



Visible light–crosslinked histamine-functionalized hyaluronic acid and gelatin hydrogels for wound healing applications

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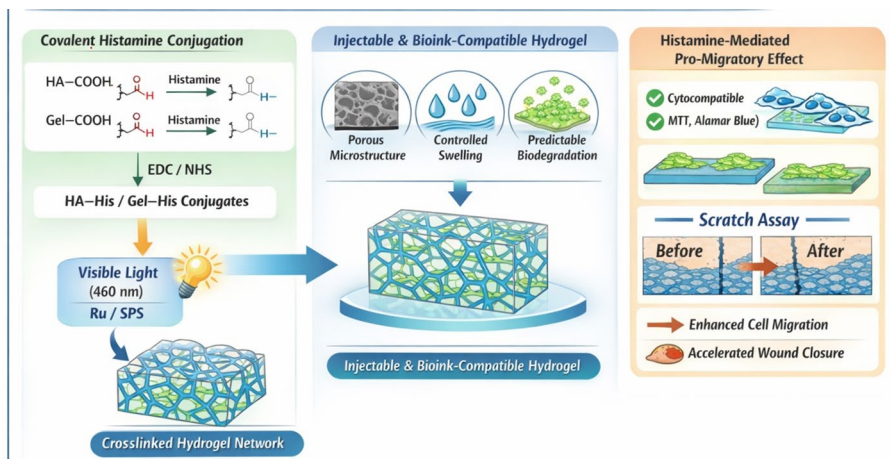
Received: 30 January 2026 / Revised: 30 May 2026 / Accepted: 1 June 2026
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Abstract

Histamine-functionalized hyaluronic acid (HA) and gelatin (Gel) hydrogels were developed via covalent conjugation and visible light–mediated Ru/SPS photopolymerization for potential wound healing applications. Histamine was successfully grafted onto HA and Gel backbones via EDC/NHS coupling, and hydrogel networks were formed under 460 nm visible light irradiation. Structural confirmation was achieved through FTIR and ¹H-NMR analyses, while the degree of substitution (DS) was determined to be approximately 11% for HA–His conjugates. The prepared hydrogels exhibited rapid swelling behavior, reaching equilibrium within 120 min and attaining approximately 1.5-fold weight increase. Biodegradation studies revealed an initial mass loss of ~20–25% within 5 days in PBS, while enzymatic degradation with lysozyme resulted in accelerated degradation beginning at day 3. Rheological analysis demonstrated shear-thinning behavior with a dominant elastic response ($G' > G''$), indicating structural stability suitable for injectable and 3D bioprinting applications. In vitro cytocompatibility assays (MTT and Alamar Blue) showed no significant cytotoxicity ($p > 0.05$), while scratch assay results indicated enhanced wound closure rates of 98.30% (HA–His) and 98.44% (Gel–His) after 24 h compared to control groups. SEM analysis revealed interconnected porous architectures (~4–50 μm for HA–His and ~15–45 μm for Gel–His), favorable for cell infiltration and nutrient diffusion. Overall, this study presents a covalently stabilized, visible light–crosslinked histamine-functional hydrogel platform with tunable physicochemical properties and promising in vitro wound healing performance. However, further in vivo and inflammation-specific studies are required to fully validate its clinical translational potential.

Extended author information available on the last page of the article

Graphical abstract



Keywords Histamine · Photo-crosslink · Histamine-conjugated · Visible light · Hyaluronic acid · Gelatin

Introduction

Photo-induced crosslinking offers precise spatiotemporal control over hydrogel formation and has emerged as a powerful strategy for fabricating biomaterials under cell-compatible conditions. In particular, visible light-mediated crosslinking has gained increasing attention in tissue engineering due to its reduced phototoxicity, deeper penetration, and improved cytocompatibility compared with ultraviolet-based systems, enabling in situ gelation and homogeneous network formation in biological environments [1–4]. Visible light-mediated crosslinking has recently emerged as a promising strategy for hydrogel fabrication due to its reduced cytotoxicity, improved tissue penetration, and spatiotemporal control over gelation compared to UV-based systems [1, 5–7].

Natural polymers such as hyaluronic acid (HA) and gelatin (Gel) are widely employed in hydrogel-based biomaterials due to their inherent biocompatibility, biodegradability, and ability to support cell adhesion and migration. HA plays a critical role in skin homeostasis and wound repair by regulating cell motility and extracellular matrix (ECM) organization, while gelatin, derived from collagen, provides bioactive motifs that promote cellular attachment and proliferation. Functional modification of these polymers enables the incorporation of bioactive cues and the tuning of physicochemical and mechanical properties, thereby expanding their applicability in regenerative medicine [8–11].

In parallel with material design strategies, biologically active small molecules have been explored as modulators of wound healing. Histamine is a multifunctional biogenic amine involved in inflammation, angiogenesis, and tissue repair. Upon injury, histamine is released primarily from mast cells and contributes to vasodila-

tion, increased vascular permeability, and immune cell recruitment, all of which are essential for the early stages of wound healing [12–14]. Beyond its inflammatory role, histamine has been shown to promote fibroblast and keratinocyte migration, enhance growth factor signaling, and accelerate wound closure *in vitro* and *in vivo*. Despite these beneficial effects, uncontrolled or burst release of histamine may lead to adverse inflammatory responses, underscoring the need for delivery systems that enable localized and controlled presentation [14–16].

Covalent conjugation of bioactive molecules to polymer backbones represents an effective strategy for stabilizing their presentation within hydrogel networks and minimizing rapid diffusion or systemic exposure. Previous studies have demonstrated that conjugation-based hydrogels can preserve biological activity while offering improved control over degradation and release profiles [17–19]. In this context, visible light-mediated crosslinking provides a mild and versatile approach for forming covalently stabilized networks without the need for harsh reaction conditions or enzymatic components. In addition, stimuli-responsive hydrogel systems have been extensively investigated for controlled and on-demand drug release in response to environmental cues, further enhancing their applicability in biomedical and regenerative medicine applications [20, 21].

Although the biological benefits of histamine in tissue repair are well documented in the literature, its topical use in its free form is severely limited by rapid diffusion, systemic toxicity risks, and the potential to trigger prolonged acute inflammatory responses at the wound site. Therefore, achieving a localized and controlled delivery platform remains a critical challenge. To the best of our knowledge, studies on covalently histamine-functionalized HA and gelatin hydrogels fabricated via visible-light crosslinking remain limited. To address this gap, this study introduces a novel strategy for creating a cell-friendly, localized, and controlled presentation mechanism by covalently conjugating histamine onto both HA and gelatin backbones using a ruthenium–sodium persulfate (Ru-SPS) system under visible light (460 nm). Unlike conventional histamine-loaded systems that suffer from burst release, the present approach successfully stabilizes histamine within the polymer network through covalent functionalization. This strategic design significantly minimizes the risk of systemic toxicity while fully preserving histamine's biofunctional contribution to wound healing-related cellular responses.

In this study, histamine was covalently conjugated to hyaluronic acid and gelatin to generate biofunctional hydrogel systems, which were subsequently crosslinked using a visible light-mediated photochemical strategy. The physicochemical, structural, mechanical, and biological properties of the resulting HA-His and Gel-His hydrogels were systematically evaluated. HRP-mediated crosslinking was included solely for comparative assessment in short-term cell viability and proliferation studies. By integrating histamine functionality with visible light-driven hydrogel fabrication, this work aims to establish a cytocompatible and biologically supportive platform for wound healing and soft tissue engineering applications.

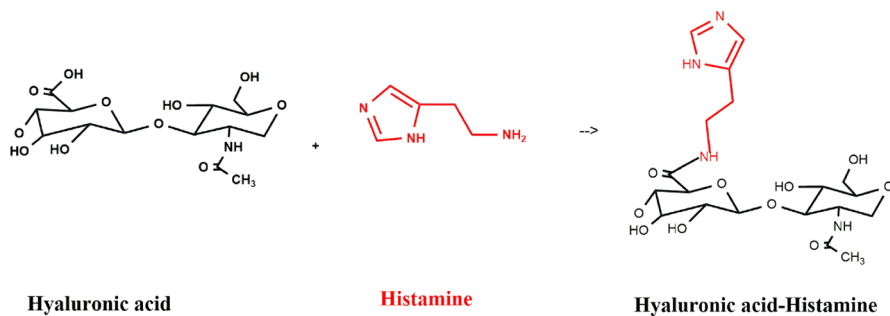


Fig. 1 Schematic representation of the synthesis of HA–His conjugates. HA was chemically conjugated with histamine through covalent linkage, resulting in HA–His structures. The red-highlighted segments indicate the histamine moieties attached to the HA backbone

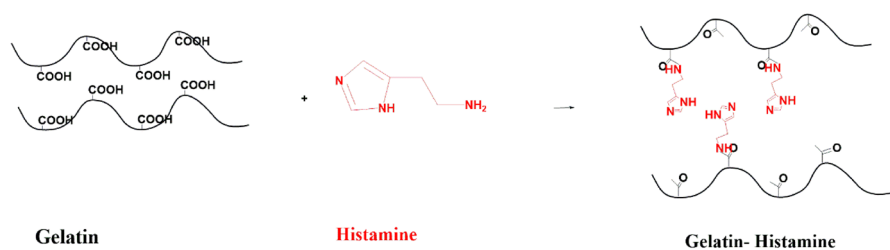


Fig. 2 Schematic representation of the synthesis of Gel–His conjugates. Gel was chemically conjugated with histamine through covalent linkage, resulting in Gel–His structures. The red-highlighted segments indicate the histamine moieties attached to the Gel backbone

Materials and methods

Synthesis of HA–His conjugates

HA, widely used in the literature for conjugate synthesis and known for its biocompatibility, was selected for this study. To prepare the conjugate illustrated in Fig. 1, 2.5 mmol of HA was first dissolved in 100 mL of distilled water. Subsequently, 2.5 mmol of EDC-HCl and 2.5 mmol of NHS were added to initiate the conjugation reaction with 1 mmol of histamine. Throughout the reaction, the pH of the mixture was maintained at 4.7, and the solution was incubated overnight at room temperature under magnetic stirring. Upon completion, the pH was adjusted to 7.0. The solution was then transferred into dialysis tubing and dialyzed against distilled water for four days. Finally, the purified solution was lyophilized to obtain the dry conjugate [17].

Synthesis of gel–his conjugates

Gel was modified with histamine to form a conjugate, as depicted in Fig. 2. For this purpose, Gel was dissolved at 10% (w/v). After complete dissolution, histamine was added at a 2:1 ratio, followed by the addition of EDC and NHS to initiate the reaction. The pH of the solution was adjusted to 4.7, and the mixture was stirred at room

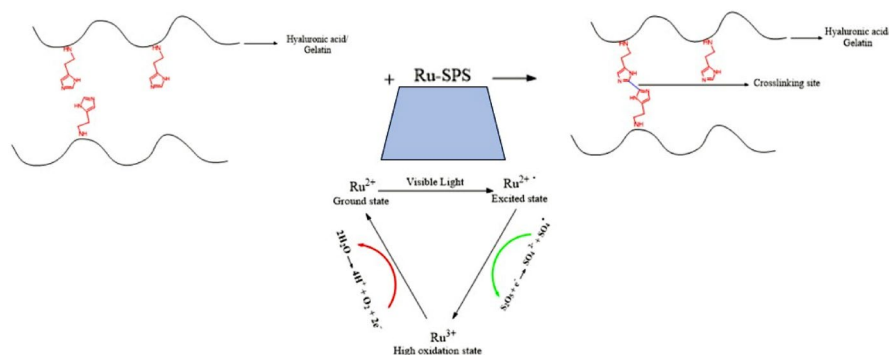


Fig. 3 Crosslinking mechanisms of histamine: schematic representation of the photoinitiated conjugation of histamine with HA and Gel using Ru-SPS as the photoinitiator. Upon visible light irradiation, Ru-SPS undergoes excitation from the ground state (Ru^{2+}) to the excited state, followed by oxidation to Ru^{3+} , generating reactive species that facilitate crosslinking between histamine and the polymer backbones. The resulting crosslinked structures establish stable conjugation sites within the hyaluronic acid/gelatin matrix with HA and Gel

Table 1 Experimental parameters related to photopolymerization conditions. In this study, systematic optimization experiments were carried out at different Ru and SPS concentrations to optimize hydrogel formation and crosslinking efficiency. Light exposure time and light source geometry were kept constant to determine the optimum conditions

Ru concentration	SPS concentration	Light intensity	Distance from source	Exposure time	Hydrogel thickness
0,1 mM	5 mM	460 nm	1 cm	15 s.	5 mm

temperature for 24 h. After 24 h, the pH was adjusted to 7.0, and the solution was transferred into dialysis tubing and dialyzed against distilled water for four days. The purified solution was then lyophilized.

Crosslinking of the conjugates

The synthesized conjugates were subjected to crosslinking under blue light with a wavelength of 460 nm for varying durations, and parameters such as photoinitiator concentration and visible light exposure time were optimized accordingly. The crosslinking mechanisms are illustrated in Fig. 3. Ruthenium (Ru)–sodium persulfate (SPS) was employed as the photoinitiator system [17, 22, 23]. Optimized values are shown in Table 1.

Characterization of conjugates

To evaluate their suitability as scaffolds for tissue engineering applications, the fabricated hydrogels were comprehensively characterized. Physicochemical properties, mechanical stability, and microstructural features were systematically assessed using a combination of analytical techniques. These evaluations provided essential insights

into the hydrogels' structural integrity, water retention capacity, and potential for supporting cell adhesion and proliferation.

Swelling tests

A swelling test was performed to determine the water retention capacities of the hydrogels ($n=3$). The samples were initially dried at 37 °C for 1 h, after which their initial dry weights (W_0) were measured. Subsequently, the specimens were immersed in distilled water for various time intervals up to 180 min. At each designated time point, the water-absorbed samples were removed, gently placed on filter paper for a brief period to remove excess surface water, and their swollen weights (W_S) were immediately recorded. % Swelling Degree = $[(W_S - W_0) / W_0] \times 100$.

Biodegradation study

The biodegradation profiles of the hydrogels were evaluated under both hydrolytic and enzymatic conditions ($n=3$). For hydrolytic degradation, phosphate-buffered saline (PBS) was used, while lysozyme-containing solutions were employed for enzymatic degradation. The initial dry weight of each scaffold (W_0) was recorded, and samples were incubated in 7.5 mL PBS or 7.5 mL lysozyme solution at 37 °C with gentle shaking (75 rpm). Degradation was monitored at predetermined time intervals (days 1, 3, 5, 10, 20, and 30). At each time point, samples were removed, surface liquid was blotted, dried, and the final dry weight (W_t) was measured. Mass loss over time was calculated to quantitatively assess the degradation rate and stability of the hydrogels under biological conditions.

Rheological behavior of conjugates

The rheological properties of the conjugates were evaluated using a rheometer to assess their viscosity behavior in response to varying shear rates. Hydrogel samples were subjected to different shear rates, and the resulting viscosity profiles were recorded. The obtained data provided insights into behavior, structural stability, and processability of the materials, particularly relevant for 3D bioprinting and other biomedical applications, thereby contributing to the understanding of their functional performance.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy is a widely used technique for identifying functional groups in organic and inorganic compounds, based on the characteristic vibrations of chemical bonds at specific wavenumbers. In this study, the chemical composition of visible light-crosslinked hydrogel samples was analyzed using FTIR. Samples were powdered and measured directly in Attenuated Total Reflectance (ATR) mode over the 4000–600 cm^{-1} range, which provides detailed information on vibrations of functional groups such as O-H, N-H, C-H, C=O, and C-O. This analysis allowed the assessment of structural integrity, chemical stability, and potential new bond forma-

tion after crosslinking. Spectral data also revealed molecular-level changes resulting from the conjugation process, contributing to understanding the functional properties and design efficiency of the developed hydrogel systems.

¹H-NMR

To elucidate the chemical structures of the conjugates and confirm the synthesis processes, ¹H-NMR analysis was performed. Lyophilized conjugate samples (10 mg each) were dissolved in 1 mL of deuterated solvent (D₂O) to obtain homogeneous solutions. Spectra were recorded over a chemical shift (δ) range of 0–15 ppm. The resulting spectra were analyzed to identify characteristic proton signals, enabling structural verification and assessment of successful modifications.

Surface morphology analysis

The surface morphology, microstructural features, and surface composition of the hydrogels were examined using Scanning Electron Microscopy (SEM). Before imaging, samples were lyophilized and coated with a thin carbon layer to enhance conductivity and achieve high-resolution images. The coated samples were mounted on SEM stubs and imaged at magnifications of 100x, 250x, and 500x. The obtained images were analyzed to assess porosity, surface smoothness, and microstructural organization of the hydrogels.

Mechanical test

The mechanical properties of the hydrogel samples were evaluated by uniaxial compression testing. Cylindrical hydrogel specimens (height: 5 mm, diameter: 12 mm) were prepared for each formulation and divided into two groups: (i) directly tested samples and (ii) samples pre-immersed in PBS for 1 min before testing ($n=2$ for each group). Compression analyses were performed at room temperature (25 °C) using a Shimadzu AGS-X 5 kN universal testing machine at a crosshead speed of 1 mm/min. The rheological and mechanical characterization protocol was conducted in accordance with the ASTM F2150 standard for biomaterial scaffolds. During testing, compressive force values were continuously recorded, and stress–strain curves were generated to evaluate the deformation behavior of the hydrogels. The elastic modulus and compressive strength parameters were subsequently calculated from the obtained stress–strain data for comparative analysis between the formulations.

Cell–material interaction studies

Within the scope of this study, mouse fibroblast cells (L929) were used as a model to evaluate cell–material interactions of the conjugates.

Cytotoxicity assay

Before cell culture, the hydrogels were sterilized by immersion in 80% ethanol for 1 h, followed by thorough washing with sterile PBS to remove residual ethanol. To further ensure sterility, the samples were incubated in culture medium supplemented with 5% antibiotics, and subsequently washed again with sterile PBS. Sterilized hydrogels ($n=3$) were then transferred into 15 mL centrifuge tubes containing appropriate medium and incubated at 37 °C under 5% CO₂ conditions. The cytotoxicity assessment was performed indirectly, following an elution-based approach, by exposing fibroblast cells to the hydrogel-conditioned medium rather than direct contact with the materials.

For cytotoxicity evaluation, cells were seeded into 48-well culture plates at a density of 1×10^4 cells per well. MTT assays were performed at 24 h and 48 h after cell seeding. At each time point, the medium in each well was replaced with hydrogel-conditioned medium, and incubation was continued. MTT reagent was prepared at a 1:15 ratio in culture medium, added to the wells, and incubated for 4 h. Following incubation, an isopropanol-HCl solution was added to dissolve the formazan crystals. The resulting solutions were transferred into 96-well plates, and absorbance was measured at 570 nm using a microplate reader.

Cell proliferation assay (Alamar Blue)

Cell proliferation on the fabricated hydrogels was assessed using the Alamar Blue assay. The effect of different hydrogel groups on cellular proliferation was evaluated on days 1, 3, and 5 ($n=3$). Hydrogels were placed in 48-well plates, and 5×10^4 cells were seeded onto each hydrogel. To promote initial cell attachment, the plates were incubated at 37 °C under 5% CO₂ for 30 min before the addition of culture medium.

At the predetermined time points, the culture medium was removed and replaced with a resazurin solution prepared at a 1:15 dilution in DMEM-F12 or DMEM-Low Glucose medium. The plates were incubated for 4 h to allow viable cells to reduce resazurin to resorufin, generating a fluorescent signal indicative of metabolic activity. Following incubation, the supernatants were transferred to 96-well plates, and absorbance was measured at 570 nm using a microplate reader. Proliferation values were calculated as the mean of three independent experiments, and the results were presented graphically.

In vitro wound healing assay (Scratch Test)

An in vitro scratch assay was performed to evaluate wound healing as a model system. For scaffold sterilization, the samples were immersed in 80% ethanol for 2 h, followed by three washes with PBS. Subsequently, they were incubated in culture medium supplemented with 1% antibiotic for 24 h, completing the sterilization process.

To investigate the effect of histamine on wound healing and to assess potential alterations in cell migration, 1×10^6 L929 fibroblast cells were seeded into each well of 6-well culture plates, with both control and experimental groups prepared

in triplicate ($n=3$). The control groups received standard culture medium, whereas the experimental groups were treated with scaffold-containing medium. All cultures were maintained in an incubator at 37 °C with 5% CO₂.

After the formation of a uniform cell monolayer, a linear scratch was introduced at the center of each well using a sterile 200 µL pipette tip. Wound closure was subsequently monitored at 1, 3, 5, 7, and 12 h under a light microscope for both control and experimental groups. At each time point, the scratch area was quantified using ImageJ software, and the corresponding values were employed for comparative analysis of closure dynamics.

SEM analysis

To assess cell–material interactions, including morphology, adhesion, and spreading behavior, cells cultured on the scaffold surfaces were examined using SEM, providing complementary evidence to biocompatibility assays. For this purpose, 1×10^4 cells were seeded onto each hydrogel sample, and cell–material interactions were assessed on days 1 and 3. At the designated time points, the samples were washed twice with phosphate-buffered saline (PBS) to remove residual medium, followed by fixation in 3% glutaraldehyde solution for 30 min. Subsequently, the samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and further treated with hexamethyldisilazane (HMDS) to preserve cell–material integrity before lyophilization. The lyophilized specimens were then sputter-coated with carbon, mounted on SEM holders, and imaged at magnifications of 100x, 250x, 500x, 1000x, and 5000x. SEM analysis enabled a comparative evaluation of cell adhesion, morphological features, and the influence of the porous structure of the hydrogels on cellular behavior.

Results and discussion

Characterization studies

Conjugates of histamine with HA and Gel were crosslinked under visible light, and the swelling behavior of the biomaterials was evaluated by measuring weight changes over time. The hydrogels exhibited a pronounced swelling behavior within the initial 30 min, followed by a gradual approach to equilibrium. After 2 h, the samples reached a plateau, attaining approximately 1.5 times their original weight, indicating a rapid yet controlled water uptake characteristic of the materials. The water retention capacity of biomaterials is critically important for their ability to form gel-like structures and for their applicability in tissue engineering. The swelling test indicated that the polymeric network facilitated water absorption and swelling within the hydrogel matrices.

Conjugates of hyaluronic acid (HA) and gelatin functionalized with histamine were crosslinked under visible light, and their swelling behaviors were evaluated via time-dependent weight changes. The samples exhibited a rapid swelling profile within the first 30 min, and their weights reached a plateau at the end of 120 min, at which point

the biomaterials reached approximately 1.5 times their initial weight. This swelling test demonstrated that the interconnected spaces between the polymer layers successfully allowed for water entrapment, establishing the fluid retention capacity required for stable gel-like structures in tissue engineering scaffolds (Fig. 4A).

To put these findings into perspective, alternative crosslinking strategies in the literature yield distinctly different swelling profiles. For instance, in a study utilizing enzymatically crosslinked HA-serotonin (HA-Ser) hydrogels, a 2% (w/v) concentration swelled to 0.5 times its initial state within 3 days before stabilizing, whereas a 1% (w/v) concentration contracted to nearly 35% of its initial volume. Furthermore, in photo-crosslinked methacrylated HA systems, the hydrogels required approximately 26 h to reach 1.4 times their initial weight, maintaining similar equilibrium swelling ratios regardless of the methacrylation degree due to incomplete photopolymerization under limited exposure times. In comparison, the visible light-crosslinked serotonin/histamin-HA-gelatin matrices synthesized in this work achieved a comparable swelling equilibrium (1.5-fold) within a significantly shorter timeframe (120 min), demonstrating highly efficient water absorption kinetics [24, 25]. These findings support the notion that the developed biomaterials may exhibit comparable characteristics in terms of water retention capacity and swelling profiles.

The biodegradation behavior of the prepared hydrogels was investigated for up to 30 days under both hydrolytic and enzymatic conditions using different media [26]. The hydrogels crosslinked under visible light exhibited an initial rapid mass loss by day 5 in PBS, followed by a more sustained and gradual degradation phase. In the presence of lysozyme, enzymatic degradation commenced earlier, with a pronounced mass loss observed on day 3, subsequently transitioning into a controlled degradation

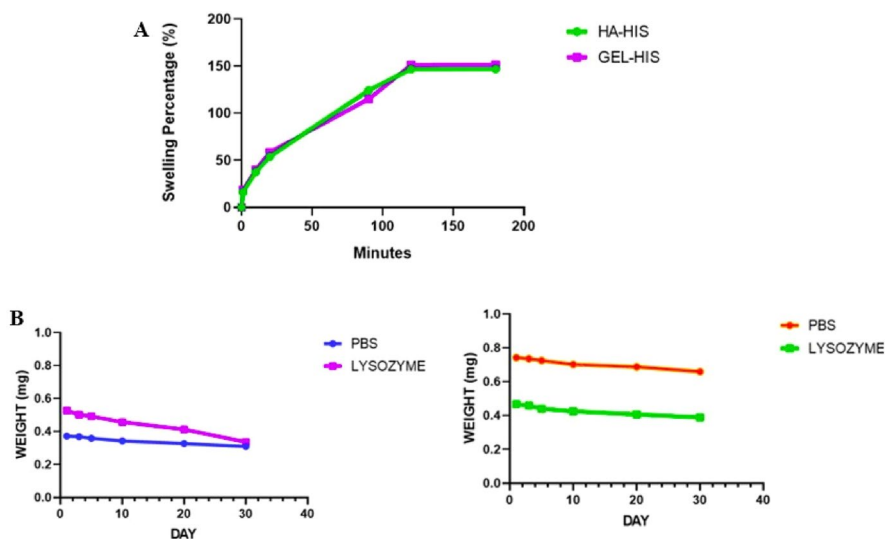


Fig. 4 (A) Swelling profiles of HA-His and Gel-His conjugates, demonstrating the time-dependent fluid absorption capacity and network stability of the hydrogels. (B) Biodegradation behavior of hydrogels prepared to illustrate their structural integrity and degradation kinetics under physiological conditions

profile similar to that observed in PBS (Fig. 4B). Similarly, previous studies have shown that hydrogels synthesized from HA-tyramine conjugates undergo complete enzymatic degradation within two weeks [27]. Light-responsive hydrogel systems have been reported to enable on-demand drug release through controlled network degradation or photochemical activation, further supporting the tunable degradation behavior observed in the present study [28, 29].

Lysozyme was selected as a preliminary enzymatic degradation model because it is associated with the inflammatory wound microenvironment and is released by activated immune cells, particularly neutrophils and macrophages, during the early stages of wound healing. These inflammatory cells contribute to tissue remodeling, debris clearance, and regulation of the wound healing response through the secretion of various proteolytic and antimicrobial enzymes [30, 31]. Furthermore, lysozyme has been widely investigated in wound healing-related biomaterial systems due to its antimicrobial and wound-supportive properties [32]. Therefore, lysozyme was considered suitable for the preliminary evaluation of hydrogel stability under inflammation-related conditions. Nevertheless, we agree that hydrogel component-specific enzymes, such as hyaluronidase for HA and collagenase/gelatinase for gelatin, would provide a more biologically representative degradation profile. Accordingly, detailed degradation studies using these enzymes have been planned for future investigations.

Rheological characterization analyses were performed to evaluate the suitability of the conjugate-based hydrogels developed in this study for fabrication using three-dimensional (3D) bioprinting technologies. Rheological measurements conducted on hydrogel solutions prepared at 1% concentration revealed a significant decrease in viscosity with increasing shear rate. This finding indicates that the hydrogels exhibit shear-thinning behavior, which is critical for bioinks used in bioprinting applications. The viscosity analysis results of hydrogels crosslinked under visible light are presented below (Fig. 5A).

Shear-thinning behavior is a critical parameter for inks and bioinks intended for use in three-dimensional (3D) printing applications. The reduction in viscosity under increasing pressure and corresponding shear stress facilitates smooth flow through the printer nozzle, enabling a successful 3D printing process. The samples examined exhibited higher viscosity at low shear rates, a rheological characteristic that allows the material to deposit uniformly and reproducibly onto the printing substrate.

Viscoelastic properties play a crucial role in evaluating the mechanical durability and structural stability of hydrogels. Accordingly, frequency sweep analyses were conducted to investigate the viscoelastic behavior of the developed hydrogels. During the tests, rheological responses of the materials under dynamic strain were assessed across a range of frequencies, with analyses performed under isothermal conditions at a constant temperature. This approach eliminated the influence of temperature, enabling the isolated evaluation of frequency-dependent effects on the material behavior.

Rheological frequency analyses performed on hydrogel samples crosslinked via visible light photochemistry revealed significant insights into their viscoelastic properties. The results demonstrated that, across all frequency ranges, the storage modulus (G') values were significantly higher than the loss modulus (G'') values. In particular, the G' values were approximately twice as high as the G'' values, indicat-

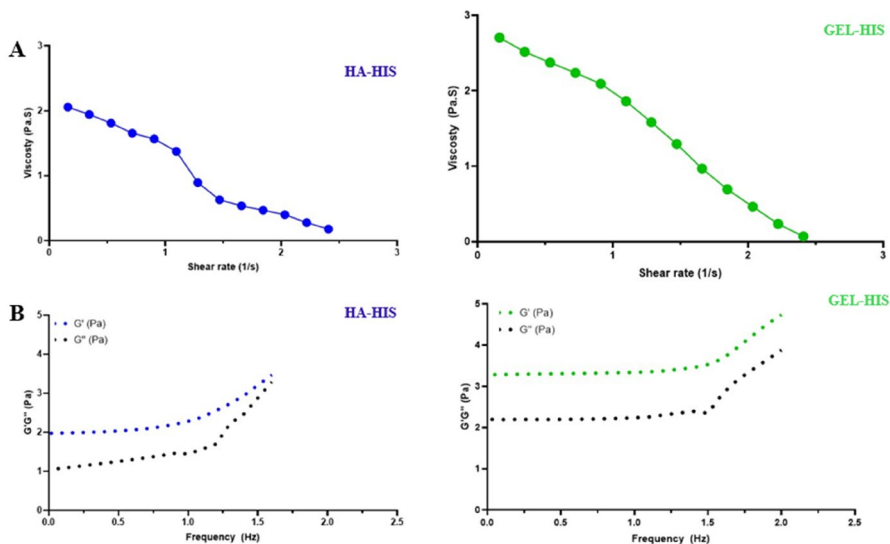


Fig. 5 (A) Viscosity analysis of crosslinked hydrogels, indicating the effect of conjugate composition on the rheological characteristics of the polymer networks. (B) Viscoelastic properties of crosslinked hydrogels, reflecting the elastic and viscous responses that govern their mechanical performance

ing that the crosslinked hydrogels exhibited predominantly elastic rather than viscous behavior (Fig. 5B).

This observation suggests that the materials behave in a solid-like manner by storing a substantial portion of the applied mechanical energy and are prone to elastic deformation. The dominance of the G' modulus confirms the resistance of these hydrogels to shape deformation under stress, highlighting their ability to recover elastically rather than undergo viscous flow [33]. The prevalence of such elastic properties is of critical importance in tissue engineering applications that require cell encapsulation, structural support, and mechanical integrity. In biomedical contexts where mechanical stability is essential, these hydrogels-exhibiting predominantly elastic behavior-hold significant potential as functional and durable scaffolding materials for engineered tissues.

FTIR analysis was performed to characterize the chemical structures of the conjugates and to identify their functional groups. FTIR spectroscopy analyses confirmed the chemical structural modifications of conjugates formed between histamine molecules with HA and Gel. In the HA conjugates, the Amide I ($1650\text{--}1700\text{ cm}^{-1}$) and Amide II ($1500\text{--}1600\text{ cm}^{-1}$) bands indicated the formation of covalent amide bonds, demonstrating successful conjugation. Additionally, the broadband observed in the $3300\text{--}3200\text{ cm}^{-1}$ range supported the presence of hydroxyl ($-\text{OH}$) and amino ($-\text{NH}$) groups, revealing the structural effects of the modification. In gelatin-based systems, similar spectral changes were observed; increased intensities and shifts in the Amide I and Amide II bands confirmed the covalent interaction of histamine with the carboxyl groups of Gel and the formation of new amide bonds. Aromatic C-H deformation vibrations ($900\text{--}600\text{ cm}^{-1}$) in both systems illustrated the impact of conjugation on aromatic structures (Fig. 6). These findings demonstrate that HA and gelatin-based

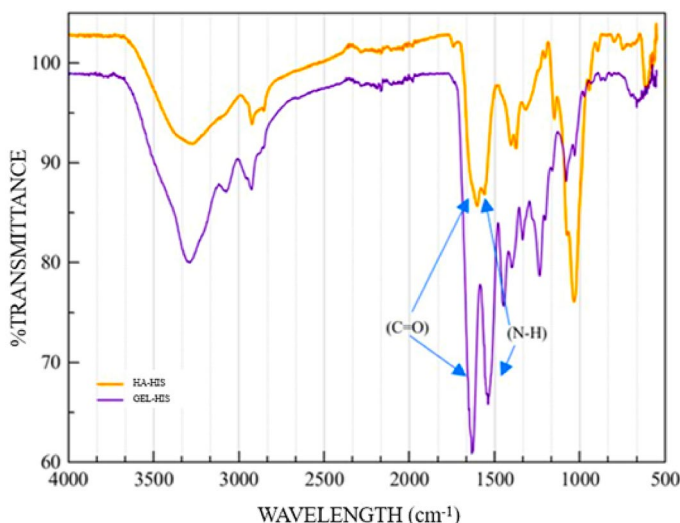


Fig. 6 FTIR spectra of HA-His and Gel-His conjugates, confirming successful chemical modification and the presence of characteristic functional groups

biomolecules were effectively functionalized with histamine, achieving successful chemical modifications in their structures [34].

The successful chemical modification and structural layout of the biopolymer backbones were systematically investigated via overlaid ^1H -NMR spectroscopy, as shown in Fig. 7. In the HA-His spectrum, the characteristic resonance peak at $\delta \sim 1.91$ ppm was successfully attributed to the N-acetylglucosamine methyl protons of the hyaluronic acid backbone. The complex signals observed in the $\delta \sim 3.0$ – 4.5 ppm region corresponded to the overlapping resonances of the polymer pyranose ring protons and the methylene ($-\text{CH}_2-$) groups of the conjugated histamine. Crucially, the degree of substitution (DS) of histamine onto the HA backbone was quantitatively determined by comparing the integral area of the histamine imidazole ring protons ($\delta \sim 6.98$ or 7.56 ppm, 1H) with the sharp N-acetyl singlet reference (set to 3.00), yielding a DS of approximately 11%. In the Gel-His spectrum, the characteristic aliphatic amino acid proton signals ($\delta \sim 0.8$ – 2.3 ppm) intertwined with histamine-related methylene resonances, corroborating the chemical modification of the gelatin structure. Furthermore, the successful covalent functionalization of gelatin was confirmed by the appearance of characteristic histamine imidazole proton signals in the aromatic region at $\delta \sim 7.00$ ppm and $\delta \sim 7.62$ ppm. While the inherent structural complexity and overlapping side-chain signals of the gelatin backbone restrict a precise numerical DS integration, the distinct broadening of these imidazole peaks provides robust qualitative evidence of stable covalent conjugation rather than a simple physical mixture. The coexistence of the respective polymer backbone signals along with the characteristic histamine proton shifts indicates the successful formation of HA-His and Gel-His conjugates, consistent with similar amine-functionalized natural polymer modifications reported in the literature [19, 35–38].

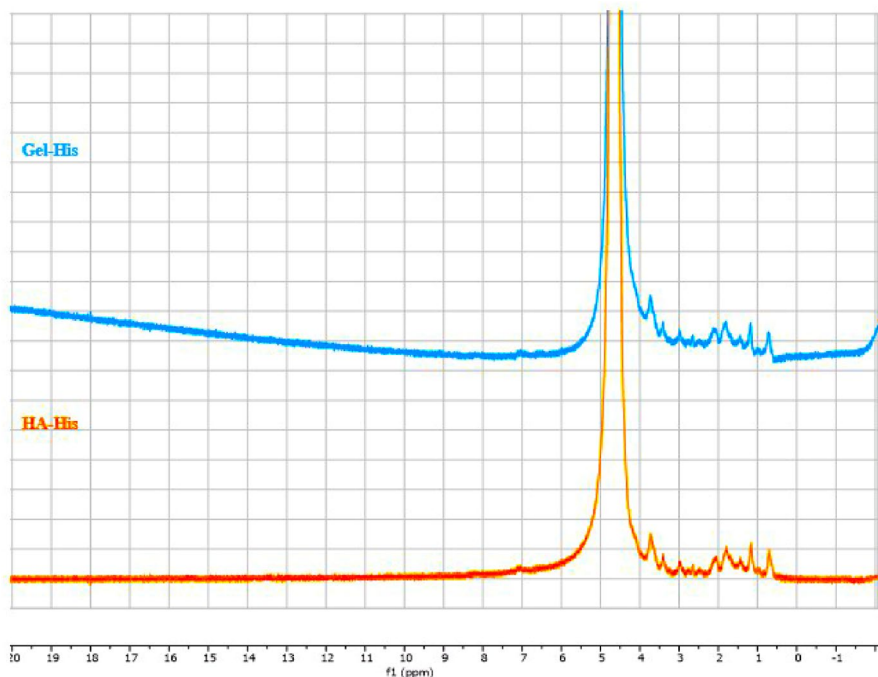


Fig. 7 ^1H -NMR spectra of the hydrogels, providing structural verification of conjugate formation and polymeric network configuration

SEM analyses revealed the presence of a porous surface topology in all examined hydrogel samples. Although pore sizes varied among the samples, the resulting structures are of a scale suitable for providing a microenvironment conducive to cell adhesion and proliferation. To the best of our knowledge, there is no detailed structural characterization study in the literature explicitly focusing on hydrogels prepared from the hyaluronic acid (HA)- or gelatin-based histamine conjugates utilized in this work. However, as the closest benchmark, studies investigating gelatin conjugates formulated with tyramine -another biogenic amine (BA)- have reported an average pore diameter of approximately $68\ \mu\text{m}$ across five different samples. The lower pore sizes obtained in the present study compared to these literature values could be attributed to the inherent differences in the molecular structures and molecular volumes of the respective BAs (tyramine and histamine). Specifically, it can be inferred that histamine, being a smaller and more flexible molecule, acts as a primary factor influencing the crosslinking density and, consequently, the resulting porosity [39]. Similarly, in the conjugates of HA with tyramine, pore sizes were observed to vary depending on the ratios of HA and tyramine [27].

The microstructural characteristics of the hydrogels were investigated by SEM analysis, demonstrating that the crosslinking method had a significant influence on pore size and pore size distribution (Fig. 8). Hydrogels crosslinked via visible light (VL) were found to exhibit a more open, interconnected, and cell-permissive porous architecture compared to those prepared using the HRP enzymatic system.

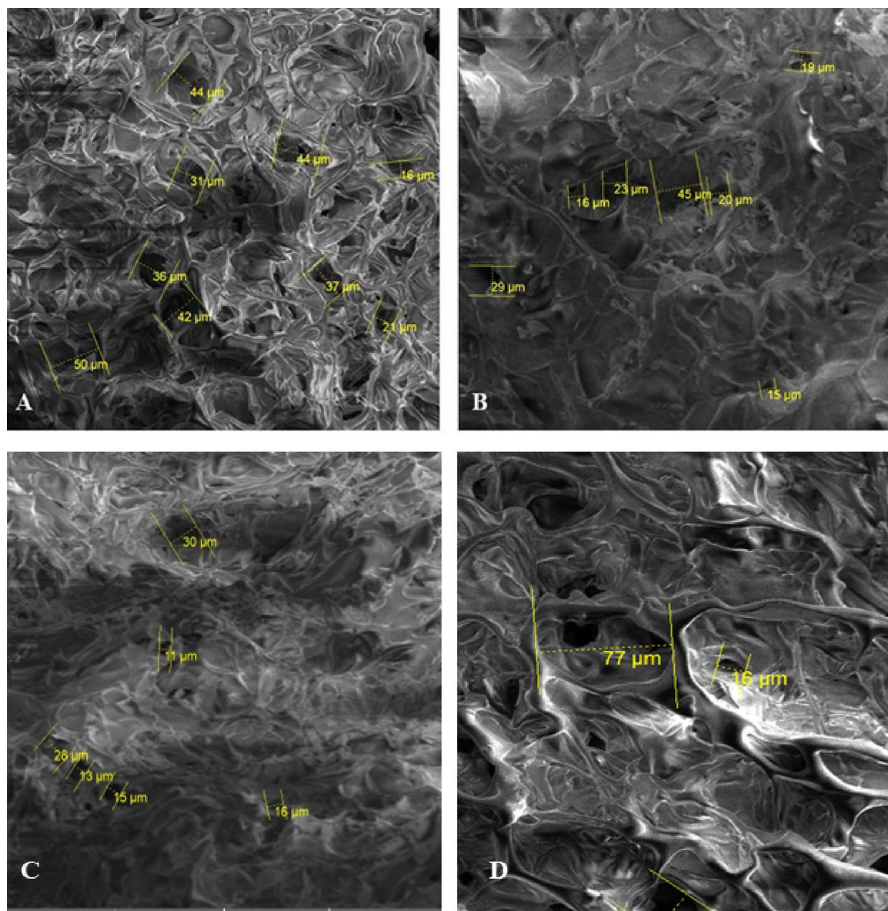


Fig. 8 SEM images showing the pore sizes of the hydrogels: (A) HA-His VL, (B) Gel-His VL, (C) HA-His HRP, (D) Gel-His HRP

HA-His VL hydrogels (Fig. 8A) displayed an interconnected porous network with a broad pore size range (~4–50 μm), reflecting a relatively loose and heterogeneous structure. This type of open porous morphology is considered conducive to cellular infiltration into the hydrogel matrix and to supporting three-dimensional cell organization. Similarly, Gel-His VL hydrogels (Fig. 8B) contained well-defined pores with a relatively homogeneous distribution (~15–45 μm), providing a microstructure that is advantageous for cell adhesion as well as efficient nutrient and oxygen diffusion.

In contrast, hydrogels crosslinked using the HRP enzyme exhibited a denser and more compact network morphology (Fig. 8C–D). In these samples, pore sizes were generally smaller and more irregular, and partially closed structures were observed in certain regions. Such a dense microarchitecture may restrict cellular infiltration and hinder the homogeneous distribution of cells within the hydrogel.

Overall, the SEM findings indicate that visible light-mediated crosslinking yields a more open, interconnected, and biologically functional porous structure. These fea-

tures are expected to facilitate cell localization within the hydrogel pores, enhance cell–material interactions, and suggest that visible light–based systems represent a more efficient approach for tissue engineering applications.

SEM analyses demonstrated that the crosslinking strategy has a pronounced influence on the microstructural organization of the hydrogels. Both HA-His and Gel-His hydrogels crosslinked via visible light (VL) exhibited a more open, interconnected, and porous network architecture compared to their counterparts prepared using the HRP enzymatic system. In visible light–crosslinked systems, a broader distribution of pore sizes was observed, indicating the formation of a more accessible and permeable hydrogel matrix. Similar tunability of pore morphology under visible light conditions has been reported for GelMA hydrogels, where pore sizes ranging from ~26 to 103 μm were achieved by varying formulation parameters under visible light initiation, and these structures supported the growth and function of primary cardiomyocytes *in vitro* [40].

Open and interconnected porosity has been widely recognized as advantageous for cellular attachment and infiltration in hydrogel scaffolds, with light-mediated hydrogels showing enhanced cell–material interactions compared to more densely crosslinked networks [41].

In contrast, hydrogels crosslinked using the HRP enzyme displayed a denser and more compact network structure, with partially closed pores evident in certain regions. This microarchitecture is likely associated with a higher effective crosslinking density, which may restrict homogeneous cell infiltration and limit cell spreading within the hydrogel matrix. While HRP-mediated crosslinking enables efficient network formation, the resulting compact structure may be less favorable for applications requiring extensive cell–matrix interactions. The impact of crosslinking modality on network density and pore morphology has also been noted in studies comparing photocrosslinked structures with alternative crosslinking strategies, emphasizing the role of initiation and network formation kinetics in determining scaffold architecture [42].

The open and interconnected porous structure observed in visible light–crosslinked hydrogels is particularly conducive to supporting cell localization within the hydrogel pores and promoting three-dimensional cellular organization. Appropriate pore sizes combined with a continuous network architecture are expected to facilitate sustained diffusion of nutrients and oxygen, thereby contributing to a microenvironment that supports cell viability and proliferation. These features align with previous observations in visible light–crosslinked systems, where the biocompatibility and tunable porosity of the hydrogel promoted desirable cellular responses [40].

It should be noted that the microstructural assessment in this study is based on qualitative SEM observations, and the lack of quantitative pore size and distribution analysis represents a limitation. Nevertheless, the clear morphological differences observed between the two crosslinking strategies underscore the ability of visible light–mediated crosslinking to tailor hydrogel microstructure toward a more biologically functional configuration. Visible light crosslinking has been explored previously as a viable approach for forming natural polymer–based hydrogels *in situ* without significant cytotoxic effects on human mesenchymal stem cells [22].

Overall, these findings highlight visible light–based crosslinking as a controlled, cell-friendly approach, offering significant potential for hydrogel-based tissue engi-

neering and cell culture applications. Future studies incorporating quantitative morphometric analyses and direct correlations with cellular behavior will further clarify the full capabilities of visible light–crosslinked hydrogel systems. Recent advances in visible-light crosslinkable and multi-stimuli responsive hydrogels suggest strong potential for next-generation smart biomaterials in tissue engineering and drug delivery applications [7, 21].

The mechanical properties of the hydrogels were examined, and the findings revealed that these materials possess limited structural durability. Based on the data obtained from mechanical tests, the materials exhibited low values, particularly in terms of compressive strength and elasticity.

The stress-strain behavior demonstrated that the materials underwent slow and continuous deformation under applied load, predominantly within the linear elastic region. This indicates that the materials have a high deformation capacity within the elastic zone and can maintain stable behavior under loading conditions within a certain range of flexibility.

The ultimate compressive strength (UCS) data, supported by graphical analysis, revealed the maximum compressive stress that the hydrogel samples could withstand without failure. The low UCS values obtained indicate that the materials possess a highly porous, soft, and flexible structure. This suggests that, rather than being suitable for load-bearing structural applications, these hydrogels are more appropriate for specific uses with lower mechanical loading requirements, such as in fillers, insulation, suspension systems, or as biomaterials [43].

The elastic modulus values reflect the stiffness of the materials within the elastic deformation region, indicating the extent to which they deform under stress. The obtained low elastic modulus values demonstrate that the hydrogels possess a highly flexible structure and can undergo significant deformation even under low stress. This mechanical property highlights a key advantage of hydrogels in biomaterial applications where soft tissue-like behavior is desirable.

Graphical results are shown in Figs. 9 and 10.

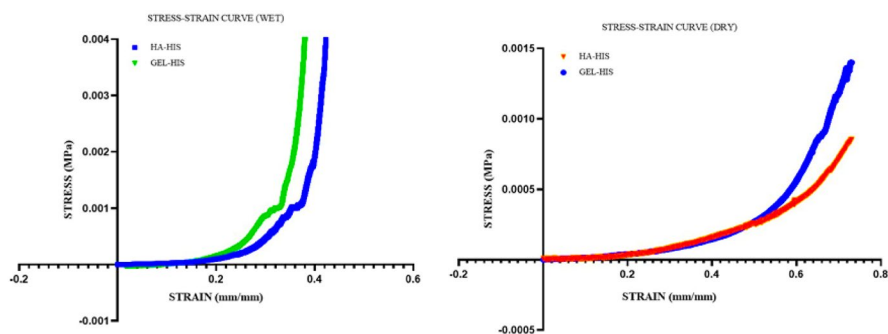


Fig. 9 Stress–strain curves obtained from the compression test of the hydrogels. The curves illustrate the mechanical response of HA-His and Gel-His conjugate hydrogels under compressive loading, highlighting differences in elasticity and strength related to the network structure

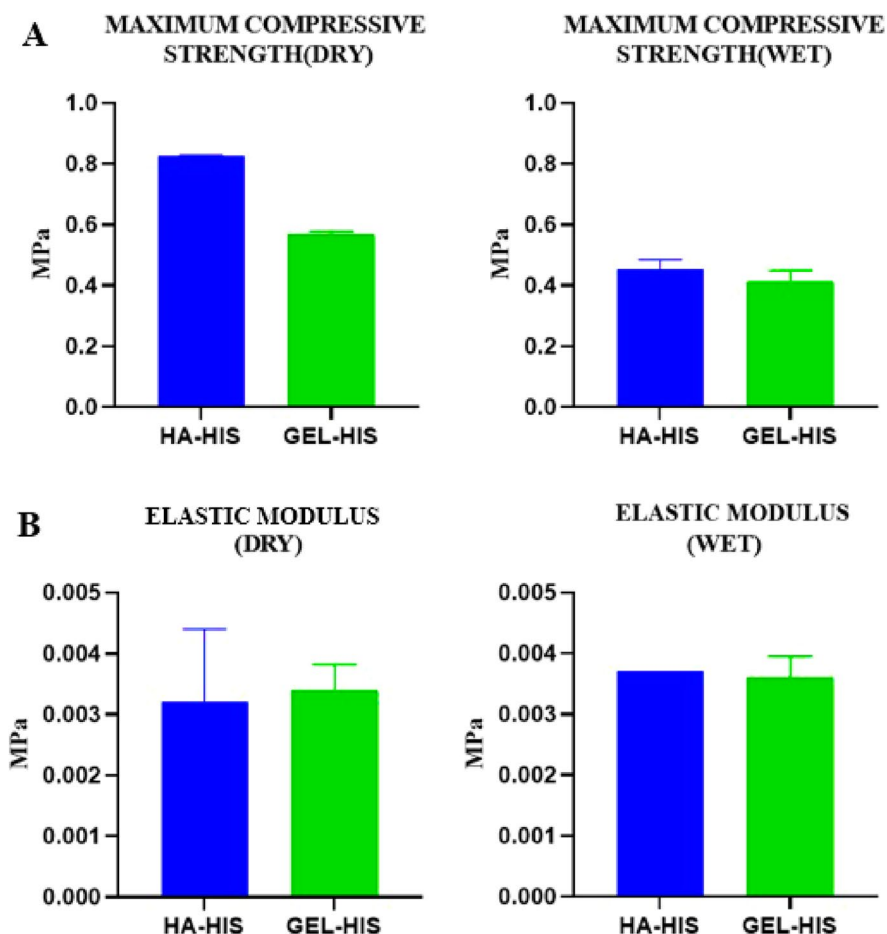


Fig. 10 (A) Graphs depicting the unconfined compressive strength (UCS) values of the hydrogel samples. (B) Graphs showing the elastic modulus values of the hydrogel samples

Cell–material interaction studies

The cellular analyses presented in Fig. 11 illustrate the effects of different crosslinking strategies on cell proliferation and viability in HA-His and Gel-His hydrogels. Cell proliferation was evaluated using the Alamar Blue assay (Fig. 11A), while the MTT assay was employed to assess potential cytotoxic effects and short-term cell viability at 24 and 48 h (Fig. 11B).

One-way ANOVA of the Alamar Blue data revealed no statistically significant differences in cell proliferation among the experimental groups and the control ($p > 0.05$). This finding indicates that neither visible light– nor HRP-mediated crosslinking adversely affected cellular proliferation, with proliferative activity being maintained at comparable levels across all groups. In particular, the proliferation pro-

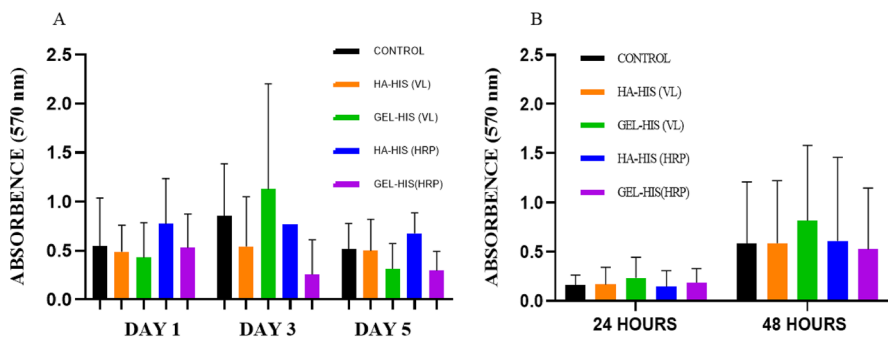


Fig. 11 Evaluation of cell proliferation and viability on HA-His and Gel-His hydrogels prepared using different crosslinking strategies. **(A)** Alamar Blue assay results showing cellular proliferation in control and hydrogel groups. **(B)** MTT assay results assessing

files observed in the visible light-crosslinked hydrogels suggest that this approach provides a biocompatible microenvironment that supports cell growth.

Similarly, the MTT assay results demonstrated no significant differences in cell viability among the groups after 24 and 48 h of incubation ($p > 0.05$). These results indicate that the tested hydrogel systems did not suppress short-term cellular metabolic activity and did not induce detectable cytotoxic effects. The comparable MTT absorbance values across groups further support the cellular safety of the applied crosslinking strategies.

The absence of statistically significant differences should not be interpreted as a lack of biological relevance. Rather, the preservation of cell proliferation and viability at levels comparable to the control group clearly demonstrates the cytocompatible nature of the developed hydrogel systems. In the context of tissue engineering and cell-based applications, maintaining physiological cellular behavior without inducing cytotoxic or inhibitory effects is a critical prerequisite for the successful implementation of newly developed biomaterials. From this perspective, the present findings highlight visible light-mediated crosslinking as a biologically balanced and cell-friendly approach.

Taken together, the combined evaluation of Alamar Blue and MTT assays confirms that the hydrogel systems support both cellular proliferation and viability without adverse effects. Nevertheless, further studies involving extended culture periods and time-dependent quantitative analyses of cell behavior would provide additional insight into the long-term biological performance of visible light-based hydrogel platforms.

In the experimental study, the Scratch Assay test was applied as an *in vitro* model to evaluate wound healing. The *in vitro* scratch assay is a simple, cost-effective, and widely used method for examining cell migration. This technique involves creating an artificial gap on a confluent cell monolayer, followed by the migration of cells at the edges to close the gap and the re-establishment of cell-cell junctions.

In this study, L929 cells were seeded into 6-well plates, and the closure rate of the wound model was assessed over 24 h between the control and experimental groups. Microscopic images of the wells were captured, and changes in the wound areas over

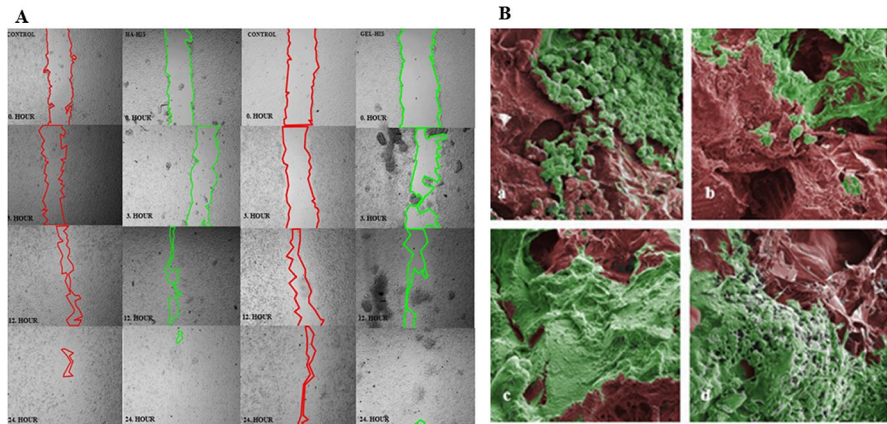


Fig. 12 (A) Microscopic images of the scratch assay results showing the time-dependent progression of cell migration in control and experimental groups. (B) SEM images of cell-cultured hydrogels on days 1 and 3: (a) HA-His, Day 1; (b) Gel-His, Day 1; (c) Gel-His, Day 3; (d) HA-His, Day 3. In the images, the green-colored regions correspond to the adhered and spread cells, whereas the red-colored regions indicate the hydrogel surface. The morphological differences observed between days 1 and 3 illustrate the progressive attachment and spreading of cells on both HA-His and Gel-His hydrogel scaffolds

Table 2 Quantitative comparison of wound areas measured at different incubation times (0, 3, 12, and 24 h) in cell culture–based in vitro wound models generated by scratch assay

Label	Area	Mean	Min	Max	Perim.	Median	Label	Area	Mean	Min	Max	Perim.	Median
ha-his cont 0	591354	181.68	55	254	5066.87	183	ha-his 0	610833	175.29	0	247	4587.66	187
ha-his cont 3	480384	135.48	28	228	4395.85	140	ha-his 3	552312	159.57	20	240	4629.93	183
ha-his cont 12	196803	168.78	22	226	4504.17	173	ha-his 12	131088	148.07	9	245	3929.60	154
ha-his cont 24	30249	197.31	138	233	1370.62	198	ha-his 24	10374	192.34	41	218	514.65	193
gel-his cont 0	711738	210.25	71	244	4430.0	211	gel-his 0	901395	189.77	0	255	4825.54	192
gel-his cont 3	657327	213.89	64	243	4352.43	209	gel-his 3	449922	194.53	2	240	6073.80	197
gel-his cont 12	315555	210.14	29	239	4260.53	209	gel-his 12	253530	139.44	0	221	4414.09	138
gel-his cont 24	120150	177.72	53	231	3672.52	179	gel-his 24	14013	206.25	49	234	556.84	207

time were analyzed using the ImageJ software. Microscopic images of the control and experimental groups are presented in Fig. 12. Table 2 presents the quantitative comparison of wound areas measured throughout the 24-h incubation period.

The scratch assay results revealed a significantly higher wound closure rate in both HA-His and Gel-His groups compared to the control groups. After 24 h, the HA-His group exhibited a wound closure rate of 98.30%, and the Gel-His group showed 98.44%, both exceeding the closure rates of the corresponding control groups, which were 94.88% and 85.77%, respectively. These findings support the notion that his-

tamine-containing formulations promote enhanced cell migration and accelerated wound healing.

As observed in the scratch assay results (Fig. 12), the histamine-modified hydrogels significantly accelerated vertical cell migration and promoted faster closure of the model wound area compared to the control groups. This enhanced cellular response may be associated with the unique presentation mode of the conjugated histamine moieties. As the hydrogel matrix undergoes gradual degradation, particularly through local enzymatic cleavage mediated by cell-secreted enzymes, histamine-containing fragments may be released into the wound microenvironment. In parallel, covalently immobilized histamine groups remaining on the scaffold surface may support cell attachment and migration through histamine receptor-mediated signaling pathways and integrin-related cell–matrix interactions. Such a dual-mode bioactive presentation could provide sustained biological stimulation while avoiding the rapid burst-release behavior commonly observed in physically loaded delivery systems [44–47].

The observed pro-healing effects of histamine are consistent with its reported antioxidant properties and its ability to modulate the inflammatory response. Histamine's capacity to neutralize free radicals and support cell proliferation may positively contribute to wound healing processes. Indeed, a study by et al. demonstrated that histamine increased nasal fibroblast migration, and this effect was confirmed through scratch assay analyses [15].

Owsiak and colleagues investigated the effects of histamine stimulation on keratinocyte function using the scratch assay method. The results of the study demonstrated that histamine treatment modulated proinflammatory responses in keratinocytes and enhanced cellular functions associated with wound healing. Furthermore, it was reported that histamine reduced apoptosis at the *in vitro* level, thereby accelerating the wound healing process [16].

The minimal differences observed between the HA-His and Gel-His groups highlight the influence of the carrier matrix on wound healing. The higher closure rates seen in hyaluronic acid-based systems can be attributed to the well-known properties of this polymer in supporting cell migration. On the other hand, the gelatin-based formulations are thought to provide a more controlled and stable release of histamine, thereby eliciting a comparably effective healing response.

The morphological characteristics of cells cultured on the materials, as well as their adhesion and spreading behaviors on the tissue scaffold surfaces, were thoroughly examined using SEM (Fig. 12). Analyses conducted on day 1 revealed that cells in all hydrogel groups successfully adhered to the surface and exhibited morphological spreading. By day 3, the images showed a marked increase in cell proliferation and a denser distribution across the surface. This increase indicates effective interactions between the cells and hydrogels, suggesting the creation of a favorable microenvironment and supporting the biocompatibility of the materials.

SEM images particularly highlighted the positive impact of the porous surface structures on cell morphology. These structures facilitate cell adhesion, support cytoskeletal organization, and consequently contribute to cell proliferation, migration, and potential differentiation processes.

Conclusion

In this study, histamine-functionalized hyaluronic acid (HA–His) and gelatin (Gel–His) hydrogels were successfully synthesized via covalent conjugation and fabricated using visible light-mediated Ru/SPS crosslinking. The resulting hydrogels demonstrated well-defined physicochemical, structural, mechanical, and biological properties suitable for tissue engineering applications.

Quantitative characterization revealed rapid swelling behavior, reaching equilibrium within 120 min with an approximate 1.5-fold increase in weight. Biodegradation studies showed initial mass loss of ~20–25% within 5 days under hydrolytic conditions, while enzymatic degradation in lysozyme-containing media initiated earlier at day 3, indicating environment-sensitive degradation behavior. Rheological analyses confirmed shear-thinning properties and a dominant elastic response ($G' > G''$), supporting processability for injectable and 3D bioprinting applications.

Biological evaluation demonstrated high cytocompatibility, with no statistically significant differences in cell viability or proliferation between experimental and control groups ($p > 0.05$). Moreover, scratch assay results indicated enhanced wound closure rates exceeding 98% within 24 h for both hydrogel systems, suggesting that histamine functionalization contributes positively to cell migration and wound closure behavior.

Importantly, histamine was covalently immobilized within the polymer network (DS ~11% for HA–His), indicating that its biological effect is likely mediated through a combination of surface-bound bioactive presentation and gradual release of histamine-containing degradation fragments, rather than burst release.

Despite these promising findings, several limitations should be acknowledged. First, degradation was evaluated only under PBS and lysozyme conditions, which do not fully replicate the complexity of wound microenvironments (e.g., hyaluronidase- and collagenase-rich conditions). Second, the study is limited to in vitro assessments, and no in vivo inflammatory or wound healing models were included. Third, long-term cellular responses and immune modulation effects were not investigated. Finally, quantitative crosslinking density and full structure–property correlations remain to be further elucidated.

Therefore, while the present study demonstrates a cytocompatible and functionally active hydrogel platform with promising in vitro wound healing performance, further in vivo validation and biologically relevant enzymatic degradation studies are required to confirm its translational potential. Although the direct comparison between visible light- and HRP-mediated crosslinking was limited to short-term cellular assays, the visible light-based approach offers notable practical and biological advantages, including spatiotemporal control over gelation, avoidance of enzymatic components, and compatibility with in situ and light-triggered fabrication strategies. These features make visible light-mediated crosslinking a versatile and controllable platform for hydrogel fabrication.

Overall, the findings demonstrate that histamine-functionalized HA and gelatin hydrogels prepared via visible light-mediated crosslinking provide a cytocompatible and biologically supportive microenvironment capable of maintaining cell viability, proliferation, and migration. While further studies incorporating long-term cell cul-

ture and in vivo validation are warranted, the present results underscore the potential of visible light–crosslinked hydrogel systems as promising candidates for wound healing and soft tissue engineering applications.

Acknowledgements This work was supported by the Scientific and Technological Research Council of Türkiye (TÜBİTAK) under Project No. 123M287. The authors gratefully acknowledge TÜBİTAK for the financial support provided for this research.

Author contributions FÖ: Investigation, methodology, data curation, formal analysis, visualization, writing – original draft, writing – review & editing. SO: Conceptualization, supervision, funding acquisition, writing – review & editing. All authors reviewed the manuscript.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). This work was supported by the Scientific and Technological Research Council of Türkiye (TÜBİTAK) with project code 123M287. This study was conducted as part of the PhD thesis of Fulya ÖZDEMİR under the supervision of Prof. Dr. Sedat ODABAŞ.

Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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