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Article ID: IJB026330356

Citation: Hwang J, Kim Y, Park JS, Kong M, Kim HS, Lee KY. Three-dimensional bioprinting of biomimetic bilayer hydrogels with engineered mechanical and cellular heterogeneity for wound healing. *Int J Bioprint*. 2026. doi: 10.36922/IJB026330356

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RESEARCH ARTICLE

Volume X Issue X (2026)

doi: 10.36922/IJB026330356

Three-dimensional bioprinting of biomimetic bilayer hydrogels with engineered mechanical and cellular heterogeneity for wound healing

Running title: Heterogeneous bilayer hydrogel wound patches

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Citation: Hwang J, Kim Y, Park JS, Kong M, Kim HS, Lee KY. Three-dimensional bioprinting of biomimetic bilayer hydrogels with engineered mechanical and cellular heterogeneity for wound healing. *Int J Bioprint*. 2026. doi: 10.36922/IJB026330356

Received: April 30, 2026

Revised: August 12, 2026

Accepted: August 25, 2026

Published online: August 25, 2026

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Abstract

In view of the global wound care burden on healthcare systems, there is an urgent need for advanced therapeutic solutions to promote wound healing. While conventional bilayer dressings statically define layer mechanics, they risk structural failure upon deformation. To address this limitation, we present a self-healing biomimetic bilayer hydrogel system that utilizes hyaluronic acid (HA) derivatives to emulate the mechanical and cellular heterogeneity of native skin tissue. The hydrogels were prepared by synthesizing carbodihydrazide-conjugated HA (HA-CDH) and crosslinking it with oxidized diol-modified HA in the presence of adipic acid dihydrazide. By adjusting the HA-CDH concentration, the stiffness of the bilayer hydrogel was optimized to effectively replicate the distinct mechanical properties of the epidermis and dermis. This bilayer structure with cell-specific stiffness and different cell types promoted cell-specific proliferation and upregulated key wound healing markers such as collagen type I and α -smooth muscle actin. Furthermore, the hydrogel exhibited excellent shear-thinning and dynamic self-healing properties that ensure high shape fidelity during extrusion-based 3D bioprinting of customized wound patches. In a full-thickness mouse wound model, these cell-laden bilayer patches promoted wound repair by significantly suppressing initial inflammatory responses, enhancing neovascularization, and facilitating balanced extracellular matrix remodeling without fibrotic thickening. The proposed self-healing bilayer hydrogel system stands as a promising, structurally dynamic solution for effective wound healing and has potential for further applications in tissue engineering.

Keywords: Hydrazide-modified hyaluronate; Oxidized hyaluronate; Heterogeneity; Self-healing; 3D bioprinting

1. Introduction

The global wound care burden poses an escalating challenge to healthcare systems, with millions of patients suffering from full-thickness skin defects annually.^[1, 2] While traditional autologous skin grafting remains a clinical standard, its utility is severely constrained by limited donor site availability, large wound surface areas, and donor site morbidity.^[3] To overcome these tissue shortages, diverse bioengineered acellular and cell-laden skin matrices have been commercially developed.^[4, 5] However, achieving true functional and biomimetic skin repair remains challenging because native skin is inherently heterogeneous and comprises structurally stratified layers with distinct cellular compositions and mechanical environments.^[6]

Therefore, multilayer systems that regulate the structural and mechanobiological cues are essential for effective skin tissue engineering. Specifically, epidermal keratinocytes and dermal fibroblasts — the primary cellular architects of skin — thrive in drastically divergent physical microenvironments.^[6-10] Keratinocytes migrate and proliferate more efficiently on stiff matrices reminiscent of the thin epidermis, whereas fibroblasts favor softer, compliant matrices.^[11-13] Consequently, deploying a mechanically uniform monolayer scaffold creates a suboptimal environment that inevitably compromises one cell type at the expense of the other, which leads to delayed re-epithelialization or disordered fibrotic healing.

Multilayer scaffold systems have been proposed to bridge this mechanobiological gap.^[14-19] However, most prior designs are functionally limited because their mechanical profiles are statically defined during crosslinking.^[20] During clinical application and active patient movement, these stratified constructs are highly vulnerable to localized tearing, compression, or interfacial delamination. Once fractured, a static bilayer scaffold loses its defined mechanical boundary, which fundamentally disrupts layer-specific cell behaviors and inhibits coordinated tissue repair. Therefore, achieving successful barrier restoration requires a self-healing platform that establishes physical and mechanical stratification and also dynamically preserve and heal its internal architectural boundaries under stress. Additionally, this self-healing system is advantageous for extrusion-based 3D printing over conventional mold-cast techniques because the rapid and precise production of patient-specific wound patches can be fabricated with an adjustable microarchitecture.

Based on these challenges, we hypothesized that creating a structurally stratified bilayer hydrogel tailored to cell-specific stiffness thresholds would optimize individual skin cell behavior, and furthermore, that incorporating autonomous self-healing capabilities within this system would dynamically preserve the mechanical boundaries at the layer interface under physical deformation, thereby collectively accelerating coordinated in vivo wound repair. To test this hypothesis, we propose an advanced, 3D-bioprinted biomimetic bilayer hydrogel platform characterized by structural heterogeneity and autonomous self-healing capabilities (Figure 1a). Hydrogels were engineered by synthesizing carbodihydrazide-conjugated hyaluronic acid (HA-CDH) and crosslinking it with oxidized diol-modified HA (odHA). Hyaluronic acid (HA) was selected as the underlying polysaccharide backbone due to its inherent biocompatibility and its critical, native role in stimulating cell migration and suppressing hyper-inflammatory signals during early granulation phases.^[21-24] By integrating adipic acid dihydrazide (ADH) into the network, we introduce dynamic, reversible hydrazone bond exchanges that impart robust self-healing and rapid shear-thinning properties suitable for 3D bioprinting (Figure 1b). This design yields three distinct advantages including tailored mechanical heterogeneity, cellular heterogeneity, and dynamic structural integrity to directly connect our hypothesis to the experimental design.

In this study, we systematically characterize the rheological profiles, self-healing kinetics, and shape fidelity of this responsive bioink system. Furthermore, we validate the localized mechanobiological responses of encapsulated primary cells in vitro and evaluate the therapeutic efficacy of the cell-laden bilayer patches in a full-thickness mouse wound model to demonstrate its clinical potential for advanced wound repair.

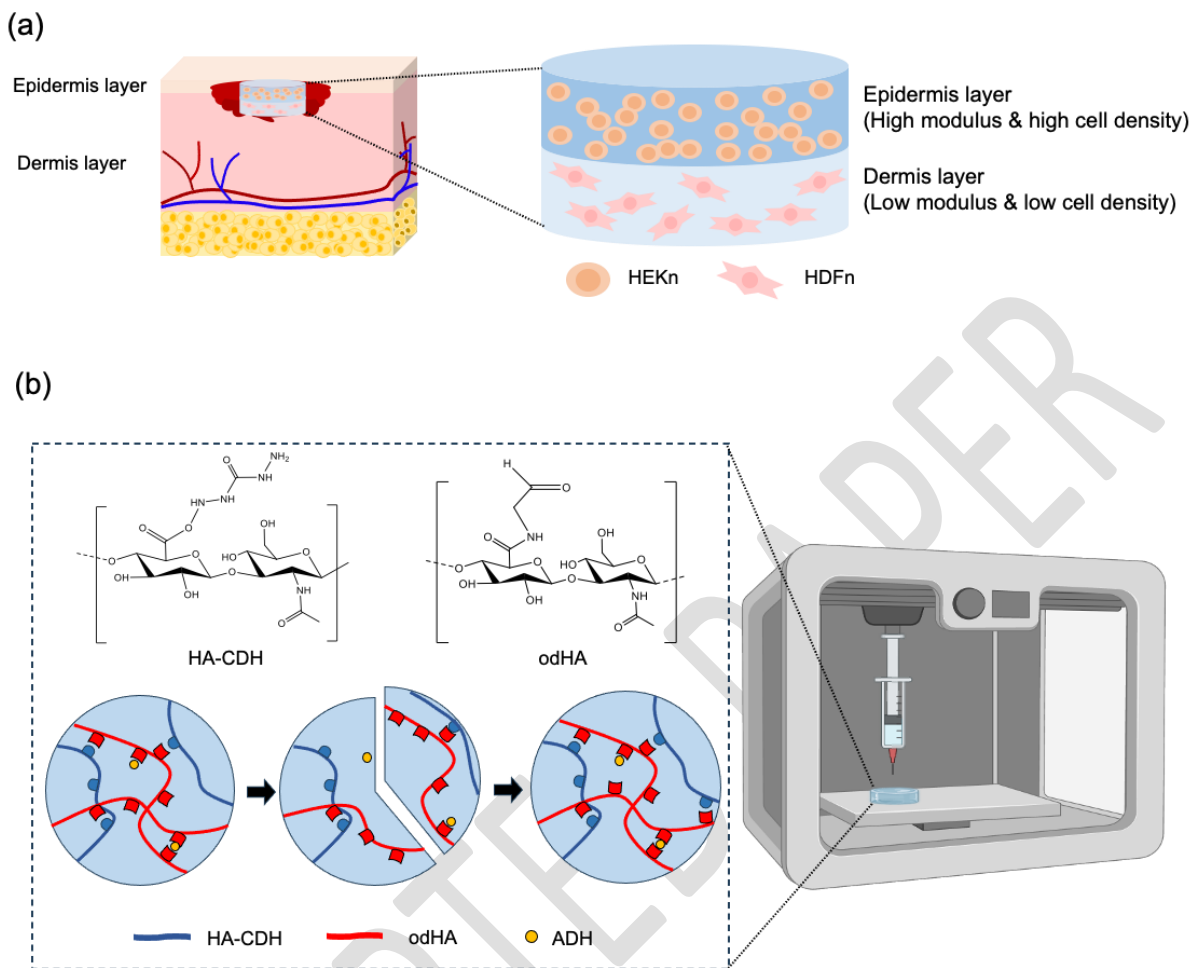


Figure 1. Biomimetic bilayer hydrogel system for wound healing. (a) Schematic of the wound healing process using a 3D-printed bilayer wound patch composed of self-healing hydrogels encapsulating human keratinocytes, neonatal (HEKns) and dermal fibroblasts, neonatal (HDFns). (b) Schematic for self-healing behavior of 3D-printable carbodihydrazide-modified HA (HA-CDH)/oxidized diol-modified HA (odHA)/adipic acid dihydrazide (ADH) hydrogels.

2. Materials and methods

2.1. Materials

HA (MW 700 kDa) was purchased from Lifecore (Chaska, MN, USA). Carbodihydrazide (CDH) and 3-amino-1,2-propanediol were provided by TCI (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was procured from Proteochem (Hurricane, UT, USA). 1-Hydroxybenzotriazole hydrate (HOBt), sodium periodate (NaIO_4), ADH, and dimethyl

sulfoxide (DMSO) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Dulbecco’s phosphate-buffered saline (DPBS), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin (PS) were provided by Gibco (Grand Island, NY, USA). EpiLife™ medium (MEPI500CA), human keratinocyte growth supplement kit (HKGS Kit, S001K), trypsin/ethylenediaminetetraacetic acid solution, trypsin neutralizer solution, and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The EZ-Cytox kit was supplied by DoGenBio (Seoul, Korea). Sodium dodecyl sulfate (SDS) was purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from Amresco (Solon, OH, USA).

2.2. Synthesis of HA derivatives

HA (1 g) was dissolved in deionized water (0.5% w/v) for 24 h. HOBt (0.3567 g) dissolved in DMSO and CDH (1 g) was then added to the HA solution. EDC (0.101 g) was added to the HA solution after 30 min ([HOBt]:[EDC] = 1:0.2, molar ratio), and the pH was adjusted to 6.0. The reaction proceeded for 24 h to obtain HA-CDH. The reaction solution was dialyzed against a 0.1 M NaCl solution for 48 h, followed by additional dialysis in deionized water for 24 h (molecular weight cutoff = 3500 g/mol).^[36]

To synthesize odHA, HA (1 g) was first dissolved in deionized water (0.5% w/v) for 24 h. HOBt (0.3567 g) and 3-amino-1,2-propanediol (0.2 g) were added to the HA solution, and the pH was then adjusted to 6.0. EDC (0.1515 g) was added to the HA solution ([NH₂]:[HOBt]:[COOH] = 1:1:1, molar ratio) to obtain diol-modified HA (dHA). After 24 h, dialysis was performed in a dilute HCl solution (pH 3.5) containing 0.1 M NaCl for 48 h, followed by a further 24 h of dialysis in deionized water (molecular weight cutoff = 3500 g/mol) and lyophilization. The lyophilized dHA (1 g) was dissolved in deionized water (0.5% w/v) for 24 h and was oxidized with sodium periodate (0.56 g) for 10 min ([HA]/[NaIO₄] = 1:0.5, molar ratio). The oxidation was quenched using ethylene glycol (0.736 mL). The resulting solution was subjected to dialysis using deionized water for 48 h (molecular weight cutoff = 3500 g/mol). All the HA derivatives were lyophilized and stored at –20 °C.^[37]

2.3. Characterization of HA derivatives

Fourier transform infrared (FTIR) spectroscopy was used to confirm the synthesis of HA derivatives (Nicolet 6700, Thermo Scientific, Waltham, MA, USA).^[38] FTIR spectra were acquired within the wavenumber range of 800–2000 cm^{-1} (number of scans: 32, resolution: 4 cm^{-1} , scan rate: 4 mm/s). The synthesis of odHA was confirmed using ^1H nuclear magnetic resonance (NMR) spectroscopy (VNMRS 600 MHz, Varian, Palo Alto, CA, USA; D_2O , 4.65 ppm).^[39]

The degree of hydrazide substitution in HA-CDH was determined using a TNBSA assay. Sodium bicarbonate was dissolved in deionized water to prepare the reaction buffer (pH 8.5). HA and HA-CDH were dissolved in a sodium bicarbonate buffer (1 mg/mL), and 0.25 mL of 0.01% (w/v) TNBSA solution was added to 0.5 mL of the buffer solution. The sample solution was incubated at 37 °C for 2 h. Subsequently, 10% SDS solution (0.25 mL) and 1 N HCl (0.125 mL) were added to the sample solution. The absorbance at 340 nm was measured using a spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA), and the degree of substitution (DS) was calculated using BSA as the standard.

odHA was dissolved in deionized water to a concentration of 10 mg/mL, and an excess amount of *t*-butyl carbazate (TBC) was added to the solution. The solution was stirred for 1 h, followed by the addition of sodium cyanoborohydride (NaBH_3CN) ($[\text{TBC}]:[\text{NaBH}_3\text{CN}] = 1:1$, molar ratio). After 24 h, the solution was dialyzed against deionized water (molecular weight cutoff = 3500 g/mol) and lyophilized. The degree of aldehyde modification of odHA was quantified by ^1H NMR spectroscopy (VNMRS 600 MHz, Varian, Palo Alto, CA, USA; D_2O , 4.65 ppm).

2.4. Determination of molecular weight

Molecular weight characterization of HA, HA-CDH, and odHA was performed by size-exclusion chromatography in combination with multi-angle laser light scattering (SEC-MALLS). The analysis utilized a Shimadzu chromatographic platform (Kyoto, Japan) fitted with a PL Aquagel-OH MIXED-H column (7.5 × 300 mm; Tosoh Bioscience, San Francisco, CA, USA), in combination with a multi-angle light scattering detector and a differential refractive index detector (DAWN HELEOS II and Optilab Rex, Wyatt Technology, Santa Barbara, CA, USA). During SEC-MALLS analysis, DPBS served as the chromatographic carrier solvent, delivered at 0.5

mL/min, while the refractive index increment (dn/dc) of the HA-based derivatives was defined as 0.167 mL/g.^[39]

2.5. Hydrogel preparation and characterization

Both HA-CDH and ADH were dissolved in DPBS for 24 h, followed by mixing with an odHA solution (1.0%, w/v) to form hydrogels. The ratio between HA-CDH and odHA was varied, while the ADH concentration was maintained at 0.1% (w/v). The gelation time of the hydrogel was 30 min.

Rheological measurements were performed using a rotational viscometer (Bohlin Gemini 150; Malvern, Worcestershire, UK) equipped with a 20 mm parallel plate. The diameter and thickness of the hydrogel disks were 20 and 1 mm, respectively. All hydrogel samples were carefully placed between the plates at a frequency of 1 Hz to avoid air bubbles. All the tests were conducted at 25 °C.

2.6. Evaluation of self-healing behavior of hydrogels

The self-healing properties of the hydrogels were demonstrated by preparing disks with a diameter of 10 mm and thickness of 1 mm. The discs were cut into pieces, reattached, and allowed to heal for 30 min. An amplitude sweep was performed with strain levels ranging 1–1000%. Subsequently, a strain beyond the gel's deformation point was applied for 100 s to disrupt the gel network, followed by a 1% strain for 100 s to monitor the recovery.

2.7. 3D printing

The 3D structures were fabricated using an extrusion-based 3D printer (Invivo, Seoul, Korea). A 3D model was designed using Tinkercad (Autodesk) and printed using an extrusion-based 3D printer. The hydrogel was loaded into a 10 mL syringe and allowed to rest for 30 min before being printed using a 25-gauge needle. The motor pressure was set to 300 N and the infill density was maintained at 80%. The printing speed was varied from 5 to 15 mm/s. The fidelity was evaluated

by printing cylindrical models with a diameter of 10 mm and a height of 5 mm at speeds ranging 5–15 mm/s. The diameter and height of each printed result were measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) and compared with those of the designed model. To assess the effect of cell incorporation on printing fidelity, the printability index (*Pr*) value was calculated from the pore geometry of printed grid structures using the same image analysis protocol.

2.8. Cell culture and in vitro cytotoxicity evaluation

Primary human epidermal keratinocytes, neonatal (HEKn) (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in EpiLife™ medium with HKGS and 1% PS at 37 °C in a 5% CO₂ atmosphere. Primary human dermal fibroblast, neonatal (HDFn) cells (ATCC, Manassas, VA, USA) and HaCaT cells (CLS Cell Lines Services GmbH, Germany) were cultured in DMEM supplemented with 5% FBS, 1% PS at 37 °C in a 5% CO₂ atmosphere.

The in vitro cytotoxicities of HA, HA-CDH, and odHA were assessed using an EZ-Cytox kit. Briefly, HEKn and HDFn were seeded in a 96-well plate at a density of 5.0×10^3 cells/well and cultured at 37 °C in a 5% CO₂ atmosphere. After 24 h of incubation, 10 µL of HA, HA-CDH, or odHA solutions were added to each well and incubated for an additional 24 h. Subsequently, 10 µL of an EZ-Cytox solution was added to each well, and the plate was incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. The absorbance was measured at 450 nm using a spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA).

To determine the effect of the extrusion process on cellular survival, the viability of encapsulated cells was evaluated before and after printing. HEKn cells (2.5×10^6 cells/mL) and HDFn cells (2.5×10^5 cells/mL) were incorporated into HA-CDH 1.5 and HA-CDH 0.5 hydrogels, respectively. Post-printing cell viability was compared against non-printed controls for 24 h using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.9. Proliferation assay in vitro

Hydrogels encapsulating HEKn and HDFn (1.0×10^5 cells/mL) were prepared at various compositions and cultured for 7 d at 37 °C in a 5% CO₂ atmosphere. An MTT solution was diluted to 0.1% in the medium and applied to the hydrogels, followed by incubation for 5 h. The solution was then removed, and DMSO was added to dissolve the formazan crystals. After 10 min, the solution in the plate was transferred to a new 96-well plate, and absorbance was measured at 540 nm using a spectrophotometer.^[33] Cell viability within the hydrogels was further assessed using a LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen). After 7 d of culture, the hydrogel/cell constructs were stained with calcein AM and ethidium homodimer-1 according to the manufacturer's instructions, and fluorescence images were obtained using a confocal laser scanning microscope (Leica Microsystems, Germany).

2.10. Gene expression analysis in vitro

HEKn and HDFn were encapsulated in hydrogel disks (diameter = 10 mm, thickness = 1 mm) at densities of 2.5×10^6 cells/mL and 2.5×10^5 cells/mL, respectively, and incubated at 37 °C in a 5% CO₂ atmosphere for 7 d. After incubation, the relative gene expression levels in HEKn and HDFn cells were quantified using reverse transcription polymerase chain reaction (RT-PCR, Takara) and real-time PCR (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA). For real-time PCR analysis, hydrogels were homogenized (T 10 basic ULTRA-TURRAX, IKA, Staufen, Germany). The homogenized RNA was extracted using the RNAiso kit (Takara, Kusatsu, Japan), and the expression levels of collagen type I (Col1) and α -smooth muscle actin (α -SMA) were analyzed by comparison with GAPDH using RT-PCR and real-time PCR.

The primer sequences used for HEKn and HDFn cells were as follows (Cosmogenetech, Seoul, Korea):^[34, 35]

GAPDH, 5'-GAA GGT GAA GGT CGG AGT-3', 5'-GAA GAT GGT GAT GGG ATT TC-3';
Col1, 5'-CAG CCG CTT CAC CTA CAG C-3', 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3';
 α -SMA, 5'-CCG ACC GAA TGC AGA AGG A-3', 5'-ACA GAG TAT TTG CGC TCC GAA A-3'.

2.11. Evaluation of wound healing performance in vivo

All in vivo procedures were performed in compliance with the animal welfare regulations of Hanyang University and received formal approval from the university's Institutional Animal Care and Use Committee (IACUC) [2024-0149A]. BALB/c nude mice (ORIENTBIO, Korea, 8 weeks, 20–24 g, male, n = 4 per group) were anesthetized using isoflurane, and a full-thickness wound (diameter = 10 mm) was created in the dorsal area of the mice using a biopsy punch (Acu-Punch 10 mm). Wound sites were treated with HA-CDH 0.5 hydrogel with HDFn cells, HA-CDH 0.5 hydrogel with HEKn cells, and bilayer hydrogel (HA-CDH 0.5 as the lower layer with HDFn, HA-CDH 1.5 as the upper layer with HEKn) ([HDFn] = 2.5×10^5 cells/mL, [HEKn] = 2.5×10^6 cells/mL). A bilayer patch without cells was also prepared. The control group was covered with a Tegaderm film (3M, 6 × 7 cm). Photographs of the wound sites were taken on days 0, 1, 3, 5, 7, and 14. Wound contraction (%) was calculated as follows:

$$\text{Wound contraction (\%)} = [W_0 - W_n]/W_0 \times 100,$$

where W_0 represents the wound size on day 0, and W_n represents the wound size on day n. Wound size was measured using ImageJ software program.

The wound samples were obtained from the mice and immersed in 4% paraformaldehyde solution for over 24 h, followed by a dehydration process (wherein the tissue was passed through ethanol concentrations of 70%, 80%, and 95% twice, and 100% three times for 30 min at each step). Subsequently, the tissue was immersed in paraffin three times for 30 min to prepare for dehydration and paraffin processing; subsequently, the tissue was embedded in a paraffin block and was sliced into sections with a thickness of 7 μ m using a microtome (RM2145, Leica, Germany), followed by hematoxylin and eosin (H&E) and Masson's trichrome staining. To evaluate neovascularization, immunohistochemical staining for CD31 was performed using a rabbit polyclonal anti-CD31 antibody (ab124432, Abcam; 1:500) overnight at 4 °C, followed by incubation with an HRP-conjugated goat anti-rabbit IgG secondary antibody (ab205718, Abcam; 1:5000) for 1 h at room temperature. Collagen area fraction and CD31-positive microvessel area were quantified from Masson's trichrome and immunohistochemically stained sections, respectively. The dermal gap was quantified using ImageJ software program by measuring the distance between wound edges

in H&E-stained tissue sections. Measurements were performed on central wound sections, and at least three sections per sample were analyzed to obtain average values.

2.12. Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple-group comparisons and an unpaired two-tailed Student's *t*-test for two-group comparisons. A *P*-value of less than 0.05 was considered statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

3. Results and discussion

3.1. Synthesis and characterization of HA-CDH and odHA

FTIR analysis was conducted to confirm the synthesis of HA-CDH and odHA (Figure S1). The conjugation of CDH to HA resulted in a reduction of the carboxylic acid group, as indicated by a decrease in the peak intensity at 1410 cm⁻¹ (O–H bending, carboxylic acid). This change reflected the reaction between the carboxylic acid of HA and the hydrazide of CDH.^[35] The introduction of hydrazide groups to HA-CDH was confirmed by the appearance of a peak at 1710 cm⁻¹ (C=O stretching, amide), which was absent in unmodified HA.^[31, 36] For odHA, the addition of aldehyde groups was confirmed by a characteristic peak at 1730 cm⁻¹ (C=O stretching, aldehyde).^[35] This functionalization was attributed to the oxidation of the diol groups of dHA using sodium periodate to generate aldehyde-modified odHA.^[32]

The DS of odHA was determined using ¹H NMR spectroscopy. The aldehyde groups in odHA were reacted with TBC, allowing their quantification using ¹H NMR (Figure S2). The *t*-butyl methyl protons were observed exclusively in TBC-derivatized odHA at 1.37 ppm. The methyl protons of *N*-acetyl groups in the HA backbone appeared at 1.89 ppm. Weak signals in the range of 4.3–4.4 ppm were attributed to the anomeric protons of the HA backbone. Overlapping signals between 3.2 and 3.7 ppm were assigned to the sugar ring protons derived from the repeating units of *N*-acetyl-*D*-glucosamine and *D*-glucuronic acid.

Quantitative analysis indicated that odHA exhibited an aldehyde functionalization level of 26.2%, corresponding to the number of aldehyde groups per 100 repeating saccharide units (Table 1). For HA-CDH, the DS, representing the density of hydrazide groups per 100 saccharide repeats, was calculated as 17.3% based on the TNBSA assay (Table 1). Additionally, the molecular weights of HA, HA-CDH, and odHA were evaluated by SEC-MALLS (Table 2). Partial oxidation of dHA mitigated the reduction in the molecular weight typically associated with backbone oxidation, thereby preserving overall chain integrity. HA-CDH showed an increased M_w , indicating the successful conjugation of CDH without degradation of the HA backbone. According to the manufacturer, the HA used in this study was highly purified and had a narrow molecular weight distribution.

Table 1. Theoretical and actual DS values of HA derivatives.

Sample	Theoretical DS (%)	Actual DS (%)
odHA	30.0	26.2 ^a
HA-CDH	20.0	17.3 ^b

^a Determined by ¹H NMR spectroscopy

^b Determined by the TNBSA assay

Table 2. The molecular weights of HA, HA-CDH, and odHA determined by size-exclusion chromatography with multi-angle laser light scattering (SEC-MALS).

Sample	M_w ^a (g/mol)	PDI ^b
HA	5.4×10^5	1.05
HA-CDH	9.6×10^5	1.27
odHA	5.8×10^5	1.63

^a Weight-average molecular weight

^b Polydispersity index (M_w/M_n)

3.2. Preparation and characterization of hydrogels

The hydrogels were prepared using HA-CDH and odHA. HA-CDH with a DS of 17.3% and odHA with a DS of 26.2% were selected based on a previous study in which these DS values were optimized to provide an appropriate crosslinking density while maintaining the mechanical stability and self-healing properties of the HA-based hydrogel system.^[32] The ratio of [HA-CDH] to [odHA] was varied to obtain hydrogels with different mechanical properties. ADH was used to enhance the self-healing properties of the HA-CDH/odHA hydrogels while maintaining the same concentration in the gels ([ADH] = 0.1% (w/v)).^[29] Hydrogels prepared at [HA-CDH]:[odHA] = 0.5:1.0, 1.0:1.0, and 1.5:1.0 were coded and referred to as HA-CDH 0.5, HA-CDH 1.0, and HA-CDH 1.5, respectively.

Each sample was dissolved in DPBS overnight at room temperature before use.^[29, 31] The storage shear modulus (G') and loss shear modulus (G'') of hydrogels were measured as a function of HA-CDH content (Figure 2a). The G' values increased at higher HA-CDH contents, with measured values of 265.3 ± 10.9 Pa for HA-CDH 0.5, 542.9 ± 34.3 Pa for HA-CDH 1.0, and 893.3 ± 20.6 Pa for HA-CDH 1.5 (Figure 2b). To assess the G' value in the presence of cells, HaCaT cells, immortalized human keratinocytes frequently used in dermatological research,^[37] were encapsulated in hydrogels at a density of 2.5×10^6 cells/mL. The G' values were influenced by the HA-CDH content in the gels, despite the inclusion of cells, and the modulus differences of the HA-CDH hydrogels (at varying HA-CDH ratios) were found to be similar (Figure 2b).

Proliferation of keratinocytes is more effective in hydrogels with higher mechanical stiffness, and proliferation of fibroblasts is effective in those with lower mechanical stiffness.^[11, 12, 38] Additionally, the epidermal layer has a higher modulus than the dermal layer in the skin tissue.^[38] Thus, the epidermis layer was modeled using HA-CDH 1.5 ($G' = 893.3 \pm 20.6$ Pa), and the dermis layer was implemented with HA-CDH 0.5 ($G' = 265.3 \pm 10.9$ Pa) in this study to mimic the structure of natural skin. The hydrogel system was designed to facilitate wound healing by creating an environment that promotes the proliferation of multiple cell types.

Conventional HA hydrogels often fail to achieve significant elastic modulus changes without excessive amounts of polymers, unless they are combined with synthetic materials.^[28] Additionally,

HA hydrogels composed of acyl hydrazone bonds degraded rapidly and disappeared before sufficient wound regeneration occurred. Therefore, HA-CDH has been used to address the challenges associated with conventional HA-based hydrogels.^[25-27] HA-CDH was used to cross-link odHA to achieve a controllable elastic modulus by tuning the HA-CDH ratio.

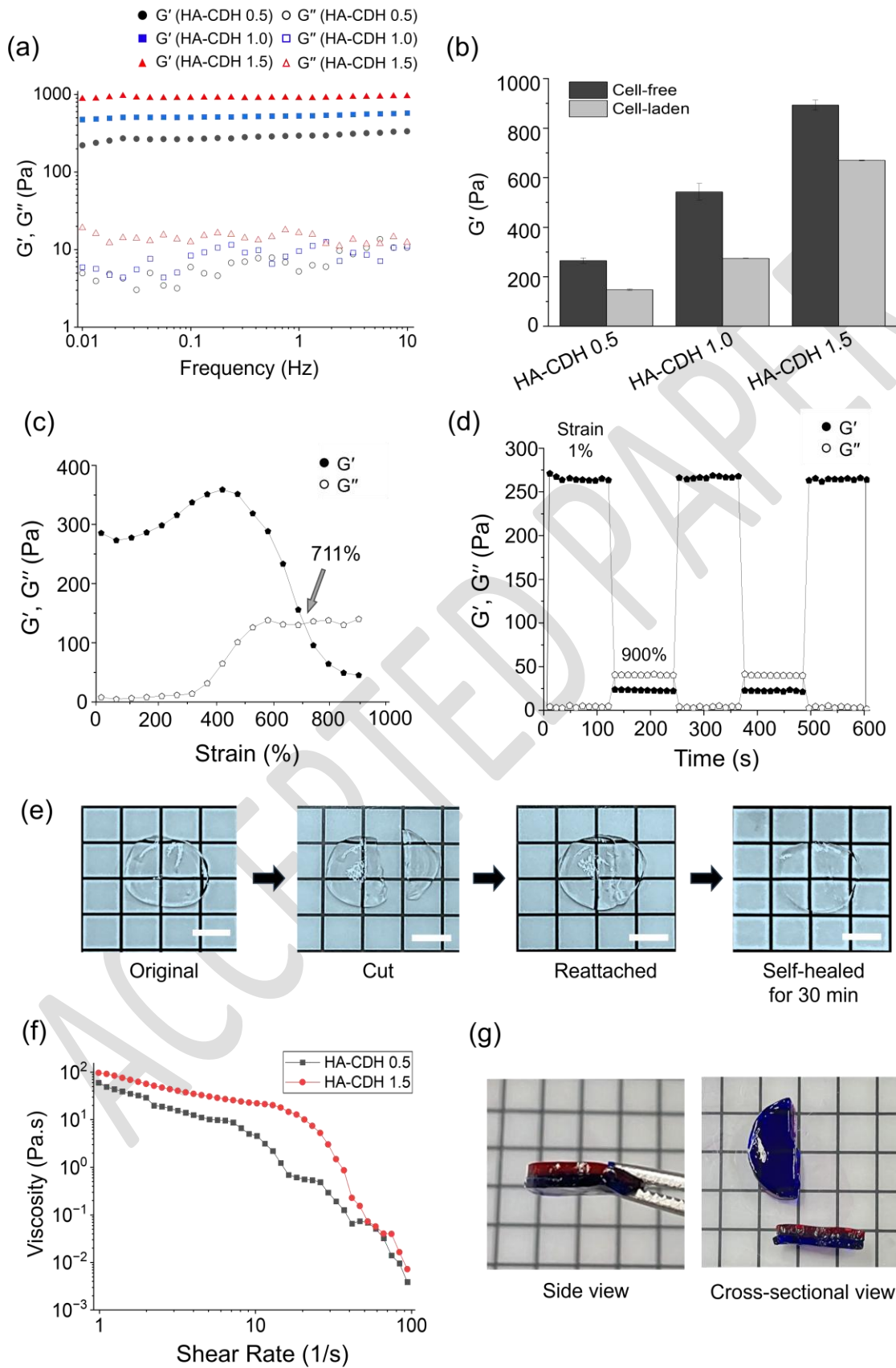


Figure 2. Viscoelastic and self-healing properties of hydrogels. (a) Storage shear modulus and loss shear-modulus values of HA-CDH 0.5, HA-CDH 1.0, and HA-CDH 1.5 hydrogels at various frequencies. (b) Storage shear modulus values of HA-CDH 0.5, HA-CDH 1.0, and HA-CDH 1.5 hydrogels at 0.1 Hz. The hydrogels were prepared in the absence (black bars) and presence (gray bars) of HaCaT cells (cell density of 2.5×10^6 cells/mL, $n = 4$). (c) Amplitude sweep test and (d) repeated self-healing behavior of HA-CDH 0.5 hydrogels assessed using a rotational viscometer. (e) Self-healing behavior of HA-CDH 0.5 hydrogels after cutting and reattachment for 30 min (scale bar, 5 mm). (f) Changes in viscosity of HA-CDH 0.5 and HA-CDH 1.5 hydrogels as a function of shear rate. (g) Representative side and cross-sectional views of the self-healed bilayer hydrogel construct composed of HA-CDH 0.5 and HA-CDH 1.5 hydrogel disks, distinctly dyed to demonstrate interfacial integration.

3.3. Self-healing properties

Self-healing behavior was evaluated using a rotational viscometer.^[31, 39] The flow point, identified from the amplitude sweep test, represents the transition where the G' and G'' values cross related to the structural failure of the hydrogel. The flow rates of HA-CDH 0.5, HA-CDH 1.0, and HA-CDH 1.5 hydrogels were recorded at 711%, 811%, and 843%, respectively (Figures 2c and S3). During repeated cycles of strain variation from 1% to 900%, the G'' values surpassed the G' values at 900% strain, indicating gel failure. However, when the strain was reduced to 1%, the G' values were rapidly restored in the ADH-containing hydrogels, demonstrating their self-healing capability (Figures 2d and S3). This effect is attributed to the introduction of additional mobile hydrazide groups, which facilitate dynamic bond exchange within the network. Particularly, ADH participates in competitive acylhydrazone bond formation with aldehyde groups in odHA, enhancing bond exchange dynamics. Although hydrazone bonds between HA-CDH and odHA are reversible, their bond exchange is limited within the polymer network, resulting in limited self-healing. The incorporation of ADH promotes network rearrangement and enables effective structural recovery after deformation. By contrast, consistent with previous reports,^[40] the hydrogel without ADH exhibited only partial recovery, reaching approximately 70% of the initial G' value after deformation, indicating limited self-healing capability (Figure S4). When the hydrogel disks were divided into two pieces and reattached, the cracks disappeared and healed completely within 30 min at room temperature (Figures 2e and S5). The self-healing ability was maintained even after complete breakage, demonstrating that the hydrogel could recover despite the damage.

Furthermore, to evaluate the suitability of the hydrogel for extrusion-based printing, its flow behavior was analyzed. The viscosity decreased with increasing shear rate, indicating typical shear-thinning behavior (Figure 2f). Notably, HA-CDH 1.5 consistently exhibited higher viscosity than HA-CDH 0.5 across the entire shear rate range, reflecting stronger network interactions at higher polymer content. This shear-thinning behavior is advantageous for extrusion-based 3D printing, as it allows smooth extrusion under shear while maintaining structural integrity after deposition.

The shear-thinning and self-healing behaviors of the hydrogel are fundamentally governed by the same dynamic crosslinking chemistry. High shear rates during nozzle passage disrupt the reversible bonds, inducing shear-thinning behavior that facilitates smooth extrusion. Upon deposition, the immediate removal of shear stress enables the rapid re-association of these dynamic bonds, driving the self-healing process that restores network elasticity (G') and preserves construct fidelity.

To evaluate macroscale integration, a bilayer construct was assembled from HA-CDH 0.5 and HA-CDH 1.5 hydrogel layers. Side and cross-sectional imaging demonstrated a continuous bilayer structure without delamination (Figure 2g), further validating the self-healing capability of the dynamic network.

3.4. 3D printing

The optimal printing speed of the hydrogel was determined by measuring the filament diameter and shape fidelity. The HA-CDH 0.5 hydrogel filament exhibited discontinuous extrusion at a printing speed of 15 mm/s, whereas it maintained a continuous linear shape at printing speeds of 5 and 10 mm/s (Figure 3a). Similarly, the HA-CDH 1.5 hydrogel filament showed linear extrusion at 5 and 10 mm/s; however, at 5 mm/s, the filament became thicker, reducing the precision of printing. The filament was extruded discontinuously at a speed of 15 mm/s (Figure S6). Based on these observations, 3D printing was conducted at a printing speed of 10 mm/s for both the HA-CDH 0.5 and HA-CDH 1.5 hydrogels.

The diameters and heights of the 3D models printed using hydrogels were measured, and the shape fidelity was evaluated by assessing their similarity to the designed model. A cylindrical 3D model

with a diameter of 10 mm and height of 5 mm was printed, and the shape fidelity of the hydrogels was determined. For the HA-CDH 0.5 hydrogel, the model printed at a speed of 5 mm/s exhibited a height comparable to that of the designed model, but had an enlarged diameter, resulting in a thicker shape than originally intended. At 15 mm/s, the printed model exhibited a reduced height and an elongated diameter, indicating poor fidelity to the original design. However, the model printed at 10 mm/s displayed excellent similarity to the designed dimensions, confirming that this speed was the most suitable (Figure 3b). Regarding the HA-CDH 1.5 hydrogel, printing at a speed of 5 mm/s resulted in noticeable deviations in both the diameter and height of the printed structures compared with the intended design. Increasing the speed to 15 mm/s resulted in the printed models being taller than intended, and the diameters were reduced, thus reducing the overall fidelity. By contrast, printing at 10 mm/s produced models that closely matched the original dimensions (Figure S6), and this speed proved to be the most suitable for both HA-CDH 0.5 and HA-CDH 1.5 hydrogels. Both hydrogels demonstrated successful 3D printing by fabricating various 3D models (Figure 3c). Additionally, an adjustable infill density of the 3D-printed structures for both the HA-CDH 0.5 and HA-CDH 1.5 hydrogels was achieved at the optimized printing speed (Figure 3d).

To evaluate the printability of the bioinks, grid constructs were fabricated using both cell-free and cell-laden HA-CDH 0.5 and HA-CDH 1.5 hydrogels (Figure 3e). Printing fidelity was quantified using the *Pr* value derived from grid pore geometry, where a *Pr* value near 1.0 reflects an ideal square pore structure. The *Pr* values of the cell-laden formulations were comparable to their cell-free counterparts (Figure 3f), confirming that cell encapsulation did not adversely affect printing performance. These findings demonstrate that HDFn-laden HA-CDH 0.5 and HEKn-laden HA-CDH 1.5 hydrogels retain excellent printability and construct fidelity.

By utilizing 3D printing technology, hydrogels tailored to the shape of the wound area can be printed to form patient-specific wound-healing hydrogels.^[14, 17] These patient-customized hydrogel patches not only conform to the unique shape of the wound but also provide targeted support to enhance the healing process.^[41]

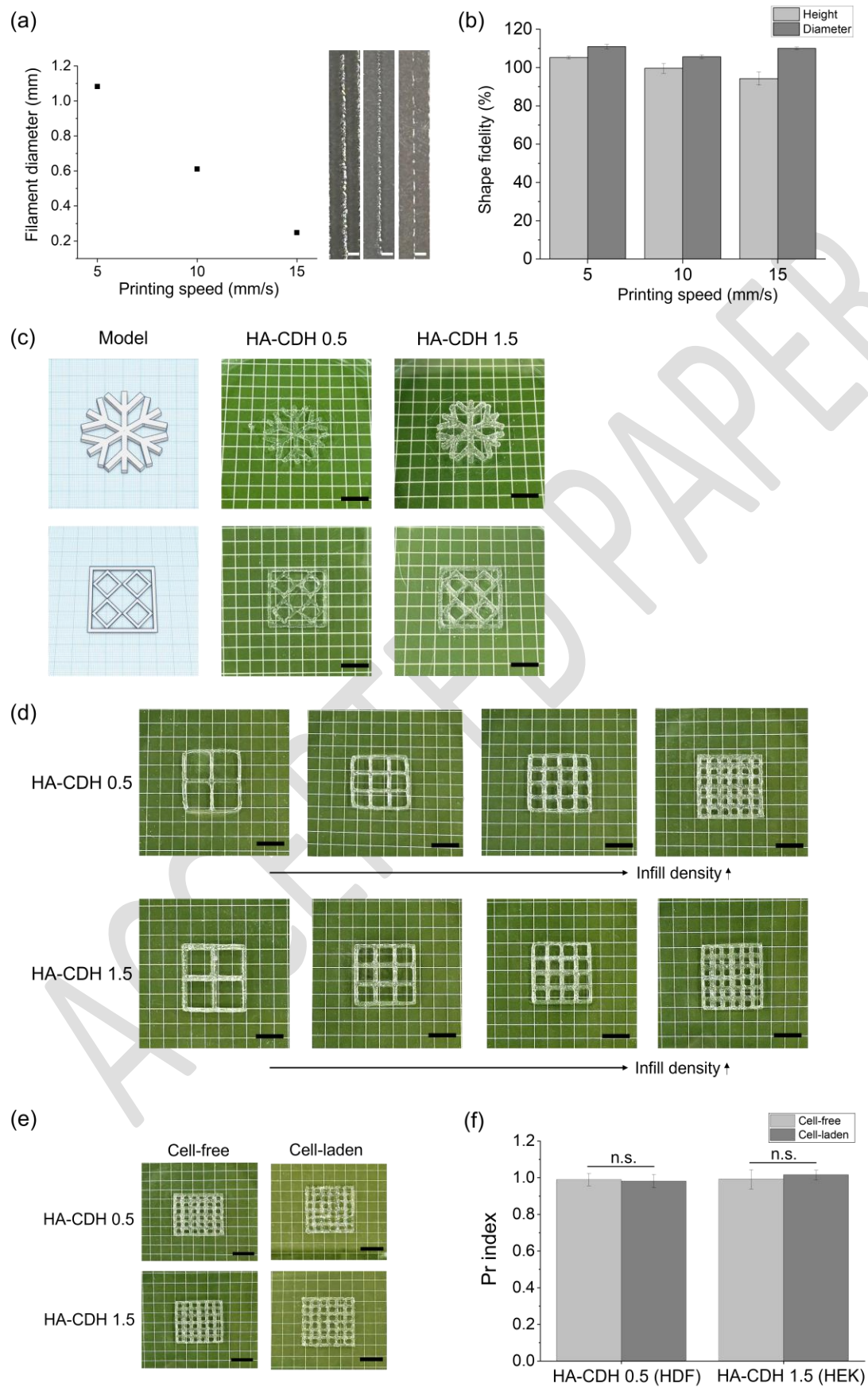


Figure 3. 3D printability and shape fidelity of hydrogels. (a) Changes in filament diameters of HA-CDH 0.5 hydrogels depending on the printing speed and images of the filaments (scale bar, 2 mm). (b) Shape fidelity of HA-CDH 0.5 hydrogels printed at various printing speeds ($n = 4$). (c) Images of 3D-printed structures using HA-CDH 0.5 and HA-CDH 1.5 hydrogels (scale bar, 10 mm). (d) Changes in infill density of 3D-printed constructs (scale bar, 10 mm). (e) Representative images of 3D-printed cell-free and cell-laden grid constructs (scale bar, 10 mm). HEK_n cells and HDF_n cells were incorporated into HA-CDH 1.5 and HA-CDH 0.5 hydrogels, respectively. (f) Printability index (Pr) values of the cell-free and cell-laden constructs ($n = 4$; Student's t -test; n.s., not significant).

3.5. Cell viability and proliferation assays

The cytotoxicity of HA-CDH and odHA, the key components used in hydrogel preparation, was evaluated using HEK_n and HDF_n cells at various polymer concentrations. No significant toxicity was observed in any of the tested solutions (Figure S7).

The proliferation of HEK_n and HDF_n cells encapsulated within the hydrogels was assessed using an MTT assay. Each cell type was encapsulated within the hydrogels and cultured for 7 d. The MTT assay was performed at predetermined time points, and the absorbance at 540 nm was measured. These results indicate distinct patterns of proliferation in HEK_n and HDF_n cells. For HEK_n cell proliferation, an increase in HA-CDH concentration affected an increase in proliferation. While HA-CDH 0.5 exhibited minimal changes in absorbance on day 7 compared with that on day 1, HA-CDH 1.5 exhibited an approximate two-fold increase, indicating enhanced proliferation (Figure 4a). Conversely, HDF_n proliferation decreased at higher HA-CDH concentrations (Figure 4b). These findings suggest that HA-CDH 0.5 is optimal for enhancing HEK_n proliferation, whereas HA-CDH 1.5 is more conducive for HDF_n cells, enabling the formulation of hydrogels that are favorable to each cell type. To evaluate the effect of extrusion-based bioprinting on cell survival, the viability of encapsulated cells within printed hydrogels was compared against non-printed controls. Both HEK_n cells encapsulated in HA-CDH 1.5 and HDF_n cells encapsulated in HA-CDH 0.5 maintained their viability after printing (Figures 4c and 4d), confirming that the shear stress associated with the printing process did not significantly compromise cellular viability. To confirm cell viability within the hydrogels, a LIVE/DEAD assay was further performed after 7 d of culture. Confocal microscopy images showed that most HEK_n and HDF_n cells remained viable, with no apparent signs of cytotoxicity within the hydrogels (Figures 4e and 4f). These results are consistent with the proliferation trends observed in the MTT

assay, indicating that the hydrogel provided a cytocompatible microenvironment that supported cell survival.

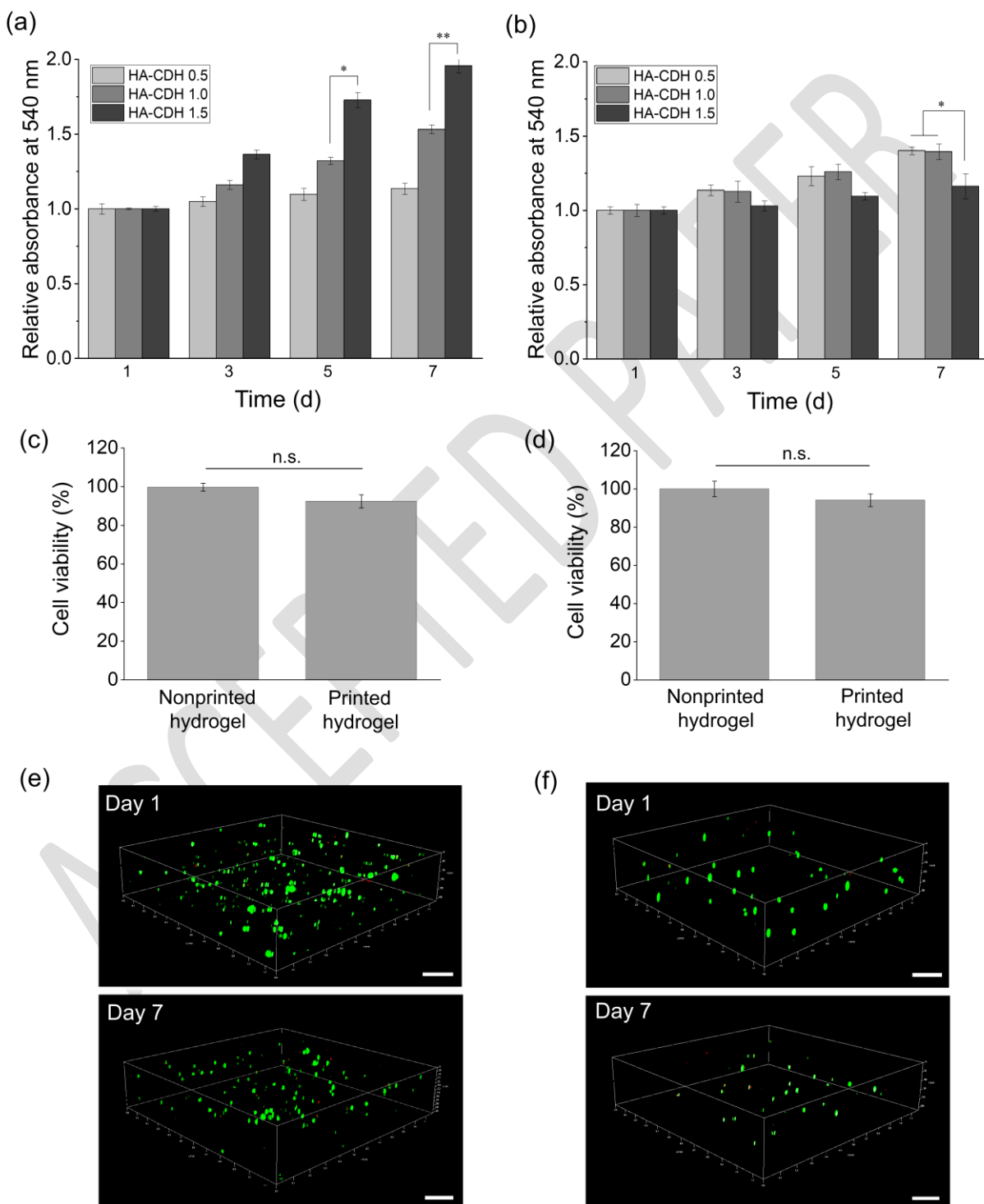


Figure 4. Cell proliferation and viability within hydrogels. Proliferation assays of (a) HEKn and (b) HDFn cells encapsulated in various HA-CDH hydrogels and cultured at 37 °C in a 5% CO₂ atmosphere. Relative absorbance on day 1 was used as the baseline for comparing the cell proliferation ([cell] = 1.0×10^5 cells/mL; n = 3; one-way ANOVA with Tukey's post-hoc test; * $P < 0.05$, ** $P < 0.01$). Relative viability of (c) HEKn cells encapsulated in HA-CDH 1.5 hydrogels and (d) HDFn cells encapsulated in HA-CDH 0.5 hydrogels in non-printed and 3D-bioprinted groups. Data were normalized to the corresponding non-printed cell-laden hydrogel group ([cell] = 1.0×10^5 cells/mL; n = 3; Student's *t*-test; n.s., not significant). Confocal microscopic images of (e) HEKn and (f) HDFn cells cultured within HA-CDH 1.5 and HA-CDH 0.5 hydrogels, respectively (scale bar, 100 μ m).

3.6. In vitro analysis of quantified gene expression

Col1 plays an important role in wound healing by promoting keratinocyte migration and supporting epidermal regeneration.^[42, 43] Incorporating keratinocytes into a collagen matrix can reduce inflammation and accelerate wound healing.^[44, 45] Thus, Col1 expression is a valuable indicator of the healing ability of hydrogel-wound patches.^[45] Additionally, α -SMA is a marker of activated fibroblasts and contributes to wound contraction during the fibrosis phase.^[46] Thus, α -SMA expression is often used to evaluate the activity of fibrogenic cells involved in tissue remodeling and wound closure.^[34] In this study, the gene expression levels of Col1 and α -SMA of HEKn and HDFn cells encapsulated within hydrogels were analyzed.

After encapsulating HEKn or HDFn cells in a hydrogel and cultivating them for 7 d, we analyzed the relative gene expression levels using RT-PCR (Figures 6a and 6b) and qPCR (Figures 6c–f). In the hydrogel with encapsulated HEKn cells, increasing HA-CDH concentrations resulted in a gradual increase in the expression levels of Col1 and α -SMA (Figures 6c and 6d). Conversely, in the hydrogel with encapsulated HDFn cells, higher expression levels of Col1 and α -SMA were observed at the lower HA-CDH concentration of 0.5, as determined by real-time PCR (Figures 6e and 6f).

These results indicate that encapsulating HEKn cells in high-modulus hydrogels (e.g., HA-CDH 1.5) and HDFn cells in low-modulus hydrogels (e.g., HA-CDH 0.5) can provide a microenvironment that is conducive to wound healing. Bilayer systems with different physical properties are useful for closely replicating the structural characteristics of the skin, and can enhance tissue regeneration compared with conventional approaches.

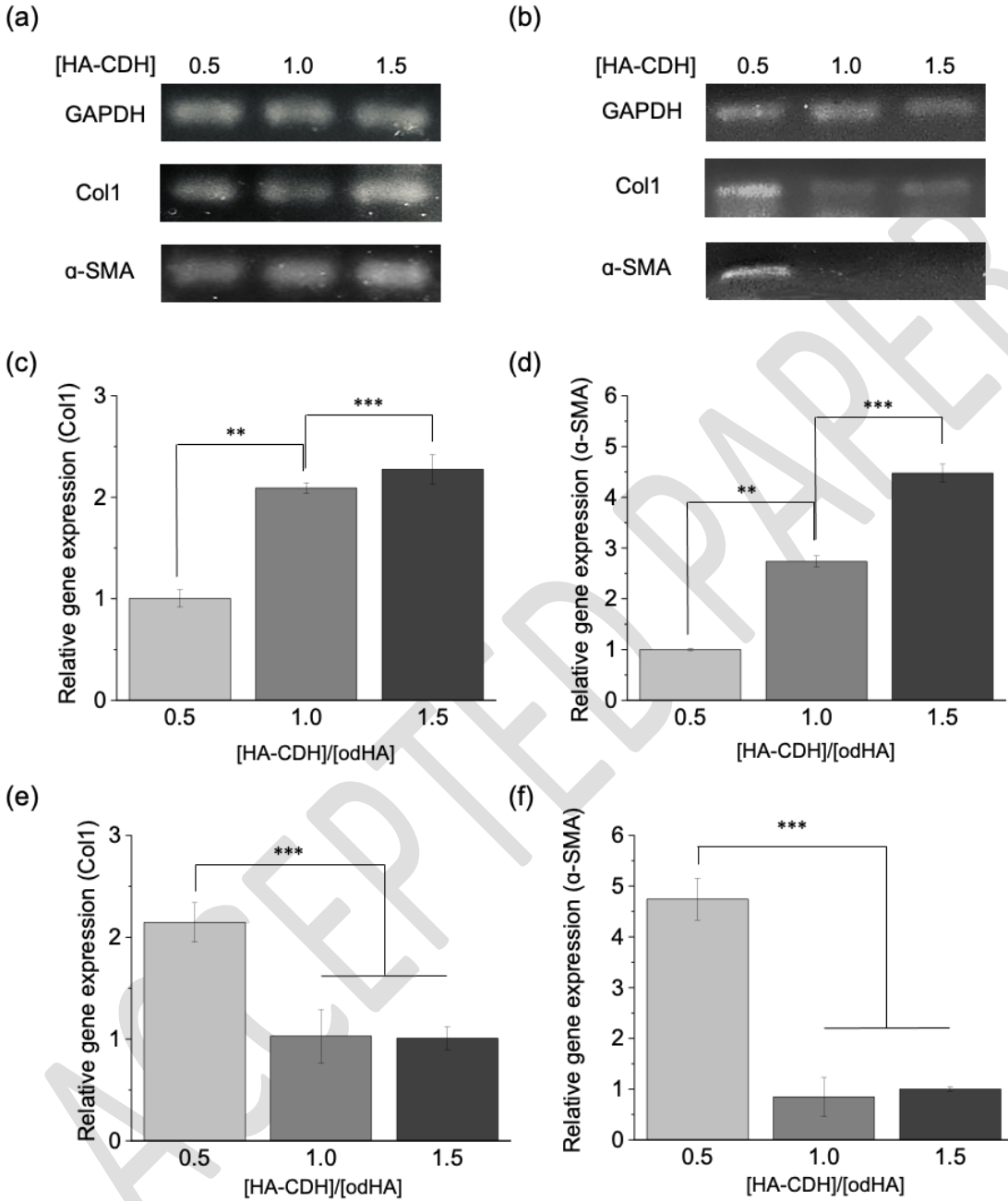


Figure 5. Gene expression of wound healing markers. Expressions of Col1 and α -SMA in (a) HEK293T and (b) HDFn cells encapsulated within hydrogels and cultured for 7 d in vitro. Quantitative expression levels of (c) Col1 and (d) α -SMA in HEK293T cells. Quantitative expression levels of (e) Col1 and (f) α -SMA in HDFn cells ($n = 4$; one-way ANOVA with Tukey's post-hoc test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.7. Transplantation of primary cells encapsulated in hydrogels in vivo

The wound-healing efficacy of a bilayer hydrogel encapsulating human primary cells was evaluated in a mouse model. A full-thickness wound was created on the backs of the mice, and the healing process was monitored after hydrogel implantation (Figures 6a and S8). A reduction in wound area over time was observed more clearly in the hydrogel-treated groups than in the control group (Tegaderm only, no hydrogel). Bilayer hydrogels with different mechanical properties and cell densities were prepared and used for transplantation. The upper layer was constructed using HA-CDH 1.5 hydrogel encapsulating HEK cells (2.5×10^6 cells/mL) to emulate the epidermis. The lower layer was prepared with HA-CDH 0.5 hydrogel encapsulating HDF cells (2.5×10^5 cells/mL) to emulate the dermis layer. On day 7, an inflammatory reaction characterized by yellowish discoloration of the surface was observed in all groups except the bilayer group (seeded with cells). By contrast, the bilayer group (seeded with cells) exhibited no signs of inflammation. By day 14, the wounds had completely healed, indicating superior recovery compared with the healing states of other groups. The monolayer groups (HA-CDH 0.5 and HA-CDH 1.5) also exhibited considerable wound healing, although scabs were still present at the wound sites. However, the bilayer group (with cells) only exhibited residual wound traces, suggesting a more advanced healing process. Subsequently, the wound areas of the studied mice were quantified. Treatment of wound sites with bilayer hydrogel patches effectively reduced the wound area compared with the monolayer and bilayer hydrogel (without cells) groups, and almost complete recovery was observed (Figure 6b).

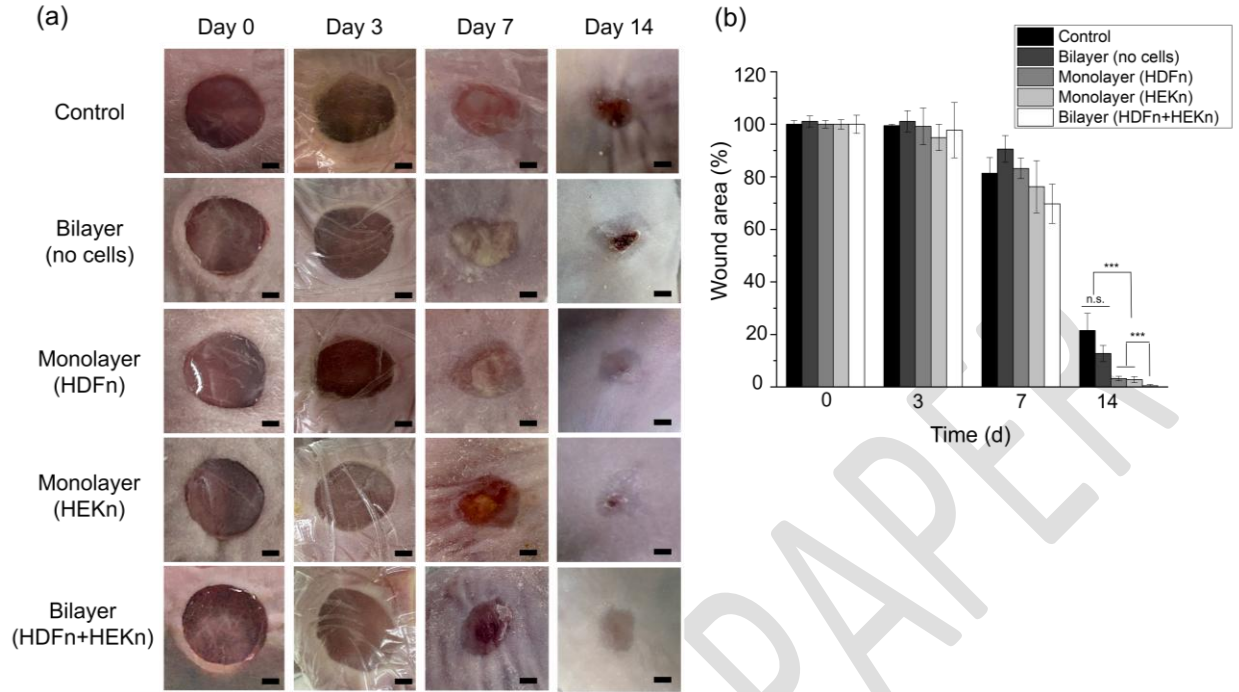


Figure 6. In vivo evaluation of wound healing efficacy using various hydrogel patches encapsulating primary human cells. (a) Images of wound sites (scale bar, 2 mm) and (b) quantified wound area over time (n = 4; one-way ANOVA with Tukey's post-hoc test; n.s., not significant; *** $P < 0.001$).

H&E staining revealed distinct group differences (Figure 7). In the control group (untreated wound), numerous red blood and immune cells were observed within the dermal layer below the red line, indicating incomplete wound healing (Figure 7a). Similarly, the bilayer group (no cells) exhibited many red blood and immune cells within the dermal layer owing to incomplete wound closure. In these images, the yellow line denotes the epidermal–dermal junction, whereas the red line indicates the boundary of the regenerating dermal region. By contrast, the monolayer group containing either HDFn or HEKn exhibited relatively fewer red blood cells, but immune cells were still present, and clear differences in epidermal and dermal thickness were observed compared with normal mouse skin. In the HDFn group, cells within the dermal layer were markedly denser and thicker than those in the other groups. Similarly, HEKn cells exhibited a pronounced thickening of the epidermal layer. Notably, both monolayer groups (HDFn and HEKn) exhibited regenerated skin layers that were thicker than those in the normal mouse tissue. Conversely, in the bilayer hydrogel group that contained both HDFn and HEKn cells, neither red blood cells nor immune

cells were detected. The thickness of each layer closely resembled that of the normal tissue (intact skin tissue) without excessive thickening. Compared with the other groups, the absence of red blood cells and immune cells, along with a layer thickness similar to that of normal mouse tissue, suggests excellent skin tissue recovery.^[47, 48]

Dermal gap analysis demonstrated a substantial reduction in gap size in the cell-containing groups. Among them, the bilayer group encapsulating HDFn and HEKn exhibited the greatest improvement in dermal closure compared to other groups, showing enhanced tissue continuity (Figures 7d and S9). Neovascularization, an important indicator of internal tissue regeneration,^[49] was further evaluated using H&E-stained tissue sections. Quantitative analysis revealed enhanced vascular formation in the cell-laden groups compared with that in the control and acellular bilayer groups (Figure 7e). The HDFn/HEKn-laden bilayer group exhibited the most prominent vascularization, suggesting effective angiogenic support within the regenerated tissue. Consistently, CD31-staining showed enhanced vascularization in the cell-laden groups, and quantitative analysis demonstrated a significantly higher CD31-positive area in the bilayer group (Figures 7c and 7g).

Masson's trichrome staining was also performed to evaluate collagen deposition and extracellular matrix remodeling within the regenerated dermal tissue.^[50, 51] Quantitative analysis of the collagen area fraction revealed an increased and more uniformly distributed collagen in the cell-laden groups (Figures 7b and 7f). Notably, the HDFn/HEKn-laden bilayer group exhibited well-organized collagen fibers without excessive accumulation, indicating balanced collagen remodeling rather than fibrotic thickening, closely resembling normal dermal architecture.

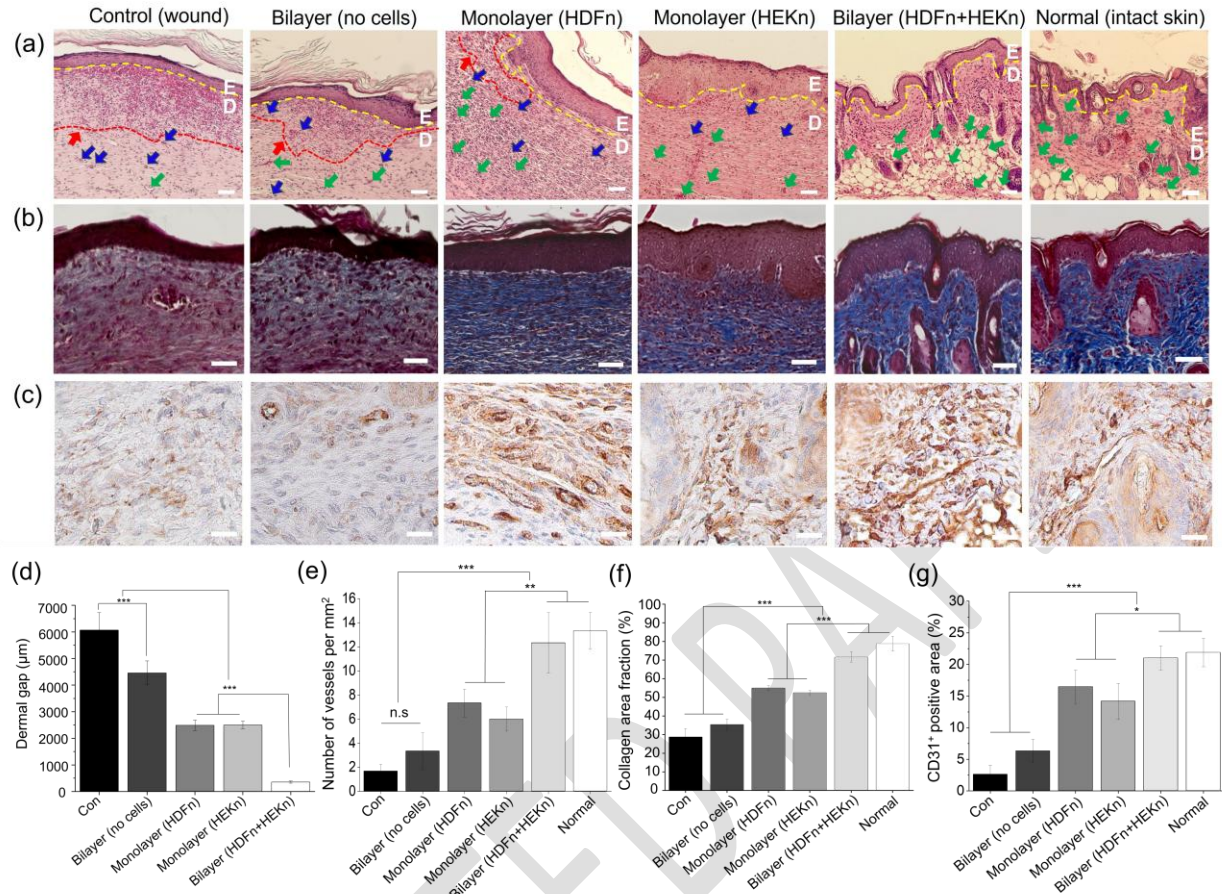


Figure 7. Histological and immunohistochemical analyses of skin tissue. Representative images of skin tissue sections stained with (a) H&E (blue arrow, immune cell; red arrow, red blood cell; green arrow, blood vessel; E, epidermis layer; D, dermis layer; yellow line, boundary of epithelium; red line, incomplete healing area; scale bar, 20 μm), (b) Masson's trichrome staining (scale bar, 50 μm), and (c) CD31 immunohistochemical staining (scale bar, 50 μm). Quantitative analysis of (d) dermal gap and (e) number of blood vessels in the H&E-stained tissue sections. (f) Collagen area fraction quantified from the dermal tissue sections after staining with Masson's trichrome. (g) CD31-positive area quantified from CD31-stained tissue sections (n = 4; one-way ANOVA with Tukey's post-hoc test; n.s., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

This research demonstrates that a biomimetic bilayer hydrogel provides more effective wound healing than conventional Tegaderm or monolayer hydrogels comprising encapsulated single-cell types. Three distinct advantages validated in this study include: (i) Tailored mechanical heterogeneity allowed precise control over the elastic modulus simply by altering the HA-CDH concentration, successfully mimicking the rigid epidermis and soft dermis within a single,

integrated construct; (ii) Cellular heterogeneity provided idealized, localized mechanical stiffnesses that concurrently optimize human keratinocyte and dermal fibroblast proliferation and gene expression in vitro; (iii) Dynamic structural integrity by self-healing nature allowed seamless interfacial crosslinking between the two layers, preventing delamination while facilitating smooth extrusion-based 3D bioprinting into custom, defect-conforming wound patches.

The lower number of red blood cells in the cell-encapsulated hydrogel group compared with the cell-free group suggests that the initial inflammatory response subsided.^[52] This reduction in blood cells, along with the decreased wound area in the cell-encapsulated hydrogel groups, indicates favorable wound healing. Finally, when HDFn and HEKn cells were encapsulated in the hydrogel and transplanted into a bilayer hydrogel (emulating a modulus difference), the recovery closely resembled that of native tissue.

Commercially available wound healing patches require removal, which can be inconvenient and uncomfortable.^[53, 54] By contrast, bilayer hydrogels composed solely of HA are biodegradable within the body; thus, hydrogel removal is not necessary. However, this study evaluated therapeutic effects only in acute wounds. Therefore, additional research is required to evaluate long-term treatment outcomes in chronic wound cases caused by diabetic conditions or burns. Additionally, this study aimed to simulate only the stiffness differences between tissues rather than the entire complex tissue environment. Future research should focus on replicating the intricate structure of skin tissue more accurately, advancing toward full-thickness models instead of limiting them to bilayer models to improve their resemblance to natural tissues. Additionally, comprehensive long-term biocompatibility and translational safety profiles are essential prior to clinical translation. Moreover, since HA hydrogels generally lack strong intrinsic antimicrobial activity, integrating functional additives such as chitosan, antimicrobial peptides, silver nanoparticles, polyphenols, or photothermal/photodynamic agents represents a crucial and logical next step to translate this platform into chronic, infected wound models.

4. Conclusion

This study bridges a critical gap in biomimetic tissue engineering by establishing that mechanical and cellular stratification must be paired with dynamic structural recovery to achieve effective

tissue repair. Moving beyond traditional static scaffolds, the unique scientific contribution of this work lies in the development of a structurally dynamic polysaccharide platform that simultaneously satisfies cell-specific mechanobiological thresholds and exhibits autonomous interfacial self-healing. By demonstrating that the dynamic rearrangement of acylhydrazone bonds safeguards layer-specific mechanics effectively under physical deformation, this research provides a clear framework to guide diverse cellular behaviors concurrently within a single, integrated matrix.

Looking forward, this responsive bioink system opens clear avenues for advanced therapeutic and translational applications. While we have validated its efficacy in acute full-thickness defects in the present work, future studies will target chronic and compromised wound environments such as diabetic ulcers or severe burn models where dynamic mechanical protection and sustained cellular support are clinically paramount. Additionally, because this platform replicates the rigid-to-compliant transition of the native epidermis and dermis precisely, it may hold significant promise as a high-fidelity, in vitro human skin modeling tool. This approach may then be expected to support high-throughput screening of dermatological therapeutics and cosmetic formulations as well as to provide a robust alternative to animal testing and advance the field of personalized regenerative medicine.

Acknowledgments

None.

Funding

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIT) [RS-2024-00355200].

Conflict of interest

The authors declare they have no competing interests.

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Writing—review & editing: Kuen Yong Lee

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

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