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Biomimetic heterogeneous 3D bioprinting of a functionally zonal meniscus

Running title: Heterogeneous 3D-bioprinted meniscus

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Abstract

Meniscus injuries are difficult to heal because of the limited vascularization and intrinsic structural heterogeneity of native meniscal tissue. Although 3D bioprinting has emerged as a promising strategy for meniscus tissue engineering, most existing scaffolds remain homogeneous and fail to recapitulate the zonal mechanical and biological characteristics of the native meniscus. In this study, a dual-nozzle 3D bioprinting strategy was developed to fabricate a biomimetic heterogeneous meniscus scaffold with region-specific mechanical and biological properties. Two hydrogel-based bioinks with distinct physicochemical and rheological characteristics were designed to mimic the inner and outer regions of the native meniscus. By integrating region-specific bioinks and cell-laden printing through a dual-nozzle extrusion system, heterogeneous meniscus scaffolds with stable structural fidelity and controllable spatial distribution were successfully fabricated. Rheological analysis demonstrated favorable shear-thinning behavior and printability of both bioinks, enabling continuous extrusion and shape maintenance during the printing process. Mechanical characterization and atomic force microscopy further confirmed the successful establishment of zonal mechanical heterogeneity within the printed constructs. In vitro biological evaluations showed that the bioprinted scaffolds supported high cell viability, sustained proliferation, and favorable cytoskeletal spreading during long-term culture. More importantly, region-specific extracellular matrix expression was observed, with significantly enhanced type I collagen expression in the outer region and elevated type II collagen expression in the inner region, consistent with the native meniscal phenotype. Overall, this study demonstrates that dual-nozzle 3D bioprinting can effectively reconstruct the zonal mechanical and biological heterogeneity of native meniscus tissue. The proposed heterogeneous hydrogel scaffold provides a promising in vitro platform for investigating biomimetic meniscus tissue engineering and may serve as a foundation for future in vivo studies.

Keywords: 3D bioprinting; Meniscus; Heterogeneity

1. Introduction

The meniscus is a fibrocartilaginous tissue that plays a critical role in load transmission, shock absorption, joint lubrication, and knee stability¹⁻³. Due to its limited intrinsic vascularization and low self-healing capacity, meniscus injuries are difficult to repair, particularly in the inner avascular region^{4,5}. Partial meniscectomy and suture repair remain the most commonly used clinical treatments⁶; however, these strategies often fail to restore the native biomechanical function and hierarchical structure of the meniscus, thereby accelerating cartilage degeneration and increasing the risk of osteoarthritis progression^{7,8}. Consequently, the development of functional biomimetic substitutes for meniscus regeneration has become an important research focus in tissue engineering and regenerative medicine⁹⁻¹¹.

Native meniscus tissue exhibits pronounced regional heterogeneity in terms of cellular composition, extracellular matrix distribution, and biomechanical properties^{9,12}. The outer red zone is highly vascularized and mainly composed of fibroblast-like cells and type I collagen fibers, providing superior tensile strength and structural stability⁹. In contrast, the inner white zone is avascular and enriched with fibrochondrocytes and type II collagen, contributing to compressive resistance and load buffering^{13,14}. In addition, the meniscus possesses spatially distributed mechanical gradients and anisotropic collagen architectures that are essential for maintaining physiological joint function^{13,15,16}. Therefore, successful meniscus regeneration requires not only geometric reconstruction, but also the simultaneous restoration of zonal mechanical and biological heterogeneity¹⁷.

Recent advances in tissue engineering have promoted the development of hydrogel-based meniscus scaffolds owing to their high water content, tunable physicochemical properties, and favorable cytocompatibility¹⁸. In particular, photocrosslinkable hydrogels such as gelatin methacryloyl (GelMA) and methacrylated hyaluronic acid (HAMA) have demonstrated considerable potential for cell-laden bioprinting applications¹⁹⁻²¹. For example, several studies have utilized GelMA-based bioinks to fabricate cell-laden meniscus constructs with favorable printability and cytocompatibility, enabling the formation of biomimetic three-dimensional microenvironments for fibrochondrocyte growth and extracellular matrix deposition^{22,23}. In addition, decellularized extracellular matrix (dECM)-based bioinks have also been explored to

enhance the bioactivity and tissue-specific inductive properties of engineered meniscus scaffolds^{24–26}.

To better mimic the structural complexity of native meniscus tissue, increasing efforts have been devoted to the development of gradient and multi-material bioprinting strategies²⁷. For instance, gradient scaffolds incorporating region-specific growth factor delivery or spatially controlled collagen distributions have been reported to promote zonal tissue regeneration^{28,29}. Moreover, multi-nozzle extrusion systems and microfluidic-assisted bioprinting approaches have enabled the fabrication of heterogeneous constructs with tunable material compositions and layered architectures^{30,31}. Despite these advances, most previously reported meniscus scaffolds remain structurally homogeneous or only partially reproduce the native zonal characteristics. The simultaneous reconstruction of regional mechanical heterogeneity and zonal extracellular matrix phenotypes remains challenging²⁸. As a result, engineered constructs often exhibit insufficient biomechanical performance and limited capability to maintain region-specific cellular functions^{28,32}.

Three-dimensional bioprinting has emerged as a powerful strategy for the fabrication of complex tissue constructs owing to its capability for precise spatial control over biomaterials, living cells, and bioactive factors³³. Unlike conventional scaffold fabrication techniques, 3D bioprinting enables the layer-by-layer deposition of cell-laden bioinks into predefined architectures, thereby allowing the reconstruction of tissue-specific geometries and heterogeneous microenvironments^{34,35}. This spatially controlled manufacturing approach is particularly advantageous for engineering complex tissues with hierarchical organization, such as cartilage, osteochondral tissue, vascularized tissue, and meniscus^{34,36}.

Recent advances in multi-material and multi-nozzle bioprinting technologies have further expanded the capability of 3D bioprinting in mimicking native tissue heterogeneity³⁷. By integrating different biomaterials, cell types, and bioactive components within a single construct, bioprinted scaffolds can achieve region-specific mechanical properties and biological functions^{38,39}. For example, gradient bioprinting strategies have been employed to fabricate osteochondral constructs with distinct cartilage and bone phases, while microfluidic-assisted printing approaches have enabled the generation of continuous compositional gradients and multilayered tissue architectures^{40,41}. In addition, extrusion-based bioprinting has demonstrated

excellent adaptability for processing hydrogel bioinks with high cell density and tunable rheological properties, making it one of the most widely used techniques for soft tissue engineering applications^{20,30}.

Despite these advances, the fabrication of biomimetic meniscus constructs remains highly challenging because of the intrinsic structural and functional heterogeneity of native meniscus tissue^{15,42,43}. Most previously reported bioprinted meniscus scaffolds still exhibit relatively homogeneous architectures and insufficient regional differentiation in both mechanical and biological properties²⁸. Therefore, the development of heterogeneous bioprinting strategies capable of simultaneously reconstructing zonal mechanics and extracellular matrix characteristics remains an important objective in functional meniscus tissue engineering⁴⁴.

In this study, we developed a dual-nozzle extrusion-based 3D bioprinting strategy to fabricate a biomimetic heterogeneous meniscus scaffold with region-specific mechanical and biological properties. Two hydrogel bioinks with distinct physicochemical characteristics were designed to mimic the outer and inner regions of the native meniscus. By integrating spatially controlled dual-bioink printing and region-specific cell encapsulation, heterogeneous meniscus constructs with stable structural fidelity were successfully fabricated. Specifically, GelMA/HAMA/SilMA composite hydrogels with distinct mechanical properties were combined with synovial fibroblasts (SFs) and meniscal fibrochondrocytes (MFCs) to mimic the outer red zone and inner white zone, respectively. The resulting heterogeneous constructs were systematically evaluated *in vitro* in terms of physicochemical properties, printing fidelity, micromechanical heterogeneity, cellular behavior, and region-specific extracellular matrix deposition. This study serves as an *in vitro* proof-of-concept demonstration of a biomimetic heterogeneous bioprinting strategy and provides a platform for future investigations toward functional meniscus tissue engineering.

2. Materials and methods

2.1. Materials

These materials were utilized in this work:

Gelatin methacrylate (GelMA), purchased from SunP, Shanghai, China.

Hyaluronic acid methacrylate (HAMA), purchased from EFL, Suzhou, China.

Silk methacrylate (SilMA), purchased from EFL, Suzhou, China.

Cells: Obtained from Wanwu Biotech, Hefei, China.

Culture media: Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher Scientific, Waltham, MA, USA.

Phosphate-buffered saline (PBS), Sigma-Aldrich, St. Louis, MO, USA.

Fetal bovine serum (FBS), Sigma-Aldrich, St. Louis, MO, USA.

Trypsin-EDTA, Sigma-Aldrich, St. Louis, MO, USA.

Penicillin-streptomycin solution, Thermo Fisher Scientific, Waltham, MA, USA.

2.2. Preparation of bioinks

GelMA/HAMA/SilMA composite hydrogels were prepared by dissolving GelMA (8 wt%) and HAMA (2 wt%) in phosphate-buffered saline (PBS) at 37 °C under constant stirring until complete dissolution. SilMA was subsequently added at varying concentrations to obtain different composite hydrogel formulations. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, 0.25 wt%) was used as the photoinitiator. The precursor solutions were protected from light before use. Sterilization was achieved by filtration through a 0.22 µm membrane following solution preparation.

2.3. Rheological Tests

A rheological test was performed by a rheometer (Anton Paar, Austria). To evaluate the shear-thinning behavior and temperature-responsive viscosity of the bioinks, 270 µL of each formulation was loaded onto the rheometer plate. Viscosity measurements were conducted at 25 °C over a shear rate range of 0.01–100 s⁻¹. For temperature-dependent rheological analysis, the viscosity was recorded at a fixed shear rate of 10 s⁻¹ while the temperature was gradually reduced from 40 to 15 °C at a controlled cooling rate of 1 °C per 90 s. All rheological measurements were performed using three independently prepared hydrogel samples (n = 3 biological replicates), and representative averaged curves are presented.

Use Roman numerals in parentheses, e.g., (I), (II), (III), (IV), to list equations and formulae.

$$E = mc^2 \quad (I)$$

2.4. Scanning Electron Microscopy (SEM)

The microstructural morphology of the lyophilized GelMA/HAMA/SilMA hydrogels was characterized using a scanning electron microscope (SEM; JSM-7610F, JEOL) operated at an accelerating voltage of 5 kV. Briefly, the prepolymer solutions were cast into cubic molds and photocrosslinked by exposure to 405 nm ultraviolet light for 60 s, yielding hydrogel constructs with dimensions of $10 \times 10 \times 10 \text{ mm}^3$. The samples were subsequently stored at -80°C overnight, followed by freeze-drying for 24 h using a lyophilizer (Scientz-10N, Scientz). The lyophilized hydrogels were sectioned horizontally at the mid-plane with a razor blade and sputter-coated with a thin layer of gold before SEM observation.

2.5. Porosity Analysis by ImageJ

The porosity of the composite hydrogel system was estimated from scanning electron microscopy (SEM) images using ImageJ software (National Institutes of Health, USA). SEM images were first converted to 8-bit grayscale and subsequently binarized using an automatic thresholding algorithm to distinguish pore regions from the solid matrix. The porosity was calculated as the ratio of the pore area to the total image area according to the following equation:

$$\text{Porosity}(\%) = A_{\text{pore}}/A_{\text{total}} \times 100\%$$

For each group, at least three randomly selected SEM images were analyzed, and the average value was reported.

2.6. Elastic Modulus Tests

The compressive mechanical properties of the hydrogel samples were evaluated by uniaxial compression using a universal testing system (ZLC-2D, Jinan XLC Testing Machine Co., Ltd., China) equipped with a 100 N load cell at room temperature. Cylindrical specimens with flat and parallel end surfaces were subjected to compression at a constant crosshead speed of 1 mm min^{-1} . The hydrogels were prepared with a diameter of 15 mm and a height of 10 mm before testing.

2.7. Swelling Behavior of Hydrogels

Cylindrical hydrogel samples from each formulation group (S1–S4) were fabricated via photocrosslinking (8 mm in diameter and 2 mm in height) and accurately weighed to obtain their initial wet weight (W_0). The samples were then completely immersed in phosphate-buffered saline (PBS) in a 24-well plate and incubated at 37 °C to simulate physiological conditions. At predetermined time points (1, 2, 4, 10, 24, and 48 h), the samples were removed, and excess surface water was gently blotted with lint-free paper before reweighing to obtain the swollen weight (W_s). The swelling ratio (SR) was calculated according to the following equation:

$$SR = \frac{W_s - W_0}{W_0} \times 100\%$$

2.8. Degradation Behavior of Hydrogels

Five groups of photocrosslinked hydrogel samples were first weighed to obtain their initial weight (W_0) and then immersed in phosphate-buffered saline (PBS). The samples were incubated in a thermostatic orbital shaker at 37 °C with a shaking speed of 100 rpm to simulate physiological conditions. Every 7 days, the hydrogel samples were removed from the degradation medium, and excess surface liquid was gently blotted with filter paper prior to weighing to obtain the remaining weight at each time point (W_t). The degradation process was continuously monitored for 35 days. The degradation degree of the hydrogels was evaluated by calculating the weight loss ratio relative to the initial weight according to the following equation:

$$WLR = \frac{W_0 - W_t}{W_0} \times 100\%$$

2.9. Cell Source

The primary cells used in this study, including rabbit MFCs and synovial margin fibroblasts, were commercially purchased from Hefei Wanwu Biotechnology Co., Ltd (Hefei, China). The supplier certified that these cells were isolated from New Zealand rabbits in accordance with standard ethical guidelines for laboratory animals. Therefore, no additional ethical approval was required for the in vitro use of these commercially obtained cells in this research.

2.10. Cell Viability Assay

Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay. Cells were plated in 96-well culture plates at a density of 3,000 cells per well. At the designated time points, 10 μ L of

CCK-8 reagent (Beyotime, China) was added to each well. After incubation for 1 h at 37 °C, the absorbance was recorded at 450 nm using a multifunctional microplate reader (BioTek, Winooski, VT, USA).

2.11. Live/dead Staining

Cell viability within the composite hydrogels was evaluated using a live/dead viability assay. At the designated time point, samples were incubated with Calcein-AM (2 μ M) and Propidium Iodide (PI, 4 μ M) for 30 min at 37 °C. Fluorescence images were acquired using a confocal laser scanning microscope. Live cells were visualized in green and dead cells in red. The live cell fluorescence area was quantified using ImageJ software and normalized to the total cell area.

2.12. Bioprinting of Heterogeneous Meniscus

A dual-nozzle extrusion-based bioprinting strategy was employed to fabricate heterogeneous meniscus constructs. All printing procedures were carried out using a commercial dual-nozzle bioprinter (SUNP Biotech, China). Two independent printheads were loaded with different bioinks to enable spatially controlled deposition during a single printing process. The printing speed was set to 5 mm s⁻¹, while the extrusion rate for each nozzle was maintained at 5 μ m³ s⁻¹ to ensure continuous and stable filament formation. The printing parameters were optimized to achieve uniform strand morphology and precise pattern fidelity. During the printing process, the coordinated motion of the dual nozzles allowed for the accurate placement of distinct bioinks according to the predesigned heterogeneous meniscus model. Based on preliminary optimization experiments, stable filament formation and structural fidelity were achieved using a printing speed of 5 mm/s, an extrusion rate of 5 μ L/s, and nozzle temperatures of 22 °C (1S bioink) and 25 °C (0S bioink). These parameters were selected as the optimized printing window for heterogeneous meniscus fabrication. All constructs were printed under sterile conditions at room temperature and subsequently subjected to further crosslinking and cell culture as described above.

2.13. Cell encapsulation and hydrogel crosslinking

Primary fibroblasts were suspended in the hydrogel precursor solutions at a density of 1 $\times$ 10⁶ cells mL⁻¹. The cell-laden hydrogels were cast into cylindrical molds and subjected to UV irradiation (405 nm, 10 mW cm⁻²) for 60 s to induce photocrosslinking. The same photocrosslinking conditions were applied to all hydrogel constructs throughout the study to ensure uniform

crosslinking and experimental consistency. After gelation, constructs were transferred to fresh culture medium and incubated at 37 °C with 5% CO₂.

2.14. Immunofluorescence Staining

Immunofluorescence staining was performed to evaluate the expression and spatial distribution of specific extracellular matrix-related proteins. Briefly, samples were fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature, followed by three rinses with phosphate-buffered saline (PBS). Cell membranes were permeabilized using 0.1% (v/v) Triton X-100 for 10 min and subsequently blocked with 5% (w/v) bovine serum albumin (BSA) for 1 h to minimize nonspecific binding. The samples were then incubated with primary antibodies against target proteins at 4 °C overnight. After washing with PBS, fluorophore-conjugated secondary antibodies were applied and incubated for 1 h at room temperature in the dark. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, fluorescence images were acquired using a confocal laser scanning microscope.

2.15. Atomic force microscopy (AFM) characterization of micromechanical properties

The micromechanical properties of the hydrogel scaffolds were characterized using an atomic force microscope (AFM, NanoWizard 4XP, Bruker, Germany). To minimize the influence of surface topography and local structural heterogeneity on the measurement results, a spherical probe modified with a silica microsphere (SAA-SPH-10UM, Bruker) was employed for all indentation tests. Prior to measurement, the probe was equilibrated in the testing medium for 30 min to reduce thermal drift, and the spring constant of the cantilever ($k \approx 0.25$ N/m) was calibrated using the thermal noise method. All force-indentation measurements were performed under liquid conditions to maintain the hydrogels in their fully swollen state. During testing, the trigger force was set to 1 nN to ensure that the indentation depth remained within the linear elastic deformation range of the hydrogel samples. Force-indentation curves were acquired from the transverse cross-sections of the printed meniscus scaffolds, and the apparent Young's modulus values were subsequently calculated by fitting the obtained curves using the Hertz contact model. For heterogeneous meniscus constructs, indentation measurements were performed along the cross-sectional direction spanning from the outer red-zone region to the inner white-zone region. Sampling points were collected at intervals of 200 μ m across the interface region, and three independent measurements were obtained at each location to minimize local variability.

Homogeneous control scaffolds fabricated entirely using either 0S or 1S bioinks were evaluated under identical testing conditions for comparison. Young's modulus measurements were performed on three independently fabricated scaffolds ($n = 3$ biological replicates). Multiple indentation sites were analyzed at each predefined position, and the reported modulus values represent the averaged results from the independent samples. Error bars were not displayed in the modulus distribution profiles to preserve the visualization of the continuous spatial mechanical gradient across the scaffold.

2.16. Statistical analysis

All hydrogel characterization experiments, including rheological, mechanical, swelling, and degradation analyses, were performed using at least three independently prepared biological replicates. Data are presented as the mean $\pm$ standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. The normality of the data was assessed using the Shapiro-Wilk test, and homogeneity of variance was tested using Levene's test. For all tests, statistical significance was defined as $p < 0.05$.

3. Results

3.1. Optimization and characterization of GelMA/HAMA/SilMA composite hydrogels

GelMA is a widely used photocurable derivative of gelatin that retains arginine–glycine–aspartic acid (RGD) motifs and matrix metalloproteinase (MMP)-sensitive sites inherent to its parent collagen. These bioactive sequences endow GelMA with excellent cell-adhesive properties, thereby promoting cell attachment, spreading, proliferation, and matrix remodeling. In the context of meniscal tissue engineering, GelMA provides a biologically favorable microenvironment that supports cell viability and facilitates cell–matrix interactions essential for tissue regeneration.

HAMA is a methacrylated form of hyaluronic acid, a major glycosaminoglycan component of the native meniscal extracellular matrix, particularly associated with the fibrochondrocyte-rich regions.

Incorporation of HAMA into the hydrogel system enables partial biochemical recapitulation of the native ECM, contributing to enhanced cellular signaling, matrix synthesis, and pericellular matrix formation. Moreover, HAMA has been reported to regulate cell migration and differentiation, thereby supporting extracellular matrix reconstruction and functional tissue maturation.

SilMA, derived from methacrylated silk fibroin, is characterized by high mechanical strength, superior toughness, and low swelling behavior compared with conventional gelatin- or polysaccharide-based hydrogels. The inclusion of SilMA significantly enhances the mechanical integrity and structural stability of the composite hydrogel, rendering it particularly suitable for load-bearing applications such as meniscal tissue regeneration. By modulating SilMA content, the mechanical properties of the hydrogel can be finely tuned to achieve region-specific stiffness, thereby mimicking the mechanical gradient from the fibrous outer zone to the cartilage-like inner zone of the native meniscus.

To construct a bioink system suitable for heterogeneous meniscus bioprinting, a composite hydrogel consisting of GelMA, HAMA, and SilMA was developed.

GelMA (8 wt%) and HAMA (2 wt%) were fixed as the primary matrix components to provide cell-adhesive motifs and cartilage-mimetic biochemical cues, respectively. SilMA, known for its excellent mechanical reinforcement capability and tunable crosslinking density, was introduced as a variable component at concentrations of 0, 1, 2, 3, and 4 wt% to systematically investigate its effects on rheological behavior, thermoresponsiveness, mechanical properties, and microstructural characteristics of the composite hydrogel (**Fig.1**).

Rheological analysis demonstrated that all hydrogel formulations exhibited pronounced shear-thinning behavior, characterized by a rapid decrease in viscosity with increasing shear rate (**Figure 1a**). This property is favorable for extrusion-based bioprinting because it facilitates continuous extrusion while reducing shear-induced instability during printing. Temperature sweep measurements further revealed the thermosensitive viscoelastic behavior of the composite hydrogels (**Figure 1b**), indicating their capability to maintain structural stability under printing conditions.

SEM observations showed distinct porous microstructures among the different hydrogel formulations (**Figure 1c**). As the SilMA concentration increased, the pore morphology and internal

network structure gradually changed, suggesting that SilMA incorporation effectively regulated the microarchitecture of the composite hydrogels. Quantitative analysis further confirmed significant differences in hydrogel porosity among the groups (**Figure 1d**).

Mechanical characterization demonstrated that the compressive modulus increased progressively with increasing SilMA concentration (**Figure 1e**), indicating enhanced mechanical strength of the composite hydrogels. Meanwhile, degradation analysis showed that hydrogels with higher SilMA content exhibited slower degradation rates over the experimental period (**Figure 1f**). Swelling analysis further demonstrated that all hydrogel groups rapidly absorbed water during the initial incubation stage and gradually reached equilibrium swelling, although the final swelling ratios differed among formulations (**Figure 1g**).

Collectively, these results demonstrated that the incorporation of SilMA effectively modulated the rheological, microstructural, mechanical, degradation, and swelling properties of GelMA/HAMA composite hydrogels, thereby providing a tunable material platform for subsequent meniscus bioprinting applications.

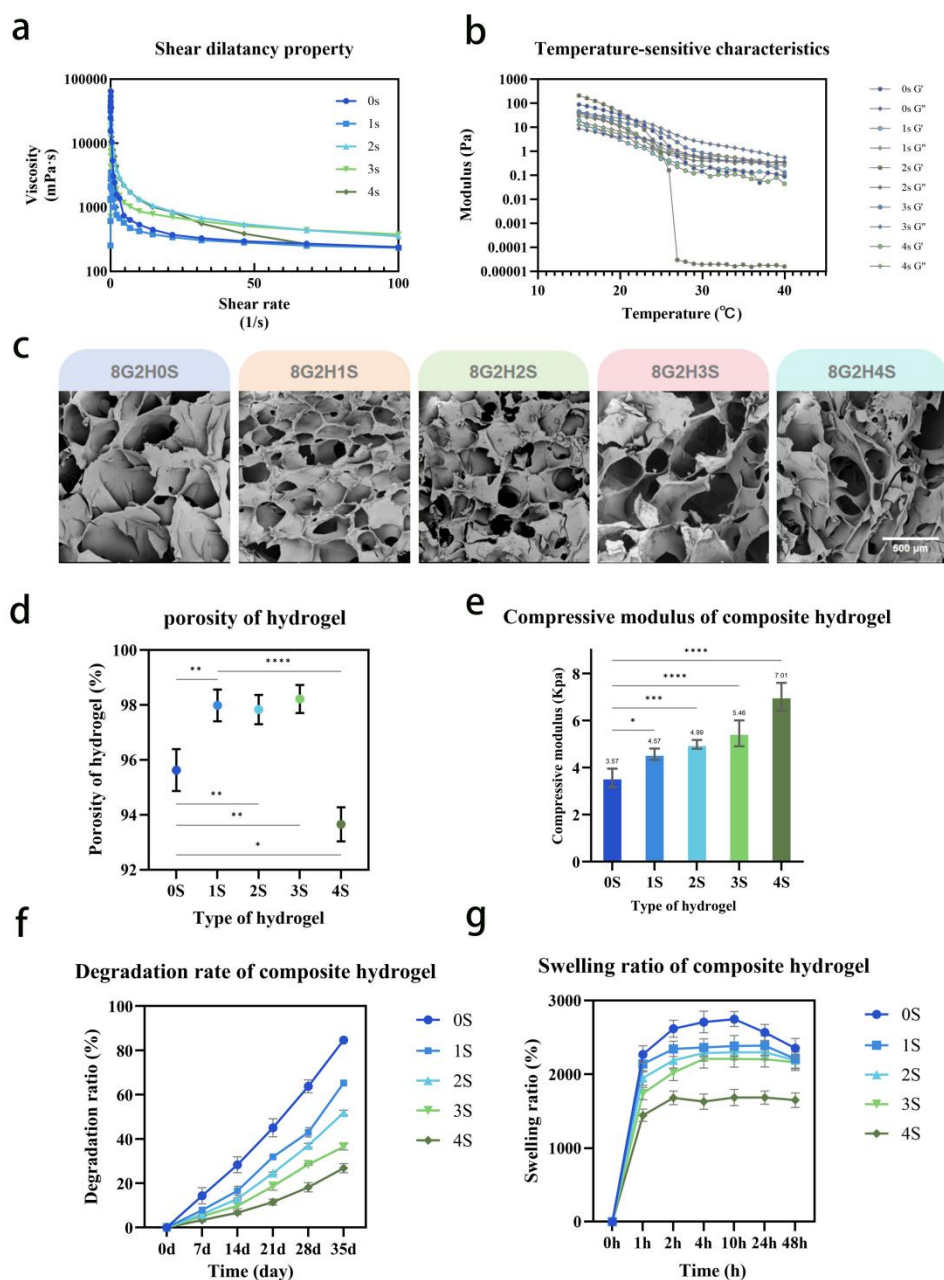


Figure 1. Physicochemical characterization of GelMA/HAMA/SilMA composite hydrogels with different SilMA concentrations. (a) Shear-thinning behavior of composite hydrogels with different SilMA concentrations under varying shear rates. (b) Temperature sweep rheological analysis showing the storage modulus (G') and loss modulus (G'') of different hydrogel formulations. (c) Scanning electron microscopy (SEM) images showing the internal porous microstructures of composite hydrogels. Scale bar = 500 μ m. (d) Quantitative analysis of hydrogel porosity. (e)

Compressive modulus of composite hydrogels with different SilMA concentrations. (f) In vitro degradation behavior of composite hydrogels over 35 days. (g) Swelling ratios of composite hydrogels at different time points. In all formulations, the concentrations of GelMA and HAMA were fixed at 8% and 2%, respectively, while SilMA concentrations were adjusted to 0%, 1%, 2%, 3%, and 4%, denoted as 0S, 1S, 2S, 3S, and 4S, respectively. Data are presented as mean $\pm$ standard deviation (SD) ($n = 3$ independent biological replicates). Statistical significance was analyzed using one-way ANOVA (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3.2 Selection of region-specific bioinks for heterogeneous meniscus bioprinting

To establish a biomimetic heterogeneous meniscus bioprinting system, the cytocompatibility of GelMA/HAMA/SilMA composite hydrogels containing different SilMA concentrations was systematically evaluated following the physicochemical and mechanical characterization described above. Live/dead staining demonstrated that cells encapsulated within all hydrogel groups generally maintained favorable survival after culture; however, noticeable differences in cellular status were observed among the formulations (**Figure 2a**). Hydrogels containing lower SilMA concentrations exhibited denser distributions of viable cells, whereas the high-SilMA groups showed markedly reduced live-cell signals.

Quantitative analysis of live fluorescence area demonstrated that the 0S, 1S, and 2S groups maintained relatively high levels of viable-cell fluorescence, reaching 96.66%, 97.39%, and 97.57%, respectively (**Figure 2b**). In contrast, the live fluorescence area decreased to 87.94% and 87.59% in the 3S and 4S groups, indicating impaired cellular status in hydrogels containing elevated SilMA concentrations.

Cell viability analysis further confirmed the concentration-dependent effect of SilMA incorporation on cellular behavior (**Figure 2c**). The 0S and 1S hydrogel materials demonstrated the highest cell survival rate, both reaching over 92.34%. However, cell viability progressively decreased with increasing SilMA concentration, declining to 67.39% in the 2S group and further dropping to 56.14% and 13.39% in the 3S and 4S groups, respectively. These findings suggested that although increasing SilMA concentration enhanced the mechanical strength of the composite

hydrogels, excessive SilMA incorporation adversely affected the cellular microenvironment and significantly inhibited cell survival and proliferation.

Combined with the mechanical characterization results shown in Figure 1, the incorporation of SilMA effectively improved the compressive properties of the composite hydrogels, with compressive modulus increasing from 3.57 kPa in the 0S group to 4.57 kPa in the 1S group and further rising to 7.01 kPa in the 4S group. Considering the intrinsic zonal heterogeneity of native meniscus tissue, both biomechanical requirements and biological compatibility were comprehensively considered during bioink selection. The 1S hydrogel provided moderately enhanced mechanical properties while still maintaining favorable cytocompatibility, whereas higher-SilMA formulations exhibited compromised cellular viability despite superior mechanical performance.

Therefore, 8G2H1S and 8G2H0S hydrogels were ultimately selected as the region-specific bioinks for heterogeneous meniscus reconstruction (**Figure 2d**). Specifically, the mechanically reinforced 8G2H1S hydrogel encapsulating primary meniscus synovial margin fibroblasts was used to mimic the outer red zone of the native meniscus, whereas the softer and more cytocompatible 8G2H0S hydrogel loaded with MFCs was used to reconstruct the inner white zone. Based on this region-specific material and cellular design, a dual-nozzle extrusion-based bioprinting strategy was subsequently established to spatially deposit the two hydrogel-cell systems into a single biomimetic meniscus construct (**Figure 2e**).

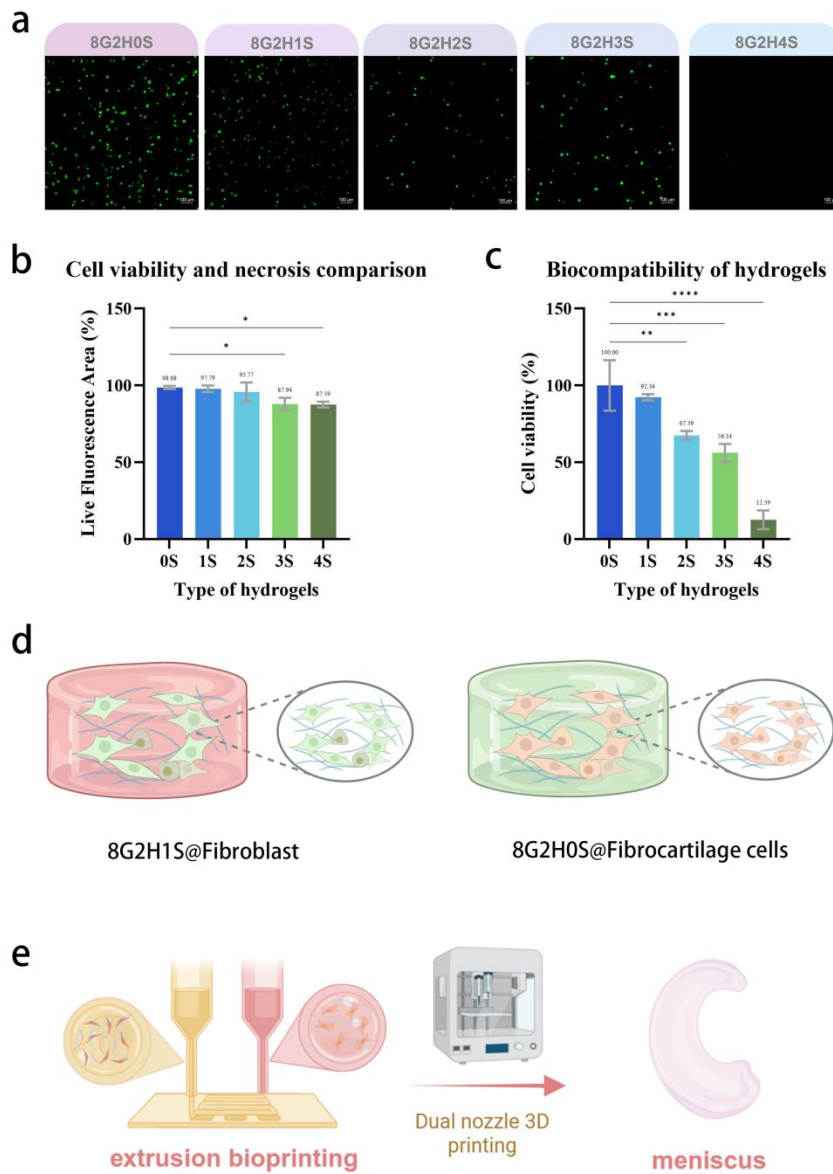


Figure 2. Establishment of a region-specific bioprinting strategy for heterogeneous meniscus reconstruction. (a) Live/dead staining of cells encapsulated within GelMA/HAMA/SilMA composite hydrogels containing different SilMA concentrations. Live cells are stained green and dead cells are stained red. Scale bar = 100 μm . (b) Quantitative analysis of live fluorescence area in different hydrogel groups. (c) Cell viability analysis of different hydrogel formulations.

(d) Schematic illustration of region-specific bioink and cell selection for heterogeneous meniscus reconstruction. The 8G2H1S hydrogel encapsulating primary meniscus synovial margin fibroblasts was used to mimic the outer red zone, while the 8G2H0S hydrogel encapsulating MFCs was used to mimic the inner white zone. (e) Schematic illustration of the dual-nozzle extrusion-based 3D bioprinting process for heterogeneous meniscus scaffold fabrication. Data are presented as mean $\pm$ standard deviation (SD) (n = 3 independent biological replicates). Statistical significance was analyzed using one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

3.3. Dual-nozzle bioprinting enables reconstruction of zonal mechanical heterogeneity

To fabricate biomimetic meniscus scaffolds with region-specific mechanical characteristics, a dual-nozzle extrusion-based bioprinting strategy was established using a BioMaker bioprinter (SUNP Biotechnology, Beijing, China). A meniscus scaffold model was first designed using SolidWorks, followed by printing path planning to enable spatially controlled deposition of distinct bioinks within predefined regions (**Figure 3a**). The dual-nozzle printing strategy allowed the independent distribution of region-specific hydrogel systems corresponding to the native red and white zones of the meniscus (**Figure 3b**).

Bioprinting was performed using 25G nozzles with an inner diameter of 0.26 mm at a printing speed of 5 mm/s and an extrusion rate of 5 μ L/s. Since the printing platform employed a mechanically driven extrusion system, the influence of bioink viscosity on the actual extrusion volume could be largely neglected, thereby ensuring stable and continuous material deposition during printing. To preserve cell viability while maintaining printing fidelity, different nozzle temperatures were applied according to the rheological characteristics of the selected bioinks. Specifically, the nozzle temperature for the 1S bioink was maintained at 22 °C, whereas the 0S bioink was printed at 25 °C. In addition, to prevent structural collapse during multilayer deposition, an interlayer ultraviolet (UV) pre-crosslinking strategy was introduced throughout the printing process. Based on these optimized printing parameters, heterogeneous meniscus scaffolds with stable structural fidelity were successfully fabricated (**Figure 3c**).

To determine whether the dual-nozzle bioprinting strategy successfully reconstructed zonal mechanical heterogeneity, the micromechanical properties of the printed scaffolds were characterized using atomic force microscopy (AFM). Young's modulus measurements were performed along the transverse section of the scaffold at 200 μm intervals. The dual-nozzle heterogeneous meniscus scaffold exhibited obvious region-dependent mechanical differences (**Figure 3d**). Specifically, the outer red-zone region fabricated using the 1S hydrogel exhibited Young's modulus values predominantly ranging from approximately 4800 to 7000 Pa, whereas the inner white-zone region fabricated using the 0S hydrogel displayed lower modulus values mainly distributed between approximately 3000 and 4500 Pa. The transition of modulus distribution between the two regions further demonstrated the successful formation of a spatial mechanical gradient within the printed construct.

To verify that the observed mechanical heterogeneity originated from the region-specific bioink distribution rather than scaffold geometry or measurement artifacts, homogeneous control scaffolds fabricated entirely using single 1S or single 0S bioinks were further evaluated. The scaffold printed solely using the 1S bioink exhibited relatively uniform Young's modulus values throughout the entire transverse section, with modulus values consistently distributed within the range of approximately 4500–6800 Pa and without obvious regional variation (**Figure 3e**). In contrast, the scaffold fabricated entirely using the 0S bioink displayed uniformly lower Young's modulus values ranging from approximately 3000–5000 Pa across the entire section (**Figure 3f**). Unlike the dual-nozzle scaffold, neither of the homogeneous control groups exhibited a clear zonal transition in mechanical properties.

Collectively, these results demonstrated that the dual-nozzle extrusion-based bioprinting strategy effectively reconstructed zonal micromechanical heterogeneity resembling the native meniscus tissue through spatially controlled deposition of region-specific bioinks.

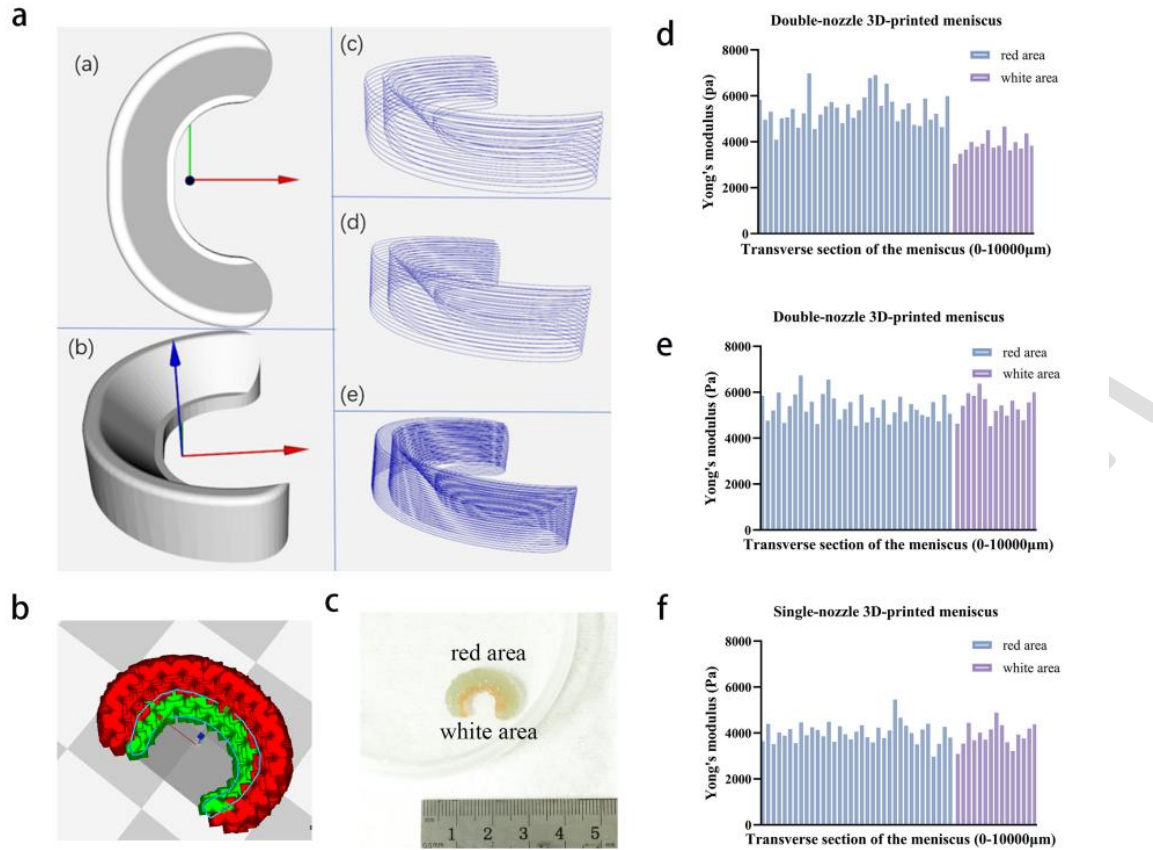


Figure 3. Dual-nozzle bioprinting and reconstruction of zonal mechanical heterogeneity in biomimetic meniscus scaffolds. (a) Computer-aided design (CAD) model and printing path planning of the meniscus scaffold generated using SolidWorks. (b) Schematic illustration of the dual-nozzle printing path for region-specific deposition of red-zone and white-zone bioinks. (c) Representative image of the dual-nozzle bioprinted heterogeneous meniscus scaffold. (d) Atomic force microscopy (AFM)-based Young's modulus distribution of the dual-nozzle bioprinted heterogeneous meniscus scaffold fabricated using 1S hydrogel for the red zone and 0S hydrogel for the white zone. (e) AFM-based Young's modulus distribution of the meniscus scaffold bioprinted using single 1S bioink. (f) AFM-based Young's modulus distribution of the meniscus scaffold bioprinted using single 0S bioink. Young's modulus profiles were obtained from three independently fabricated scaffolds ($n = 3$ biological replicates), with multiple measurement sites analyzed at each position. Data are presented as averaged modulus values across the scaffold cross-section.

3.4. Optimization of a universal coculture medium for heterogeneous meniscus reconstruction

The native meniscus exhibits pronounced zonal heterogeneity from the vascularized outer red zone to the avascular inner white zone, accompanied by gradual transitions in cellular phenotype and metabolic microenvironment. To mimic the heterogeneous cellular microenvironment of the native meniscus in vitro and establish a universal coculture system capable of supporting both region-specific cell populations, synovial fibroblast culture medium (fc medium) and meniscal fibrochondrocyte culture medium (cc medium) were prepared and systematically evaluated.

Considering the spatial transition of nutrient composition and cytokine distribution within native meniscus tissue, four culture medium formulations were designed using gradient mixing ratios, including pure fc medium, Mix2 medium (fc:cc = 2:1), Mix1 medium (fc:cc = 1:2), and pure cc medium.

The metabolic activity of SFs isolated from the outer meniscus region was first evaluated under two-dimensional culture conditions (**Figure 4a**). Relative cell viability remained stable in pure fc medium and Mix2 medium, reaching 96.2% and 93.7%, respectively, with no significant differences observed between groups. Interestingly, SFs cultured in Mix1 medium also maintained comparable cellular activity (~101%), indicating that SFs possessed strong adaptability to variations in culture medium composition. Notably, SF viability further increased to 110.3% in pure cc medium and showed significant differences compared with the control group. These findings suggested that SFs exhibited relatively low dependence on specific nutritional microenvironments and could maintain stable biological activity across a broad range of medium compositions.

In contrast, MFCs derived from the inner meniscus region displayed markedly different responses to the culture conditions (**Figure 4b**). In pure fc medium and Mix2 medium, MFC viability was severely suppressed, remaining at approximately 65%. However, when the proportion of cc medium increased, MFC viability significantly improved. Both Mix1 medium and pure cc medium supported substantially enhanced cellular activity, indicating that MFCs strongly depended on chondrogenic microenvironmental cues for survival and metabolic maintenance.

Collectively, these results demonstrated that SFs possessed relatively broad environmental adaptability, whereas MFCs exhibited strong dependence on cartilage-inductive culture conditions. Therefore, the selection of a coculture system for heterogeneous meniscus reconstruction should prioritize the maintenance of MFC viability and phenotypic stability.

Based on the comprehensive metabolic activity analysis of both cell types, pure cc medium was ultimately selected as the universal coculture system for subsequent *in vitro* culture of heterogeneous meniscus constructs. This strategy effectively preserved the biological activity of MFCs without causing adverse effects on SF survival, thereby providing a more suitable microenvironment for long-term cellular maintenance and region-specific tissue remodeling within the heterogeneous meniscus scaffold.

3.5. *In vitro* cell viability within the 3D bioprinted heterogeneous meniscus constructs

To evaluate whether the interconnected porous architecture of the bioprinted hydrogel scaffolds could effectively support nutrient diffusion and metabolic waste exchange for long-term cell survival in a three-dimensional microenvironment, cell viability within the constructs was dynamically assessed using Calcein-AM/PI Live–Dead staining during *in vitro* culture. Cell-laden scaffolds were collected at days 1, 3, 5, and 7 post-printing, gently rinsed with PBS, and incubated with Calcein-AM and PI staining solution for 30 min at 37 °C in the dark prior to confocal imaging.

Representative Live–Dead staining results for both the outer red-zone and inner white-zone regions are shown in Figure 4c. Throughout the 7-day culture period, the majority of encapsulated cells within the constructs exhibited strong and uniformly distributed green fluorescence signals, while only a limited number of scattered dead cells with red fluorescence were observed. These findings indicated that the mechanical extrusion-based printing process and the 405 nm UV-assisted rapid pre-crosslinking strategy did not induce substantial irreversible cellular damage during scaffold fabrication.

Moreover, the favorable cellular survival observed throughout the constructs suggested that the hydrogel network possessed suitable mass transport capability, enabling efficient nutrient diffusion and metabolic waste removal within the three-dimensional microenvironment. Importantly, the density of viable cells within the constructs gradually increased with prolonged

culture time (**Figure 4d,e**), indicating that the encapsulated cells not only maintained high viability but also underwent continuous proliferation during in vitro culture.

Collectively, these results demonstrated that the optimized printing parameters and hydrogel system exhibited excellent cytocompatibility and provided a favorable three-dimensional microenvironment capable of supporting long-term cellular survival and proliferation within the heterogeneous meniscus scaffolds.

3.6 Long-term proliferation of meniscus cells within the 3D bioprinted hydrogel scaffolds

To further evaluate the capability of the heterogeneous hydrogel scaffolds to support long-term cellular proliferation, the metabolic activity and proliferation behavior of encapsulated meniscus cells were quantitatively monitored for up to 15 days in vitro using the CCK-8 assay. Conventional two-dimensional (2D) culture was included as the control model for comparative analysis. At each predetermined time point, CCK-8 reagent was added and incubated for 2 h prior to absorbance measurement at 450 nm. The proliferation results were normalized to the initial value at day 1.

As shown in Figure 4 g, meniscus cells encapsulated within the 3D bioprinted hydrogel scaffolds exhibited sustained and stable proliferative behavior throughout the entire culture period. During the early culture stage (days 1–3), cellular proliferation remained relatively moderate, which was likely associated with the adaptation of cells from conventional 2D culture conditions to the three-dimensional hydrogel microenvironment. From day 5 onward, the metabolic activity of the cells increased markedly, indicating successful establishment of cell–matrix interactions and progressive remodeling within the hydrogel network. Throughout the 15-day observation period, the 3D group maintained a continuous upward proliferation trend without obvious plateau formation or proliferative decline. By day 15, the relative cell proliferation level reached nearly six-fold compared with the initial state.

In contrast, cells cultured under conventional 2D conditions exhibited a typical proliferation pattern characterized by rapid expansion followed by progressive decline (**Figure 4f**). During the initial culture stage, cells underwent logarithmic growth and rapidly increased in metabolic activity, reaching a peak proliferation level of approximately 1.6-fold by day 9. However, due to the limited surface area and restricted spatial environment of 2D culture plates, cells rapidly reached confluence and subsequently experienced contact inhibition. As culture time increased, the

combined effects of spatial overcrowding and localized nutrient depletion led to a sharp reduction in proliferative activity beginning around day 7. By days 13 and 15, the metabolic activity of the 2D-cultured cells had decreased dramatically and approached minimal levels, indicating substantial loss of cellular viability.

The striking difference between the proliferation profiles observed in the 2D and 3D culture systems demonstrated that the bioprinted hydrogel scaffolds effectively overcame the physical limitations associated with conventional planar culture. The interconnected three-dimensional hydrogel network provided a more biomimetic microenvironment capable of supporting sustained cellular survival and long-term proliferation. Such favorable long-term proliferative capacity is expected to facilitate subsequent extracellular matrix deposition and promote the histological maturation of engineered meniscus tissue constructs.

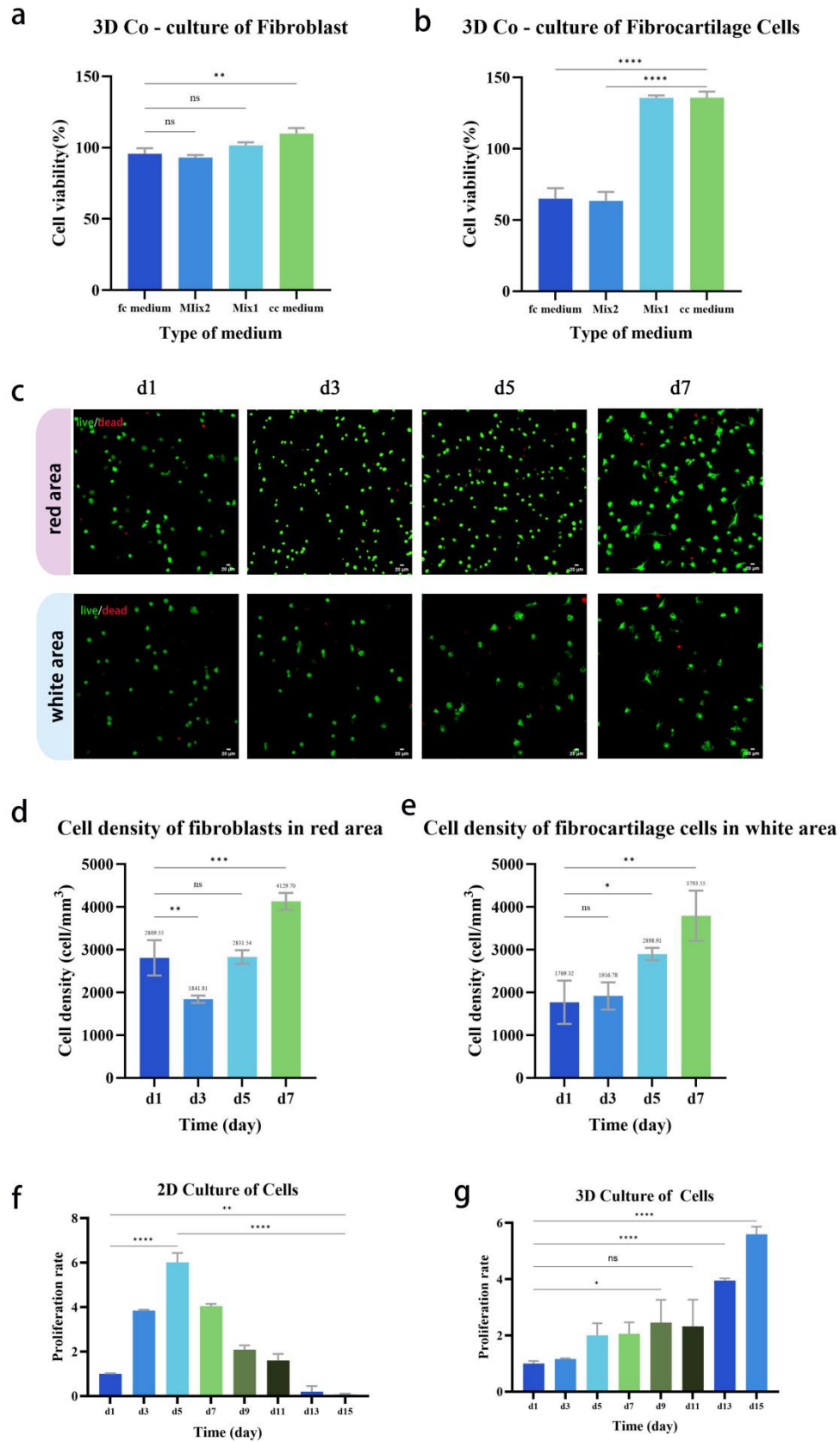


Figure 4. In vitro biocompatibility, cell proliferation, and morphological spreading of region-specific cells within the 3D bioprinted heterogeneous meniscus constructs. (a) Representative Live/Dead confocal images of meniscus MFCs in the inner zone (S0) and SFs in the outer zone (S1) at days 1, 3, and 7 post-printing. Live cells are stained green and dead cells are stained red. (b) Representative confocal images showing the cytoskeletal organization (F-actin/DAPI staining) of encapsulated MFCs and SFs at days 1, 3, and 7. F-actin is stained red and cell nuclei are stained blue. (c) Quantitative analysis of cell viability for the inner and outer zones at prescribed time points based on Live/Dead images. (d) Cell proliferation profiles of MFCs and SFs within the 3D bioprinted constructs over 7 days of culture, assessed via CCK-8 assay. Data are presented as mean $\pm$ SD (n = 3). Statistical significance is indicated as *p < 0.05, ***p < 0.001, and ****p < 0.0001.

3.7 Region-specific cytoskeletal remodeling within heterogeneous meniscus constructs

Representative F-actin/DAPI staining images of encapsulated cells within the heterogeneous meniscus constructs are shown in **Figure 5a**. Both SFs in the outer 1S region and MFCs in the inner 0S region exhibited progressive cytoskeletal remodeling during the 7-day in vitro culture period, indicating successful adaptation to the three-dimensional hydrogel microenvironment.

In the outer red-zone region, SFs initially displayed rounded or slightly spread morphologies at day 1, with F-actin signals mainly localized along the cell periphery. As culture time increased, SFs gradually extended pronounced pseudopodia and developed increasingly organized stress fibers. By day 7, cells exhibited elongated spindle-like morphologies with strong directional F-actin organization and extensive cytoskeletal spreading throughout the surrounding hydrogel matrix. The long cellular protrusions and enhanced actin organization suggested that SFs actively interacted with and remodeled the surrounding 3D microenvironment, consistent with the characteristic phenotype of fibroblast-like cells in native meniscus red-zone tissue.

In contrast, MFCs encapsulated within the inner 0S hydrogel exhibited comparatively restrained spreading behavior throughout the culture period. Although gradual cytoskeletal extension and stress fiber formation were also observed over time, the cells generally maintained more rounded or polygonal morphologies with relatively shorter cellular protrusions compared with SFs. By day 7, MFCs displayed improved cytoskeletal organization and stable cell–matrix interactions while

still preserving the typical morphological characteristics associated with fibrochondrocyte-like phenotypes.

The distinct morphological differences observed between the outer and inner regions suggested that the heterogeneous hydrogel microenvironment successfully guided region-specific cellular behaviors even under shared coculture conditions. Notably, the more extensive spreading and longer pseudopodia observed in SFs indicated that the mechanically reinforced 1S hydrogel provided a favorable microenvironment for fibroblastic cytoskeletal remodeling and migration, whereas the relatively confined morphology of MFCs within the softer 0S hydrogel more closely resembled the native cellular characteristics of the avascular white-zone meniscus tissue.

Collectively, these findings demonstrated that the dual-nozzle bioprinted heterogeneous meniscus constructs effectively supported region-specific cytoskeletal organization and phenotypic maintenance, thereby establishing a biomimetic cellular microenvironment for subsequent extracellular matrix remodeling.

3.8 Region-specific extracellular matrix reconstruction within heterogeneous meniscus constructs

Following confirmation of the favorable cytocompatibility and long-term proliferative capacity of the 3D bioprinted hydrogel scaffolds, the functional reconstruction of extracellular matrix (ECM) within the heterogeneous meniscus constructs was further evaluated. The biomechanical function of native meniscus tissue is highly dependent on its zonally organized ECM composition, in which the outer vascularized red zone is primarily enriched with type I collagen (COL I) to resist circumferential tensile stress, whereas the inner avascular white zone contains abundant type II collagen (COL II) to withstand compressive loading. Therefore, the successful region-specific deposition of these two representative collagen subtypes was considered a critical indicator for evaluating biomimetic meniscus reconstruction.

To investigate fibrous matrix reconstruction within the outer region, the expression of COL I, a major structural protein of native meniscus red-zone tissue, was systematically evaluated. Immunofluorescence staining revealed pronounced COL I-positive signals within the outer 1S region of the heterogeneous constructs (**Figure 5b**). The COL I fluorescence signals exhibited

dense and continuous network-like distributions surrounding the encapsulated cells and throughout the extracellular regions, indicating active matrix secretion by SFs within the 3D hydrogel microenvironment. In contrast, only weak and sparsely distributed COL I fluorescence was observed in the inner OS region, suggesting relatively limited type I collagen synthesis by MFCs under the white-zone microenvironment.

To quantitatively evaluate regional COL I deposition, fluorescence images were analyzed using ImageJ under identical imaging parameters. After channel separation and background subtraction, the average fluorescence intensity within defined regions of interest (ROIs) was quantified and normalized to the corresponding DAPI-positive nuclei counts to minimize the influence of cell density differences. Quantitative analysis demonstrated that COL I expression in the inner white-zone region was only approximately 20% of that observed in the outer red-zone region (**Figure 5d**), with statistically significant differences between groups. These findings confirmed the successful establishment of red-zone-dominant COL I deposition resembling the ECM distribution characteristics of native meniscus tissue.

Subsequently, the expression of COL II, a representative marker associated with fibrochondrocyte phenotype maintenance, was further analyzed to evaluate cartilage-like matrix reconstruction within the inner region. Similar immunofluorescence staining and quantitative analysis procedures were applied to ensure experimental consistency. As shown in **Figure 5c**, strong and continuous COL II-positive fluorescence signals were predominantly observed within the inner OS region. The fluorescence signals were mainly distributed around cells and throughout the surrounding extracellular matrix, demonstrating characteristic cartilage-like ECM deposition. In contrast, the outer 1S region exhibited substantially weaker and more scattered COL II fluorescence signals, indicating limited type II collagen secretion within the fibroblast-dominant microenvironment.

Quantitative fluorescence analysis further confirmed that the normalized COL II intensity in the inner white-zone region was significantly higher than that in the outer red-zone region (**Figure 5e**). These results suggested that the inner-zone hydrogel system provided a more favorable microenvironment for maintaining fibrochondrocyte-related matrix secretion and promoting cartilage-like phenotype preservation.

Collectively, these findings demonstrated that the dual-nozzle bioprinted heterogeneous meniscus constructs successfully established region-specific extracellular matrix remodeling characterized

by COL I-rich outer regions and COL II-rich inner regions. The spatially heterogeneous matrix deposition patterns closely resembled the biological characteristics of native meniscus tissue, indicating that the engineered constructs were capable of guiding zonal cellular behavior and biomimetic tissue-specific ECM reconstruction under shared coculture conditions.

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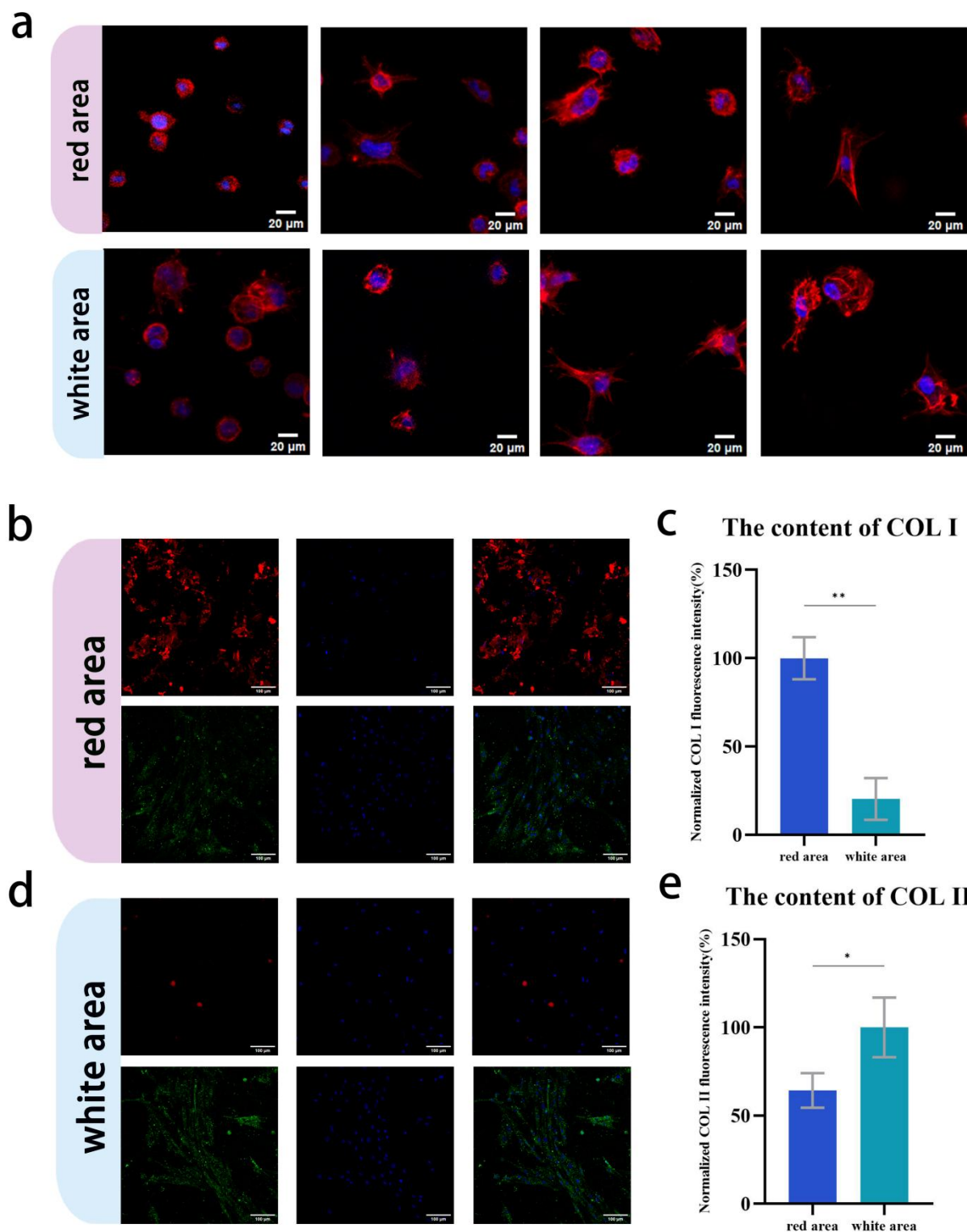


Figure 5. In vitro heterogeneous extracellular matrix (ECM) reconstruction and zone-specific differentiation within the 3D bioprinted meniscus constructs. (a) Representative confocal images

showing the cytoskeletal network (F-actin/DAPI staining) of encapsulated cells in the interconnected inner (0S) and outer (1S) zones after 7 days of shared co-culture. F-actin is stained red and cell nuclei are stained blue. (b, c) Representative immunofluorescence images of (b) Type I collagen (COL I, stained green) and (c) Type II collagen (COL II, stained green) across the macro-interface and within individual region-specific zones at day 7. Cell nuclei are counterstained with DAPI (blue). (d, e) Quantitative analysis of the relative fluorescence intensities for (d) COL I and (e) COL II deposition within the inner (S0) and outer (S1) zones. Data are expressed as mean $\pm$ SD (n = 3). Statistical significance is defined as *p < 0.05, p < 0.01, and ***p < 0.001.

4. Discussion

The native meniscus is a highly heterogeneous fibrocartilaginous tissue characterized by distinct regional variations in extracellular matrix composition, cellular phenotype, vascularization, and mechanical properties. Such structural and biological heterogeneity is essential for maintaining the complex biomechanical functions of the knee joint. However, most previously reported meniscus scaffolds have primarily focused on overall shape reconstruction or bulk mechanical enhancement, while insufficiently addressing the zonal microenvironmental differences required for functional tissue regeneration. In the present study, a dual-nozzle extrusion-based bioprinting strategy was developed to fabricate heterogeneous meniscus scaffold with region-specific mechanical and biological characteristics. By integrating tunable GelMA/HAMA/SilMA composite hydrogels with region-specific cell populations, this study successfully reconstructed biomimetic zonal microenvironments capable of guiding spatially regulated extracellular matrix remodeling in vitro.

One of the key findings of this study was the balance achieved between mechanical reinforcement and cytocompatibility through modulation of SilMA concentration within the composite hydrogel system. Increasing SilMA content effectively enhanced the mechanical properties of the hydrogels, which is consistent with previous studies demonstrating that methacrylated silk fibroin can significantly improve the structural stability and stiffness of photocrosslinked hydrogel systems. However, excessive SilMA incorporation negatively affected cellular spreading and proliferation, particularly when the concentration exceeded 2%. This phenomenon may be associated with increased network density, reduced pore size, and restricted cellular motility within highly crosslinked hydrogel environments. Therefore, rather than simply maximizing mechanical

strength, the present study emphasized the importance of balancing biomechanical performance with cellular adaptability during bioink optimization. Based on this strategy, 0S and 1S hydrogels were ultimately selected to mimic the relatively softer inner white-zone and mechanically reinforced outer red-zone microenvironments of native meniscus tissue, respectively.

Another important aspect of this study was the successful reconstruction of micromechanical heterogeneity through spatially controlled dual-nozzle bioprinting. Native meniscus tissue exhibits pronounced regional mechanical gradients that are critical for distributing compressive and tensile stresses during joint movement. In this work, AFM analysis demonstrated clear regional differences in Young's modulus between the outer and inner regions of the heterogeneous meniscus scaffold, whereas homogeneous control scaffolds fabricated using single bioinks did not exhibit such spatial transitions. These findings confirmed that the observed mechanical heterogeneity originated from the region-specific hydrogel distribution rather than geometric artifacts introduced during the printing process. Compared with conventional homogeneous hydrogel scaffolds, the establishment of region-specific micromechanical properties may provide more physiologically relevant biomechanical cues for regulating cell behavior and matrix remodeling within engineered meniscus tissues.

Importantly, the heterogeneous microenvironment established in this study also successfully guided region-specific cellular responses under shared coculture conditions. Cytoskeletal staining revealed that SFs within the outer 1S region developed elongated spindle-like morphologies with extensive pseudopodia, whereas MFCs encapsulated within the inner 0S region maintained relatively rounded or polygonal morphologies resembling native fibrochondrocyte phenotypes. More importantly, distinct extracellular matrix deposition patterns were observed after in vitro culture, characterized by enhanced COL I expression within the outer region and elevated COL II deposition within the inner region. These results suggested that the spatially heterogeneous hydrogel microenvironment itself was sufficient to direct region-specific cellular behaviors and matrix remodeling, even without the need for independently separated culture systems. Such findings further highlight the importance of integrating material composition, mechanical cues, and region-specific cell populations for biomimetic fibrocartilage tissue engineering.

The region-specific extracellular matrix deposition observed in this study suggests that the heterogeneous hydrogel microenvironment was capable of providing biological and mechanical

cues that supported zonal cellular functions in vitro. However, native meniscus tissues continuously experience complex physiological loading conditions within the knee joint, and mechanical stimulation plays a critical role in regulating cell phenotype, collagen organization, and extracellular matrix remodeling. Previous studies have demonstrated that cyclic compression, tensile loading, and shear stress can significantly influence fibrocartilage maturation and functional tissue development. Therefore, although the current constructs successfully reproduced early-stage zonal characteristics under static culture conditions, their long-term functionality may be further enhanced through the incorporation of physiologically relevant mechanical stimulation strategies.

Although the present study demonstrated favorable in vitro biological performance and successful reconstruction of zonal extracellular matrix heterogeneity, several limitations still remain. First, the current evaluation was limited to in vitro culture conditions, and the long-term in vivo performance of the heterogeneous meniscus constructs remains unclear. Future studies should further investigate tissue integration, inflammatory responses, extracellular matrix maturation, and cartilage-protective effects using clinically relevant animal models, such as rabbit or sheep meniscus defect models. In addition, native meniscus tissues are continuously subjected to complex dynamic mechanical loading environments, including cyclic compression, tensile stress, and shear stimulation. However, the present study mainly evaluated cellular responses under static culture conditions. Dynamic mechanical stimulation has been reported to play a critical role in collagen alignment, fibrocartilage maturation, and mechanotransduction-mediated extracellular matrix remodeling. Therefore, future work incorporating bioreactor-based dynamic loading systems may further enhance tissue maturation and improve the functional properties of heterogeneous meniscus scaffold.

Overall, this study demonstrates that dual-nozzle bioprinting combined with region-specific hydrogel-cell systems represents a promising strategy for reconstructing biomimetic heterogeneous fibrocartilage tissues. The engineered constructs successfully reproduced key structural, mechanical, and biological characteristics of native meniscus tissue in vitro, providing a potential platform for future functional meniscus regeneration and broader heterogeneous tissue engineering applications.

5. Conclusion

In this study, a dual-nozzle extrusion-based bioprinting strategy was successfully developed for the fabrication of biomimetic heterogeneous meniscus constructs with region-specific mechanical and biological characteristics. By integrating GelMA/HAMA/SilMA composite hydrogels with tunable physicochemical properties, region-specific bioinks were established to mimic the distinct microenvironments of the native outer red zone and inner white zone.

Comprehensive material characterization demonstrated that increasing SilMA concentration effectively enhanced the mechanical properties of the composite hydrogels, whereas excessive SilMA incorporation adversely affected cellular viability and proliferation. Based on the balance between biomechanical performance and cytocompatibility, 8G2H1S and 8G2H0S hydrogels were selected as the region-specific bioinks for reconstruction of the outer red zone and inner white zone, respectively. Combined with SFs and MFCs, the dual-nozzle bioprinting strategy enabled spatially controlled deposition of heterogeneous hydrogel-cell systems within a single meniscus construct.

The engineered heterogeneous meniscus scaffolds exhibited favorable structural fidelity, long-term cytocompatibility, and sustained cellular proliferation *in vitro*. More importantly, atomic force microscopy analysis confirmed the successful reconstruction of zonal micromechanical heterogeneity resembling native meniscus tissue. Under shared coculture conditions, encapsulated cells maintained region-specific cytoskeletal morphologies and extracellular matrix secretion patterns, characterized by enhanced COL I deposition in the outer region and elevated COL II expression in the inner region.

Collectively, these findings demonstrate that the proposed dual-nozzle bioprinting platform successfully established a biomimetic zonal microenvironment capable of guiding region-specific cellular behavior and extracellular matrix remodeling. The engineered constructs reproduced key structural, micromechanical, and biological features of native meniscus tissue *in vitro*, highlighting the feasibility of integrating region-specific hydrogel formulations and cell populations within a single heterogeneous meniscus scaffold.

Nevertheless, the present study represents an *in vitro* proof-of-concept demonstration, and further investigations are required to evaluate long-term tissue maturation, functional integration, and

regenerative performance in vivo. Future studies incorporating animal models and bioreactor-based dynamic mechanical stimulation, including cyclic compression, tensile loading, and shear stress, may further promote extracellular matrix maturation and improve the functional performance of engineered meniscus tissues. These efforts will contribute to the future translation and development of biomimetic meniscus tissue engineering strategies.

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Conflict of interest

The authors declare they have no competing interests

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Ethics approval and consent to participate

This study did not involve human participants or animal subjects. All experiments were conducted in accordance with institutional guidelines.

Consent for publication

Not applicable.

Availability of data

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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