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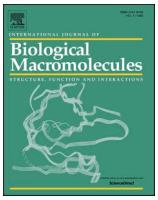

Effect of different crosslinking agents on hybrid chitosan/collagen hydrogels for potential tissue engineering applications

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ABSTRACT

Tissue engineering (TE) demands scaffolds that have the necessary resistance to withstand the mechanical stresses once implanted in our body, as well as excellent biocompatibility. Hydrogels are postulated as interesting materials for this purpose, especially those made from biopolymers. In this study, the microstructure and rheological performance, as well as functional and biological properties of chitosan and collagen hydrogels (CH/CG) crosslinked with different coupling agents, both natural such as D-Fructose (F), genipin (G) and transglutaminase (T) and synthetic, using a combination of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride with N-hydroxysuccinimide (EDC/NHS) will be assessed. FTIR tests were carried out to determine if the proposed crosslinking reactions for each crosslinking agent occurred as expected, obtaining positive results in this aspect. Regarding the characterization of the properties of each system, two main trends were observed, from which it could be established that crosslinking with G and EDC-NHS turned out to be more effective and beneficial than with the other two crosslinking agents, producing significant improvements with respect to the base CH/CG hydrogel. In addition, *in vitro* tests demonstrated the potential application in TE of these systems, especially for those crosslinked with G, T and EDC-NHS.

1. Introduction

Biomedical research has experienced an exponential growth in the last decades driven by the development of novel biomaterials and their impact on healthcare [1]. Notably, one of the major advances in tissue engineering (TE) is the development of smart biomaterials, induced pluripotent stem cells (iPSC), three-dimensional (3D) bioprinting technologies, and dynamic culture methods. In addition, emerging technologies such as extracellular vesicles, genetic engineering and artificial intelligence have also contributed to various therapeutic strategies for patient care [2]. TE relies on the application of engineering and life science principles and strategies through a fundamental understanding of the structure-function relationships of normal and pathological tissues. The main goal of TE is to develop biological substitutes to restore, maintain, or improve tissue function [3,4].

Hydrogels have been considered excellent candidates for this purpose due to their semi-solid phase, inherent flexibility and remarkable absorption properties. They can absorb water up to 1000 times their dry weight without loss of structural integrity [5,6]. In addition, hydrogels offer other attractive features for TE, such as their remarkable biocompatibility, adhesion, mechanical performance, environment responsiveness and self-healing properties that can be modulated and controlled by their composition and/or preparation methods [7,8].

Biopolymers obtained *via* eco-friendly methodologies that minimize resource waste and respect the environment have been the trend to follow in recent years [9,10]. Common examples of biopolymers used in TE applications include polypeptides, polysaccharides (PSAs) and nucleic acids [1,11,12]. Among polysaccharides, chitosan (CH) is one of the most widely used and well-known biopolymers for hydrogel fabrication owing to its structure and versatility. Its composition is based on

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the repetition of D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-(1,4) bonds [13]. It is obtained by partially deacetylating chitin, the world's most abundant natural aminopolysaccharide, through strong alkaline treatment at high temperatures [13–15]. CH has plenty remarkable properties, including biocompatibility, biodegradability, mucoadhesiveness and preventive properties [16–18]. Nevertheless, CH still presents several inconvenient for its application in TE, mainly associated with insufficient mechanical properties and biocompatibility issues in some cases, since its surface is positively charged. This, together with its high degree of deacetylation, leads to inhibition of cell growth and increased toxicity [19].

On the other hand, regarding protein-based hydrogels, collagen (CG) emerged at the beginning of the millennium as an extremely versatile biopolymer that has been widely used in the biomedical field [20]. CG is the most abundant protein in animal tissues, accounting for 30 % of the total protein content. It can be found predominantly in tendons, cartilage, skin, ligaments and bones [21]. CG consists of a triple helix formed by three polymeric chains of *ca.* 1000 amino acids connected by peptide bonds [22,23]. When considering CG-based scaffolds, it is a relevant matter to bear in mind the properties of the raw material and the denaturation degree, as scaffolds can be constructed using native CG or the denatured form (gelatin), which can be obtained through acidic or alkaline processing and thermal denaturation. In spite of the good biological properties of CG-based scaffolds, there are still several drawbacks to overcome, such as their poor mechanical performance and the possibility of denaturation once implanted [24].

Many studies have combined CH and CG as composites or blends for several biomedical applications. In general, there is a good synergy between both biopolymers that allows obtaining materials that address the mentioned drawbacks that these polymers present separately and, therefore, exhibit suitable properties [19,25–27]. However, there is room for improvement, especially in terms of mechanical performance [19]. With this perspective, the most common and effective method to improve the properties of hydrogels is to resort to chemical crosslinking, as it results in covalently crosslinked networks [1,28].

The selection of an appropriate the crosslinking agent is crucial to obtain CH/CG-based hydrogels with enhanced properties [29,30]. Therefore, chemical crosslinking can be carried out using crosslinking agents of natural or synthetic origin to form strong covalent bonds. On the one hand, synthetically derived chemical crosslinkers form stronger covalent interactions but may pose cytotoxicity risks and potential biocompatibility issues due to the presence of unreacted crosslinkers within the scaffolds. On the other hand, the most beneficial features of natural crosslinkers are their availability and sterling biocompatibility [18,31–33].

One of the foremost common crosslinking agents for CG is a combination of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) [34]. EDC is a carboxyl- and amine-reactive zero-length crosslinker that first reacts with the carboxyl group to form an O-acylisourea intermediate. This intermediate reacts quickly with an amino group, which can be either from CH or from some amino acid that contains residues with amino groups from collagen. An amide bond is formed, thereby releasing an isourea byproduct. The O-acylisourea intermediate is unstable in aqueous solution and will be hydrolyzed if it does not react with the amine group. Hydrolysis of the intermediate regenerates the carboxyl group and releases the N-substituted urea. Therefore, NHS or sulfo-NHS is required for stabilization because it reacts with the labile reactive O-acylisourea ester intermediate to form a semi-stable amine-reactive NHS ester that is stable for several hours at pH 7.4 [35,36]. In any case, despite its popularity, EDC/NHS has been shown to induce only low levels of integrin-specific cell binding under higher crosslinking conditions. This behavior is detrimental, especially in TE, as it reduces adhesion to the extracellular matrix (ECM), and needs to be controlled [37].

D-fructose (F) is a renowned carbohydrate found in some fruits, cereals, vegetables and honey [38]. It is obtained by hydrolyzing starch to glucose, using microbial enzymes. Subsequently, it is submitted to an isomerization process to become fructose, more specifically D-fructose [39–41]. Reducing sugars, including F, can be used as chemical crosslinking agents as they can undergo the so-called Maillard reaction [18,39], which is a mild reaction between amino groups and the carbonyl groups of these reducing sugars forming a Schiff base [42–45].

Despite its high cost, genipin (G) is one of the most commonly used natural crosslinking agents due to its good biocompatibility, biodegradability and stability of the resulting crosslinked products [46,47]. G (methyl 1-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta [c]pyran-4-carboxylate) is produced by the hydrolysis with β-glucosidase of geniposide, isolated from the fruits of *Gardenia jasminoides*. In addition, compared to other chemical crosslinkers, G is significantly less toxic [18,48,49]. It is generally accepted that G reacts only with primary amine groups [46], making it particularly fascinating for crosslinking reactions with proteins or CH, for example [18]. In addition, another singularity of G is the ability to form bifunctional crosslinks with these kind of molecules [50].

Transglutaminase (T) is a widely used crosslinking agent for protein-based hydrogels [51–56]. It is a natural enzyme present in almost all living organisms and is used to catalyze the formation of ε - γ (- glutamyl) lysine isopeptide bond within and between protein molecules. It utilizes the γ - carboxamide group of a glutamine residue in the polypeptide chain as the donor of the acyl group, and the ε - amino group of a lysine residue in polypeptide chain as the receptor of the acyl group [52,57–59]. The effect of transglutaminase as a crosslinking agent has also been studied in chitosan-gelatin interpenetrated polymer networks (IPNs), as reported by Zhang et al. [60].

The main objective of this work is to evaluate the effect of these crosslinking agents, including three natural crosslinkers (genipin, D-fructose and transglutaminase) and synthetic EDC/NHS, on the structure and properties of CH/CG-based hydrogels. Many other crosslinking agents could have been included to broaden the comparative assessment, *i.e.*, tannic acid or citric acid as natural crosslinkers or glutaraldehyde or glyoxal as synthetic crosslinking agents. Nevertheless, with the selected candidates, the main objective of this work is sufficiently covered, using more natural crosslinking agents rather than synthetic ones and using EDC-NHS as a representative of synthetic crosslinkers mainly due to its capacity to crosslink chitosan and collagen chains. To reach this goal, several amounts of the crosslinkers were added to appraise their impact on the crosslinking degree and thus, in their microstructural, rheological, functional and biological properties of each resulting hydrogel. Finally, hydrogels with the best performances will be selected to determine their potential application in TE.

2. Experimental

2.1. Materials

Chitosan (CH) and collagen (CG) were used as raw materials. Specifically, low molecular weight CH (MW = 130,000 g·mol^{-1}, deacetylation degree around 80 %) was supplied by Sigma-Aldrich S. A. (Germany) and type I CG (pork source, protein content higher than 90 %, denaturation degree around 75 %), provided by Protein Solutions (Essentia, Denmark). On the other hand, 0.05 M acetic acid (Panreac Química S. A., Spain) was chosen as solvent. D-(−)-Fructose (≥99 %, MW = 180.16 g/mol), genipin (>98 %, extracted from *Gardenia jasminoides* plant, MW = 226.23 g/mol), commercial transglutaminase enzyme Probind TX (100 units/g as enzymatic activity), and both EDC (commercial grade, MW = 191.7 g/mol) and NHS (98 %, MW = 115.09 g/mol) were used as crosslinking agents. D-fructose, EDC and NHS were supplied by Sigma Aldrich S. A. (Germany). Genipin was provided by Guangxi Shanyun Biochemical Science and Technology Co. (Liuzhou, China), and transglutaminase was supplied by BDF Ingredients (Barcelona, Spain).

2.2. Fabrication of hydrogels

Hydrogels were prepared based on the second protocol indicated in a previous work where CH/CG hydrogels were prepared using two protocols and subsequently characterized. It was found that the second protocol conferred the best properties to the hydrogels [19]. In this modified protocol, an additional step was included, which involved the incorporation of the different crosslinking agents. Thus, they were mixed with the raw materials and dissolved *via* agitation (maintaining a temperature of 50 °C) to improve chain mobility and interactions [61,62]. Thus, the new protocol consisted of preparing 20 mL of 1.5 wt% CH/CG solutions with 1:1 ratio in 0.05 M acetic acid solution. The pH value was adjusted to 3.2 and then the respective crosslinking agent (F, G, T and E-N, the last one in a 5:2 ratio) was added in different concentrations (0.5, 1.0 and 2.0 wt% respect the total biopolymer content). The purpose was to investigate the influence of the crosslinker amount in the properties of the resulting hydrogels. Subsequently, the solutions were magnetically stirred at 50 °C for 1 h. Afterwards, all samples were placed in the refrigerator at 4 °C for 2 h. This step was followed by an increase in the pH value, from 3.2 to 5–7 by adding 4 M NaOH. Finally, the samples were returned to the refrigerator and kept there for 24 h.

2.3. Characterization of the hydrogels

As previously commented in the Introduction section, microstructural, rheological, functional and biological properties of each hydrogel were measured, according to Fig. S1 and the subsequent analyses.

2.3.1. Fourier-transform infrared spectroscopy (FTIR)

Hydrogels containing an intermediate concentration of the crosslinking agent (1 wt%) and their respective reagents were analyzed by FTIR with a Hyperion-1000 spectrophotometer (Bruker, USA). These hydrogels were previously freeze-dried (<15 Pa for 24 h), in order to remove the solvent by sublimation, using a freeze-dryer (TELSTAR, Spain). ATR measurements were carried out obtaining the infrared profiles from 4000 to 500 cm^{-1} (4 cm^{-1} opening and 200 scans of acquisition).

2.3.2. Rheology

To assess the hydrogels' rheological performance, different shear tests were carried out using an AR 2000 oscillatory rheometer (TA Instruments, New Castle, DE, USA) equipped with parallel serrated plate-plate geometry (diameter: 40 mm). Mainly three types of rheological tests were performed:

- **Strain sweep tests**: Tests from 0.1 to 100 % strain (at 1 Hz and 20 °C) a constant frequency of 1 Hz and 20 °C were performed with the aim of determining the critical strain.
- **Frequency sweep tests**: Tests from 0.02 to 20 Hz (at a constant strain of 2 % and 20 °C) were carried out measuring G′ and G″ (elastic and viscous moduli, respectively). Furthermore, in order to facilitate the comparison among different systems, representative values of G′ and tan δ (tan(δ) = G″/G′) at 1 Hz (G'_1 and $\tan(\delta)_1$) were selected as representative and tabulated.
- **Temperature ramp tests**: The hydrogels were subjected to a temperature ramp from 10 to 40 °C, increasing temperature at a heating rate of 5 °C/min. These trials were performed with uniform frequency (1 Hz) and strain (2 %). The utility of these tests is to study the properties and stability of the hydrogels both at appropriate temperatures for storage and at body temperature.

2.3.3. Microstructure

To analyze the morphology of the hydrogel samples, they were previously freeze-dried (<15 Pa for 24 h), removing the solvent by sublimation, using a freeze dryer (LyoQuest, TELSTAR, Barcelona, Spain). Then, the samples were coated with a thin layer of palladium–gold and subsequently observed through scanning electron microscopy (SEM) in a Zeiss EVO microscope (USA). FIJI Image-J software (National Institutes of Health, USA) was used to determine the pore size distribution and the mean pore size of the selected hydrogels.

2.3.4. Swelling properties

A tea-bag method was followed according to Zhang et al. [63], though with slight modifications. An initial mass of hydrogel sample (W_0) between 0.4 and 0.6 g was weighed. Then, the hydrogel was placed inside the tea-bag and immersed in abundant distilled water for 24 h, measuring at different times (0.5, 1, 4 and 24 h). At each measurement, the tea-bag carefully rubbed with a dry cloth to remove excess liquid. Next, the bag was weighed (W_2). An empty bag underwent the same protocol, measuring its weight as W_1. The swelling capacity at t time (S_t) is calculated using Eq. (1):

$$S_t = \frac{(W_2 - W_1 - W_0)}{W_0} \times 100 \quad (1)$$

2.3.5. Biological assessment

In vitro cytotoxicity assays of the hydrogels were carried out using the CyQUANT™ LDH cytotoxicity assay (Invitrogen™ from Thermo Fisher Scientific, USA) [64]. Different cell lines from a commercial supplier (ATCC®, USA) were used, including U937 (human leukemia monocytic cells), Vero E6 (normal monkey kidney epithelial cells), U2OS (human osteosarcoma epithelial cells), Jurkat (human T leukemia cells) and HeLa (human cervical carcinoma epithelial cells). Each cell line was seeded at 1×10^5 cells/well into Nunc flat-bottomed 96-well plates (ThermoFisher Scientific, USA) using complete D-10 for HeLa, Vero E6 and U2OS cell lines or R-10 for U937 and Jurkat cell lines [Dulbecco's modified Eagle medium (DMEM, D10) or Roswell Park Memorial Institute (RPMI, R10) supplemented with 10 % of fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine]. The protocol used was the same followed by González-Ulloa et al. [65]. The cytotoxicity was measured by fluorescence in a CLARIOstar® (BMG LABTECH, Germany). Each hydrogel concentration (wt%) was measured in triplicate and the tests were repeated thrice independently. The cell viability (% Cell viability) was calculated using the following Eq. (2):

$$\%\text{Cell viability} = 100 - \left(\left[\frac{\text{Compound} - \text{treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100 \right) \quad (2)$$

Additionally, cell viability values were also checked by the trypan blue method [66].

To obtain more information about their *in vitro* biocompatibility, the hemocompatibility of the hydrogels was determined in human Red Blood Cells (RBCs) following the protocol from González-Ulloa et al. [65]. The hemolysis percentage (% Hemolysis) was calculated following Eq. (3):

$$\%\text{Hemolysis} = \frac{\text{Compound} - \text{treated Hemoglobin release} - \text{Spontaneous Hemoglobin release}}{\text{Maximum Hemoglobin release} - \text{Spontaneous Hemoglobin release}} \times 100 \quad (3)$$

2.4. Statistical analyses

At least three repetitions were performed for each measurement to ensure statistical reliability. Statistical analyses were performed using PASW Statistics for Windows (version 18: SPSS Inc., Endecott, NY, USA) using *t*-tests and one-way analysis of variance (p and <0.05). We calculated the standard deviation of the selected parameters. Significant differences were determined at the 95 % confidence level (p and <0.05) and are marked with different letters in different tables.

3. Results and discussion

3.1. FTIR characterization

FTIR measurements were conducted on the raw materials, CH/CG hydrogel without crosslinking and selected hydrogels, 1 wt% of each crosslinking agent, representing the intermediate system, were performed. The obtained FTIR spectra for both bare CH and CG, which had the expected profile (similar to what was found in literature) [67,68], are represented, as well as the CH/CG hydrogel, in each representation of Fig. 1. The most important bands for the crosslinking comparative study were identified in the spectra of CH and CG. The amide A and B peaks, corresponding to the stretching vibrations of N—H and O—H, were observed in the wide band in the range of 3600 to 3020 cm^{-1} and 2929 cm^{-1}, respectively. Additionally, there are also other characteristic bands associated with the amide I, II and III bands in each spectrum. The corresponding absorption bands are detected at 1630 cm^{-1} for amide I, 1559 and 1532 cm^{-1} in relation to amide II for CH and CG, respectively. Finally, amide III is represented by the region from 1414 to 1204 cm^{-1} for CH and from 1454 to 1237 cm^{-1} for CG. These bands are associated with C═O stretching vibrations coupled to N—H bending vibration, N—H bending vibrations coupled to C—N stretching vibrations and to C—N stretching and N—H in-plane bending from amide linkages, respectively [67–71]. When these two biopolymers were combined to form the hydrogel, a spectrum similar to that of the raw biomaterials used was obtained. Nonetheless, the intensity of the bands was intermediate compared to bare CH and CG profiles and some of the main bands and peaks were slightly displaced. These observations indicate that the obtention of a structure that integrates and combines both biopolymers was achieved, being a physically-crosslinked blended hydrogel [72].

In addition to those mentioned above, Fig. 1A shows the spectra obtained for F and the crosslinked system with 1 wt% of F (CH/CG F 1). The most notable evidence of the consecution of the reaction is the presence of the representative bands of C═N bonds (R—CH═N—R′ at 1642 cm^{-1}, being R or R′ conjugated) [73], indicating the formation of the imine bond, as suggested in a previous study [18]. Moreover, several bands show an increment in intensity, such as the bands of ν_{OH} and ν_{NH} band (3600–3000 cm^{-1}), δ_{NH} (1565 cm^{-1}) and δ_{CH2OH} (1407 cm^{-1}), possibly related to the crosslinking reaction and the subsequent increase in alcohol groups in the crosslinked structure of the resulting hydrogel [40,73,74].

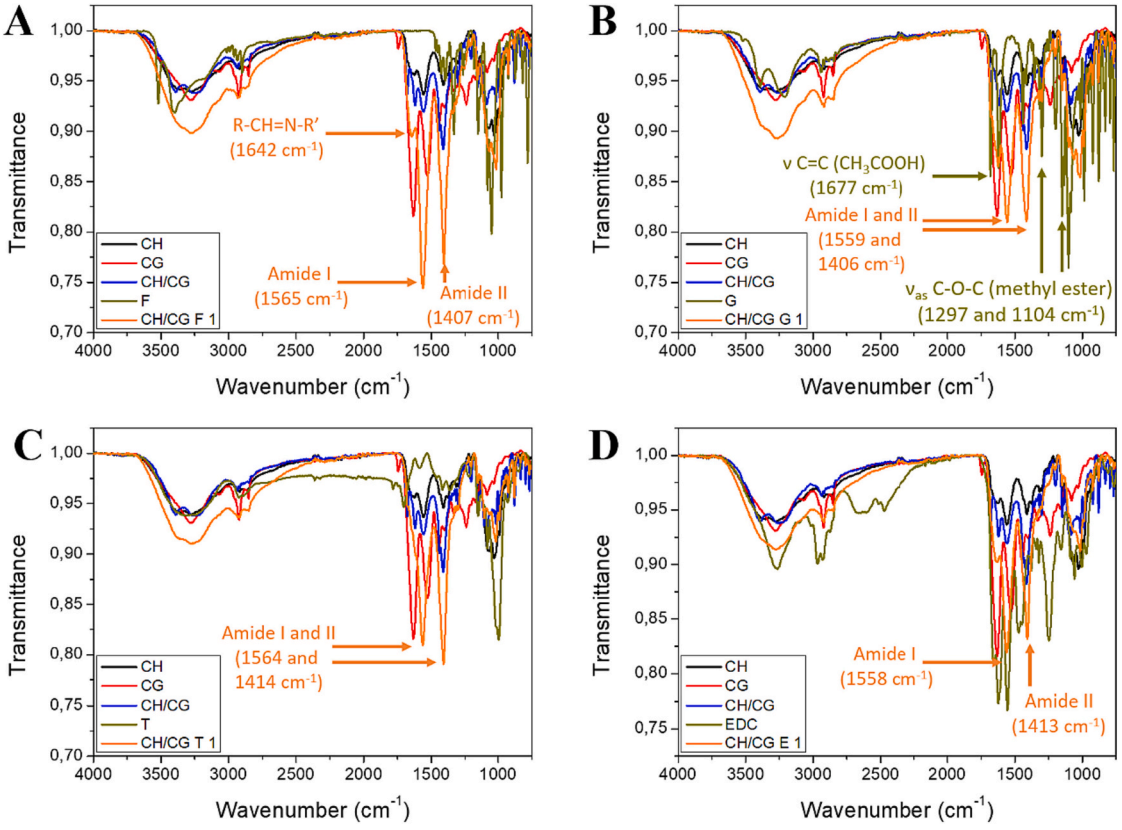

Fig. 1. FTIR spectra of chitosan (CH), collagen (CG), CH/CG hydrogels chemically non-crosslinked and crosslinked with 1 wt% of D-Fructose (F) (A), genipin (G) (B), transglutaminase (C) and EDC/NHS (D). The spectrum of each crosslinking agents is included in their corresponding figures.

Table 1
Critical strain, elastic modulus and loss tangent measurements at 1 Hz of CH/CG hydrogels crosslinked with different amounts of D-fructose, genipin, transglutaminase (Tgase) and EDC/NHS. 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	13.1^{a}	150^{A}	0.082 ± 0.006^{I}
Fructose	0.5	20.6^{b}	$194^{A,B}$	0.090 ± 0.010^{I}
	1.0	33.4^{c}	242^{B}	0.067 ± 0.002^{II}
	2.0	20.7^{b}	$219^{A,B}$	0.073 ± 0.005^{II}
Genipin	0.5	20.8^{b}	245^{B}	0.069 ± 0.002^{II}
	1.0	19.2^{b}	341^{C}	$0.073 \pm 0.009^{I,II}$
	2.0	$\geq 100^{d}$	478^{D}	0.055 ± 0.002^{III}
Tgase	0.5	10.7^{a}	$188^{A,B}$	0.085 ± 0.007^{I}
	1.0	14.9^{a}	285^{C}	0.081 ± 0.003^{I}
	2.0	13.2^{a}	$260^{B,C}$	0.087 ± 0.002^{I}
EDC/NHS	0.5	19.7^{b}	$268^{B,C}$	0.081 ± 0.004^{I}
	1.0	18.5^{b}	312^{C}	0.086 ± 0.003^{I}
	2.0	29.3^{c}	529^{E}	0.080 ± 0.002^{I}

Regarding Fig. 1B, G and the crosslinked hydrogel with 1 wt% of G (CH/CG G 1) spectra replace the ones of the reaction with F. In this case, there are several factors that prove the occurrence of the proposed reaction, such as the disappearance of the signal attributed to the stretching of the C═C bond of the carboxymethyl group (1677 cm^{-1}), the increase in the intensity of the amide bands I and II (1559 and 1406 cm^{-1}, respectively) and 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}), which also leads to the broadening of the amide I band, as reported in a previous study [18].

In Fig. 1C, the effect that T exerts on the structure, which is slightly different to the one produced by the other crosslinking agents, can be observed. This is mainly due to the fact that, as an enzyme, T would only react with CG, thus forming semi-IPN hydrogels [60]. However, only minor changes were observed in the spectrum of the hydrogel crosslinked with 1 wt% of T (CH/CG T 1) when compared to the obtained for CH/CG, primarily a slight broadening and increase in the intensity of the amide bands I and II (1564 and 1414 cm^{-1} in this case). These changes could be attributed to the conformational rearrangement induced by T in the hydrogel structure, possibly stabilizing it, as reported by Wu et al. [75].

Fig. 1D includes, apart from the parent and the CH/CG spectra, the

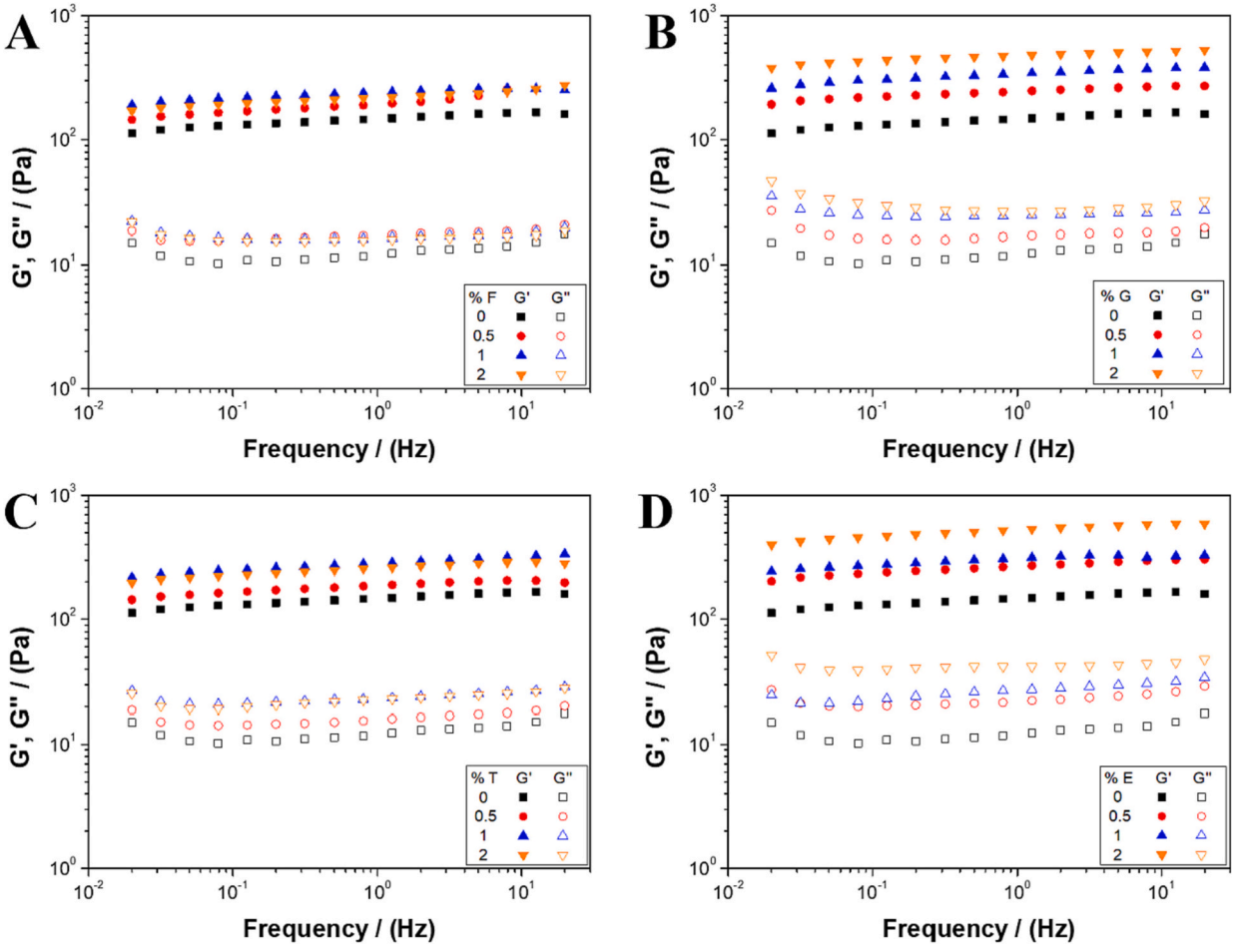

Fig. 2. Frequency sweep tests of CH/CG hydrogels crosslinked with different amounts (0.5, 1 and 2 wt%) of D-fructose (A), genipin (B), transglutaminase (C) and EDC-NHS (D).

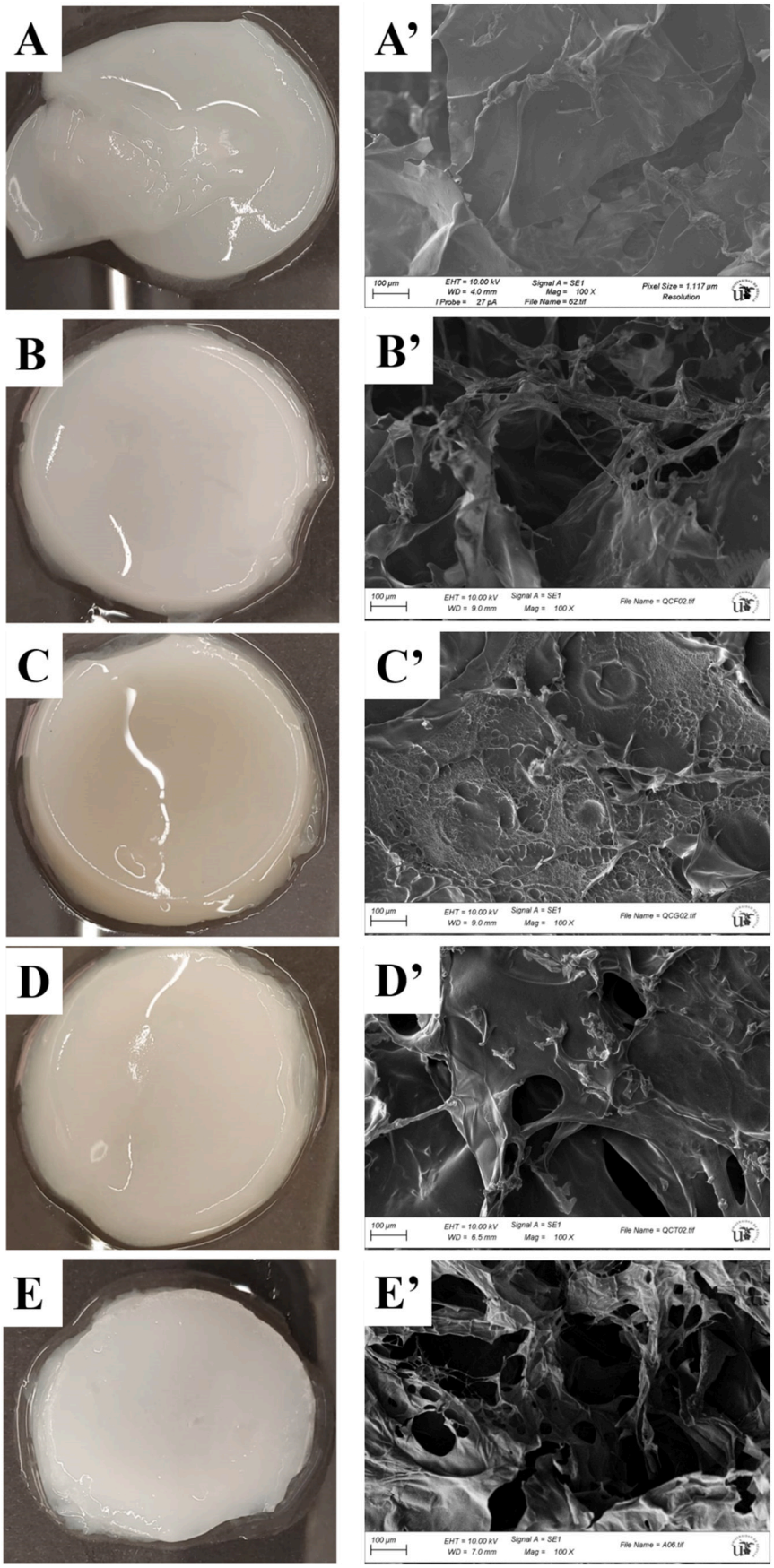

Fig. 3. Macroscopic and SEM images of the reference CH/CG hydrogel (A, A′, respectively) and the chemically crosslinked hydrogels with 1 wt% D-fructose (B and B′), genipin (C and C′), transglutaminase (D and D′) and EDC/NHS (E and E′).

Table 2
Porosity, mean pore size and swelling degree (SD) after 24 h of assay of both chemically non-crosslinked CH/CG hydrogel and selected crosslinked hydrogels with D-fructose, genipin, transglutaminase (Tgase) and EDC/NHS.

Crosslinker	Crosslinker amount (wt%)	Porosity (%)	Mean pore size (μm)	SD 24 h (%)
None	0	43.5 ± 6.5	122 ± 85.6	−7.1 ± 0.3
Fructose	1	48.9 ± 12	108 ± 96.5	−2.0 ± 0.3
Genipin	1	38.1 ± 2.8	58.1 ± 26.8	59 ± 0.2
Tgase	1	46.3 ± 1.4	119 ± 82.6	−8.2 ± 0.2
EDC/NHS	1	35.9 ± 4.5	165 ± 86.5	86 ± 0.5

obtained profiles of EDC and the corresponding crosslinked hydrogel (CH/CG E 1). The modification with EDC/NHS enables crosslinking of the components of the resulting hydrogel. This is evident from the increased transmittance values in the characteristic bands of amides (amides I, II and amide A), as there is an increase in the transmittance values. This could be explained by the activation of carboxylic acid groups of glutamic or aspartic acid residues of CG, which undergo the crosslinking reaction, forming new isopeptide bonds between these groups and amine groups of CG or/and CH, as previously reported by other authors [76–78].

In summary, the main variations observed in the FTIR analyses are principally reduced to the changes in signal and position of the amide I, II and III bands, compared to the ones obtained for the CH/CG chemically non-crosslinked hydrogel. No new characteristic bands appear or disappear, except the case of G.

3.2. Rheological evaluation

Once it has been established that the crosslinking agents successfully undergo the coupling reactions between the biopolymers, it must be verified that these reactions contribute to the desirable properties of the resulting hydrogels. Therefore, rheological characterization was performed for each system, starting with a strain sweep test to determine the critical strain, whose values are included in Table 1. After determining the critical strain, frequency sweep tests were carried out to evaluate the stability of the hydrogel structure when subjected to progressive increases in frequency. The results of these tests are shown in Fig. 2.

In all cases, G′ values are significantly higher than the G″ values, indicating the elastic response of the hydrogel systems and a more solid-like behavior rather than liquid-like. In addition, both G′ and G′ values slightly increased with increasing frequency, resulting in a hydrogel with low frequency dependence. [79,80]. It also can be observed that regardless of the crosslinking agent and its concentration used, higher G′ values were obtained compared to the CH/CG hydrogel without chemical crosslinking. This indicates that the addition of each crosslinking agent improves the rheological performance of the hydrogels. Nevertheless, two different tendencies can be observed in Fig. 2. The first trend is observed in Fig. 3A and C, where increasing the amount of crosslinking agent up to 1 wt% leads to higher G′ values. Nonetheless, further increasing the concentration of the crosslinker (2 wt%) does not necessarily result in further improvement, possibly due to saturation of the hydrogel network [17,81]. On the other hand, the second trend observed in Fig. 3B and D represents the opposite effect. Increasing the amount of crosslinking agent up to 2 wt% is beneficial for the rheological performance, obtaining even higher G′ values. These results could be attributed to the formation of a network with a more effective crosslinking with these particular compounds [47,82].

To better understand the effect of each crosslinker on CH/CG hydrogels, the values of critical strain, elastic modulus, and loss tangent (G'_1 and $\tan(\delta)_1$) at 1 Hz are summarized in Table 1.

Comparing the results in Table 1 with the data shown in Fig. 2, it is evident that the trends observed in the frequency sweep tests is consistent with the values obtained for critical strain. Although it is true that in the case of T an improvement in the critical strain is not achieved, an increase in the values of G′ is achieved with 1 and 2 wt%, though without significant differences. Crosslinking with F offers a similar result, although with slight differences, since even though the trend observed for the values of G′ is the same as that of T, the 1 wt% hydrogels exhibit greater deformability and a more solid-like behavior, as indicated by the values of critical strain and $\tan(\delta)_1$.

On the other hand, the results obtained from the reactions with G and EDC/NHS are perfectly consistent with those observed in Fig. 2. Increasing the amount of both crosslinkers leads to improvements in all the rheological properties, reaching the maximum values of G′ and critical strain with 2 wt%. This could be attributed to an increase in the crosslinking degree with these two crosslinkers, as it has been proved that increasing the amount of crosslinking agent (especially with genipin) can lead to an improvement of the Lineal Viscoelastic Range (LVR) and in G′ [83]. In addition, it should be noticed that the highest resistance would be obtained by the EDC/NHS crosslinked hydrogel, by reaching a G′ value of 529 Pa. However, the G crosslinked hydrogel not only exhibits the lowest value of loss tangent at 1 Hz (implying a greater solid behavior), but also the greater deformability among all the hydrogels, as it did not break with progressive increases in deformation (up to 100 %). Additionally, is worth mentioning that loss tangent results offered negligible differences between each system, being all of them below 0.1, meaning that hydrogels with excellent solid behavior were obtained [84].

G-crosslinked hydrogels offer a remarkable improvement in rheological performance [85], as well as with EDC/NHS [82]. In this sense, F exerts a beneficial effect on the rheological properties of CG and CH, as it does in other biopolymeric-based hydrogels [39,86]. At the same time, T, as an enzyme, would only act on the crosslinking and, thus, the rheological performance of CG [87]. Different effects of G and T in the rheological performance were observed by Pérez-Puyana et al. on CH/CG aerogels crosslinked with glutaraldehyde, G and T, where T offered a better rheological performance than G, though with higher G′ values, as these structures were freeze-dried [88]. However, it is worth mentioning that CH and CG concentration [19] and molecular weight have an important impact on the resulting rheological properties, as reported by Shagdarova et al., where CH of 700 kDa, doubling the one used in this work, and CG hydrogels crosslinked with G, exhibited G′ values around 10^3 Pa [89]. Similar results were obtained from the G′ values to those obtained in other crosslinking studies of CH hydrogels with phenolic compounds [90] or tannic acid [91].

To evaluate the rheological stability of these hydrogels against temperature, ramp tests were also performed (Fig. S2). In all cases, the hydrogels have good stability within the tested temperature range, with slight alterations in storage and loss moduli around 30 °C. This indicates that these hydrogels can preserve their properties during storage at low temperatures and within the body at physiological temperatures.

3.3. Microstructural evaluation

Fig. 3 provides both the microscopic and macroscopic views of the chemically non-crosslinked CH/CG hydrogel (Fig. 3A and A′) and the crosslinked hydrogels with each crosslinking agent at 1 wt%, selected as the intermediate and more representative concentration. From a macroscopic point of view, there were no noticeable differences

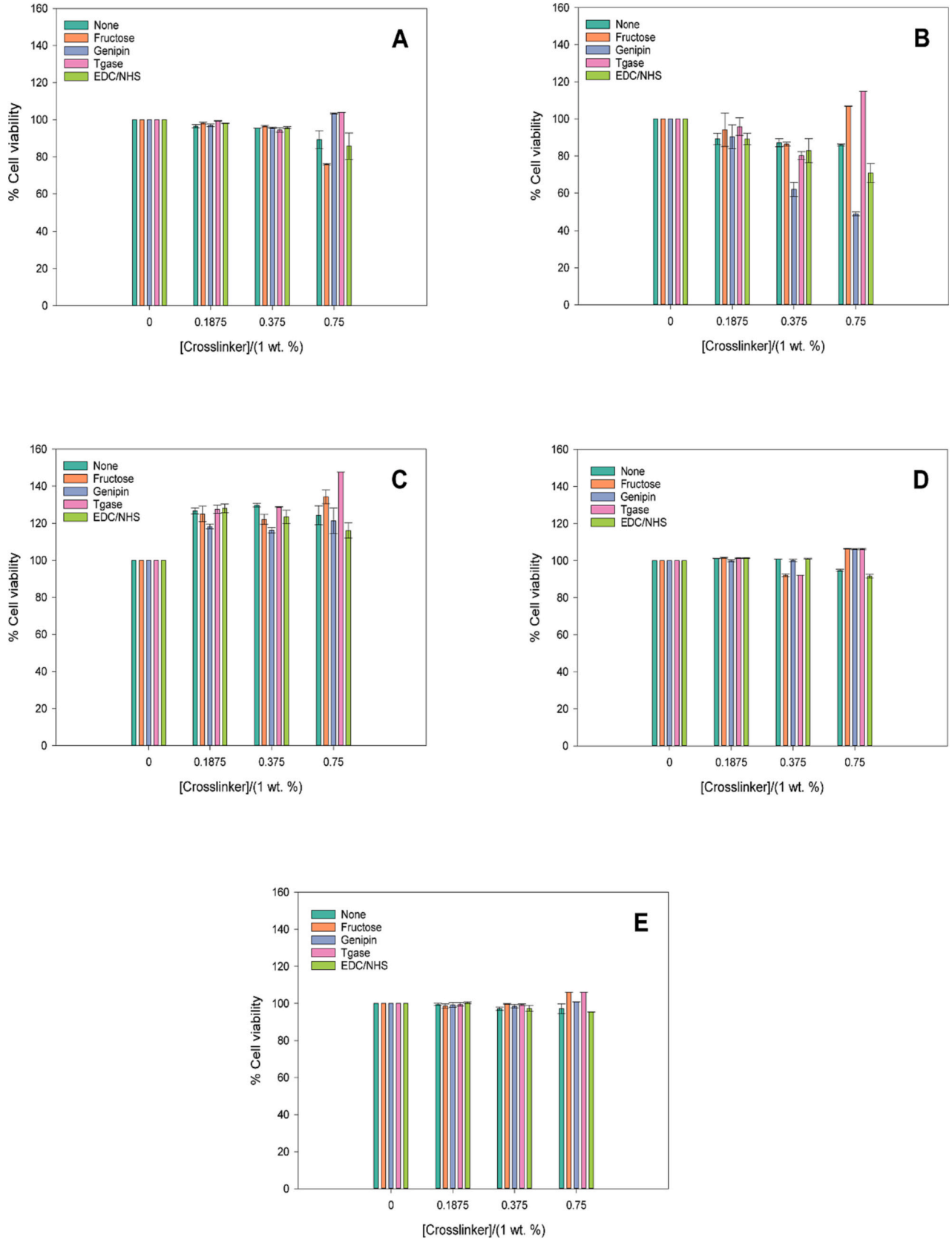

Fig. 4. *In vitro* cytotoxicity results obtained in the hybrid CH/CG hydrogels at 1 wt% without any crosslinker or crosslinked with D-fructose, Genipin, Transglutaminase (Tgase) and EDC/NHS: Vero E6 (A); HeLa (B); U2OS (C); U937 (D); and Jurkat (E) cell lines evaluated for 48 h.

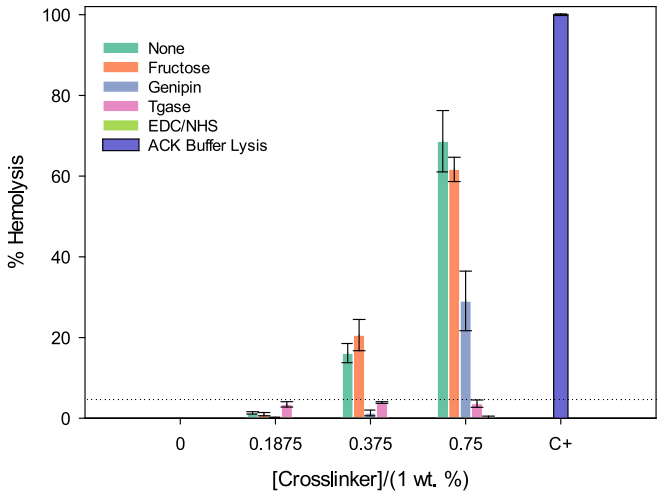

Fig. 5. *In vitro* hemolysis results obtained for the hybrid CH/CG hydrogels developed at 1 wt% without any crosslinker (None) or crosslinked with D-fructose, Genipin, Transglutaminase (Tgase) and EDC/NHS.

between the systems, which presented the characteristic white coloration. Nevertheless, the hydrogel crosslinked with G exhibited a brownish coloration. Typically, G produces prominent and characteristic blue colored products [92,93]. However, as reported by Yang et al., these hydrogels can turn brown depending on the pH when changing it from 3.2 (white) to approximately 5 [94].

Besides, the results obtained by SEM analysis did not reveal many differences between the systems analyzed either. The microstructure of the CH/CG hydrogel (Fig. 3A′) is quite similar to that reported in previous studies by the authors [19,47]. When comparing it with the microstructures of the chemically crosslinked hydrogels (Fig. 3B′–E′), in all cases, micrographs of homogeneous structures are observed, not so much in pore size as in pore distribution, being apparently the most heterogeneous structure, the one crosslinked with F (Fig. 3B′). The only exception was the crosslinked structure with G (Fig. 3C′), which showed a reduction in porosity and, *a priori*, in pore size, resulting in a more homogeneous structure compared to the other hydrogels.

In any case, to facilitate and support the comparison between systems, the values of porosity and mean pore size are shown in Table 2, together with the values obtained in the swelling tests after 24 h for the selected systems.

First of all, there are no significant differences between the systems in relation to porosity or pore size, except in the case of the hydrogel crosslinked with G. In this case, it can be seen that both the porosity value and the mean pore size are lower and more homogeneous compared to the others. This indicates that the structure of the CH/CG G 1 hydrogel has a more compact and solid structure, which is consistent with the rheological characterization results. The other structure that presented very interesting rheological properties is the one crosslinked with 1 wt% of EDC/NHS (CH/CG E 1) would also present a lower porosity value *a priori*. However, its mean pore size value was larger than that of the G-crosslinked system and any other in this study. This suggest that other factors, such as the nature of the crosslinking bond, may come into play and should be consider [95,96]. Otherwise, crosslinking with F and T would not offer significant differences with the chemically non-crosslinked hydrogel by means of porosity and mean pore size.

Regarding the results of the swelling tests, it can be seen that, after 24 h of testing, they followed a similar trend to what observed in the rheological characterization. The results of the crosslinked hydrogels with F and T displayed similar behavior between them, but different from that obtained when using G and E. In the former cases, a negative SD was observed, indicating a loss of water absorption capacity during the test, as occurs with the CH/CG hydrogel without any crosslinker. On the contrary, the hydrogels crosslinked with G and E present a greater water absorption capacity than their initial state, making them promising candidates for biomedical applications. In fact, this further suggests that crosslinking with G and E may be more efficient than with F and T, at least in CH/CG-based hydrogels.

3.4. Biological evaluation

In order to evaluate preliminarily the biocompatibility of the novel hybrid CH/CG hydrogels prepared as potential scaffolds for TE applications, *in vitro* cytotoxicity assays were carried out for each of the systems studied at 48 h (Fig. 4). CH/CG hydrogels without any crosslinker exhibited a similar behavior to a previous study reported by the authors [19], where a synergistic effect was observed between both biopolymers. Noticeably, no cytotoxicity effects were observed for the crosslinkers at 1 wt% in these CH/CG hydrogels evaluated, except in HeLa cells up to a value of 0.375 wt% (Fig. 4B). This phenomenon is due to the presence of the positive surface charge in chitosan, together with its high degree of deacetylation, which inhibits cell growth and increased toxicity [19]. In addition, the influence of T and EDC/NHS coupling to CH/CG hydrogel network was examined. The incorporation of these new crosslinking agents in these hybrid hydrogels did not exert any substantial *in vitro* cytotoxic effect, as expected [97,98]. Nevertheless, EDC/NHS-crosslinked (synthetic) CH/CG hydrogels are more toxic than the same hydrogels coupling with Tgase (a natural enzyme) due to reduced cell adhesion that leads to a specific type of apoptosis known as anoikis [99] and subsequently decreased cellular survival rate [37].

To obtain further information regarding their blood compatibility, an *in vitro* hemolytic test was performed to evaluate the hemolytic effect of the hydrogels. Fig. 5 shows the hemolysis data obtained for each hydrogel studied. CH, as explained above, is a positively charged biopolymer that can interact with the negatively charged cell membrane of red blood cells. In this sense, previous studies have suggested that electrostatic interactions with phospholipids of red blood cell membranes cause the formation of complexes that interfere with the correct functioning of these cells [100]. Consistent with the recent findings of the authors (data not published) in the presence of D-fructose and genipin as crosslinker agents, similar results were found. F-crosslinked hydrogels led to a higher hemolysis rate than G-crosslinked hybrid hydrogels, as can also be seen in Fig. 5. To support this idea, high F concentrations could produce a dysregulation of the glycolytic pathway [101]. Nevertheless, crosslinking with G reduces its hemolytic effect, as was previously described by Gao *et al* [92]. No hemolytic effect was observed for hydrogels coupled with both Tgase and EDC/NHS, as reported by other authors [102,103]. In particular, Tgase promotes the catalytic formation of intermolecular ε-(γ-glutamyl)lysine isopeptide bonds in protein substrates and regulates biological processes such as homeostasis, wound healing, phagocytosis, and bone and matrix remodelling *via* Factor XIIIa (an active Tgase) [104].

4. Conclusions

The chitosan/collagen hydrogel was successfully crosslinked with the mentioned compounds, namely D-fructose, genipin, transglutaminase and EDC-NHS, as proved by FTIR measurements. Two main trends were observed in almost each characterization assay. For F and T, the rheological properties improved with increasing the amount of crosslinker up to 1 wt%. However, a further increase to 2 wt% was not beneficial since the critical strain and G'_1 values fell compared to those obtained for 1 wt%. It can be concluded that, *a priori*, crosslinking with F and T would not be very effective since there are minimal differences in the structure, which aligns with the results of the rheological tests, whose improvement, compared to the gel without crosslinking, exists, but it is very slight. In addition, the negative swelling degree values obtained for each hydrogel, which may indicate a lack of desired

cohesion in the structure, which was lost after 24 h submerged in water. Nonetheless, there is a great difference between these crosslinking agents in terms of their biological performance. F, as a fundamental regulator of the glycolytic pathway, is harmful because it increases the synthesis of advanced glycation end products (AGEs) and reactive oxygen species (ROS) (resulting in increased cytotoxicity). In contrast, T, being a natural enzyme, makes a very beneficial contribution to the proliferation of most of the cell lines assessed and without inducing hemolysis.

On the other hand, regarding G and EDC-NHS showed significant improvement in the rheological characterization with increasing the amount of crosslinking agent (much more than F and T), reaching their maximum when including 2 wt% of crosslinking agent. Besides, although without much difference, both present a more compact and homogeneous structure than the rest of the hydrogels tested and improve the swelling properties. In terms of biological performance, both crosslinking agents enhanced the hydrogel's capacities without crosslinking by improving cell proliferation in all cell lines and reducing the hemolysis rate.

When comparing the effect of each crosslinker, it is evident that among the four, the least effective and beneficial is F. It is true that T fails to form a cohesive and compact semi-IPN structure, without any remarkable improvement in the rheological performance, but the biological improvement that T provided must be taken into account. Among the two crosslinkers considered the best crosslinkers in this study, it is challenging to determine which is superior, since both offer different contributions and, consequently, different properties (all of them very interesting for TE). G produces hydrogels with a more solid and deformable character, supported by their structure and promotes cell proliferation in most of the tested cell lines. However, EDC-NHS presents hydrogels with better elastic moduli and, therefore, higher resistance, a somewhat more porous structure with enhanced swelling capacity. Additionally, despite its synthetic origin, it significantly reduces the hemolysis rate compared to that obtained by G. That being said, both crosslinkers, although different, provide compelling results for the application of these hydrogels in TE.

CRediT authorship contribution statement

Pablo Sánchez-Cid: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **María Alonso-González:** Investigation, Validation, Writing – review & editing. **Mercedes Jiménez-Rosado:** Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Mohammed Rafii-El-Idrissi Benhnia:** Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing. **E. Ruiz-Mateos:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Francisco J. Ostos:** Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Alberto Romero:** Conceptualization, Funding acquisition, Resources, Supervision, Validation. **Víctor M. Perez-Puyana:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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