Journal of Molecular Liquids 384 (2023) 122224

Contents lists available at ScienceDirect

Journal of Molecular Liquids

journal homepage: www.elsevier.com/locate/molliq

Hybrid polymeric Hydrogel-based biomaterials with potential applications in regenerative medicine

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ARTICLE INFO

Keywords:
Collagen
Gelatin
Hydrogels
Rheological properties
Biocompatibility

ABSTRACT

In the field of regenerative medicine, the use of biomaterials as scaffolds that provide structural integrity is key for tissue regeneration. In this context, hydrogels are considered a great option, due to their elastic properties and capacity to absorb large amounts of water while preserving their structure. Notably, both collagen and gelatin are considered good candidates for use due to their high biocompatibility. In particular, gelatin is a collagen derivative that has better biological properties at the expense of poorer mechanical properties. Therefore, the main objective of this work was the development and characterization of polymeric hydrogels based on collagen and gelatin. In this sense, hydrogels with different collagen/gelatin ratios were elaborated using cooling as the gelation method. Subsequently, different studies were carried out in order to evaluate their mechanical, thermal and microstructural properties, as well as their biocompatibility. The results showed that hydrogels formed from the mixture of collagen and gelatin retain, to a large extent, the good viscoelastic properties of collagen, while showing low levels of cytotoxicity and hemocompatibility similar to those obtained for gelatin. However, owing to the nature of the materials used, the thermal characteristics are not ideal for use in biomedicine, thus further studies are required to overcome these drawbacks.

1. Introduction

Regenerative medicine is one of the most promising and innovative fields of medicine, which is focused on developing novel therapies and strategies to heal damaged or diseased tissues, or organs. Noticeably, the tremendous scope of regenerative medicine could revolutionize patient care in the treatment of oncological, neurological and cardiovascular diseases, wound healing, and degenerative and genetic disorders, among others [1]. According to the Alliance for Regenerative Medicine [2], there were 2,093 active clinical trials at the end of June 2022, with 53% of them in Phase II. Cell therapies is the largest category of ongoing clinical trials (46%), followed by cell-based immuno-oncology (34%) and gene therapies (18%). Tissue engineering strategies only represent the remaining 2% (32) of active clinical trials.

Nevertheless, millions of deaths happen worldwide every year owing to the loss or failure of tissues or organs. Therefore, to explore the most advanced approaches for regenerating or improving damaged tissues or organs is needed. Tissue engineering, a new emerging field, is a biomedical engineering discipline included within regenerative medicine, which is based on the use of three-dimensional (3D) biomaterials for mimicking several characteristics of the extracellular matrix (ECM) constituents such as proteins, proteoglycans, fibronectin and laminin present within all tissues and organs, thereby enhancing the functional restoration of damaged tissues [3]. ECM not only provides essential physical scaffolding for cellular components, but it also plays a key role in numerous biochemical and biomechanical processes involved in tissue morphogenesis, differentiation and homeostasis. Despite the fact that each tissue possesses an ECM with a unique composition, morphology and phenotype, it is fundamentally composed of water, polysaccharides and proteins [4–6].

Given the complexity and composition of native ECM, polymeric biomaterials are considered one of the cornerstones of tissue engineering. These biomaterials can be broadly classified as synthetic and natural polymers [7]. Synthetic polymers, such as polyglycolic acid, poly(lactic-

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https://doi.org/10.1016/j.molliq.2023.122224
Received 24 December 2022; Received in revised form 31 March 2023; Accepted 28 May 2023
Available online 1 June 2023

co-glycolic acid) poly(ethylene glycol), and poly(ethylene terephthalate), are especially attractive due to their ability to control structure, strength, and degradation rate when they are manufactured. Natural polymers include naturally occurring polymers (silk and chitosan), purified ECM proteins (collagen and fibronectin), and ECM obtained by decellularization of various tissues. Natural polymers have a high immune recognition due to the presence of integrins with affinity for cell surface receptors that initiate cell adhesion. However, they also have some disadvantages, such as their low mechanical properties and biodegradability, and problems related to manufacturing [8].

Since the pioneering work of Wichterle and Lim, where a hydrated hydroxymethyl methacrylate (HEMA) network with the crosslinker Ethylene glycol dimethylacrylate (EGDMA) was prepared for contact lenses in the 1960 s [9], hydrogels have gained special research interest. Hydrogels are polymeric networks, which are characterized by absorbing a significant amount of water (more than 20% of their dry weight) without dissolving or losing their structural integrity. These biomaterials, which are insoluble in water despite having polar groups, have a 3D structure that allows them to swell in the presence of a fluid, thus increasing their volume and obtaining a soft and elastic consistency, although maintaining their shape until reaching a physicochemical balance [10–12].

Regarding the raw material, most recent studies concerning scaffolds for tissue engineering have been focused on the use of biopolymers, especially proteins and polysaccharides [13]. These biopolymers present several advantages, such as biocompatibility, which is crucial to produce biomaterials that prevent foreign body responses, and biodegradability, which allows producing temporary biomaterials [14,15]. Among these biomaterials, gelatin and collagen can adopt a large number of biological structures, contributing to a high level of biocompatibility and biodegradability. Both biopolymers can also lead to high tensile strength and contain cell adhesion sequences [16].

Although most studies have used unitary systems based on either collagen or gelatin, a potential alternative lies in the mixture of these two raw materials to combine their qualities and thereby produce a hybrid biomaterial [17]. In this sense, some studies show that the fabrication of hybrid systems improves their potential application [18,19]. For example, Pottathara *et al.* studied ternary systems based on gelatin, collagen and hydroxyapatite [20], and Rajasree *et al.* evaluated the influence of chitosan on collagen/gelatin composites [21]. In the same line, Redmond *et al.* reported the potential application of collagen/gelatin scaffolds for breast cancer research, and Kavukcu *et al.* studied the influence of curcumin nanoparticles on the properties of gelatin-collagen scaffolds [22,23]. However, the use of binary hydrogels based on gelatin and collagen has not been explored in depth.

In this context, the main objective of this work was the development of hybrid hydrogels. Different collagen/gelatin ratios were used to evaluate the synergistic effect of their combination. The expected outcome of the study was the improved properties of the hybrid systems based on collagen and gelatin compared to the unitary systems, in terms of their rheological, microstructural and biological properties.

2. Materials and methods

2.1. Materials

Hydrogels were formed by the combination of two biopolymers: collagen (CG) and gelatin (GE). For this, pork type I collagen (M.W. = 300 kDa and isoelectric point at a pH range of 5–6) supplied by Protein Solutions (Essentia Protein Solutions, Denmark), and type B gelatin (M. W. = 180 kDa and isoelectric point at a pH range of 5–6) provided by Henan Boom Gelatin Co. Ltd. (China) were used as raw materials. In addition, acetic acid, supplied by Panreac Química S.A. (Spain), was used as solvent.

2.2. Processing of hydrogels

The raw materials were processed by a gelation process to fabricate hydrogels. The gelation process followed was described in previous studies [24]. The hydrogels were produced at 1.5 %w/v from different solutions in acetic acid (0.05 M at pH 3.2), considering the solubility of both raw materials in acidic media [25]. These parameters were selected as the optimal ones to achieve homogeneous and stable hydrogels [24]. Different ratios of CG/GE were used: 100/0, 75/25, 50/50, 25/75 and 0/100. The different solutions were centrifuged at 10,000 rpm for 7 min, maintaining the temperature at 4 °C. Subsequently, a gelation step was performed under specific conditions (4 °C for 2 h).

2.3. Characterization of hydrogels

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR was carried out in a Hyperion Spectrometer (Bruker, USA) within attenuated total reflection (ATR) target. Hydrogel samples were freeze-dried to avoid the interference of the water peak in the FTIR spectrum, weighed and analysed. These measurements allow knowing the bonds that make up the systems, since they emit at different wavelengths. Thus, this analysis allows evaluating the differences between the systems. The infrared spectra were obtained at 4000–1250 cm^{-1} with an opening of 100 cm^{-1}.

2.3.2. Rheological evaluation

The viscoelastic properties of the hydrogels were determined by using three types of rheological tests carried out with an AR 2000 rheometer (TA Instruments, USA).

Strain sweep tests: Measurements between 0.0001 and 40% strain at a constant frequency of 1 Hz and 25 °C were performed to determine the linear viscoelastic range and the critical strain.

Frequency sweep tests: The measurements were carried out in a frequency range between 0.02 and 20 Hz at a specific strain for each system (within the linear viscoelastic range) and 4 °C. In these tests, the elastic and viscous moduli (G' and G", respectively) were obtained, together with the loss tangent (tan δ). The values of G' and tan δ at 1 Hz were calculated (named as G'_1 and tan δ_1, respectively) for each system.

Temperature ramps: Finally, the hydrogels were exposed to a temperature range of 4–40 °C in order to evaluate their thermal stability. These tests were performed at a constant strain of 2% and 1 Hz. The critical temperature was obtained as the temperature range in which the hydrogel lost its stability.

2.3.3. Microstructural characterization

A Zeiss EVO scanning electron microscope (Germany) was used to perform the microstructural characterization of the different hydrogels. The Cryo-SEM technique was used, whereby the sample is subjected to a treatment with liquid nitrogen to fix the sample [26]. After the fixation of the samples, they were metallized with a thin layer of gold/palladium to improve their conductivity and improve their visualization in the microscope. Images were obtained at 30X and 80X at an acceleration voltage of 10 kV.

2.3.4. In vitro cytotoxicity assays

The cytotoxicity of the hydrogels was estimated in vitro using the CyQUANT™ LDH cytotoxicity assay [27]. Several cell lines from commercial supplier (ATCC®, USA) were used, Vero E6 (normal monkey kidney epithelial cells), HeLa (human cervical carcinoma epithelial cells), U937 (human leukemia monocytic cells), U2OS (human osteosarcoma epithelial cells), and Jurkat (human T leukemia 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 and Vero E6 cell lines or R-10 for U937, Jurkat and U2OS cell lines [Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute (RPMI) supplemented with 10% of fetal bovine serum (FBS),

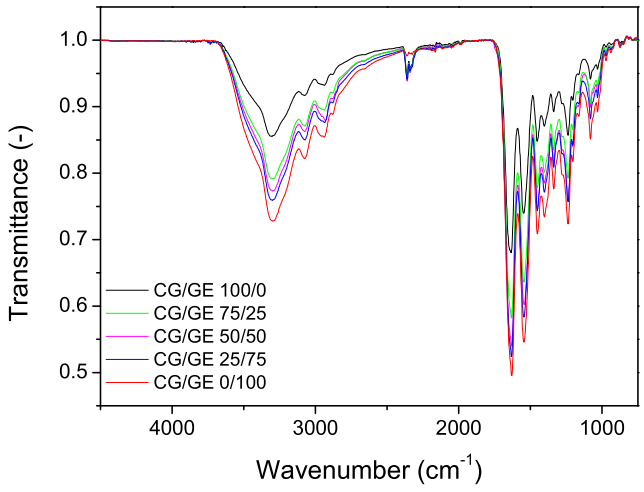

Fig. 1. FTIR profiles of the hybrid hydrogels at different ratios of CG/GE.

penicillin, streptomycin, and L-glutamine)], incubated at 37 °C in 5% CO_2, and used the following day (75 to 90% confluence). The FBS used in all experiments was heat inactivated (56 °C, 30 min) prior to use to eliminate complement activity. Hydrogel blends at different concentration (wt. %) values were added to each well and the plates were incubated for 36 h at 37 °C in 5% CO_2. Control D-10 or R-10 medium alone was used as negative control. 10 μL of 10X Lysis Buffer and 10 μL of sterile ultrapure water was added to each set of triplicate wells, which were used as the Maximum LDH Activity and Spontaneous LDH activity, respectively. Then, the medium from each well was collected by centrifugation of the plate and used to test the cytotoxicity of the hydrogels using a CyQUANT™ LDH Cytotoxicity Assay Kit according to the manufacturer's suggestion (Invitrogen™ from Thermo Fisher Scientific, USA). 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 was calculated using Equation (1):

$$\%\text{Cell viability} = 100 - \left(\left[\frac{\text{Compound} - \text{treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}}\right] \times 100\right) \quad (1)$$

Cell viability values were also checked by trypan blue method [28] and no significant differences were observed.

2.3.5. In vitro hemolysis assays

The hemocompatibility of the hydrogels was determined in Red Blood Cells (RBCs). To this end, a blood sample was extracted from 3 healthy human donors in vacutainer tubes containing EDTA (BD, Franklin Lakes, NJ, USA), obtained at the Regional Center for Blood Transfusion and Tissue Bank Sevilla- Huelva (Seville, Spain). RBCs were isolated by centrifugation at 1800 rpm for 5 min. The supernatant was discarded, and RBCs were washed twice with PBS 1X. Subsequently, 1 mL of the washed RBCs was suspended in 9 mL of 1X PBS and carefully homogenized in order to obtain a stock solution. The hydrogel blends were evaluated at the same concentration (wt. %) values used for in vitro cytotoxicity assays. ACK Lysing Buffer (Gibco™ from Thermo Fisher Scientific, USA) and 1X PBS (Gibco™ from Thermo Fisher Scientific, USA) were used as positive and negative controls, respectively. To evaluate the hemolytic effect, 125 μL of the RBCs stock solution was seeded with 125 μL of the different systems into Nunc flat-bottomed 96-well plates (ThermoFisher Scientific, USA), and were incubated at 37 °C in 5% CO2 for 4 h. Then, the 96-well plates were centrifuged, and the supernatants were transferred to another flat-bottomed 96-well plates. Each hydrogel concentration (wt. %) was measured in duplicate and the tests were repeated thrice independently. Finally, absorbance was read at 540 nm, and hemolysis percentage was calculated following Equation (2):

$$\%\text{Hemolysis} = \frac{\text{Compound} - \text{treated Hemoglobin release} - \text{Spontaneous Hemoglobin release}}{\text{Maximum Hemoglobin release} - \text{Spontaneous Hemoglobin release}} \times 100 \quad (2)$$

Table 1
Rheological parameters obtained for the different systems processed.

CG/GE ratio	Critical Strain (%)	G'$_1$ (Pa)	tan δ_1	Critical temperature (°C)
100/0	0.402 ± 0.001	208 ± 8	0.022 ± 0.001	32–35
75/25	0.064 ± 0.001	229 ± 16	0.023 ± 0.006	32–35
50/50	0.400 ± 0.001	156 ± 32	0.021 ± 0.001	32–35
25/75	0.401 ± 0.001	42 ± 6	0.06 ± 0.03	32–35
0/100	0.400 ± 0.001	0.06 ± 0.04	7 ± 5	26–29

3. Results and discussion

3.1. FTIR measurements

Fig. 1 shows the FTIR spectra of the different hybrid hydrogels. Firstly, it is important to highlight the similarity among all the profiles. This is due to the great resemblance that exists between both proteins, since gelatin is essentially denaturalized collagen. In addition, as both materials are proteins, all the systems show the five characteristic signals for this type of materials: a first signal around 3400–3300 cm^{-1} (Amide A signal) referred to the N—H stretching, followed by another signal at 3000–2900 cm^{-1} (Amide B), which indicates C—H stretching (characteristic of the CH_2 bond). Then, three main signals appeared at about 1650 (Amide I), 1550 (Amide II) and 1200 cm^{-1} (Amide III), which correspond to C═O stretching, N—H bending coupled with C—N stretching and N—H bending, respectively. These signals correspond to those obtained in other studies [29,30].

As was mentioned above, all the systems present a similar profile with minor differences in the intensity of these signals, which suggests that there are slight differences in the structure of each of the systems evaluated. Other than qualitative findings on Amide I band position, which will allow a better differentiation between both biopolymers

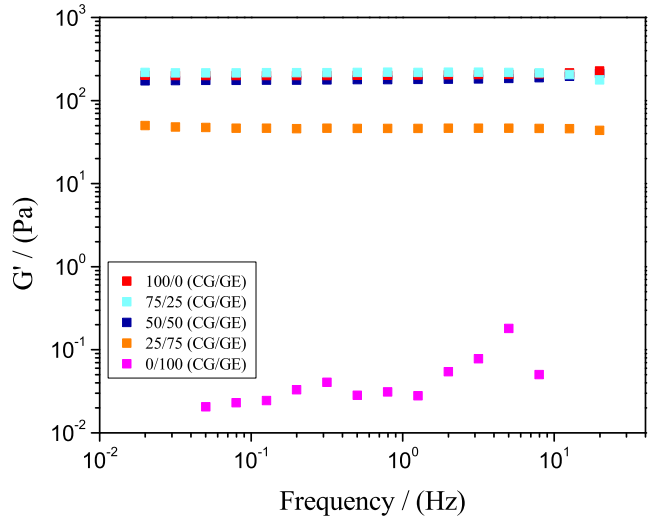

Fig. 2. Frequency sweep tests obtained by the different systems processed.

[30]. These results provide evidence of the lower triple helix integrity with the presence of gelatin B in the hybrid hydrogels investigated as a representative model of denatured collagen.

3.2. Rheological characterization

Table 1 shows the critical strain of the different hybrid hydrogels. As can be observed, the critical strain remains stable, except for the 75/25 (CG/GE) system, which shows a considerably lower value. This could be due to the synergy between the two materials at this concentration, leading to the formation of a highly compacted structure compared to the other hydrogels, which translates into a system with a higher rigidity (less elastic deformability). Nevertheless, the elastic modulus (G'_1) and loss tangent (tan δ_1) of this system do not present significant differences with respect to the 100/0 system, so the synergistic effect does not produce changes in these properties.

Fig. 2 depicts the frequency sweep tests performed on the different hybrid hydrogels. These systems present a significant degree of stability, remaining nearly invariable throughout the frequency range, which is a typical behavior for hydrogels [31]. However, the sole exception to this pattern is the CG/GE (0/100) unitary system, which provides further evidence regarding the weak structural characteristics of gelatin.

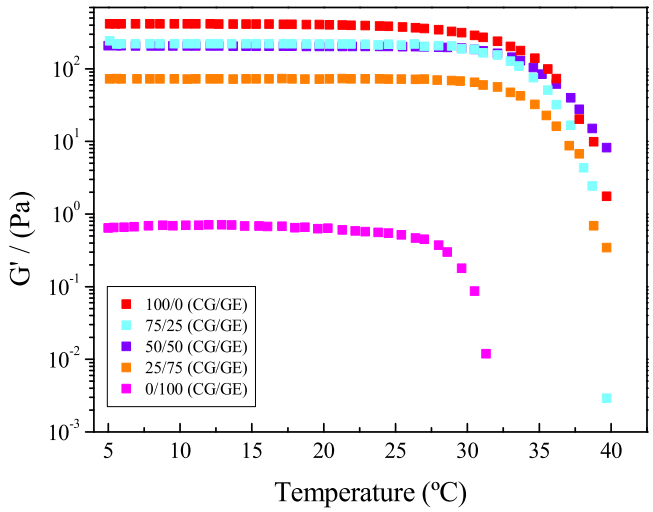

Fig. 3. Temperature ramp test carried out by the different systems processed.

Another noteworthy feature is the absence of significant differences between the systems with a collagen percentage over 50% in terms of stability.

In addition, for a meaningful comparison of the systems, G' and tan δ values at 1 Hz (G'_1 and tan δ_1) were included in Table 1. Considering the loss tangent for the different hydrogels (tan δ_1), the hydrogel systems show a clear solid character, except, once again, for the CG/GE (0/100) unitary system, whose loss tangent value denotes that this system exhibits a predominant liquid character (G''>G').

Regarding thermal stability, Fig. 3 shows the results obtained from the temperature ramp tests carried out on the different hydrogels. None of the systems under evaluation preserved their structure over 35 °C. While the addition of collagen improved thermal stability, it does not appear to be sufficient to maintain its structure at near human body temperatures. To provide a better contrast, the critical temperature ranges are shown in Table 1. As was previously mentioned, the addition of collagen seems to improve the structural properties of the hydrogels, leading to a higher thermal stability. Nevertheless, this improvement does not suffice to preserve its properties at body temperatures. The reason behind this is that polymer-based hydrogels produced by cooling have thermal stability up to 30 °C [32]. Previous studies using additional steps (i.e. electron irradiation) have demonstrated that the gelation method can alter the mechanical properties of the hydrogels formed, despite using the same material, allowing a thermal stability [33].

This indicates that the gelation technique, in accordance with the gelatin properties, are responsible for the poor mechanical and thermal properties of the 0/100 (CG/GE) system.

3.3. Microstructural characterization

Fig. 4 shows the microstructure of the hydrogels produced. There is a remarkable resemblance between both unitary systems, probably due to the great similarity between these two proteins (since gelatin is obtained from collagen). The structure obtained is homogeneous and does not show a pronounced degree of porosity, with the existing pores being derived from the rupture of the 3D structure. The gelatin-based hydrogel (CG/GE, 0/100) presented a higher porosity, probably due to the breakage caused by its poor structural properties. This structure differs from that described in other studies using collagen [34], which is likely due to the gelation technique applied.

On the other hand, the binary systems appear to have a heterogeneous arrangement, with a pronounced porosity degree. It is interesting to mention that the pores are remarkably diverse among themselves, probably due to the fact that they are formed by breaks in the hydrogel structure. Thus, the layered pattern is intensified, as can be observed in Fig. 4***D***. Furthermore, the addition of gelatin to the mixture increases both the presence of pores and the layered disposition.

In summary, there is a clear difference in microstructure between unitary systems (CG/GE 100/0 and 0/100), which are homogeneous and less porous, and binary systems (CG/GE 75/25, 50/50 and 25/75), which are highly heterogeneous and more porous. In addition, a clear trend in the increase of porosity can be observed regarding the addition of gelatin.

3.4. Biological characterization

Fig. 5 shows the results of the cytotoxicity assay after 36 h of incubation. Overall, the systems did not produce cytotoxicity. Nevertheless, an exception to this feature was found in the CG unitary system, since the higher the collagen concentration the lower the cell viability of several of the epithelial cell lines used such as HeLa and Vero E6 cell lines. This trend has been previously reported in other studies [35,36]. Notably, the addition of collagen stimulates the monocyte-derived macrophages (MDMs)-mediated innate immune response and wound healing [37], which might increase the production of pro-inflammatory cytokines and diminish cell viability in U937 cells. However, this

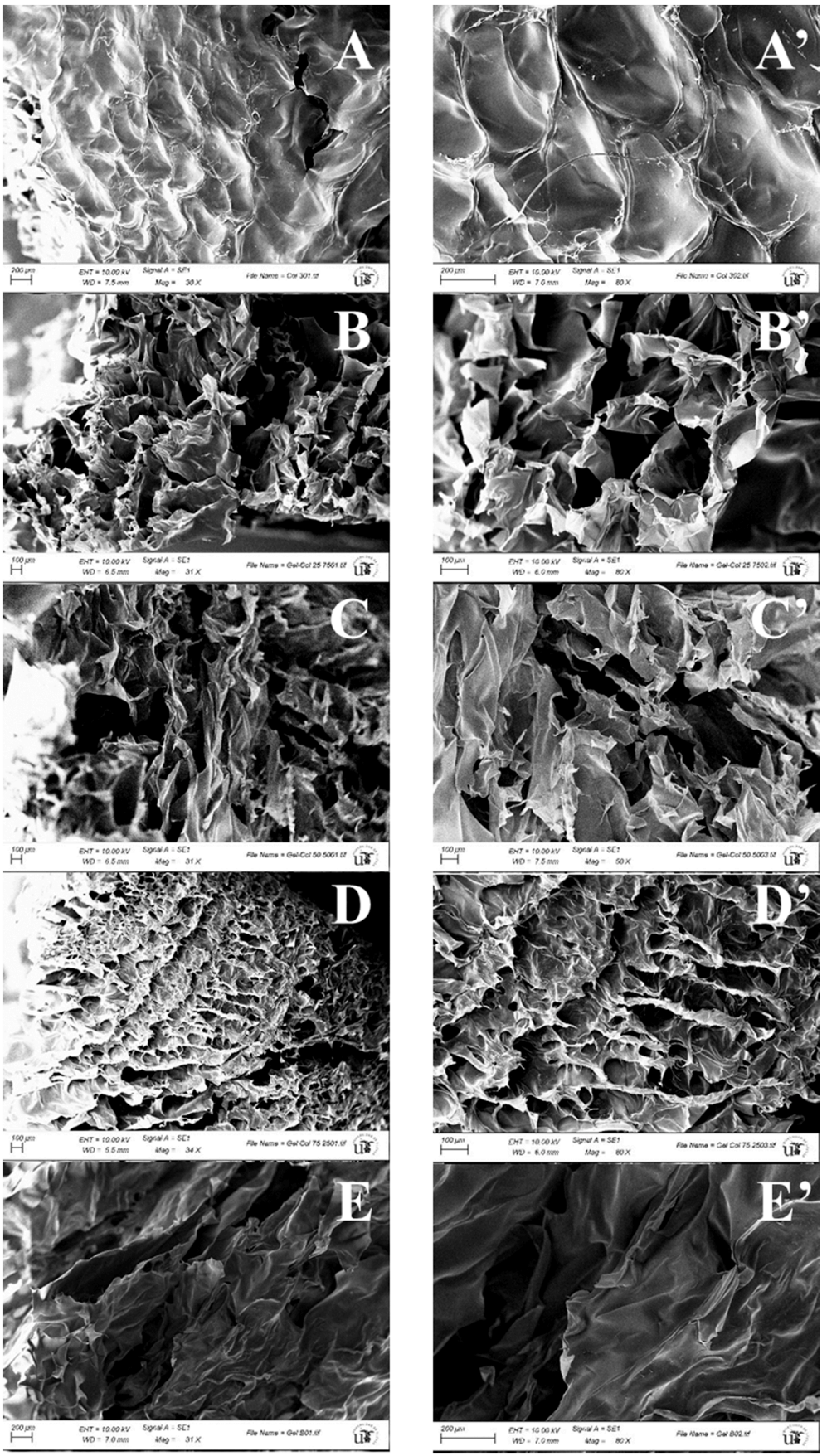

Fig. 4. ***Microstructural images of the selected hydrogels:*** CG/GE 100/0 (**A, A'**), CG/GE 75/25 (**B, B'**), CG/GE 50/50 (**C, C'**), CG/GE 25/75 (**D, D'**), CG/GE 0/100 (**E, E'**).

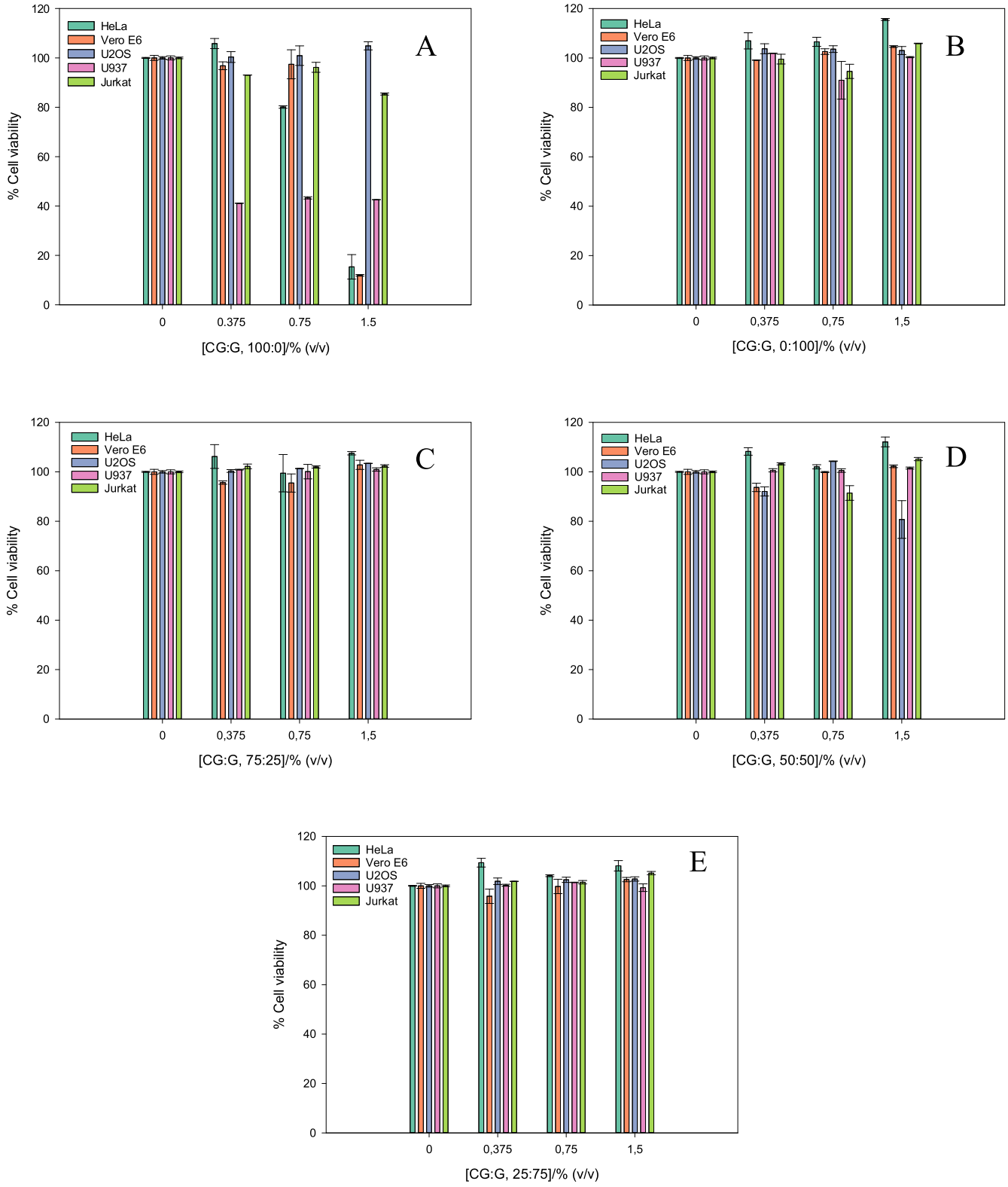

Fig. 5. ***In vitro cytotoxicity results obtained in the prepared hydrogels:*** CG/GE 100/0 (**A**), CG/GE 0/100 (**B**), CG/GE 75/25 (**C**), CG/GE 50/50 (**D**), CG/GE 25/75 (**E**).

behavior is not reported for all cell lines. U2OS cell viability does not decrease at high collagen concentrations. This may be explained by the fact that the bone matrix is partly composed of collagen; since U2OS is a bone-type cell line, it proliferates properly in a collagen-containing matrix. This non-cytotoxic activity of collagen on U2OS was reported in a previous study [38].

On the other hand, the addition of gelatin, even in small proportions, reduced cytotoxicity effect of collagen. As was already mentioned, gelatin substantially improved the biocompatibility of the systems, even for gelatin ratios below 50%. A previous study shows similar results and

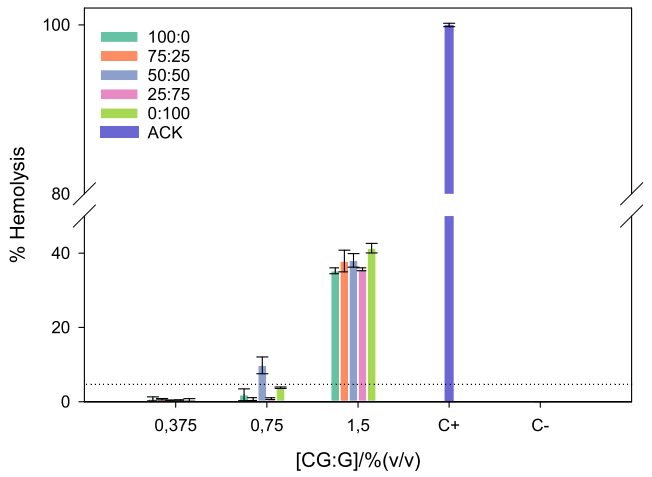

Fig. 6. *In vitro* hemolysis assay results obtained in the prepared hydrogels.

suggests that the reason for this lies in the primary structure of gelatin, which has a certain number of amino acid sequences that promote cell adhesion and growth. Although these sequences also appear in collagen, they are more common in gelatin [39].

Fig. 6 shows the hemolysis percentage resulting from the interaction between the different systems with the erythrocytes. The graph indicates the minimum percentage of hemolysis that a substance must produce in order to be considered hemolytic (5%) [40]. The first aspect that stands out is the high hemolytic activity of all systems at higher concentrations, regardless of the CG/GE ratio. Nevertheless, the hemolysis rate decreased significantly as the hydrogel concentration decreased, indicating that the systems are hemocompatible at medium–low concentration. It is suggested in another study that the presence of amino acid residues can trigger electrostatic interactions with phospholipids present in erythrocyte membranes, forming complexes that interfere with the correct functioning of those cells [41]. According to the literature, the presence of amine groups in the polymer structure leads to high hemolytic activity [42]. It is worth mentioning that, at 0.75% (w/v), 50/50 (CG/GE) exceeded the minimum level of hemolysis, which was not the case for the other systems.

As can be observed, the interaction between both biopolymers at the same ratio resulted in the formation of a system that exhibited a lower biocompatibility compared to the other systems elaborated. A possible explanation could be that the synergy between gelatin and collagen allows developing a structure where amine groups are exposed, resulting in an increased number of interactions with cell membranes that may be ultimately responsible for the toxicity of the resulting hydrogels.

The results obtained in this research demonstrate that interactions between both materials during the gelation process result in the formation of systems which, at suitable concentrations up to 0.75 (wt. %), show high biocompatibility for healthy and cancer cell lines, as well as for erythrocytes. Therefore, this suggests that, pending further evidence to support these ideas, they could be considered as potential candidates for use within the field of regenerative medicine.

4. Conclusions

The use of collagen and gelatin at different ratios for the elaboration of hydrogels by cooling leads to the development of hydrogels that possess simultaneously good mechanical properties associated with collagen, as well as those biological properties associated with gelatin. Thus, the synergy between both biomaterials enables such systems to present both favorable characteristics from each of the materials used for their formation. This occurs even when the ratio of one of these materials is considerably low (<50%). This research lays the foundation for future work focused on the production of hydrogels based on combinations of collagen and gelatin that can be used as potential biomaterials for regenerative medicine.

CRediT authorship contribution statement

G. González-Ulloa: Methodology, Validation, Software, Formal analysis, Investigation, Writing – original draft. **M. Jiménez-Rosado:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **M. Rafii-El-Idrissi Benhnia:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **A. Romero:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition. **E. Ruiz-Mateos:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition. **F.J. Ostos:** Methodology, Validation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **V. Perez-Puyana:** Methodology, Validation, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

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

The authors are unable or have chosen not to specify which data has been used.

Acknowledgements

This study was financially supported by MCIN/AEI/10.13039/501100011033/ FEDER, UE, through the project PID2021-124294OB-C21. The authors thank their financial support. The authors also acknowledge the post-doctoral grants of F.J. Ostos and V. Perez-Puyana (PAIDI-DOCTOR, Junta de Andalucía with European Social Fund, DOC_00963 and DOC_00586). The authors also thank CITIUS for granting access to and their assistance with the microscopy service.

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