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Effects of Genipin Crosslinking on the Structural and Rheological Properties of Chitosan/Collagen Hydrogels for Biomedical Applications

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Chitosan/collagen hydrogels have gained prominence in biomedical applications due to their biocompatibility and ability to promote tissue regeneration. However, rheological instability may limit their applicability. This study investigated the structural and rheological properties of chitosan/collagen hydrogels (99:1, 97:3, and 95:5%) crosslinked with 0.1% genipin. The samples were characterized by Fourier Transform Infrared Spectroscopy (FTIR), injectability, viscosity, swelling, biodegradation, and blood clotting time. Genipin crosslinking improved structural stability, increased viscosity and extrusion resistance, and provided slower and more predictable mass loss under physiological and enzymatic conditions. The swelling assay showed a progressive reduction in water uptake with increasing collagen content, further accentuated by genipin, suggesting a denser and more stable polymeric network. Additionally, the hydrogels accelerated blood coagulation, reinforcing their potential use as hemostatic agents.

Keywords: *Rheology, hemostatic, biopolymers.*

1. Introduction

Hydrogels have emerged as key materials in the biomedical field, particularly in wound treatment, offering a broad spectrum of applications and innovative solutions to complex clinical challenges. These three-dimensional, porous, and hydrated polymeric networks are characterized by their high water retention capacity and ability to conform to biological environments, features that are essential for facilitating wound healing and tissue regeneration processes[1].

Among the main biopolymers used in hydrogel development, chitosan and collagen stand out due to their biological functionality and biocompatibility. Chitosan, a cationic polysaccharide derived from the deacetylation of chitin, abundant in the exoskeletons of crustaceans, is recognized for its biocompatibility, bioadhesiveness, antimicrobial activity, pro-hemostatic action, and capacity to stimulate cell proliferation[2]. Collagen, in turn, is the predominant structural protein of the extracellular matrix and connective tissues and is widely valued for its low immunogenicity, favorable interaction with cellular receptors, and ability to promote tissue regeneration and wound healing[3].

To overcome these limitations, chemical crosslinking strategies have been employed to reinforce the mechanical performance and viscoelastic behavior of hydrogels. Among natural crosslinkers, genipin, extracted from *Genipa americana* or *Gardenia jasminoides*, has gained increasing attention due to its low cytotoxicity and effective crosslinking ability[4,5]. Studies demonstrate that genipin-crosslinked chitosan hydrogels present enhanced gel strength and reduced gelation time under physiological conditions when compared to non-crosslinked hydrogels[6]. Moreover, genipin has proven effective in improving the mechanical and rheological performance of hybrid chitosan/collagen matrices, favoring their use in tissue engineering[7]. These modifications include increased elastic modulus (G′), reflecting greater resilience and structural stability of the formed networks[8].

Beyond structural reinforcement, genipin-crosslinked chitosan/collagen hydrogels also show promise in modulating hemostatic responses, an essential aspect of their biomedical utility. Chitosan inherently exhibits procoagulant properties due to its positive charge, which promotes erythrocyte

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aggregation and platelet adhesion. The incorporation of genipin not only stabilizes the polymeric network but may also alter surface chemistry and porosity, thereby influencing interactions with blood components and clot formation dynamics[9]. These features are particularly relevant in applications involving wound healing, tissue repair, and injectable systems, where control of bleeding and rapid hemostasis are critical[10-14].

Furthermore, genipin-crosslinked hydrogels have been successfully developed as injectable, in situ-forming biomaterials with favorable viscoelasticity and biocompatibility, reinforcing their potential in minimally invasive therapies[15].

Given these promising characteristics and the ongoing challenges related to rheological performance, this study aims to evaluate the influence of collagen concentration on the structural and rheological properties of chitosan/collagen hydrogels crosslinked with genipin. By elucidating the role of collagen in the formulation, the objective is to optimize the mechanical and functional performance of these hydrogels, contributing to the development of more effective and safer therapeutic platforms for wound treatment.

2. Experimental

2.1. Materials

Medium molar weight chitosan (280 kDa) and 13% acetylation degree (87% deacetylation degree) produced by CERTBIO (Northeast Biomaterials Evaluation and Development Laboratory); Type I collagen powder obtained from bovine Achilles tendon supplied and purchased by Sigma Aldrich; Glacial acetic acid 99.8% PA 1000 mL; Ammonium hydroxide 30% PA 1000mL provided and purchased by Neon; Genipin 98% supplied and purchased by Challenge Bioproducts Co.

2.2. Preparation of genipin-crosslinked chitosan/collagen hydrogels

The chitosan solution was prepared by dissolving 2 g of chitosan in 100 mL of 1% acetic acid, under mechanical stirring for two hours. After this period, the chitosan solution (Q2) was left to stand at room temperature (25 ºC) for one hour. At the same time, the collagen was prepared, in which 2 g of collagen was added and dispersed in 100 mL of 1% acetic acid by sonication using the Eco Sonics ultrasonic processor.

Subsequently, the chitosan and collagen solutions were combined in the proportions of chitosan/collagen (99:1, 97:3, and 95:5%), being coded as CS/COL1, CS/COL3, and CS/COL5, respectively. These mixtures were subjected to constant mechanical stirring (600 RPM) at room temperature (25 ºC) for three hours.

Genipin was solubilized in pure ethyl alcohol and then incorporated into chitosan/collagen hydrogels (CS/COL1, CS/COL3, and CS/COL5) at a proportion of 0.1%, under constant mechanical agitation (600 rpm) and heating at 40 ºC for three hours. The resulting samples were coded as CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP, stored in Falcon tubes under refrigeration (~5 ºC), and subsequently characterized.

2.3. Characterizations

The hydrogels were subjected to several characterizations, including Fourier Transform Infrared Spectroscopy (FTIR), injectability, viscosity, and *in vitro* blood clotting time test.

FTIR analyses were performed using a Perkin Elmer Spectrum 400 FT-IR/FT-NIR spectrophotometer (range 4000–650 cm^{-1}, with 16 scans and 4 cm^{-1} resolution, using ATR absorbance mode), to investigate the chemical interactions and functional structures of the hydrogels.

The injectability/ejection force test was conducted using 5 mL polypropylene syringes (BD, Becton Dickinson Ind. Cirúr. Ltda.). The syringes were coupled to an Instron 3366 universal mechanical testing machine operating in compression mode, with a 500 N load cell and a constant speed of 10 mm/min at room temperature (25 °C).

The viscosity of the hydrogels was evaluated on a HAAKE MARS III parallel plate rheometer with a modular PP 35 Ti rotor system (Thermo Scientific). Measurements were performed in triplicate, with an oscillatory frequency ranging from 0.1 to 10 Hz, at 0.1% strain, 0.5 mm gap, and room temperature.

The swelling capacity of the hydrogels was assessed to determine their fluid uptake ability, a key property for biomedical applications. Samples were initially frozen at –20 °C, lyophilized, neutralized, cut into 1 cm^2 squares, and dried at 40 °C for 24 hours. After initial weighing (Pi), samples were immersed in distilled water for 30 and 60 minutes, gently blotted with filter paper to remove surface moisture, and reweighed (Pf). The swelling degree (Gi) was calculated using Equation 1. All measurements were performed in triplicate.

$$Gi(\%) = \left(\frac{P_F - P_i}{P_i} \right) x100 \quad (1)$$

The biodegradation assay was performed to evaluate the mass loss of the hydrogel under physiological and enzymatic conditions. Samples were frozen at –80 °C, lyophilized, neutralized, cut into 1 cm^2 squares, and dried at 40 °C for 24 h. Initial dry mass (Pi) was recorded. Samples were then immersed either in PBS (pH 7.0) to assess dissolution or in PBS with lysozyme (pH 7.3) to evaluate enzymatic degradation. Incubation was performed at 37.5 °C for 7 days. After incubation, samples were rinsed with distilled water, dried again at 40 °C for 24 h, and weighed (Pf). Mass loss percentage was calculated using Equation 2. All measurements were performed in triplicate:

$$Mass\ Loss(\%) = \left(\frac{P_i - P_f}{P_i} \right) x100 \quad (2)$$

In the in vitro blood coagulation test, adapted from Fang et al.[16], 250 µL of K3 EDTA-anticoagulated blood was added to 24-well plates, followed by the addition of 1000 µL of hydrogel into each well. Complete clot formation was observed at predefined time intervals (10, 20, 30, 40, 50, 60, 120, and 180 seconds). To remove unclotted blood components, 2 mL of PBS solution was added to the wells. Clotting time was determined by complete clot formation. Chitosan hydrogel (2% w/v) was used as a positive control, while K3 EDTA-anticoagulated blood served as a negative control.

3. Results and Discussion

3.1. Fourier Transform Infrared Spectroscopy (FTIR)

When analyzing the spectra of the raw materials (chitosan, collagen, and genipin), Figure 1a, the presence of characteristic bands is observed that corroborate studies[17-19]. These studies addressed, respectively, the structural and compositional changes in archaeological human bone collagen using the FTIR-ATR technique; the structural aspects of chitosan/PVA spheres cross-linked with glutaraldehyde subjected to different heat treatments; and the in vitro evaluation of the biodegradability of chitosan–genipin hydrogels.

In the chitosan spectrum, the absorptions at 3370 cm-1, 2920 cm-1 (attributed to the -OH, -NH and -CH groups), 1640 cm-1 and 1550 cm-1 (related to amide I and II), in addition to the vibrations at 1070 cm-1 and 1010 cm-1 (characteristics of the saccharide structure) stand out[18]. In the collagen spectrum, there are absorptions at 1240 cm-1, 1550 cm-1, and 1650 cm-1 related to amide III, amide I, and amide I, respectively; at 2920 cm-1, of amide B and a broad band 3500 – 3300 cm-1 related to the vibrations of amide A and OH[17]. The characteristic peaks of genipin are observed at 1680 cm-1 and 1618 cm-1, which represent the elongation of the carboxymethyl group (C=C) and the aromatic elongation (C=C), respectively[19].

When combining chitosan and collagen at different concentrations, it is possible to notice small changes in the intensity and width of characteristic peaks at various wavelengths, as observed in Figure 1b. Specifically, higher concentrations of collagen resulted in higher intensities in peaks associated with collagen, such as those related to proline and hydroxyproline, while the characteristic peaks of chitosan showed less pronounced variations in their intensities[20-22].

The addition of genipin at low concentration (0.1% w/w) did not result in significant changes in the positions of the groups in the infrared spectrum in the samples with chitosan and different concentrations of collagen (1, 3, and 5% w/w) (Figure 1c). The band at 1550 cm-1 was associated with the secondary amide, which may be the result of the reaction between the carboxymethyl groups of genipin and the hydroxyl and amino groups of chitosan. In addition, other changes were observed at 1400 cm-1, 1380 cm-1, and 1100 cm-1, suggesting the formation of new C-O-C bonds and interactions with the lysine or hydroxylysine groups of collagen[19-23].

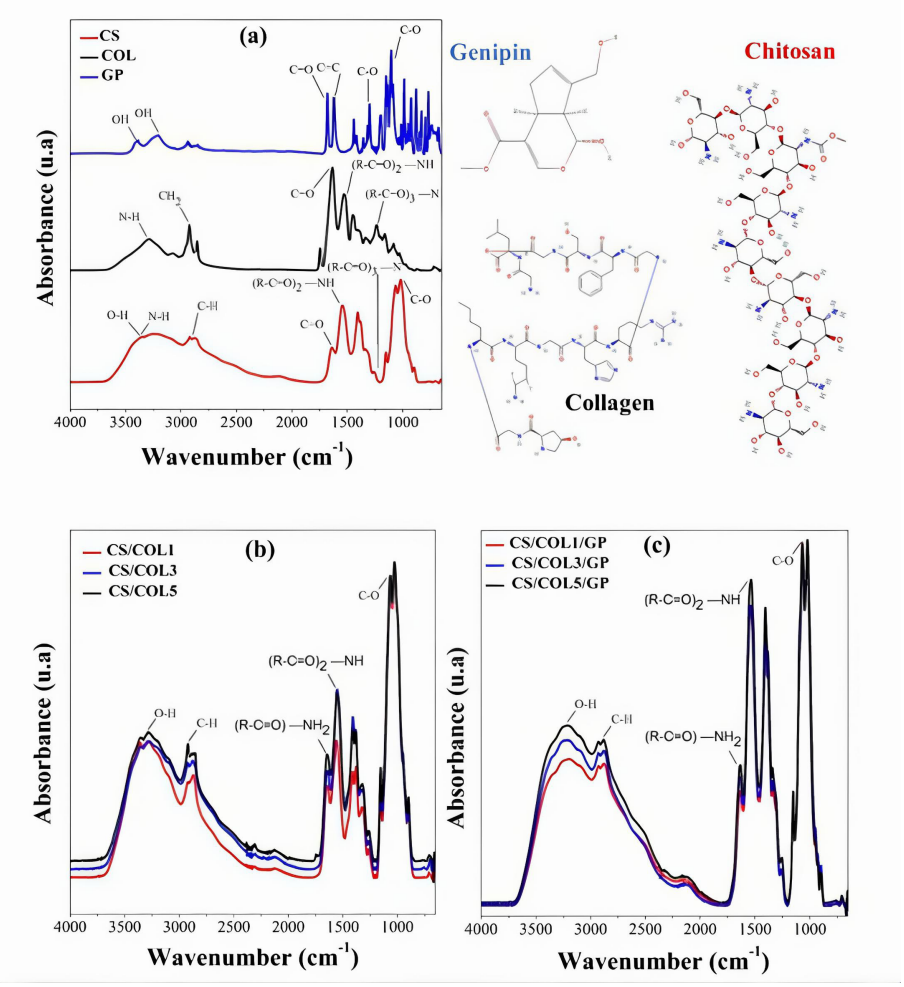

Figure 1. FTIR spectra of the raw materials (a); of the chitosan/collagen samples with 1, 3, and 5% (b), and the chitosan/collagen/genipin samples (c).

The results obtained by FTIR demonstrated the effects of genipin crosslinking on the molecular structure of chitosan/collagen hydrogels. Spectroscopic analysis revealed significant intermolecular interactions between the components, with changes in the intensity and width of the characteristic peaks, especially at the wavelengths associated with protein structures. The addition of genipin, even at low concentrations, promoted the formation of new C-O-C bonds and interactions with collagen functional groups, suggesting a structural modification that can directly influence the rheological properties of the hydrogel. These findings reinforce the role of genipin as a crosslinking agent in the stabilization of the polymer matrix, evidencing its potential to modulate the mechanical characteristics and biodegradability of hydrogels, making them promising for biomedical applications, corroborating Reay et al.[19].

3.2. Rheological analysis

The rheological tests conducted allowed an analysis of the viscoelastic properties of the non-crosslinked chitosan/collagen hydrogels identified as CS/COL1, CS/COL3 and CS/COL5 (Figure 2a), in addition to the samples crosslinked with genipin, CS/COL1/GP, CS/COL3/GP and CS/COL5/GP (Figure 2b). The influence of the collagen concentration and the degree of crosslinking was evident in the mechanical parameters analyzed, providing important information about the structural dynamics of these polymeric materials, as also reported by Muzzareli[24], who studied genipin cross-linked chitosan hydrogels as biomedical and pharmaceutical aids.

Analysis of the storage modulus (G′) revealed that the structural rigidity of the hydrogels was greater in genipin-containing samples (comparing Figure 2b with Figure 2a). The CS/COL5/GP hydrogel exhibited the highest G′ values (Figure 2b), confirming its superior rigidity, which corroborates the findings of Mi et al.[25]. The CS/COL3/GP and CS/COL1/GP samples displayed progressively lower G′ values (Figure 2b), suggesting reduced structural cohesion within these crosslinked samples. Conversely, the non-crosslinked samples (Figure 2a) demonstrated diminished G′ values, reinforcing the hypothesis that crosslinking plays a pivotal role in the mechanical stability of these biomaterials, as reported by Muzzarelli[24].

Complex viscosity (|η*|) is a rheological parameter quantifying a material's resistance to flow under oscillatory deformation. The studied systems exhibited non-Newtonian, pseudoplastic (or shear-thinning) behavior, characterized by a progressive decrease in viscosity with increasing deformation frequency (Figures 2a and 2b). Comparatively, the non-crosslinked samples (CS/COL1, CS/COL3, and CS/COL5, as depicted in Figure 2a) displayed lower |η*| values, indicating diminished resistance to oscillatory flow. This finding underscores the role of genipin in the structural stabilization of the hydrogels, consistent with Mi et al.[25], who evaluated genipin-crosslinked chitosan membranes.

Gel point analysis, often identified by the crossover of the storage (G′) and loss (G″) moduli (i.e., G′=G″) or when G′ surpasses G″, provides insights into hydrogel network formation. For the genipin-crosslinked samples (CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP; Figure 2b), G′ was significantly higher than G″ across the entire frequency range analyzed (0.1 Hz to 100 Hz). This indicates that these samples were already in a well-formed gel state, with the effective gel point occurring either at frequencies below the tested range or at an earlier curing stage. Conversely, the non-crosslinked samples (CS/COL1, CS/COL3, and CS/COL5; Figure 2a) exhibited frequency-dependent behavior. At lower frequencies, the loss modulus (G″) exceeded the storage modulus (G′), indicating a predominantly viscous character. As frequency increased, a crossover point where G′=G″ was observed for all these samples. At frequencies beyond this crossover, G′ surpassed G″, characterizing a transition towards predominantly elastic behavior or the formation of a frequency-induced 'weak gel' structure.

The loss factor (tanδ=G″/G′) values for the crosslinked hydrogels are presented in Figure 3. Although this figure specifically details the behavior of the crosslinked samples, it is generally understood that crosslinking can enhance structural cohesion. Chiono et al.[26] investigated the influence of composition and genipin crosslinking on chitosan (CS) and gelatin (G) blends for biomedical applications, where mechanical testing revealed that stiffness increased with both CS content and genipin concentration.

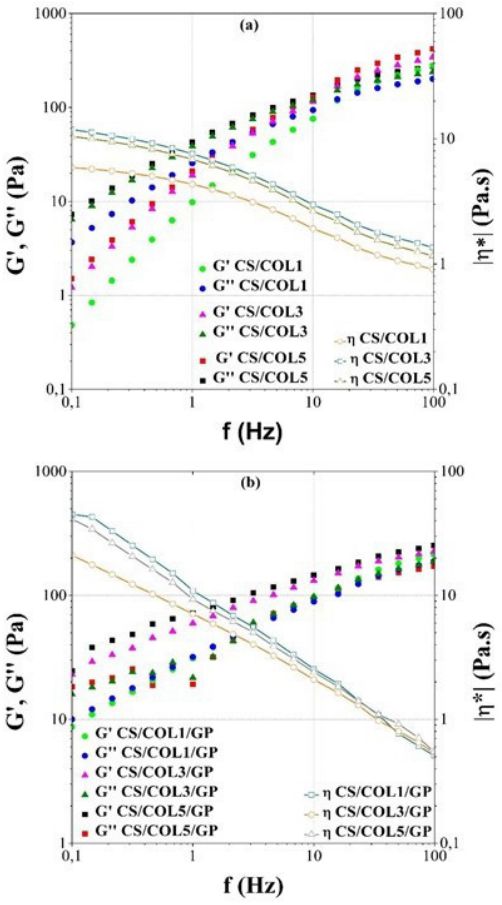

Figure 2. Representation of storage modulus (G′), loss modulus (G″), and complex viscosity (|η|) as a function of frequency for chitosan/collagen hydrogels: (a) non-crosslinked formulations – CS/COL1, CS/COL3, and CS/COL5; and (b) genipin-crosslinked formulations – CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP.

The CS/COL5/GP hydrogel exhibited the highest tanδ values across the entire frequency range, indicating a more viscous and less elastic behavior relative to the other crosslinked samples, potentially attributable to variations in crosslinking density or network architecture. The CS/COL1/GP and CS/COL3/GP samples presented tanδ values lower than 1 and also lower than those of CS/COL5/GP, indicating a predominantly elastic behavior combined with a measurable capacity for energy dissipation. This balance between high stiffness and moderate damping may be indicative of a stable and efficient polymeric network.

A more stable and efficient polymeric network in terms of mechanical energy dissipation refers to a three-dimensional crosslinked polymer structure exhibiting enhanced resistance to degradation, superior ability to maintain structural integrity, and more effective distribution of applied mechanical stresses. The stability of such a polymeric network is related to crosslinking density and the nature of intermolecular interactions, including covalent bonds, hydrophobic interactions, and hydrogen bonds. Furthermore, efficiency in mechanical energy dissipation is directly linked to the material's capacity to absorb and distribute applied forces, thereby minimizing structural failure, mechanical fatigue, and permanent deformation. This characteristic is crucial for biomedical applications, such as in biomaterials for tissue engineering and implantable devices, where the material's mechanical strength and durability are critical factors for performance and safety[27].

The results obtained corroborate studies on polymeric biomaterials, demonstrating that crosslinking with genipin can modulate the rheological properties of chitosan/collagen hydrogels, providing more structured and mechanically resistant polymeric networks[24-26].

3.3. Injectability

The injectability of the CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP hydrogels (Figure 4) was investigated using uniaxial compression tests, with analysis of the force-deformation relationship during extrusion. Chemical composition and crosslinking density are recognized factors influencing flow behavior under pressure, thereby modulating the mechanical strength of the polymeric networks[28].

The CS/COL1/GP hydrogel exhibited the lowest injection resistance (Figure 4). This finding is consistent with its rheological profile (Figure 2), as it presented the lowest storage modulus (G′) and complex viscosity (|η*|) among the genipin-crosslinked formulations, indicative of a comparatively less rigid and viscous structure. The incidental presence of air bubbles within the syringe during the testing of this specific sample could also be a potential contributing factor to this low flow resistance. Conversely, the CS/COL3/GP formulation demonstrated the highest injection resistance, followed by CS/COL5/GP, both requiring significantly greater extrusion forces than CS/COL1/GP (Figure 4). Rheologically (Figure 2), both CS/COL3/GP and CS/COL5/GP possessed higher G′ and |η*| values than CS/COL1/GP, denoting more developed and interconnected three-dimensional networks. Notably, while CS/COL5/GP exhibited the highest overall G′ and |η*| values, CS/COL3/GP demanded the maximum extrusion force. Such an observation suggests that although greater structural rigidity and viscosity typically increase injection resistance by restricting polymer chain mobility[21], the specific extrusion behavior under these compressive flow conditions is likely governed by a complex interplay of various network characteristics.

The presented data indicate that the injectability of these systems results from a balance between elastic (predominantly G′) and viscous (related to |η*|) properties, wherein the energy dissipated during flow may be partially offset by the elastic recovery of the network upon stress removal. Overall, all evaluated hydrogels exhibited injection forces below 30 N, the maximum value recommended for manual injection[29], which supports the viability of these formulations for applications in injectable systems.

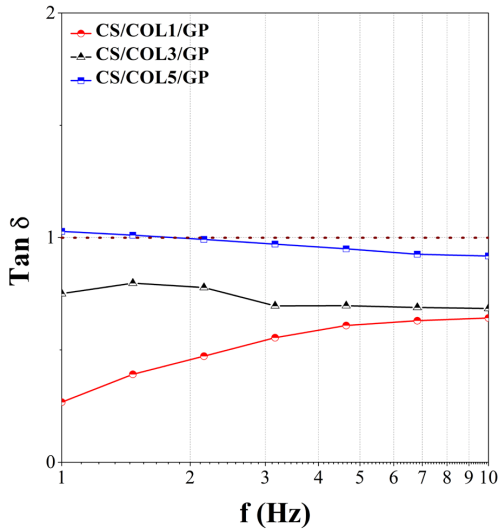

Figure 3. Loss factor (tanδ) as a function of frequency (HZ) for genipin-crosslinked chitosan/collagen hydrogels (CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP).

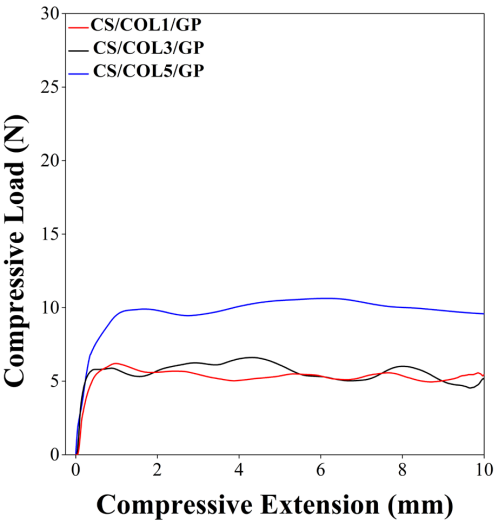

Figure 4. Injection force of chitosan/collagen hydrogel samples (1%, 3% and 5%) cross-linked with genipin.

3.4. Swelling behavior

Figure 5 shows the swelling values (%) of chitosan (CS)-based hydrogels containing different collagen concentrations, both without and with genipin (GP). It is observed that the hydrogel composed solely of chitosan exhibited the highest swelling degree, reflecting its high hydrophilicity and low crosslinking density. The progressive addition of collagen (CS/COL 1%, 3%, 5%) resulted in a gradual and statistically significant reduction in water absorption capacity. This behavior can be attributed to the increased density of intermolecular interactions between the functional groups of chitosan and collagen, such as hydrogen bonds and electrostatic interactions, which promote a more compact matrix and hinder water penetration[30,31].

The presence of the crosslinking agent genipin in the CS/COL1/GP, CS/COL3/GP, and CS/COL5/GP formulations further accentuated the reduction in swelling, highlighting the crosslinking effect of genipin on the polymeric matrix. Genipin acts by forming covalent bonds between the amino groups of chitosan and collagen chains, promoting a more stable three-dimensional structure that is more resistant to solvent uptake[32-34]. These results are consistent with the literature, which demonstrates that genipin crosslinking significantly reduces the volumetric expansion of hydrogels, while enhancing their mechanical integrity and durability in physiological conditions[10,35,36].

3.5. In vitro biodegradation

The degradation of polymeric biomaterials in physiological environments is a key factor in determining their *in vivo* performance. Table 1 shows that all tested hydrogels underwent degradation in PBS and lysozyme/PBS solutions, with degradation kinetics varying according to the composition and presence of the crosslinking agent.

The pure chitosan sample (CS) exhibited accelerated degradation in PBS, reaching 100% mass loss in just 14 days. This behavior is consistent with the literature, as chitosan, despite its biocompatibility, displays limited stability in physiological solutions due to its partial solubility in mildly acidic environments and low resistance to hydrolysis[37]. When exposed to the lysozyme solution—an enzyme present in bodily fluids—the CS sample also reached complete degradation by day 14, highlighting the low enzymatic resistance of the uncrosslinked polymeric matrix.

The incorporation of collagen (CS/COL1, CS/COL3, CS/COL5) led to a slight reduction in the degradation rate, although these samples still showed mass losses exceeding 95% after 14 days of incubation in PBS. In enzymatic medium, the same formulations also underwent rapid degradation, with complete disintegration occurring by day 14. This suggests that the simple addition of collagen was not sufficient to provide long-term structural stability to the hybrid matrix, likely due to collagen's high solubility in aqueous media and its susceptibility to lysozyme activity[38].

In contrast, the samples crosslinked with genipin (CS/COL1/GP, CS/COL3/GP, CS/COL5/GP) demonstrated significantly slower degradation in both PBS and lysozyme solutions. The CS/COL5/GP sample, for instance, showed only 65.10% mass loss after 14 days in PBS and 72.14% in the enzymatic medium. After 21 days, this formulation still retained approximately 10–12% of its original mass, indicating enhanced resistance to both hydrolytic and enzymatic degradation.

This improved stability can be attributed to the formation of additional covalent bonds between the polymer chains promoted by genipin, which restricts water diffusion and enzymatic activity by creating a denser and less degradable polymeric network[39]. This effect is particularly relevant for applications requiring controlled and sustained degradation, such as bioactive dressings or prolonged drug delivery systems.

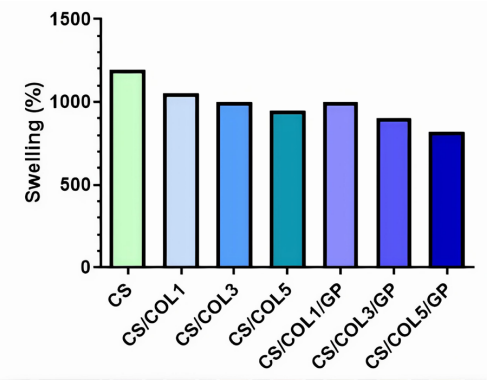

Figura 5. Swelling profile (%) of chitosan (CS)-based hydrogels incorporating different collagen concentrations (1%, 3%, and 5%), with and without the addition of the crosslinking agent genipin (GP).

Table 1. Percentage mass loss of the samples at 7, 14, and 21 days during the biodegradation assay.

Samples	Mass loss (%)					
	PBS			Lysozyme/PBS		
	7 days	14 days	21 days	7 days	14 days	21 days
CS	77.36	100.00	-	79.15	100.00	-
CS/COL1	66.96	98.13	-	67.89	100.00	-
CS/COL3	64.71	97.35	-	66.78	100.00	-
CS/COL5	63.69	95.12	-	65.89	100.00	-
CS/COL1/GP	51.86	70.78	91.17	58.79	78.68	97.34
CS/COL3/GP	48.13	69.58	90.32	55.23	76.25	95.47
CS/COL5/GP	46.12	65.10	88.67	51.49	72.14	91.18

Moreover, an inverse relationship was observed between the degree of degradation and the collagen content in the crosslinked formulations. CS/COL5/GP, with the highest collagen content, showed the greatest resistance to degradation, suggesting that increasing the collagen fraction in the presence of genipin contributes to a more stable three-dimensional network, possibly due to enhanced intermolecular interactions between the amine groups of the proteins and the aldehyde groups of genipin[40].

3.6. In vitro blood clotting time test

The in vitro blood coagulation assay evaluates the hemostatic capacity of biomedical materials by measuring the coagulation time of blood in contact with the tested material. In this study, genipin-crosslinked hydrogels were evaluated and, to quantify this property, the samples were mixed with blood in 24-well plates, and the coagulation time was determined.

The image analysis (Figure 6) reveals the effect of the different hydrogels on blood coagulation over time. It can be seen that the control sample (CS) maintains a predominantly liquid appearance for up to 60 seconds, with more evident signs of coagulation only after 90 seconds.

The highlights indicate the times at which the different hydrogels promoted faster coagulation compared to the control. For CS/COL3/GP, coagulation is already visible at 35 seconds, suggesting an early induction of the process. In CS/COL5/GP, coagulation becomes evident at 45 seconds, reinforcing that the higher concentration of collagen associated with genipin may have enhanced the hemostatic activity. At 60 seconds, it is observed that CS/COL3/GP presents a more cohesive gel compared to the control, and, at 90 seconds, coagulation in CS/COL5/GP is well established, indicating a fast and efficient response.

These results suggest that the incorporation of collagen and genipin contributes to an acceleration of the coagulation process, especially in formulations with a higher concentration of collagen (CS/COL5/GP), reinforcing its potential as an improved hemostatic agent.

The formation of blood clots in contact with different compositions of the hydrogels is shown in Figure 7. It can be observed that the control sample (CS) resulted in a smaller clot, suggesting a lower interaction between the blood and the material. In the CS/COL1/GP sample, there was an increase in clot formation, indicating an initial interaction between the blood and the hydrogel, but without a significant structural modification.

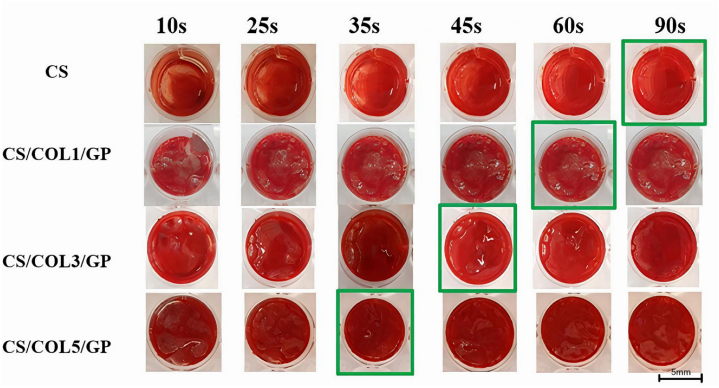

Figure 6. Evaluation of blood coagulation in the presence of different hydrogel formulations over time (10s – 90s).

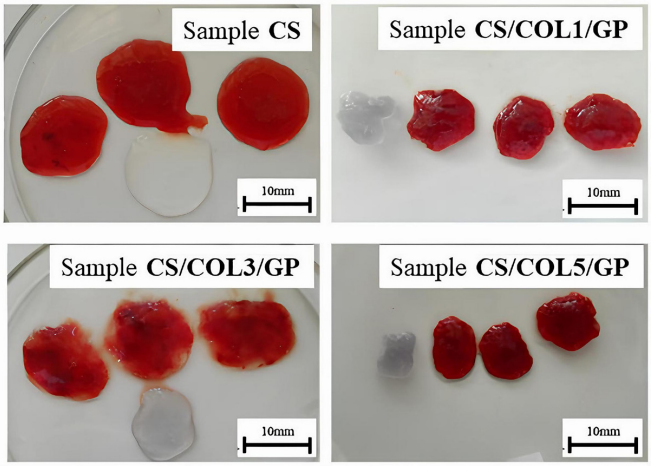

Figure 7. Images of the dynamics of the coagulation process for the CS, CS/COL1/GP, CS/COL3/GP and CS/COL5/GP hydrogels, illustrating the formation of the clot.

On the other hand, in the CS/COL3/GP and CS/COL5/GP samples, a more pronounced change was observed, with the formation of clots with a gelatinous or semi-solid texture, allowing their manipulation with tweezers. This behavior may be associated with the increased collagen concentration and the presence of genipin, which promotes greater cross-linking and mechanical stability, corroborating Zhang et al.[41] who investigated the effect of chitosan cationicity on its osteogenic and hemostatic properties cross-linked with genipin.

These results are in line with rheological observations, where CS/COL3/GP demonstrated a structural transition under increasing shear rate, allowing its manipulation. CS/COL5/GP, on the other hand, presented a clot with a semi-solid texture, suggesting greater interaction with blood and a potentially more efficient hemostatic effect, as cited by Liu et al.[42] who developed hemostatic microspheres of chitosan and gelatin cross-linked with genipin, aiming at a more effective and biocompatible alternative to traditional hemostatics.

4. Conclusions

Genipin crosslinking had a significant impact on the structural and rheological properties of chitosan/collagen hydrogels, as evidenced by changes in the physicochemical and mechanical characteristics of the materials. Genipin promoted the formation of crosslinks between chitosan and collagen molecules, increasing the structural stability of the hydrogels. FTIR analysis showed the formation of new C-O-C bonds, reflecting the interaction between the components. Furthermore, rheological tests indicated that crosslinking increased the stiffness and viscosity of the hydrogels, resulting in greater resistance to flow and dissipation of mechanical energy. The injectability of the hydrogels was directly influenced by the crosslinking density, with stiffer samples showing greater resistance to extrusion. In addition, biodegradation assays confirmed that genipin significantly delayed hydrogel degradation under physiological and enzymatic conditions, supporting its role in ensuring controlled and sustained biomaterial resorption. These results suggest that genipin crosslinking is effective in modulating the properties of chitosan/collagen hydrogels for a given biomedical application.

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Data Availability

All data generated or analyzed during this study are included in this published article.