers studied in CH hydrogels, except in HeLa cells up to a value of 0.375 wt% (Figure 6C,D). In this sense, the influence of the positive surface charge in CH blends and its high degree of deacetylation has been previously associated with increased toxicity by the authors due to cell growth inhibition.[49] In addition, F-crosslinked CH hydrogels were more cytotoxic than CH hydrogels crosslinked with G at higher hydrogel concentrations in Vero E6 cells (Figure 6A,B). F is considered a fundamental regulation factor in the glycolytic pathway; therefore, high-F concentrations lead to the synthesis of advanced glycation end products (AGEs) and reactive oxygen species (ROS), enhancing the oxidative damage and inflammation due to the imbalance between AGEs and ROS, and subsequently potentiating its toxicity.[80]

To obtain further information about its biocompatibility, in vitro hemolysis assays were conducted to determine the hemolytic effect of the hydrogels. **Figure 7** shows the hemolysis percentage resulting from the interaction between the different systems and RBCs. Generally, none of the systems were hemocompatible at higher concentrations, regardless of the crosslinker used. As was mentioned above, CH is a positively charged compound that can interact with negatively charged cell membranes, resulting in high adhesion and inhibition of cell growth. To support this idea, a previous study suggests that electrostatic interactions with phospholipids from erythrocyte membranes trigger the formation of complexes that interfere with the correct functioning of those cells.[81] Furthermore, F-crosslinked CH hydrogels produced a higher hemolysis percentage than CH hydrogels crosslinked with G, consistent with the in vitro cytotoxicity assay results. As was already described, high F concentrations could lead to a dysregulation of the glycolytic pathway. This could be the reason behind this outcome since erythrocytes lack mitochondria and their unique method of obtaining energy is glycolysis.[82] Finally, one positive behavior that emerges from our data indicates that the crosslinking with G results in a decrease in the hemolytic activity of the hydrogels (Figure 7A). This trend has been previously described by Gao et al.[70]

4. Conclusions

Chitosan hydrogels were successfully crosslinked with the proposed natural crosslinking agents, namely D-fructose and genipin, as demonstrated by the FTIR measurements. Both crosslinkers exert an important effect on the structure, which is more noticeable when their amounts are increased, consequently having a remarkable influence on the evaluated properties for each case. Regardless of the crosslinking agent, increasing the amount of crosslinker from its minimum concentration to 1 wt% results in a higher degree of crosslinking with chitosan. However, a further increase in the amount of crosslinking agent would not imply a higher crosslinking degree in any case due to saturation. This tendency was observed in most cases in the performed assays. Therefore, and with the aim of comparing the influence of both crosslinking agents, it can be established that genipin exerts a much more beneficial effect for chitosan than D-fructose since the hydrogels crosslinked with genipin, especially with the one with 1 wt%, a degree of swelling higher than that of bare chitosan is obtained, which is not achieved with D-fructose. Moreover, the rheological tests revealed that hydrogels crosslinked with genipin are more resistant and have better rheological properties than those obtained with D-fructose. This fact is attributed to crosslinking with genipin would make the structure of chitosan more compact, with less porosity. Finally, the biological in vitro assays showed that D-fructose would have a deleterious contribution to our purpose since it is a fundamental regulation factor in the glycolytic pathway, which would increase the synthesis of AGEs and ROS, consequently increasing the cytotoxicity of these scaffolds as an additional advantage of genipin crosslinking with this compound results in a decrease in the hemolytic activity of the hydrogels. Therefore, genipin proved to be a more recommendable crosslinker to obtain better chitosan-based hydrogels.

Acknowledgements

The authors would like to acknowledge CITIUS for granting access to and their assistance with the Microanalysis and Microscopy service. This study

was financially supported by MCIN/AEI/10.13039/501100011033/FEDER, UE, through the project PID2021-124294OB-C21. The authors gratefully acknowledged their financial support. This work was also possible thanks to the postdoctoral contract of Víctor M. Pérez Puyana and Francisco J. Ostos from the "Contratación de Personal Investigador Doctor" supported by the European Social Fund and Junta de Andalucía (PAIDI DOCTOR – Convocatoria 2019–2020, DOC_00586, and DOC_00963).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

F.J.O. and V.M.P.-P. contributed equally to this work. Conceptualization, P.S.-C., A.R., V.P.-P.; methodology, P.S.-C., M.J.-R., V.P.-P., G.G.-U., F.J.O.; validation, M.A.-G, A.R., M.R.B., F.J.O., V.P.-P.; formal analysis, P.S.-C., M.J.-R., G.G.-U., F.J.O., V.P.-P.; investigation, P.S.-C., M.A.-G., M.J.-R.; resources, A.R., M.R.B., F.J.O., V.P.-P.; data curation, P.S.-C., G.G.-U., F.J.O.; writing, P.S.-C., M.A.-G., G.G.-U., F.J.O.; visualization, P.S.-C., M.J.-R.; supervision, A.R., M.R.B., F.J.O., V.P.-P.; project administration, F.J.O., V.P.-P.; funding acquisition, A.R., M.R.B., F.J.O., V.P.-P. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

biocompatibility, biomedicine, chemical crosslinking, chitosan, D-fructose, genipin, hydrogels, natural crosslinking agents, rheology

Received: June 15, 2023
Revised: August 15, 2023
Published online: September 3, 2023

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