

RESEARCH ARTICLE

Hydroxyapatite–alginate–gelatin bioinks for bioprinting: Printability, microarchitecture, and dental pulp stem cell viability and morphology

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Abstract

Three-dimensional (3D) bioprinting enables the fabrication of scaffolds with highly controlled architectures; however, the extent to which modest shifts in bioink composition simultaneously influence scaffold stabilization and the resulting early cellular microenvironment remains insufficiently understood. This study investigated the influence of hydroxyapatite (HAp) concentration within a low loading range (1–2% w/v) on the printability, crosslinking stability, and microarchitecture of pre-crosslinked alginate–gelatin composite bioinks, as well as on the immediate viability and morphological adaptation of encapsulated human dental pulp stem cells (hDPSCs). Bioinks were characterized via physicochemical analysis, while printing performance was evaluated under varied operational conditions. Structural properties were assessed through micro-computed tomography (micro-CT) and swelling kinetics, whereas early cellular responses were monitored via Live/Dead staining and fluorescence-based morphometric analysis. Printability parameters were primarily dictated by nozzle diameter and layer count, with the 22G nozzle yielding larger pore sizes (1,132 vs. 940 μm) and pore areas (0.749 vs. 0.515 mm^2) than the 20G nozzle. Micro-CT confirmed highly interconnected structures with porosities of 74–78%, where the 2% HAp group promoted a highly homogeneous trabecular spacing centered between 700 and 850 μm . Incremental HAp content significantly suppressed matrix swelling kinetics in a concentration-dependent manner. Biologically, all formulations sustained high post-printing cell survival (>70%) that exceeded 90% after seven days. HAp-containing hydrogels promoted significantly higher cell viability and increased cell spreading areas. These findings underscore that small variations in HAp content influence scaffold stabilization kinetics and early stem cell behavior, while manufacturing parameters remain the primary determinants of initial geometry.

Keywords: Composite alginate-based bioinks; Bioprinting; Printability; Dental pulp stem cells; Cell viability

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1. Introduction

Three-dimensional (3D) bioprinting of cell-laden scaffolds has emerged as a cornerstone technology in tissue engineering, enabling the reproducible fabrication of constructs with highly customizable geometries that mimic native physiological microenvironments.¹ Developing biologically functional structures requires the seamless integration of multiple sequential stages, including computer-aided design, bioink formulation, optimization of printing parameters, structural stabilization, and post-printing cell culture. Among these factors, bioink formulation plays a central role as a dynamic interface between the manufacturing process and the cellular microenvironment. The composition of the bioink directly dictates not only printability and shape fidelity but also the structural and biochemical cues that govern subsequent cellular behavior.^{2–4} Consequently, even subtle compositional variations can significantly alter printing kinetics, filament deposition, scaffold stabilization, pore architecture, and the localized microenvironment within the printed structure.^{5,6}

Bioprinted scaffolds are conceptually designed to function as artificial extracellular matrices (ECMs),^{7,8} simultaneously providing mechanical support and biochemical signals to promote cell adhesion, survival, proliferation, and tissue organization. Replicating ECM-like characteristics—such as tailored porosity, interconnectivity, and cell-adhesive motifs—is therefore essential to direct early cell–matrix interactions during tissue formation. Alginate–gelatin (Alg–Gel) hydrogels are widely utilized in extrusion-based bioprinting due to their favorable rheological properties, mild crosslinking conditions, and excellent cytocompatibility.^{4,9} Within this composite system, Alg provides structural integrity and shape fidelity via ionic crosslinking, whereas Gel contributes essential cell-interactive motifs (e.g., Arg–Gly–Asp [RGD] sequences) that support cell attachment and spreading.^{10,11} Nevertheless, Alg and Gel alone exhibit limitations regarding bioactivity and structural performance, which may restrict their use in applications where enhanced osteoconductive properties are desired.¹²

To overcome these limitations, hydroxyapatite (HAp) is extensively incorporated into hydrogels as a bioactive and osteoconductive mineral filler.^{13,14} Beyond its biochemical role, HAp modulates the resulting scaffold microarchitecture.^{15,16} Specifically, variations in HAp content directly influence filament formation, pore dimensions, interconnectivity, and nutrient diffusion, thereby shaping cell–matrix interactions during the critical initial stages of culture.¹⁶ Furthermore, HAp provides material-derived signals through the localized release

of calcium and phosphate ions and surface-mediated interactions.¹⁷ These cues stimulate osteogenic-related responses—although such effects alone are insufficient to demonstrate true osteoinduction—and modulate early cell viability, spreading, and cytoskeletal organization, which are increasingly recognized as pivotal indicators of cellular adaptation within hydrogel systems.^{16,18} Consequently, the early behavior of encapsulated stem cells serves as a vital mechanistic link between initial bioink formulation and ultimate biological performance.

Human dental pulp stem cells (hDPSCs) represent a robust cell source for bone tissue engineering due to their high proliferation rates and multi-lineage osteogenic potential.^{19–21} These mesenchymal progenitors are highly sensitive to scaffold composition, architecture, and localized physicochemical cues, making them an ideal cellular model to investigate formulation-driven cell–matrix dynamics. Despite growing interest in HAp-containing composite bioinks, the extent to which modest variations in HAp concentration simultaneously affect micro-extrusion performance, multi-layered scaffold microarchitecture, and the immediate cellular microenvironment remains insufficiently understood. While previous studies have often independently examined either printability parameters or biological outcomes, few have investigated how formulation-driven changes in scaffold formation influence the initial interactions between encapsulated cells and their surrounding matrix within a single, integrated experimental framework. To address this gap, the present study investigates the effect of incremental variations in HAp content within a pre-crosslinked Alg–Gel bioink system for extrusion-based bioprinting. A pre-crosslinking strategy was specifically employed to enhance structural stability and improve process control during extrusion.²² Rather than attempting to establish the long-term efficacy of these scaffolds for bone regeneration, this work focuses exclusively on elucidating how modest compositional shifts alter bioink physicochemical properties, printing performance, multi-layer pore architecture, and the immediate post-printing viability and morphological responses of encapsulated hDPSCs.

2. Materials and methods

2.1. Formulation of pre-crosslinked cell-free bioinks

The pre-crosslinked cell-free bioinks were formulated using a blend of sodium Alg, Gel, and HAp. Sodium Alg (Sigma-Aldrich, United States of America [USA]) at 3% w/v was dissolved in ultrapure water at 40 °C under constant stirring until a homogeneous solution was obtained. Partial ionic pre-crosslinking was subsequently

induced by the controlled addition of a 20 mM CaCl_2 solution, followed by incubation for 1 h to achieve a suitable viscosity for extrusion. Subsequently, Gel (Type B, Sigma-Aldrich, USA) at 1% w/v was added and fully dissolved under continuous stirring for 1 h. HAp nanopowder particles (<200 nm, $\geq 97\%$ purity; Sigma-Aldrich, USA) were incorporated at concentrations of 1% and 2% w/v. The HAp powder was gradually added in its dry form directly to the polymeric matrix under vigorous magnetic stirring to promote uniform dispersion and prevent agglomeration. The addition was performed slowly to avoid particle clustering, followed by overnight mixing under continuous agitation to ensure complete homogeneity. To minimize particle sedimentation, all samples were used immediately after preparation. The cell-free bioink formulations were designated as Alg-Gel-HAp1% and Alg-Gel-HAp2%. An HAp-free Alg-Gel formulation was prepared using the same procedure and was designated Alg-Gel-HAp0%.

2.2. Physicochemical characterization

2.2.1. Fourier-transform infrared spectroscopy

Prior to analysis, the cell-free bioinks were dried and stored overnight in a desiccator to minimize moisture interference. Subsequently, 5 mg of each sample was thoroughly mixed with 500 mg of potassium bromide. The resulting mixture was compressed into thin pellets using a manual hydraulic pellet press. In addition to the complete composite bioinks, the individual components (Alg, Gel, and HAp) were independently analyzed under identical experimental conditions. Fourier-transform infrared (FT-IR) spectra were acquired using a Tensor 27 FT-IR spectrophotometer (Bruker, Germany). Measurements were performed over a spectral range of 200–5000 cm^{-1} with a spectral resolution of 4 cm^{-1} , collecting 128 scans per sample to optimize the signal-to-noise ratio.

2.2.2. Thermogravimetric analysis

Prior to thermal characterization, the cell-free bioinks were dried and stored overnight in a desiccator. Thermogravimetric analysis (TGA) was performed using a Simultaneous Thermal Analyzer TGA STA 6000 (PerkinElmer, USA). Samples weighing approximately 15–20 mg were placed in alumina crucibles. Along with the complete bioink formulations, the individual components were independently analyzed under identical experimental conditions. The temperature was increased from 30 to 500 $^{\circ}\text{C}$ at a constant heating rate of 10 $^{\circ}\text{C}/\text{min}$ under a nitrogen atmosphere. Weight loss was recorded as a function of temperature, and derivative thermogravimetric (DTG) curves were calculated to identify the distinct stages of thermal degradation.

2.3. Printability and shape fidelity evaluation

An extrusion-based 3D bioprinter (TissueStart, TissueLabs, Switzerland) was utilized to fabricate scaffolds designed via AutoCAD software (version 2024, Autodesk, USA). For operational printing evaluation, standardized grid scaffolds (dimensions: 10 × 10 × 2 mm) were generated with a 0/90° lay-down pattern and a strand spacing of 1 mm, utilizing two- and four-layer architectures deposited in a layer-by-layer pattern. Additionally, to evaluate the structural versatility of the bioink formulations, alternative geometries—including circular grids and circular honeycombs—were fabricated under the selected operational printing conditions identified in this study.

The cell-free bioinks were loaded into 3 mL syringes and extruded at room temperature through 20- and 22-gauge (G) conical nozzles (inner diameters of 0.610 and 0.410 mm, respectively) at extrusion speeds ranging from 1 to 3 mm/s. After printing, the scaffolds were immersed in a 100 mM CaCl_2 crosslinking solution for 15 min to induce the complete ionic gelation of the Alg phase. Shape fidelity was qualitatively evaluated by visual inspection of the printed constructs, assessing strand definition, pore uniformity, and structural integrity post-crosslinking relative to the original CAD design. Printability was quantitatively assessed using the pore printability index (Pr), calculated according to Equation 1:

$$Pr = \frac{P^2}{16A} \quad (1)$$

where P is the pore perimeter, and A is the pore area. A value of $Pr = 1$ indicates an ideal square pore geometry, reflecting high fidelity between the designed and printed structures. Values of $Pr < 1$ indicate smaller, rounded pores typically associated with over-extrusion or partial structural collapse during deposition, whereas values of $Pr > 1$ correspond to enlarged or irregular pore geometries.^{23,24}

A factorial experimental design was employed to evaluate the effects of four operational parameters (Table S1): HAp level, nozzle diameter, extrusion speed, and number of layers. Images of the printed scaffolds were acquired using a stereomicroscope (Olympus SZ2-ILST, Japan) and analyzed via Fiji/ImageJ software (University of Wisconsin-Madison, USA). For each scaffold, a region of interest corresponding to the central area of the construct was selected to ensure analytical consistency and eliminate edge effects. Images were calibrated according to the corresponding scale bar. Pore segmentation was performed by converting images to grayscale, followed by automatic thresholding using Otsu's method to distinguish open

pores from the printed filaments.²⁴ The resulting binary images were used to quantify the number of open pores and to extract morphometric parameters, including pore size (μm), pore area (mm^2), and pore perimeter (mm). At least three independent scaffolds were analyzed per experimental condition.

2.4. Micro-computed tomography analysis

Micro-computed tomography (micro-CT) analysis of the scaffolds (dimensions: $10 \times 2 \text{ mm}$ [diameter $\times$ height]) was performed using a SkyScan 1273 scanner (Bruker, Belgium) operated at a source voltage of 45 kV and a source current of 165 μA , with a voxel resolution of 5 μm . Samples were analyzed in their fully hydrated state at 10 °C to preserve their native architecture and prevent structural deformations associated with dehydration.

Three-dimensional image reconstruction was carried out using NRecon software (version 2.2.0.6, Bruker, USA) based on a cone-beam reconstruction algorithm. Standardized reconstruction parameters were applied, including a beam hardening correction of 54% to minimize metal artifacts associated with the mineral HAp phase. A Hamming filter was additionally applied during reconstruction to mitigate image noise. To ensure comparability among samples, a consistent region of interest located in the central region of each scaffold was selected, minimizing edge-related artifacts and peripheral distortions. Quantitative analysis of the reconstructed datasets was performed using CTAn software (version 1.23.0, Bruker microCT, Belgium). Image binarization was conducted via a global thresholding approach, applying identical threshold values across all samples. Furthermore, phase segmentation based on grayscale attenuation differences was performed to distinguish the radiopaque HAp phase from the surrounding polymeric hydrogel matrix. Morphometric parameters, including trabecular spacing and total porosity, were quantified from the reconstructed volumes.

2.5. Swelling behavior

The swelling kinetics of the scaffolds were evaluated through gravimetric measurements in both phosphate-buffered saline (PBS; pH 7.4) and complete culture medium at 37 °C. Three scaffold formulations were analyzed: (i) Alg-Gel-HAp0% (control), (ii) Alg-Gel-HAp1%, and (iii) Alg-Gel-HAp2%. Lyophilized scaffolds were initially weighed to determine their initial dry weight (W_0) and subsequently immersed in the respective media under static incubation. Samples were collected at predefined time points (1, 2, 3, 4, and 24 h). At each interval, scaffolds were removed from the fluid, gently blotted with filter paper to eliminate excess surface liquid, and immediately

weighed to obtain the swollen weight (W_s). The swelling ratio was determined according to **Equation 2**:

$$\text{Swelling ratio (\%)} = \frac{(W_s - W_0)}{W_0} \times 100 \quad (2)$$

2.6. Cell culture

Human dental pulp stem cells were isolated from the dental pulp of extracted human third molars obtained from healthy donors with written informed consent. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA), and incubated at 37 °C in a humidified atmosphere with 5% CO_2 . Cells at passage 10 were utilized for all experimental procedures. Upon reaching approximately 80% confluence, cells were sub-cultured using trypsin (TryPLE, Gibco, USA) according to standard protocols. Prior to experimental use, cells were enzymatically detached, counted in a Neubauer chamber, and viability was assessed via the trypan blue exclusion assay.

2.7. Formulation of cell-laden bioinks and 3D bioprinting

Prior to formulation, Alg, Gel, and HAp powders were sterilized by ultraviolet (UV) light exposure for 2 h, following procedures previously reported for *in vitro* applications.²⁵ To ensure homogeneous UV irradiation, the powders were periodically agitated manually every 20 min during the sterilization process. Subsequently, the biomaterial components were dissolved and blended under strict aseptic conditions following the same sequence described for the cell-free bioinks. Before cell incorporation, aliquots of the resulting sterile biomaterial mixture were inoculated into liquid Lysogeny Broth (LB) medium (Sigma-Aldrich, USA) and plated onto solid LB-agar plates, followed by incubation at 37 °C for 72 h to rigorously confirm sterility. No microbial growth or colony formation was detected after 72 h, validating the efficacy of the sterilization protocol (Figure S1).

Cells were incorporated into the sterile bioink matrix at a final density of 1×10^6 cells/mL using interconnected syringes coupled through a Luer-lock connector, as previously described.²⁶ The resulting cell-laden bioinks were transferred into sterile 3 mL syringes fitted with a 22G dispensing conical nozzle. Based on the operational window identified during the cell-free pre-screening, a printing speed of 3 mm/s, a 22G nozzle, and a four-layer construct architecture were selected as the standardized parameters for all cell-laden experiments. Scaffolds were directly bioprinted onto sterile 6-well plates under

these optimized conditions. The printed constructs were immediately crosslinked using a sterile 100 mM CaCl_2 solution for 15 min. After crosslinking, the constructs were washed three times with sterile PBS (pH 7.4) to eliminate excess calcium ions, and 2 mL of complete culture medium was added to each well. Finally, the bioprinted scaffolds were incubated at 37 °C in a 5% CO_2 environment. The culture medium was refreshed every two days. For biological evaluations, scaffolds containing 0% (control), 1%, and 2% HAp were analyzed at 0, 3, and 7 days post-bioprinting.

2.8. Post-printing viability of encapsulated hDPSCs

Cell viability was determined using a Live/Dead cell staining assay (calcein-AM and ethidium homodimer-1; Invitrogen, USA), following previously described protocols.^{5,27,28} The Live/Dead staining solution was prepared according to the manufacturer's instructions, utilizing 2 μM calcein-AM and 4 μM ethidium homodimer-1 diluted in sterile PBS. Scaffolds were incubated with the staining solution for 30 min at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, samples were gently washed with PBS to remove excess dye. Fluorescence images were acquired using a BX53 epifluorescence microscope (Olympus, Japan) equipped with a 4 $\times$ objective lens. For each scaffold formulation ($n = 3$), six randomly selected fields at different focal planes were captured to account for the 3D architecture of the scaffolds. Image analysis was performed individually using Fiji/ImageJ software. Live cells (green fluorescence) and dead cells (red fluorescence) were quantified, and cell viability was expressed as the percentage of live cells relative to the total number of cells detected per image.

2.9. Morphology and cytoskeletal organization of encapsulated hDPSCs

Cell morphology and cytoskeletal organization were evaluated via fluorescent cytoskeleton staining. Bioprinted scaffolds were collected after seven days of culture and fixed with 4% paraformaldehyde for 60 min at room temperature. Samples were subsequently washed twice with PBS (pH 7.4) and permeabilized with 0.05% Triton X-100 for 5 min at room temperature. The actin cytoskeleton was stained using Phalloidin-TRITC (1:1500 dilution; Thermo Fisher Scientific, USA), while cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000 dilution; Thermo Fisher Scientific, USA) for 15 min. All staining procedures were performed at room temperature in the dark. After each staining step, samples were washed three times with PBS (pH 7.4) to remove excess reagents. Fluorescence images were acquired using a BX53 epifluorescence microscope equipped with TRITC

and DAPI filter sets. Three independent replicates were analyzed for each scaffold, and representative images from three distinct regions per sample were captured using identical image acquisition parameters to ensure comparability among groups. Quantitative morphometric analysis was performed using Fiji/ImageJ software to extract the cell spreading area, aspect ratio, and circularity.

2.10. Statistical analysis

Printability statistical analyses were performed using R software (version 4.5.0; R Core Team, 2025). A factorial analysis of variance (ANOVA) was conducted to evaluate the effects of four experimental factors on the printing outcomes: HAp level (levels 1 and 2), nozzle diameter (20G and 22G), extrusion speed (1, 2, and 3 mm/s), and number of layers (2 and 4). The response variables analyzed were: number of open pores, pore size (μm), pore area (mm^2), pore perimeter (mm), and printability index.

Due to the unbalanced experimental design (three treatment combinations with $n = 1$ due to printing failures under specific parameter combinations), Type III sum of squares was employed using the `Anova()` function from the "car" package.²⁹ Effect sizes were calculated using omega-squared (ω^2), which provides a less biased estimate of the population effect size compared to eta-squared, particularly for small sample sizes.³⁰ Statistical significance and effect magnitude were interpreted separately: p values were used to assess evidence against the null hypothesis, whereas ω^2 was used to evaluate practical relevance. The interpretation of ω^2 followed established guidelines: small (0.01), medium (0.06), and large (0.14) effects.³¹ Effects with $\omega^2 \leq 0.001$ were considered negligible, even when associated p -values were statistically significant. Prior to ANOVA, the assumptions of normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. For variables that violated the normality assumption (open pores and pore area), non-parametric Kruskal-Wallis tests were performed as a complementary analysis to validate the ANOVA findings.

Principal component analysis (PCA) was conducted as an exploratory multivariate analysis to summarize patterns among the response variables and to visualize how samples were distributed across experimental groups. PCA was used for descriptive visualization rather than hypothesis testing or mechanistic inference. Prior to PCA, all variables were standardized (z -score transformation) to ensure equal contribution regardless of measurement scale. The analysis was performed using the `prcomp()` function from base R. Data manipulation and visualization were performed using the "dplyr" and "tidyr" packages³² for data wrangling, and "ggplot2"³³ with "ggpubr"³⁴ for generating publication-

quality figures. Statistical significance was set at $\alpha = 0.05$ for all tests.

Cell viability statistical analyses were undertaken with GraphPad Prism Software version 9.0.0 (GraphPad, USA). Data are presented as means $\pm$ standard deviation. Statistical analysis utilized two-way ANOVA followed by Tukey's post-hoc test, and $p < 0.05$ was considered significant.

3. Results

3.1. Fourier-transform infrared spectroscopic analysis

The chemical functional groups of the pre-crosslinked composite cell-free bioinks and their standalone components were successfully identified via FT-IR analysis (Figure 1). In the HAp spectrum, intense characteristic absorption bands were observed at 560, 600, 1,000, and 1,100 cm^{-1} , corresponding to the bending and stretching vibrations of the phosphate (PO_4^{3-}) groups. In the sodium Alg spectrum, prominent bands identified within the 1,400–1,625 cm^{-1} range were assigned to the asymmetric and symmetric stretching vibrations of its carboxylate (COO^-) anions. Additionally, the absorption bands in the 1,020–1,080 cm^{-1} region corresponded to the characteristic C–O stretching vibrations of the pyranose ring, asymmetric C–O–C glycosidic stretching, and C–C stretching of the Alg backbone. In the Gel spectrum, a well-defined band

at 1,630 cm^{-1} was identified, corresponding to the amide I vibrations (C=O stretching of CONH_2 groups). All these diagnostic spectral footprints were successfully preserved and identified in the spectra of both composite inks (Alg–Gel–HAp1% and Alg–Gel–HAp2%), confirming the structural integrity and presence of all constituent phases without the appearance of new covalent absorption bands.

3.2. Thermogravimetric analysis

The thermal stability of the composite formulations was evaluated to ensure structural integrity across a temperature range, including physiological cell culture conditions (37 °C). Figure 2A illustrates the weight loss profiles (TGA) alongside their corresponding derivative curves for the composite bioinks and individual components. No appreciable mass loss was recorded at 37 °C for any formulation. Initial detectable thermal degradation onset occurred at approximately 78 °C and 110 °C for the 1% and 2% w/v HAp formulations, respectively, primarily associated with the evaporation of bound water. As the temperature increased, successive stages of thermal degradation were recorded. The first main mass loss stage occurred below 120 °C, followed by a second stage characterized by a sharp increase in degradation rate between approximately 180 °C and 250 °C. The third thermal stage, recorded between 250 °C and 350 °C, accounted for the greatest cumulative weight loss due to the pyrolytic decomposition of the Alg and Gel polymeric backbones. The DTG profiles (Figure

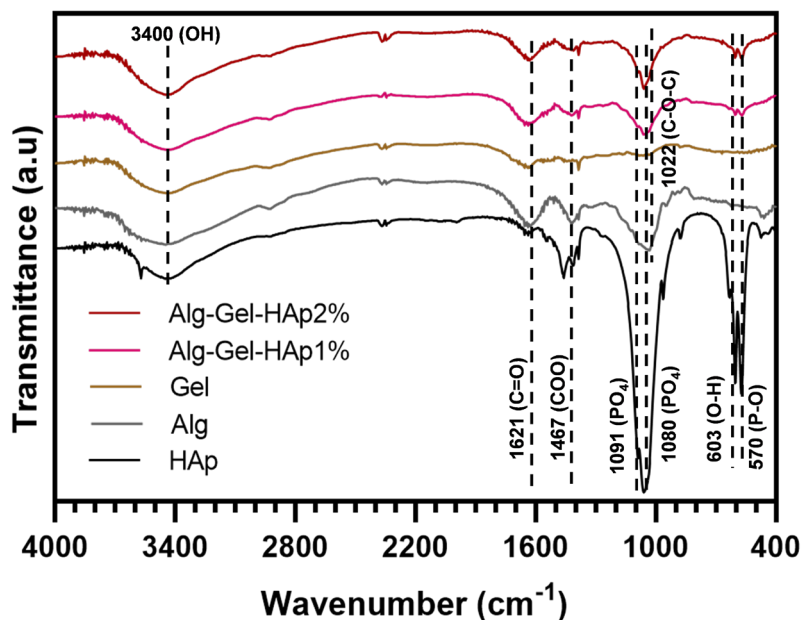


Figure 1. FT-IR analysis of functional groups in pre-crosslinked Alg–Gel–HAp inks
Abbreviations: Alg: Alginate; Gel: Gelatin; HAp: hydroxyapatite.

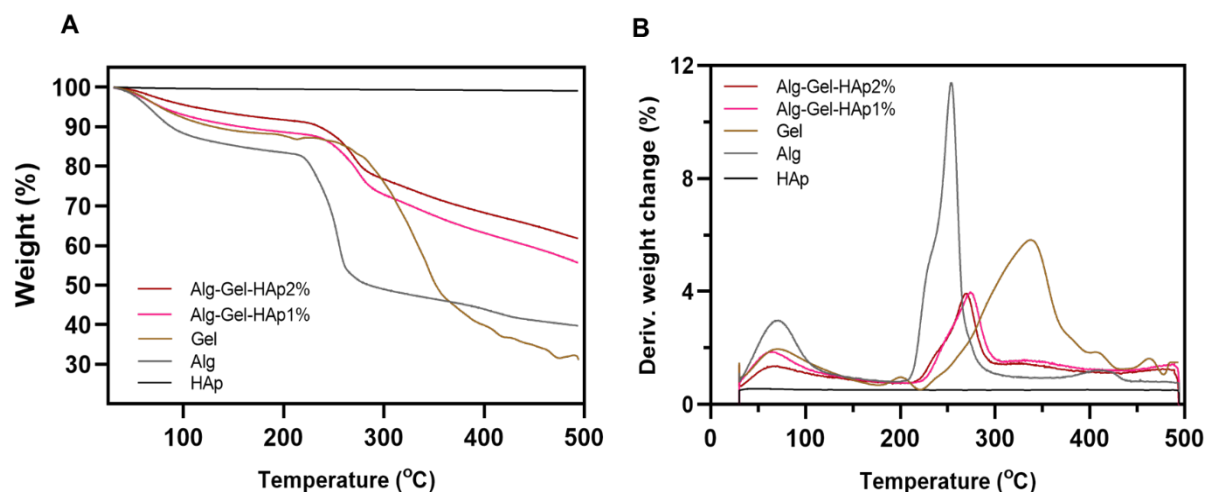


Figure 2. Thermogravimetric analysis of pre-crosslinked Alg-Gel-HAp and their individual components. (A) Mass loss profiles and (B) derivative thermogravimetric curves obtained from thermogravimetric analysis. Abbreviations: Alg: Alginate; Gel: Gelatin; HAp: hydroxyapatite.

2B) revealed a main degradation peak with a maximum degradation temperature ($T_{\max}$) close to 275 °C for both composite formulations. In contrast, Alg exhibited a lower $T_{\max}$ centered around 250 °C. This shift of the main degradation peak toward higher temperatures in the composite inks confirms an enhanced thermal stability driven by the incorporation of the inorganic phase. HAp showed no defined degradation peaks within the evaluated temperature window.

3.3. Evaluation of printability and structural fidelity of pre-crosslinked cell-free bioinks

3.3.1. Macroscopic evaluation and printing fidelity

The macroscopic morphology, shape fidelity, and structural integrity of the bioprinted scaffolds immediately post-printing and after stabilization are presented in Figure 3. The extrusion of square grid-like constructs successfully generated well-defined macrostructures featuring interconnected and visually homogeneous pore networks. However, variations in nozzle gauge, layer count, and HAp concentration led to distinct structural differences in filament definition. Constructs printed utilizing the narrower 22G nozzle displayed visibly thinner, sharper, and highly resolved filaments compared to those fabricated with the wider 20G nozzle (Figure 3A). This reduction in nozzle diameter restricted lateral material spreading, maintaining tighter geometrical tolerances and more defined pore boundaries.

Regarding layer stacking, the Alg-Gel-HAp2% formulation demonstrated superior shape retention in both 2-layer and 4-layer architectures. While the Alg-

Gel-HAp1% group exhibited minor filament relaxation and localized pore occlusion—particularly when scaling up to 4 layers with the 20G nozzle—the 2% w/v HAp group effectively preserved the squareness of the pore architecture and minimized structural sagging. To assess the geometric versatility and deposition accuracy of the formulation, alternative multi-layered complex geometries were successfully fabricated (Figure 3B). The extrusion system accurately reproduced a concentric grid-like internal pattern with continuous, open pore paths, as well as a biomimetic honeycomb architecture featuring well-resolved hexagonal pores. Both configurations maintained precise perimeter boundaries without experiencing material sagging or pore closure along the non-linear deposition paths.

3.3.2. Effects of printing parameters on scaffold characteristics

A total of 66 experimental observations were analyzed. The factorial ANOVA detected statistically significant effects for several printing parameters (Figure 4; Table S2). In relative terms, nozzle diameter showed the largest ω^2 values, followed by the number of layers. However, the absolute ω^2 estimates were small or negligible across all factors (maximum $\omega^2 = 0.034$), indicating that statistical significance should not be interpreted as strong practical or biological relevance.

Nozzle diameter was statistically significant for four out of five response variables ($p < 0.001$ for all). The largest relative effect was observed for pore area ($F = 202.50$, $p < 0.001$, $\omega^2 = 0.034$), followed by open pores ($F = 228.92$, p

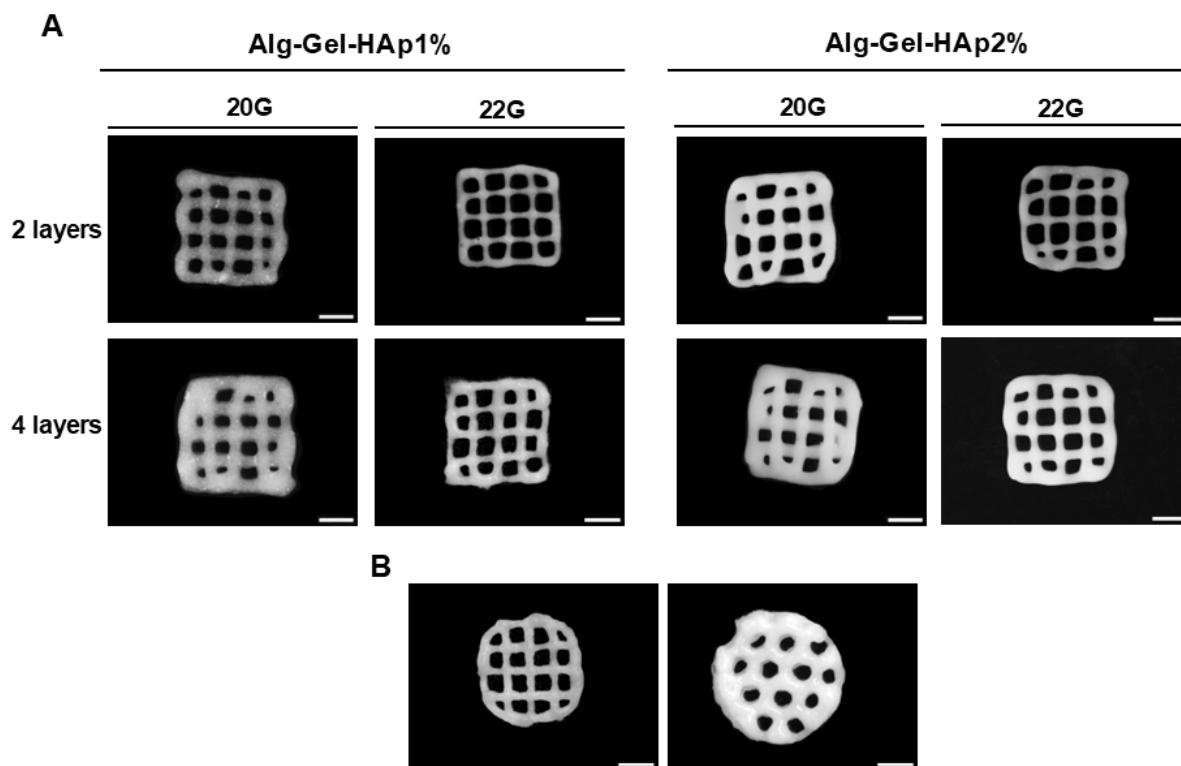


Figure 3. Morphological evaluation and printing fidelity of the bioprinted scaffolds. (A) Macroscopic top-view images of the grid-like scaffolds (dimensions: $10 \times 10 \times 2$ mm) printed under different processing conditions (from left to right: 1% and 2% of HAp), demonstrating shape fidelity and interconnected pore homogeneity. (B) Representative images of circular scaffold geometries showing geometric versatility and filament deposition accuracy (left: grid-like and right: honeycomb-like internal architectures, respectively). Scale bars: 2 mm.

< 0.001 , $\omega^2 = 0.011$), pore size ($F = 209.21$, $p < 0.001$, $\omega^2 = 0.009$), and pore perimeter ($F = 176.05$, $p < 0.001$, $\omega^2 = 0.008$). These ω^2 values indicate small effect magnitudes despite strong statistical evidence. Scaffolds printed with the smaller-inner-diameter 22G nozzle showed higher mean pore dimensions than those printed with the larger-inner-diameter 20G nozzle; mean pore size was $1,132 \mu\text{m}$ vs $940 \mu\text{m}$, and mean pore area was 0.749 mm^2 vs 0.515 mm^2 , respectively (Figure 5A).

The number of layers also showed statistically significant effects on scaffold properties, but the corresponding ω^2 estimates were small or negligible. Open pores decreased with increasing layer number ($F = 204.63$, $p < 0.001$, $\omega^2 = 0.010$), with scaffolds printed at 2 layers showing higher pore counts (mean = 15.5) than those at 4 layers (mean = 12.0) (Figure 5B). Pore area ($F = 27.80$, $p < 0.001$, $\omega^2 = 0.005$), pore perimeter ($F = 26.65$, $p < 0.001$, $\omega^2 = 0.001$), and pore size ($F = 19.83$, $p < 0.001$, $\omega^2 = 0.001$) were also statistically significant, but their effect sizes indicate limited practical magnitude. HAp level showed statistically

significant effects on printability index ($F = 21.40$, $p < 0.001$, $\omega^2 < 0.001$), open pores ($F = 55.17$, $p < 0.001$, $\omega^2 = 0.003$), and pore perimeter ($F = 7.84$, $p = 0.008$, $\omega^2 < 0.001$), but these effects were negligible to very small by ω^2 . Extrusion speed only affected pore size marginally ($F = 3.27$, $p = 0.048$, $\omega^2 < 0.001$), and this result was considered statistically weak with negligible practical magnitude.

The normality assumption was violated for open pores (Shapiro–Wilk $p < 0.001$) and pore area ($p = 0.007$). Non-parametric Kruskal–Wallis tests supported the presence of statistical differences for nozzle diameter ($\chi^2 = 21.81$, $p < 0.001$ for open pores; $\chi^2 = 38.85$, $p < 0.001$ for pore area) and number of layers ($\chi^2 = 25.68$, $p < 0.001$ for open pores; $\chi^2 = 14.63$, $p < 0.001$ for pore area). These tests were used as sensitivity checks for statistical significance; practical interpretation was based on the ω^2 effect-size estimates.

3.3.3. Exploratory multivariate visualization of printing outcomes

In the exploratory PCA, the first two components

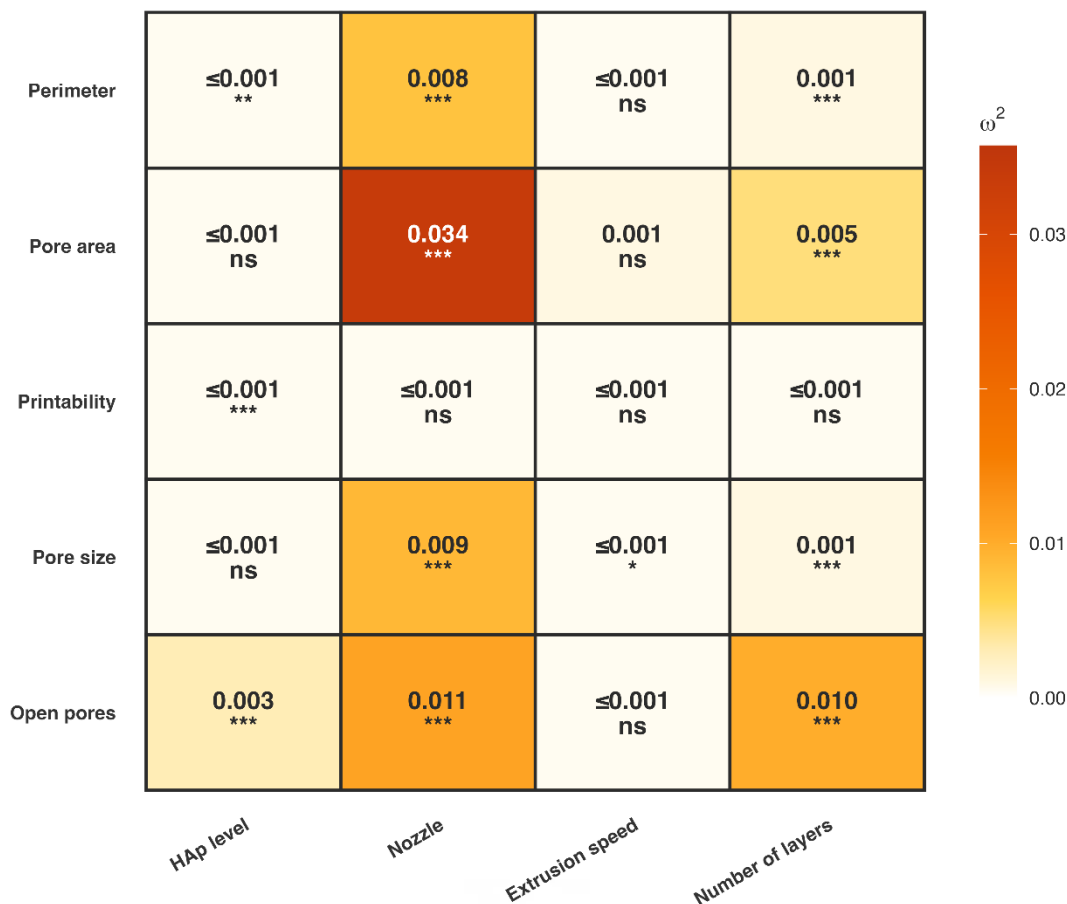


Figure 4. Heatmap of effect sizes (omega-squared, ω^2) from factorial ANOVA (Type III sum of squares) for all response variables and main experimental factors. Color intensity indicates effect magnitude, and cell values indicate ω^2 . Cells labeled ≤ 0.001 indicate negligible ω^2 estimates that were zero or ≤ 0.001 after bias correction and rounding. Asterisks indicate p -value thresholds (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant) and should be interpreted independently of effect magnitude. Nozzle diameter exhibited the largest relative effect sizes across most variables, particularly for pore area ($\omega^2 = 0.034$), followed by number of layers. However, all ω^2 values were below the conventional threshold for a medium effect, and most represented small or negligible effects.

Abbreviation: HAp: Hydroxyapatite.

summarized 89.4% of the total variance (PC1: 69.2%; PC2: 20.2%), providing a compact descriptive view of the measured scaffold properties (Figure 6). PC1 was mainly associated with pore morphometric variables, with high negative loadings for pore perimeter (−0.53), pore area (−0.53), pore size (−0.52), and open pores (−0.41). Therefore, PC1 can be interpreted descriptively as a pore-dimension gradient. PC2 was mainly aligned with printability index (loading = 0.99), suggesting that printability varied along an axis distinct from the pore morphology variables in this dataset.

The PCA biplot suggested a nozzle-associated distribution of samples along PC1. Scaffolds printed with the 22G nozzle tended to occur in the negative PC1 region, corresponding to larger pore dimensions, whereas 20G

nozzle scaffolds tended to occur in the positive PC1 region, corresponding to smaller pore dimensions. The limited overlap of the 95% confidence ellipses is consistent with the ANOVA results, but this exploratory pattern should not be interpreted as independent mechanistic evidence. HAp level showed a less distinct distribution in the PCA space, consistent with the smaller effect sizes observed in the ANOVA.

3.4. Microarchitectural characterization of bioprinted scaffolds

To evaluate the internal 3D architecture of the scaffolds, micro-CT analysis was performed. Representative 3D reconstructions allowed the visualization of the internal geometry, total porosity, and volumetric HAp distribution (Figure 7).

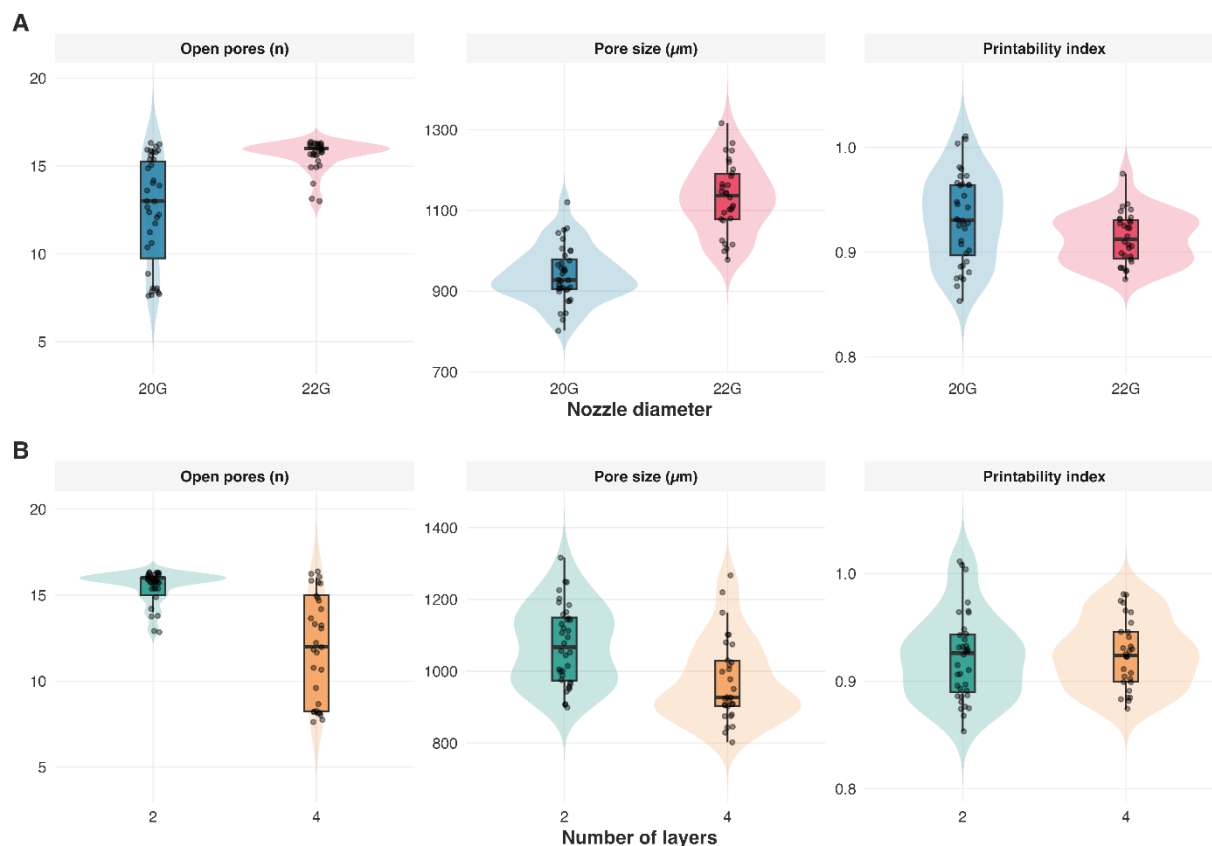


Figure 5. Effects of main experimental factors on scaffold properties. (A) Distribution of response variables by nozzle diameter (20G vs 22G). (B) Distribution of response variables by number of layers (2 vs 4). Violin plots show the probability density of the data, boxplots indicate the median and interquartile range, and individual points represent experimental observations ($n = 66$). The 22G nozzle was associated with higher mean pore dimensions (pore size: 1,132 μm vs. 940 μm ; pore area: 0.749 mm^2 vs. 0.515 mm^2), while increasing layer number from 2 to 4 was associated with lower pore count (15.5 vs 12.0) and pore size (1072 μm vs 973 μm).

The reconstructed datasets revealed a continuous network of interconnected pores distributed homogeneously throughout the scaffold volume. Cross-sectional side views confirmed structural continuity along the vertical axis, demonstrating excellent preservation of the multi-layered shape post-printing. Furthermore, the radiopaque HAp particles were uniformly dispersed throughout the hydrogel matrix, with only minor localized agglomerations (Figure 7A and 7B). Trabecular spacing distribution was evaluated as an indirect volumetric measure of effective internal pore spacing. In the Alg-Gel-HAp1% scaffolds, the spacing profile was characterized by a broad, heterogeneous plateau, exhibiting relatively balanced volume fractions across a wide range from 350 to 900 μm (Figure 7C). In contrast, the Alg-Gel-HAp2% scaffolds displayed a more concentrated spacing distribution, characterized by a pronounced peak centered tightly between 700 and 850 μm , which represented the dominant volumetric fraction (Figure 7D). Quantitative

total porosity analysis (Figure 7E) revealed that both formulations maintained highly porous internal networks, with average porosity values of 74% and 78% for the 1% and 2% w/v HAp bioinks, respectively.

3.5. Swelling behavior

The swelling kinetics of the bioprinted scaffolds are presented in Figure 8. All formulations exhibited a time-dependent increase in swelling ratio; however, the extent of hydration was significantly influenced by both the composition of the immersion medium and the HAp concentration. In general, swelling ratios were consistently higher in PBS than in DMEM throughout the 24 h incubation period. In PBS, the HAp-free scaffolds (Alg-Gel-HAp0%) displayed the highest swelling capacity, reaching approximately 460% after 24 h. The incorporation of HAp reduced water uptake in a concentration-dependent manner, with the Alg-Gel-HAp1% and Alg-Gel-HAp2% formulations reaching approximately

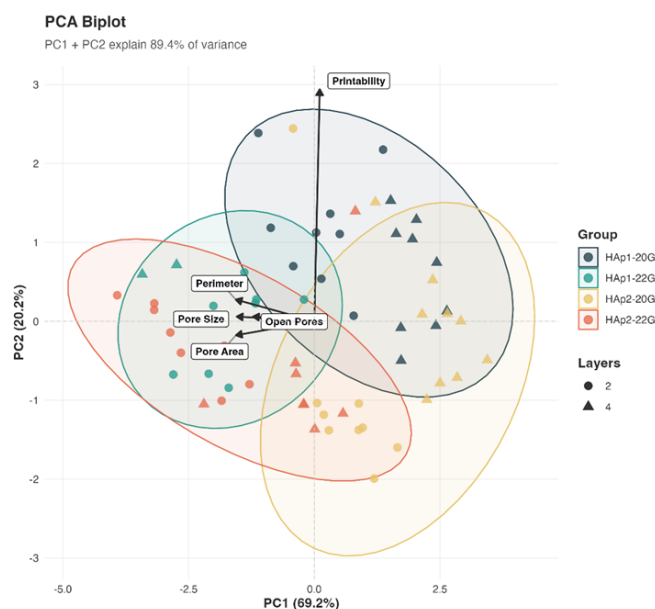


Figure 6. Principal Component Analysis (PCA) biplot of scaffold properties used as an exploratory visualization. Points represent individual samples colored by experimental group (HAp level $\times$ Nozzle diameter combination), with shapes indicating the number of layers (circles = 2 layers, triangles = 4 layers). Arrows show variable loadings on PC1 and PC2. Shaded ellipses represent 95% confidence regions for each group. PC1 (69.2% variance) is associated with pore dimensions (pore size, area, perimeter, and count), while PC2 (20.2% variance) is primarily aligned with printability index variation. The distribution of 20G and 22G samples along PC1 is consistent with the nozzle effect detected by ANOVA, but the PCA is descriptive and should not be interpreted as mechanistic or causal evidence.

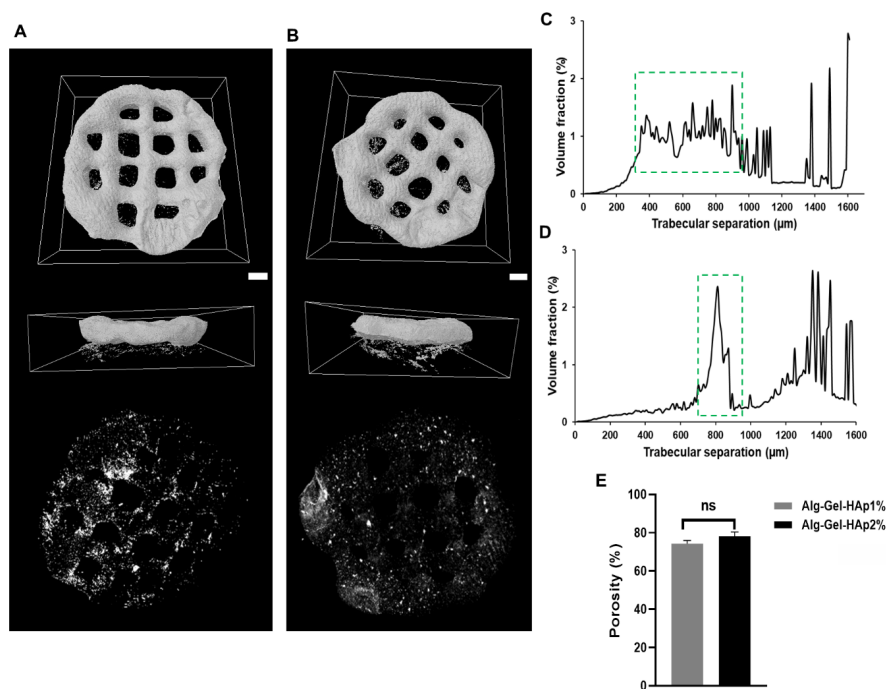


Figure 7. Microarchitecture of printed scaffolds. (A–B) Top views, side views, and HAP particle distribution in three-dimensional reconstructions of scaffolds printed with the 1% and 2% Alg–Gel–HAP bioinks, respectively. Scale bars: 200 μm ; equivalent magnification based on voxel size: 20 $\times$. (C–D) Trabecular spacing distributions for scaffolds formulated with the 1% and 2% Alg–Gel–HAP bioinks, respectively. (E) Average total porosity of the scaffolds. Dashed green boxes indicate the ranges with the highest volume fraction and, consequently, the dominant pore-size range. Results are presented as mean $\pm$ standard deviation ($n = 3$). No statistically significant differences (ns) were found in total porosity between groups (Mann–Whitney U test, $p > 0.05$). Abbreviations: Alg: Alginate; Gel: Gelatin; HAp: hydroxyapatite.

207% and 160%, respectively. A similar downward trend was observed in DMEM, although the overall swelling ratios were substantially lower across all groups. Under culture medium conditions, all groups showed a gradual, controlled increase in swelling over time, with the HAp-free control maintaining the highest values. Notably, the differences among formulations became less pronounced in DMEM compared to PBS.

3.6. Cell viability

The cytocompatibility of the bioprinted scaffolds was evaluated via a Live/Dead assay to determine the survival of encapsulated hDPSCs immediately post-bioprinting (3 h; day 0) and during *in vitro* culture (days 3 and 7). Representative epifluorescence micrographs demonstrated a clear predominance of viable hDPSCs (green fluorescence) across all formulations throughout the evaluation period, with dead cells (red fluorescence) occurring in low proportions at all time points (Figure 9). At day 0, the encapsulated cells exhibited a highly homogeneous spatial distribution within the 3D filaments. Throughout day 3 and day 7, the constructs successfully sustained this high proportion of live cells, confirming that the formulated composite matrices provided a cytocompatible microenvironment.

Quantitative cell viability analysis confirmed these

highly favorable biological trends (Figure 10). At day 0, the Alg-Gel-HAp1% group exhibited significantly higher cell viability compared to the Alg-Gel-HAp2% group ($p < 0.05$), while the HAp-free control displayed intermediate baseline values. After three days of culture, a significant increase in cell viability was observed in both HAp-loaded formulations, with the Alg-Gel-HAp2% group reaching the highest overall values. By day 7, both the Alg-Gel-HAp1% and Alg-Gel-HAp2% scaffolds sustained significantly higher cell viability compared to the HAp-free control group ($p < 0.01$), with both composite groups reaching viability levels close to 100%. Notably, the HAp-free control group exhibited higher variability across independent replicates, whereas the HAp-containing groups demonstrated a highly homogeneous and reproducible biological response over time.

3.7. Cell morphology and cytoskeletal organization

Fluorescent cytoskeleton staining revealed a uniform 3D distribution of encapsulated hDPSCs throughout the bioprinted scaffolds across all experimental conditions (Figure 11A). The cells remained well-dispersed, confirming that the incorporation of the mineral phase up to 2% w/v did not induce cell aggregation or precipitation during the extrusion process. Quantitative morphometric descriptors confirmed that the addition of the mineral

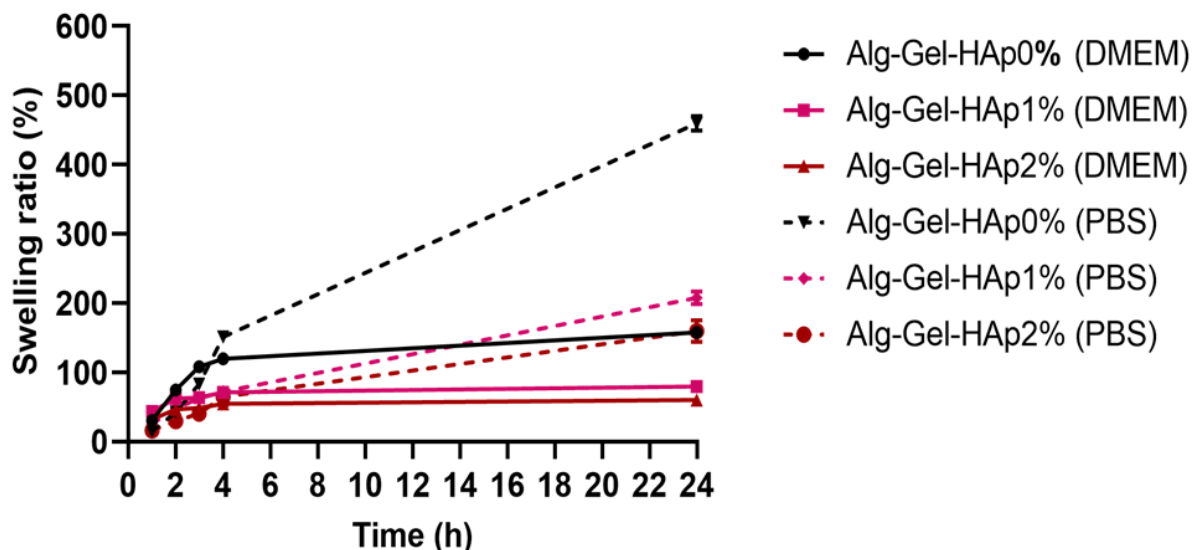


Figure 8. Swelling behavior of pre-crosslinked Alg-Gel scaffolds containing 0%, 1%, and 2% (w/v) hydroxyapatite during incubation in PBS and DMEM. Solid lines represent swelling ratios measured in PBS, whereas dashed lines represent swelling ratios measured in DMEM. Data are presented as mean $\pm$ standard deviation ($n = 3$).

Abbreviations: Alg: Alginate; Gel: Gelatin; HAp: hydroxyapatite; PBS: Phosphate-buffered saline.

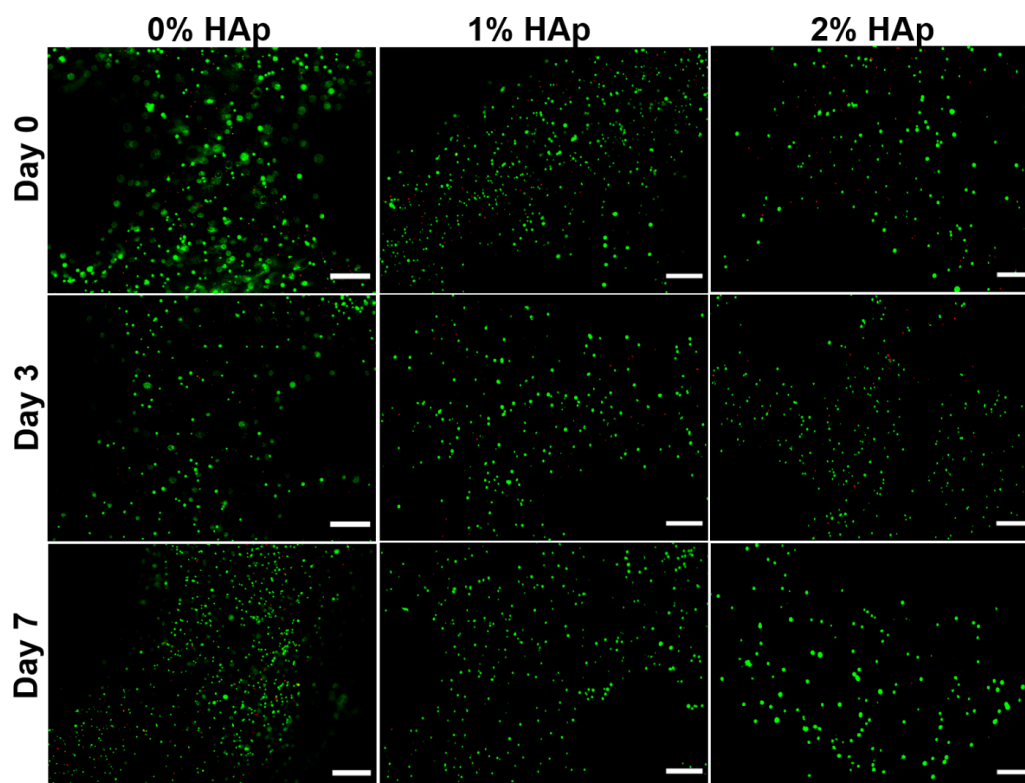


Figure 9. Live/Dead assay representative images of bioinks (loaded with 0%, 1%, and 2% HAp) on days 0 (within 3h after extrusion), 3, and 7 after bioprinting. Green dots in images represent live cells, and red dots represent dead cells. Scale bars: 200 μm , magnification: 4 $\times$.

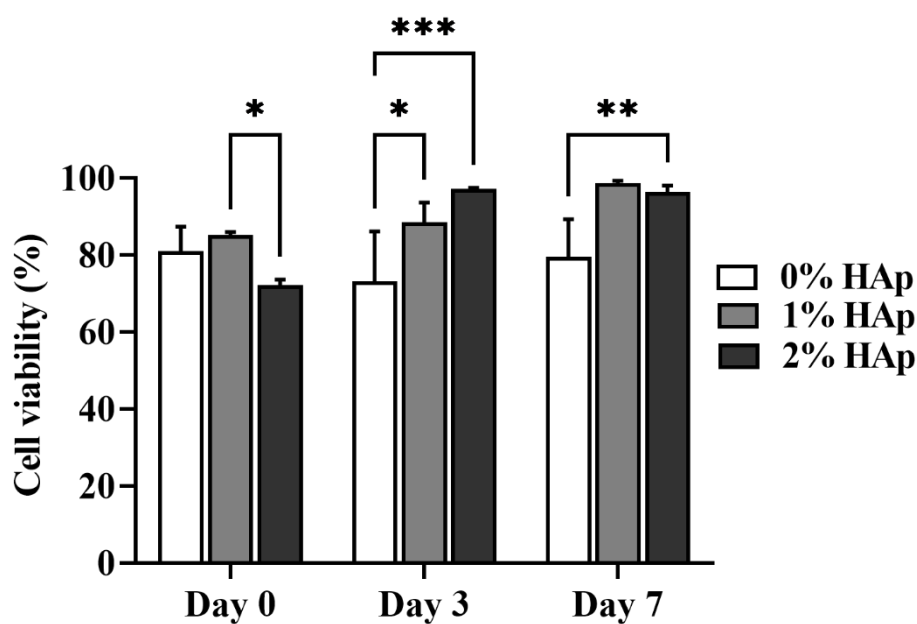


Figure 10. Quantitative evaluation of cell viability based on Live/Dead staining. Cell viability is represented as mean $\pm$ standard deviation ($n = 3$). Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. Brackets indicate pairwise comparisons between scaffold formulations at the same time point. Statistical significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Abbreviation: HAp: Hydroxyapatite.

phase significantly modulated the morphological adaptation of hDPSCs within the hydrogel matrix (Figure 11B–D). The HAp-free control group (Alg–Gel–HAp0%) exhibited a tightly restricted cell area, with values densely clustered below $200\ \mu\text{m}^2$ (Figure 11B), while retaining a predominantly spherical morphology characterized by a low aspect ratio (~ 1.15 ; Figure 11C) and a high circularity index (~ 0.92 ; Figure 11D). Conversely, the incorporation of 1% w/v HAp induced a pronounced shift in cellular adaptation, expanding the median cell spreading area to approximately $350\ \mu\text{m}^2$. This morphological shift was accompanied by a broader, more heterogeneous distribution in circularity and a noticeable increase in the aspect ratio, with several outliers exceeding 1.5, indicating localized cytoskeletal elongation and initial filopodial extension. Interestingly, increasing the HAp content to 2% w/v sustained the enlarged cell spreading area ($340\ \mu\text{m}^2$) but resulted in a reversion of both circularity (~ 0.90) and aspect ratio (~ 1.10) toward a more uniform, rounded distribution similar to the control. These findings demonstrate that

while HAp effectively dictates early cell spreading areas, its capacity to promote directional cytoskeletal elongation follows a non-linear, concentration-dependent trend within this 3D microenvironment.

4. Discussion

Extrusion 3D bioprinting represents a suitable platform to recreate the complex architecture, organization, and spatial distribution of cells and ECM found in native tissues. This approach is based on a tight interplay between multiple factors such as bioink composition, printing process parameters, scaffold architecture, and cell functionality.^{8,35} Among these, the definition of bioink components and formulation strategy are one of the initial and most critical steps in bioprinting, as material-level decisions directly affect printability, structural fidelity, and the biological microenvironment involving the encapsulated cells.³⁶ Alg, Gel, and HAp are biomaterials commonly used to fabricate bone-related scaffolds due to their complementary physicochemical and bioactive properties. The

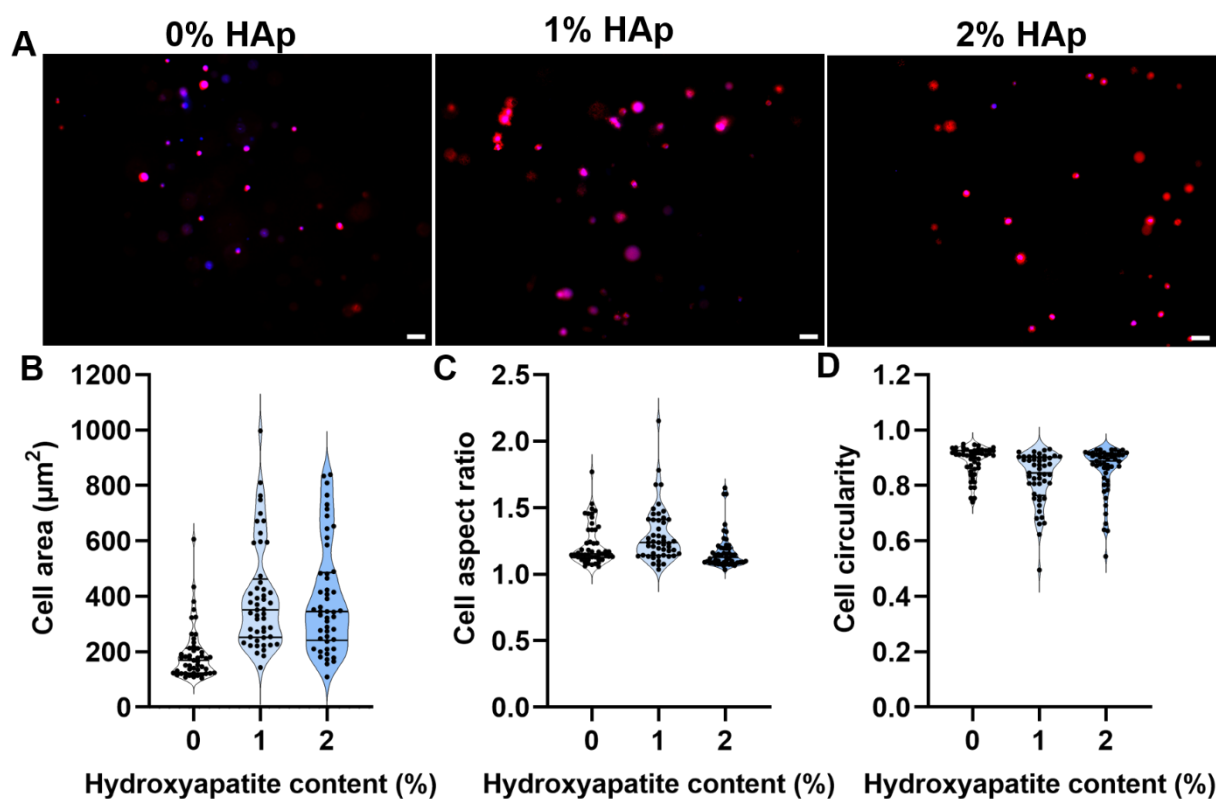


Figure 11. Morphological characterization of the human dental pulp stem cells encapsulated within 3D bioprinted scaffolds at day 7 post-printing. (A) Representative epifluorescence microscopy images showing cell morphology in experimental groups, where nuclei are stained in blue and the cytoskeleton in red. Scale bars: $80\ \mu\text{m}$; magnification: $10\times$. Quantitative analysis of cell shape descriptors, including (B) cell area, (C) aspect ratio, and (D) cell circularity ($n = 50$ cells analyzed for each parameter). Morphological measurements were performed using Fiji/ImageJ from three representative images per group. Abbreviation: HAp: Hydroxyapatite.

combination of these biomaterials has been reported as the acellular component of bioinks capable of supporting the fabrication of stable and structurally defined 3D bioprinted structures.¹⁵

The structural and chemical integrity of the constituent phases within a composite bioink directly dictates its post-printing functional performance. In this study, FT-IR analysis confirmed the successful preservation of the spectral footprints for sodium Alg, Gel, and HAp within the fabricated formulations. The absence of novel covalent absorption bands or significant chemical shifts suggests that the ink formulation process is predominantly governed by non-covalent physical entrapment and ionic crosslinking, rather than altered chemical identities. Retaining the native physicochemical state of Gel and HAp is crucial; it ensures that the biochemical motifs responsible for cell-matrix recognition and osteoconductive signaling remain structurally accessible to the encapsulated cells. This interfacial integration is further supported by the thermogravimetric profiles. The macromolecular decomposition peak shift toward higher temperatures in the composite formulations, relative to Alg, underscores an enhanced thermal stability driven by the mineral phase. This stabilizing mechanism is consistent with previous reports by Anaya-Sampayo *et al.*,¹⁶ demonstrating that highly dispersed HAp nanopowders act as effective thermal barriers that restrict the diffusion of volatile degradation products. Thermodynamically, this structural shift provides indirect evidence of strong interfacial wetting and physical compatibility between the inorganic nanoparticles and the surrounding polymeric network, a phenomenon that fundamentally alters the structural and mechanical behavior of the printed filaments.

A primary challenge in functional bioink design is balancing structural shape fidelity with stable extrusion processing. The multi-variable factorial ANOVA demonstrated that while operational parameters significantly influenced final scaffold morphometry, the absolute effect sizes remained remarkably low across all individual factors. Rather than indicating a lack of experimental control, this low practical magnitude reveals a highly desirable architectural robustness: the pre-crosslinked Alg-Gel-HAp matrix maintains structural stability and processing predictability across the entire investigated operational window, shielding the printing outcomes from minor systemic fluctuations. The dominant relative effect size of the nozzle diameter on pore morphometry aligns with microfluidic and geometric principles governing extrusion-based additive manufacturing. The narrower 22G nozzle consistently yielded wider, more defined pore dimensions by restricting

lateral material spreading and maintaining a high surface-area-to-volume ratio during deposition. Conversely, the progressive reduction in pore count and dimensions observed when scaling the architecture from 2 to 4 layers reflects a cumulative mechanical loading phenomenon. During layer-by-layer stacking, the underlying uncrosslinked filaments undergo continuous vertical compressive deformation under the weight of successive layers, inducing minor lateral relaxation and partial pore occlusion. This behavior mirrors the trends documented for collagenous matrices by Pottathara and Kokol,³⁷ highlighting the universal challenge of gravity-induced material sagging in multi-layered hydrogels.

The incorporation of incremental HAp content emerged as an effective formulation strategy to mitigate this structural relaxation. At 2% w/v HAp, the increased solid fraction allows the rigid ceramic nanoparticles to function as physical reinforcing fillers that restrict the conformational mobility of the Alg-Gel chains. This steric hindrance dampens the post-printing structural relaxation of the filament, yielding higher printability index values that closely approximate the ideal theoretical square pore geometry. The multivariate exploratory PCA further corroborated this trend, demonstrating that while spatial pore dimensions vary primarily along a nozzle-driven gradient (PC1), printability behaves as an independent operational axis (PC2) directly modulated by the solid-fraction chemistry of the ink. Previous studies have reported that HAp incorporation can improve the structural stability of hydrogel-based bioinks by reinforcing the polymeric network and enhancing shape retention after deposition.^{13,14} In this context, the higher ceramic content may restrict polymer chain mobility and reduce structural relaxation of the deposited filaments, thereby limiting filament spreading and promoting better preservation of pore geometry. Furthermore, steric interactions between HAp particles and the surrounding polymer network may contribute to increased resistance to deformation during layer-by-layer deposition, resulting in a more uniform pore architecture.

Beyond superficial shape fidelity, the internal 3D microarchitecture of a bioprinted scaffold governs its mass-transport efficiency and ultimate tissue-in-growth potential. The micro-CT analysis confirmed that both composite formulations achieved high, statistically equivalent total porosities (74–78%), aligning with the established morphological requirements for trabecular bone substitutes, which typically range between 50% and 90%.^{38,39} This indicates that the extrusion process and subsequent ionic crosslinking preserve an open, interconnected porous network capable of supporting

unobstructed nutrient diffusion and metabolic waste clearance. However, the volumetric distribution of internal spacings revealed distinct structural differences driven by composition. While the 1% w/v HAp group exhibited a broad, heterogeneous plateau indicative of irregular filament boundaries and variable pore dimensions, the 2% w/v HAp formulation produced a highly concentrated distribution centered tightly between 700 and 850 μm . This structural refinement indicates that a higher mineral content promotes a more homogeneous internal architecture by stabilizing filament dimensions uniformly across the entire 3D volume. Notably, this dominant spacing range directly matches the structural bottleneck dimensions (700–1200 μm) identified by Ghayor and Weber⁴⁰ as highly favorable for bone substitute materials. Consequently, the 2% w/v HAp formulation successfully combines high macrostructural interconnectivity with a microarchitectural uniformity that closely mimics native bone environments, although its relevance for bone regeneration remains to be experimentally demonstrated.

The swelling behavior of bioprinted crosslinked scaffolds is a foundational determinant of scaffold performance, directly governing long-term structural retention, pore interconnectivity, and mediated nutrient-waste exchange post-printing. The concentration-dependent restriction in water uptake observed upon the incorporation of the mineral phase reflects a distinct physical and thermodynamic modification of the hydrogel network. Structurally, the insoluble ceramic particles function as rigid physical fillers embedded within the hydrophilic Alg–Gel mesh. These clusters restrict the conformational mobility of the surrounding polymeric chains and increase matrix tortuosity, which effectively minimizes the free volume available for water entrapment. This mechanism and its subsequent impact on matrix density are strongly corroborated by recent investigations into the influence of HAp and Gel content on the crosslinking dynamics of Alg bioinks.¹⁶ Specifically, it has been demonstrated that the chemical interplay between the inorganic HAp phase and the Gel domains significantly modulates the internal crosslinking efficiency of the Alg matrix. The introduction of HAp creates a more compact, sterically hindered network that physically restricts polymer chain relaxation, directly leading to the concentration-dependent reduction in water uptake observed in our composite scaffolds. Thermodynamically, this trend is further driven by the volumetric substitution of the highly hydrophilic polymer mass—and its associated water-binding carboxyl and amide functional groups—with the inorganic HAp fraction, thereby dampening the overall osmotic drive for fluid absorption.

Sustaining high post-printing cell viability is a key criterion for evaluating a bioprinting process. The high survival rates recorded across all groups throughout the 7-day culture window confirm that the pre-crosslinked Alg–Gel–HAp system and the selected operational parameters do not induce irreversible mechanical lysis. The transient reduction in cell viability observed exclusively in the 2% w/v HAp group immediately post-printing (day 0) highlights a classic trade-off in rheological modification. Incorporating a higher solid mineral fraction increases the viscosity and dynamic shear stresses within the nozzle during extrusion, augmenting the susceptibility of encapsulated cells to membrane deformation and shear-induced damage. This phenomenon aligns with the findings of Kolan *et al.*,²⁵ who reported an immediate decline in cell survival upon increasing the inorganic filler content in bioprinted hydrogels. However, the complete recovery of viability close to 100% by day 7 demonstrates that this initial mechanical stress was fully reversible, confirming that the composite matrix provides a highly supportive microenvironment.

Regarding our cellular model, late-passage (P10) hDPSCs were intentionally utilized. While prolonged *in vitro* expansion can progressively diminish long-term multi-lineage differentiation kinetics and stemness retention, extensive literature confirms that late-passage mesenchymal stem cells fully retain their short-term structural survival, early cell-matrix attachment mechanics, and initial morphological responsiveness.^{41–43} This makes them a highly appropriate and sensitive model for evaluating the immediate, formulation-driven biological microenvironment within a 7-day screening window. The significant increase in viability and the marked reduction in replicate variability documented in both HAp-containing groups by day 7 further indicate that the mineral phase provides vital material-derived signals—likely through local calcium/phosphate homeostatic fluctuations and biomimetic surface-mediated interactions—that actively enhance hDPSC adaptation and survival compared to the HAp-free control group.

Cell morphology analysis provided complementary insights regarding early scaffold-cell architectural interactions. Cells in the HAp-free control group maintained a predominantly rounded morphology, characterized by relatively small projected areas, high circularity values, and low aspect ratios. This morphological profile is consistent with previous reports showing that highly crosslinked Alg matrices restrict cell spreading due to the limited availability of cell-adhesive sites and the reduced capacity for matrix remodeling. For instance, Zhang *et al.*⁵ observed that increasing Alg concentration promoted the formation

of compact cellular aggregates rather than interconnected cellular networks. Similarly, Gonzalez-Fernandez *et al.*²⁷ observed limited cell spreading in a scaffold with increased Alg crosslinking density, despite maintaining high cell viability. Interestingly, the incorporation of HAp markedly altered this morphological response. Both HAp-containing formulations promoted a substantial increase in cell area compared with the control group, indicating enhanced cell spreading within the hydrogel environment. This observation suggests that the mineral phase modifies the local microenvironment in a manner that facilitates cell-material interactions, even within the constraints imposed by the Alg network. However, the effect of HAp on cell shape was not strictly proportional to its concentration. The Alg-Gel-HAp1% formulation induced greater variability in circularity and aspect ratio, showing signs of localized cytoskeletal elongation. Conversely, cells encapsulated in the Alg-Gel-HAp2% scaffolds exhibited a more homogeneous and rounded morphology, despite maintaining a similarly enlarged cell area. This non-linear, concentration-dependent trend indicates that while HAp effectively provides biochemical anchoring cues that expand the cell area, an excessive inorganic fraction (2%) may increase local microstructural stiffness and induce steric hindrance. This structural compaction likely confines the cells, restricting their physical capacity for outward cytoskeletal elongation. Despite the increase in cell area observed following HAp incorporation, hDPSCs in all experimental groups retained a predominantly rounded configuration throughout the 7-day culture window. Several structural factors contribute to this response. First, the dual ionic crosslinking strategy employed in this study generated a dense hydrogel network that constrained extensive cytoskeletal extension and cell migration. Alg-based hydrogels provide excellent structural stability; however, their limited susceptibility to cell-mediated proteolytic degradation often restricts the development of highly elongated, spindle-like morphologies. In addition, although Gel contains RGD cell-adhesive motifs that support attachment, the accessibility of these sites may be partially hindered within the interpenetrating ionically crosslinked Alg network, limiting the establishment of the extensive focal adhesions required for pronounced elongation.

Another crucial factor to consider is that cell morphology within 3D hydrogel matrices differs substantially from the artificial stretching observed on conventional two-dimensional plastic substrates. In 3D microenvironments, rounded or partially spread morphologies are not indicative of poor cellular performance at this early experimental stage, but rather reflect the spatial confinement and physicochemical characteristics of the surrounding

mineralized matrix. Indeed, the high cell viability observed across all groups confirms that the encapsulated hDPSCs remained functional and structurally responsive within the architectural boundaries of the composite bioink.

5. Conclusion

In this study, Alg-Gel-HAp composite bioinks were successfully developed and characterized for extrusion-based bioprinting applications. The results demonstrated that scaffold macro-architecture was primarily governed by printing parameters, particularly nozzle diameter and layer number, whereas HAp incorporation acted as a structural and compositional modulator that improved shape fidelity and governed the internal uniformity of the porous network. The resulting constructs exhibited highly interconnected, open-porosity frameworks with volumetric pore distributions matching the native microarchitectural benchmarks of human trabecular bone. Physicochemically, the inclusion of the mineral phase significantly restricted hydrogel swelling through network tightening and steric hindrance, a phenomenon strongly driven by altered crosslinking dynamics and chain relaxation pathways. Notably, this structural matrix remained highly stable under physiological environments, where exposure to cell culture medium (DMEM) triggered calcium homeostatic buffering and surface passivation, successfully preventing extensive swelling-induced macrostructural distortion.

The biological evaluation confirmed that all bioink formulations supported high hDPSC viability post-bioprinting and throughout a 7-day screening window, establishing the cytocompatibility of the selected extrusion window. Moreover, HAp-containing scaffolds actively promoted enhanced cell survival and adaptive cell-spreading kinetics compared with the Alg-Gel control scaffolds without HAp, indicating that the mineral phase provides essential material-derived cues within the 3D hydrogel microenvironment. Relatively low HAp concentrations (1–2% w/v) proved sufficient to induce measurable, favorable shifts in printability, scaffold stabilization, and early cellular adaptation, highlighting the high sensitivity of the interpenetrating network to minor inorganic modifications.

Overall, these findings underscore the strict interdependence between composite bioink composition, processing performance, architectural stability, and biological response, supporting their continued evaluation as functional candidates for bone tissue engineering. Nevertheless, further studies incorporating comprehensive rheological and mechanical profiles and long-term multi-lineage osteogenic differentiation analyses remain necessary to establish the absolute regenerative capacity of

these bioprinted scaffolds.

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Conflict of interest

The authors declare no conflicts of interest.

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Writing—review & editing: All authors

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The protocol for the isolation and use of hDPSCs was approved by the Scientific Ethics Committee of the Universidad de La Frontera (Protocol No. 156_24). Informed consent was obtained from all subjects involved in the study prior to tooth extraction.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon

reasonable request.

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