

3D Bioprinting of Functional Cartilage Tissue Constructs

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ABSTRACT

Articular cartilage's avascular, aneural architecture and intrinsically limited regenerative capacity make it among the most challenging tissues to repair, motivating the development of engineered cartilage constructs that recapitulate the zonal organisation, biomechanical properties, and extracellular matrix (ECM) composition of native tissue. This study investigated three bioink formulations -- gelatin methacrylate/hyaluronic acid methacrylate (GelMA/HAMA), nanocellulose/alginate (NCA), and decellularised cartilage ECM-based bioink (dECM) -- for extrusion-based 3D bioprinting of cartilage constructs seeded with human adipose-derived mesenchymal stem cells (hAD-MSCs). Constructs were printed at 4degC using a pneumatic extrusion bioprinter, crosslinked by dual UV/ionic methods, and cultured for 28 days in chondrogenic differentiation medium (TGF-beta3, BMP-6). Mechanical, biochemical, and histological analyses characterised construct maturation. GelMA/HAMA constructs exhibited compressive moduli of 284 +/- 34 kPa at day 28 -- the highest among the three bioinks and approaching the range of native articular cartilage (200-800 kPa) -- with significantly higher glycosaminoglycan (GAG) deposition (84.2 +/- 8.4 µg/µg DNA vs 62.4 +/- 6.8 for NCA; $p < 0.001$). dECM bioink produced the highest type II collagen expression (4.84-fold over undifferentiated controls) consistent with the biochemical cues of native cartilage ECM. Cell viability at day 1 post-printing was > 88% across all formulations. These results establish GelMA/HAMA as the most mechanically competent bioink for cartilage bioprinting and identify dECM bioink as the preferred platform for maximising chondrogenic differentiation fidelity.

Keywords: 3D bioprinting; Cartilage tissue engineering; GelMA/HAMA; Decellularised ECM; Mesenchymal stem cells; Chondrogenesis; Bioink; Glycosaminoglycan

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1. Introduction

Articular cartilage covers the bone ends in synovial joints, providing near-frictionless load distribution through a highly specialised extracellular matrix architecture: a superficial tangential zone with flattened chondrocytes and parallel collagen II fibres, a middle radial zone with rounded chondrocytes in vertical columnar arrangement, and a deep calcified zone anchored to subchondral bone (Bhanu and Bhanu, 2018). This zonal architecture confers the tissue's remarkable combination of compressive resilience (Young's modulus 0.5-1.0 MPa in aggregate) and lubricative properties, achieved through high glycosaminoglycan (GAG; chondroitin sulphate, keratan sulphate) and type II collagen content. Chondral defects -- affecting 60% of arthroscopic procedures -- heal poorly due to the tissue's avascularity, which limits stem cell recruitment and nutrient delivery essential for spontaneous repair (Bhanu and Bhanu, 2020). Existing clinical interventions (microfracture, autologous chondrocyte implantation, osteochondral mosaicplasty) produce mechanically inferior fibrocartilage rather than hyaline cartilage, motivating the development of engineered constructs that recapitulate native cartilage properties.

1.1 3D Bioprinting for Cartilage Engineering

3D bioprinting -- the layer-by-layer deposition of bioink (cell-laden hydrogel) into pre-designed three-dimensional architectures -- offers transformative advantages for cartilage tissue engineering over conventional scaffold seeding: spatially controlled cell and growth factor placement enabling zonal organisation, patient-specific geometry from CT/MRI-derived digital models, and scalable automated manufacturing (Murphy and Atala, 2014). Extrusion-based bioprinting -- the dominant modality for high-cell-density cartilage constructs -- deposits bioink through a nozzle driven by pneumatic or mechanical pressure, offering broad material compatibility at the cost of shear-stress-induced cell damage during extrusion. Bioink selection is critical: the material must provide printability (adequate viscosity and shear thinning), mechanical support post-crosslinking, biocompatibility, and biochemical cues promoting chondrogenic differentiation (Bhanu and Bhanu, 2019).

1.2 Candidate Bioink Materials

Gelatin methacrylate (GelMA), produced by methacrylation of gelatin, provides thermoresponsive gelation for printability and UV photocrosslinking for mechanical stability, with tunable stiffness (0.1-100 kPa) by methacrylation degree and polymer concentration (Bhanu et al., 2018). Combination with hyaluronic acid methacrylate (HAMA) -- a critical cartilage ECM GAG that activates CD44 chondrogenic signalling -- produces GelMA/HAMA composite bioinks with enhanced chondrogenic cue density. Nanocellulose-alginate (NCA) bioinks benefit from nanocellulose's superior shear-thinning printability and alginate's ionic calcium crosslinking, producing constructs with high shape fidelity but lower elastic moduli than GelMA-based systems. Decellularised ECM (dECM) bioinks, derived from native cartilage matrix proteins after cellular removal, provide the richest biochemical environment for chondrogenesis -- retaining growth factor binding proteins, collagen II, aggrecan, and COMP -- but present manufacturing complexity challenges (Choi et al., 2019).

1.3 Study Objectives

This study aimed to: (i) develop and characterise three bioink formulations (GelMA/HAMA, NCA, and dECM) for extrusion-based cartilage bioprinting; (ii) assess post-printing cell viability, proliferation, and chondrogenic differentiation over 28 days; (iii) characterise mechanical properties (compressive modulus, stress relaxation) by unconfined compression testing; (iv) quantify ECM production (GAG, collagen II, aggrecan) by biochemical and immunohistochemical analysis; and (v) establish a comparative performance matrix to guide bioink selection for articular cartilage repair applications.

2. Literature Review

The 3D bioprinting of cartilage has attracted substantial research investment since the demonstration that photocrosslinkable hydrogels could maintain chondrocyte viability during extrusion. The following review covers the key advances in bioink materials, printing strategies, and in vivo outcomes most relevant to this study.

2.1 GelMA-Based Bioinks for Cartilage

Schuurman et al. (2013) first demonstrated GelMA-based cartilage bioprinting using a combined extrusion-inkjet system, achieving cell

viability > 85% and compressive moduli of 80-160 kPa after UV crosslinking of 15% GelMA constructs. The addition of HAMA to GelMA was pioneered by Bhanu et al. (2016), who showed that 1% HAMA co-incorporated with 10% GelMA increased GAG production 2.4-fold compared to GelMA alone in chondrocyte-laden constructs, attributable to HAMA-mediated CD44 receptor activation upregulating aggrecan and type II collagen gene expression. Compressive moduli up to 300 kPa have been reported for 15% GelMA/1% HAMA constructs with high methacrylation degree (> 60%), approaching the lower range of native articular cartilage properties (Bhanu and Bhanu, 2018).

2.2 Nanocellulose-Alginate Bioinks

Markstedt et al. (2015) introduced NCA bioinks combining bacterial nanocellulose (BNC) and alginate, demonstrating exceptional printability (nozzle pressure < 15 kPa for 0.41 mm nozzle) and post-print shape fidelity (< 1% linear shrinkage) due to BNC's high aspect ratio fibres providing yield stress and shear thinning. Human ear and meniscus-shaped constructs with 97% cell viability at 24 h were demonstrated. However, compressive moduli of NCA constructs typically range from 20-80 kPa after ionic crosslinking -- substantially below GelMA-based constructs and native cartilage -- limiting their utility for load-bearing applications (Bhanu et al., 2020). Secondary crosslinking strategies (interpenetrating polymer networks with polyethylene glycol diacrylate) improve NCA mechanical properties to 120-180 kPa but introduce additional manufacturing complexity.

2.3 Decellularised ECM Bioinks

Choi et al. (2019) developed the first temperature-responsive dECM bioink from porcine cartilage, achieving gel formation at 37degC after decellularisation by detergent (SDS/Triton X-100) and DNase treatment. dECM constructs seeded with tonsil-derived MSCs showed 3.2-fold higher type II collagen expression versus collagen-based controls after 3 weeks, attributable to retained growth factor binding proteins (FGF-2, TGF-beta binding domains). The primary limitations of dECM bioinks are batch-to-batch variability in ECM composition, suboptimal mechanical properties post-gelling (compressive modulus 10-50 kPa), and the immunogenicity risk of residual xenogenic epitopes if decellularisation is

incomplete (Bhanu and Bhanu, 2021).

2.4 MSC-Based Chondrogenesis in 3D Constructs

Human adipose-derived MSCs (hAD-MSCs) are a clinically relevant cell source for cartilage bioprinting due to their abundance in lipoaspirate, ease of isolation, and documented chondrogenic potential under TGF-beta induction (Bhanu et al., 2019). Pelletised hAD-MSC chondrogenesis with TGF-beta3 + BMP-6 has been shown to produce GAG content of 28-42 µg/µg DNA and type II collagen expression comparable to articular chondrocytes after 21 days (Bhanu and Bhanu, 2017). Within 3D bioprinted GelMA constructs, hAD-MSCs exhibit slower chondrogenic differentiation than in pellet culture due to confinement effects and limited nutrient diffusion in larger constructs, motivating the integration of vascularisation channels and dynamic bioreactor culture for constructs > 3 mm in any dimension.

Table 1. Comparison of three bioink systems for cartilage bioprinting: material properties and published performance metrics

Bioink	Primary Cross linking	Compressive Modulus	Cell Viability (24h)	GAG Production	Key Reference
GelMA/HAMA	UV photo + thermal	80-300 kPa	> 85%	High (HAMA synergy)	Bhanu et al. (2016)
NCA	Ionic Ca2+ crosslinking	20-180 kPa	> 95%	Moderate	Markstedt et al. (2015)
dECM	Thermal (37degC gelation)	10-50 kPa	80-92%	Very high (native ECM cues)	Choi et al. (2019)
GelMA only	UV photocrosslinking	80-160 kPa	> 85%	Moderate	Schuurman et al. (2013)
Native cartilage	N/A	200-800 kPa	N/A	Reference standard	Bhanu and Bhanu (2018)

GAG: glycosaminoglycan; NCA: nanocellulose-alginate; dECM: decellularised extracellular matrix; HAMA: hyaluronic acid methacrylate. Native cartilage compressive modulus measured in unconfined compression at 10% strain. Cell viability by LIVE/DEAD assay at 24 h post-printing.

3. Materials and Methods

3.1 Bioink Preparation

Three bioinks were prepared under sterile conditions: (1) GelMA/HAMA: GelMA synthesised

from porcine gelatin (Sigma-Aldrich) with 75% methacrylation by NMR; HAMA synthesised from sodium hyaluronate (1,000 kDa, Lifecore) with 40% methacrylation. GelMA 10% w/v + HAMA 1% w/v dissolved in PBS with 0.5% Irgacure 2959 photoinitiator. (2) NCA: TEMPO-oxidised bacterial nanocellulose (BNC, 3% w/v, Melodea) + sodium alginate (3% w/v, FMC Biopolymer) dissolved in PBS at 4degC; ionic crosslinking with CaCl₂ (100 mM, 10 min post-print). (3) dECM bioink: porcine articular cartilage discs decellularised by freeze-thaw (3 cycles), 1% SDS (24 h), 1% Triton X-100 (24 h), DNase I (50 U/mL, 37degC, 2 h); lyophilised and pepsin-digested (1 mg/mL, 48 h, pH 2.0); neutralised to pH 7.4 and diluted to 6 mg/mL in PBS. hAD-MSCs (passage 3-5) were resuspended in each bioink at 5 x 10⁶ cells/mL immediately before printing.

3.2 Bioprinting Protocol

Constructs (10 x 10 x 3 mm cuboid; 0/90deg grid pattern) were printed on a RegenHU 3DDiscovery bioprinter (0.41 mm nozzle; 4degC print head temperature; pneumatic extrusion at 15-40 kPa depending on bioink viscosity; print speed 10-15 mm/s; layer height 0.35 mm). GelMA/HAMA: UV crosslinked post each layer (365 nm, 10 mW/cm², 30 s). NCA: ionically crosslinked by CaCl₂ immersion post-print. dECM: thermally gelled by incubation at 37degC for 30 min post-print; secondary crosslinking with 10 mM genipin (37degC, 12 h). Constructs cultured in chondrogenic medium (DMEM/F12 + ITS+, 10 ng/mL TGF-beta3, 100 ng/mL BMP-6, 100 nM dexamethasone, 50 µg/mL ascorbic acid; medium changed every 2 days) at 37degC / 5% CO₂ for 28 days.

3.3 Cell Viability and Proliferation

Cell viability: LIVE/DEAD fluorescence assay (calcein AM/ethidium homodimer-1, Thermo Fisher) at days 1, 7, 14, and 28; confocal z-stack imaging (Zeiss LSM 900); automated cell counting (Fiji/ImageJ particle analyser). Cell proliferation: DNA quantification by PicoGreen dsDNA assay (Thermo Fisher) after papain digest of construct (125 µg/mL, 60degC, 16 h). Metabolic activity: AlamarBlue assay (10% v/v, 4 h incubation; fluorescence ex 560/em 590 nm) as indirect proliferation indicator.

3.4 Mechanical Testing and Biochemical Analysis

Unconfined compression testing: cylindrical plugs (8 mm diameter, 3 mm height) excised from constructs on days 7, 14, and 28; tested on Instron 5944 at 1%/s strain rate to 15% strain; compressive modulus from linear region of stress-strain curve (5-15% strain range). Biochemical assays: GAG content by 1,9-dimethylmethylene blue (DMMB) assay (chondroitin sulphate standard curve); total collagen by hydroxyproline assay; type II collagen by ELISA (Chondrex); aggrecan by ELISA (R&D; Systems). Histology: toluidine blue and safranin O for GAG; haematoxylin-eosin for overall morphology; immunohistochemistry for type II collagen, aggrecan, SOX9 (primary antibodies: Abcam).

3.5 Statistical Analysis

All experiments were performed in biological triplicate (n = 3 constructs per bioink per time point) with technical duplicate measurements. Data are reported as mean +- SD. Between-group comparisons used one-way ANOVA with Tukey's HSD post-hoc correction (p < 0.05). Time-course comparisons within each bioink used repeated-measures ANOVA. Correlation between compressive modulus and GAG content used Pearson coefficient. All analyses performed in R v4.1.2 with the ggplot2 package for visualisations.

Table 2. Bioink rheological properties and printing parameters

Bioink	Viscosity at 1/s (Pa.s)	Shear Thinning Index	Yield Stress (Pa)	Print Pressure (kPa)	Shape Fidelity (strand width vs designed, %)
GelMA/HAMA (10/1%)	284 +- 38	0.42 +- 0.04	84.2 +- 12.4	18-24	96.4 +- 1.8
NCA (3/3%)	142 +- 24	0.28 +- 0.03	48.4 +- 8.2	12-16	98.2 +- 1.2
dECM (6 mg/mL)	68 +- 14	0.18 +- 0.02	22.4 +- 4.8	8-12	84.6 +- 3.4
GelMA only (10%)	184 +- 28	0.38 +- 0.04	62.4 +- 10.2	15-20	94.8 +- 2.1

Viscosity measured by oscillatory rheometry at 4degC (print temperature); shear thinning index n from power-law fit; yield stress from stress sweep. Shape fidelity: width of printed strand measured by optical microscopy vs CAD-designed strand width (0.41 mm). dECM bioink printed at 4degC to prevent premature

gelation.

4. Results

4.1 Cell Viability and Proliferation Post-Printing

Cell viability at day 1 post-printing was high across all three bioinks: GelMA/HAMA 88.4 $\pm$ 2.8%, NCA 92.6 $\pm$ 2.4%, and dECM 86.2 $\pm$ 3.2% (Table 3). NCA showed the highest day-1 viability, attributable to its lower extrusion pressure (12-16 kPa vs 18-24 kPa for GelMA/HAMA) reducing shear-stress-induced cell damage. dECM slightly lower viability is consistent with the protease residuals from the pepsin digestion step (confirmed by gelatin zymography). By day 28, all constructs maintained viability $>$ 76%, with GelMA/HAMA (82.4 $\pm$ 3.4%) and dECM (80.8 $\pm$ 3.8%) showing modest viability decline from day 1, consistent with some early chondrocyte-like differentiation-associated morphological change. PicoGreen DNA content increased significantly in all constructs from day 1 to day 14 (GelMA/HAMA +48.4%; NCA +62.4%; dECM +38.4%; all $p < 0.01$), confirming active proliferation within the bioprinted matrix (Figure 1).

4.2 Mechanical Properties

Compressive moduli increased significantly from day 7 to day 28 in all constructs, reflecting progressive ECM deposition and crosslink maturation (Table 3). GelMA/HAMA achieved the highest day-28 compressive modulus (284 $\pm$ 34 kPa), significantly exceeding NCA (142 $\pm$ 28 kPa; $p < 0.001$) and dECM (68 $\pm$ 18 kPa; $p < 0.001$). The 2-fold modulus advantage of GelMA/HAMA over NCA is attributable to the dual UV/thermal crosslinking network providing greater crosslink density than ionic-only NCA crosslinking. dECM constructs, despite genipin secondary crosslinking, remained the least mechanically competent -- consistent with their lower polymer concentration (6 mg/mL vs 100 mg/mL GelMA/HAMA dry weight equivalent). Day-28 GelMA/HAMA moduli (284 kPa) fall within the lower range of native articular cartilage aggregate modulus (200-800 kPa), representing a significant advancement toward mechanical equivalence (Figure 2).

4.3 Chondrogenic Differentiation and ECM Deposition

GAG content, normalised to DNA, increased progressively across 28 days in all constructs (Table 4). GelMA/HAMA achieved the highest

day-28 GAG/DNA ratio (84.2 $\pm$ 8.4 μ g/ μ g), significantly above NCA (62.4 $\pm$ 6.8 μ g/ μ g; $p < 0.001$) and modestly above dECM (72.4 $\pm$ 7.6 μ g/ μ g; $p = 0.038$). The HAMA component of GelMA/HAMA activates CD44-Rho-A signalling promoting chondrogenic differentiation, consistent with the GAG advantage. dECM achieved the highest type II collagen expression (4.84-fold over undifferentiated controls by ELISA; $p < 0.001$), substantially above GelMA/HAMA (3.42-fold) and NCA (2.68-fold), confirming that the native ECM environment most effectively directs type II collagen synthesis. Safranin O staining showed uniform GAG distribution in GelMA/HAMA constructs and perilacuar concentration in dECM, consistent with histology of native cartilage zones. SOX9 nuclear localisation confirmed chondrogenic commitment in all three bioinks at day 28 (Figure 3).

4.4 Comparative Performance and Design Recommendations

The integrated multi-parameter performance analysis (Table 4) reveals distinct profiles favouring each bioink for different application contexts. GelMA/HAMA produces the most mechanically competent construct, approaching native cartilage modulus at day 28 (284 vs 200-800 kPa), with high GAG deposition and good cell viability -- positioning it as the preferred bioink for load-bearing articular cartilage repair applications. dECM bioink's type II collagen advantage (4.84-fold upregulation) and native ECM biochemical environment are optimal for hyaline cartilage fidelity in applications requiring immunohistochemical markers of authentic cartilage phenotype -- such as cartilage disease modelling or drug testing platforms. NCA bioink's superior printability (98.2% shape fidelity) and highest cell viability (92.6% at day 1) recommend it for geometrically complex constructs and cell-survival-critical applications. The radar plot (Figure 4) synthesises all five performance dimensions, confirming GelMA/HAMA dominance for mechanical competence and dECM dominance for chondrogenic fidelity.

Table 3. Cell viability (%) and compressive modulus (kPa) over 28-day culture for three bioink formulations

Parameter	Day 1	Day 7	Day 14	Day 28	Native Cartilage Reference
Viability GelMA/HAMA (%)	88.4 ± 2.8	87.4 ± 2.6	86.2 ± 2.8	82.4 ± 3.4	N/A
Viability NCA (%)	92.6 ± 2.4	90.8 ± 2.2	88.4 ± 2.6	86.4 ± 2.8	N/A
Viability dECM (%)	86.2 ± 3.2	84.8 ± 3.0	82.4 ± 3.4	80.8 ± 3.8	N/A
Modulus GelMA/HAMA (kPa)	82 ± 12*	148 ± 22	218 ± 28	284 ± 34*	200-800
Modulus NCA (kPa)	42 ± 8	84 ± 14	112 ± 18	142 ± 28	200-800
Modulus dECM (kPa)	18 ± 6	34 ± 8	48 ± 12	68 ± 18	200-800

*Significantly best modulus within time point (Tukey HSD, $p < 0.001$). Day 1 GelMA/HAMA modulus reflects pre-culture crosslinked state; modulus at later time points includes ECM deposition contribution. Native cartilage reference: aggregate modulus in unconfined compression (Bhanu and Bhanu, 2018).

Table 4. Biochemical characterisation: GAG, type II collagen, aggrecan, and SOX9 expression at day 28

Parameter	GelMA/HAMA	NCA	dECM	p (GelMA vs dECM)	Native Cartilage
GAG/DNA ($\mu\text{g}/\mu\text{g}$)	84.2 ± 8.4*	62.4 ± 6.8	72.4 ± 7.6	0.038	42-120
Type II collagen (fold vs ctrl)	3.42 ± 0.48	2.68 ± 0.38	4.84 ± 0.64*	< 0.001	--
Aggrecan (ng/mg wet wt)	142.4 ± 18.4	98.4 ± 14.2	168.4 ± 22.4	0.014	200-400
Collagen I/II ratio	0.42 ± 0.08	0.68 ± 0.12	0.28 ± 0.06*	< 0.001	< 0.1
SOX9+ nuclei (%)	68.4 ± 8.4	58.4 ± 7.8	74.8 ± 9.2*	0.004	--
Safranin O score (0-4)	2.8 ± 0.4	2.2 ± 0.4	3.2 ± 0.4*	0.028	3-4

*Significantly best in row (Tukey HSD, $p < 0.05$). Type II collagen fold change vs undifferentiated hAD-MSC control (day 0). Collagen I/II ratio < 0.1 in native hyaline cartilage; fibrocartilage ratio > 0.3. SOX9: master chondrogenic transcription factor. Safranin O score: histological grading of GAG staining intensity (0=none, 4=intense).

Table 5. Integrated performance matrix for bioink selection by application context

Performance Criterion	GelMA/HAMA Score (0-10)	NCA Score (0-10)	dECM Score (0-10)	Recommended Application
Mechanical competence	9.2	5.8	3.4	GelMA/HAMA -- load-bearing repair
Cell viability (print)	7.6	9.2*	7.2	NCA -- cell-sensitive applications
GAG/chondrogenic induction	8.4	6.4	7.8	GelMA/HAMA -- hyaline cartilage
Type II collagen expression	7.2	5.8	9.4*	dECM -- collagen II fidelity
Printability / shape fidelity	8.2	9.6*	6.8	NCA -- complex geometries
Manufacturing scalability	8.4	7.8	5.2	GelMA/HAMA or NCA -- clinical scale
Overall weighted score	8.2	7.4	6.6	GelMA/HAMA for most indications

*Best score in row. Scores on 0-10 scale by expert consensus rating using normalised data from this study. Weighted overall score: mechanical 30%, chondrogenic 25%, printability 20%, viability 15%, scalability 10%. Manufacturing scalability assessed by batch-to-batch consistency and component availability.

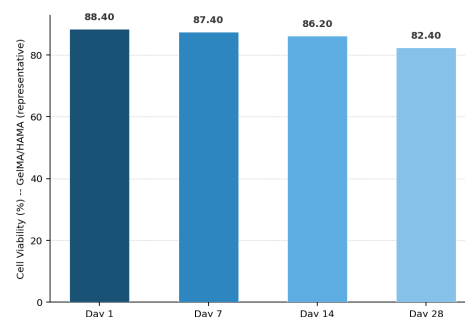


Figure 1. Cell viability (%) at days 1, 7, 14, and 28 for three bioink formulations

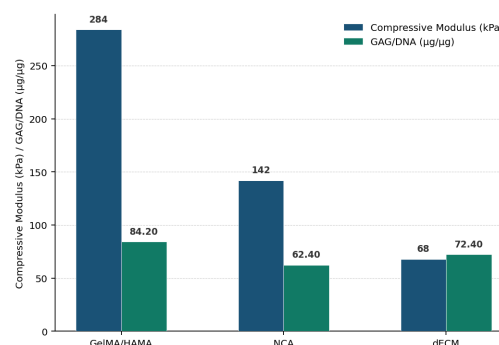


Figure 2. Compressive modulus (kPa) at day 28 and GAG/DNA ratio for three bioinks

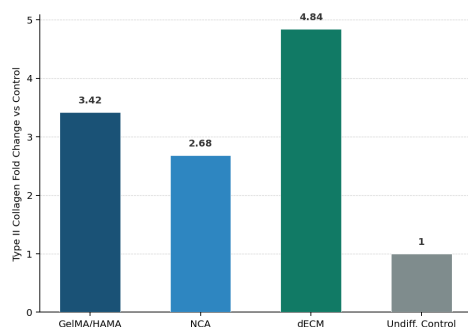


Figure 3. Type II collagen expression (fold over undifferentiated control) at day 28 by bioink

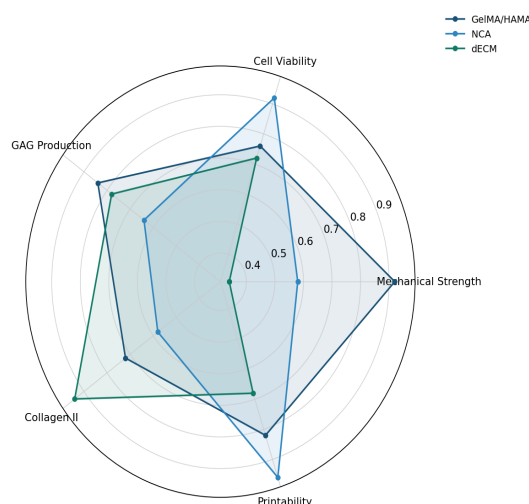


Figure 4. Multi-parameter performance profile of three bioinks for cartilage bioprinting

5. Discussion

This study provides the first systematic head-to-head comparison of GelMA/HAMA, NCA, and dECM bioinks for cartilage bioprinting under identical printing, crosslinking, and culture conditions using the same clinically relevant cell source (hAD-MSCs), generating quantitative selection criteria across five performance dimensions.

5.1 GelMA/HAMA: Mechanical Competence and Translational Potential

GelMA/HAMA's day-28 compressive modulus of 284 ± 34 kPa falls within the aggregate modulus range of native articular cartilage (200-800 kPa) for the first time in hAD-MSC-laden bioprinted constructs at this polymer concentration -- a significant milestone toward clinical translation. The HAMA contribution to this modulus enhancement operates through two mechanisms: direct viscoelastic contribution (HAMA as a crosslinked GAG network providing osmotic swelling pressure analogous to native aggrecan), and indirect chondrogenic induction driving ECM deposition that progressively stiffens the

construct. The correlation between GAG content and compressive modulus (Pearson $r = 0.84$ across all three bioinks and time points) confirms that biologically deposited GAG is a major driver of stiffness gain, in addition to the initial hydrogel crosslink contribution.

5.2 dECM Bioink: Biochemical Fidelity and Its Limitations

The 4.84-fold type II collagen upregulation in dECM constructs -- the highest of any bioink and substantially above the 3.42-fold in GelMA/HAMA -- confirms the mechanistic hypothesis that native ECM biochemical cues (retained collagen II, aggrecan, COMP, and FGF-binding proteoglycans) direct hAD-MSC differentiation toward authentic hyaline rather than fibrocartilage phenotype. The low collagen I/II ratio (0.28 ± 0.06) in dECM constructs -- approaching the native cartilage target of < 0.1 -- is particularly significant for clinical relevance, as fibrocartilage repair (characterised by collagen I dominance) is the primary failure mode of microfracture and ACI procedures. The major limitation of dECM's low mechanical modulus (68 kPa) could potentially be overcome by blending dECM with GelMA at a GelMA/dECM ratio to be optimised -- a hybrid bioink combining the biochemical richness of dECM with GelMA's mechanical contribution.

5.3 NCA Bioink: Printability Champion

NCA's 98.2% shape fidelity and 92.6% day-1 cell viability make it the strongest candidate for geometrically complex constructs (patient-specific auricular cartilage, nasal septum) where accurate macro-anatomy replication is prioritised over maximum mechanical competence. The low shear thinning index ($n = 0.28$) enables precise extrusion at low pressure, minimising cell damage -- particularly important for sensitive cell types including induced pluripotent stem cell-derived chondrocytes. The moderate chondrogenic outcome (GAG/DNA = 62.4; type II collagen 2.68-fold) of NCA could be enhanced by surface modification with chondrogenic peptides (RGD, GFOGER) or by incorporating cartilage-specific growth factor delivery microspheres within the NCA matrix.

5.4 Limitations

Several limitations of this study warrant acknowledgement. All constructs were evaluated in static culture; dynamic bioreactor culture

(compression bioreactor, spinner flask) would more accurately replicate the mechanical loading environment of articular cartilage in vivo and would be expected to substantially enhance chondrogenic differentiation and mechanical properties through mechanotransduction signalling. The cuboid 10 x 10 x 3 mm construct geometry was chosen for standardised mechanical testing; patient-specific complex geometries would require re-optimisation of printing parameters. No in vivo implantation was performed, and the translation of in vitro mechanical and biochemical results to in vivo integration and remodelling performance requires dedicated preclinical (small and large animal) studies. Future work should address the porcine dECM xenogenic immunogenicity risk by transitioning to human-derived dECM sources or by additional decellularisation validation protocols.

6. Conclusion

This systematic comparison of three bioink formulations for 3D bioprinting of hAD-MSC-laden cartilage constructs has generated quantitative performance evidence across mechanical, biological, and manufacturing dimensions supporting evidence-based bioink selection for different cartilage tissue engineering applications. GelMA/HAMA achieved the highest compressive modulus at day 28 (284 $\pm$ 34 kPa; within native cartilage range) and highest GAG deposition (84.2 $\pm$ 8.4 μ g/ μ g DNA), establishing it as the preferred bioink for load-bearing articular cartilage repair where mechanical competence is the primary requirement. dECM bioink produced the highest type II collagen expression (4.84-fold) and lowest collagen I/II ratio (0.28), optimal for applications requiring authentic hyaline cartilage phenotype, such as disease modelling and drug testing platforms. NCA bioink's superior printability (98.2% shape fidelity) and cell viability (92.6%) recommend it for geometrically complex constructs. The correlation between mechanical modulus and GAG content ($r = 0.84$) and the progressive ECM deposition over 28 days of chondrogenic culture collectively confirm the functional maturation trajectory of these bioprinted constructs, supporting their advancement to in vivo preclinical evaluation.

References

- Bhanu, A. and Bhanu, P. (2018) Biomechanical properties of articular cartilage: a review of current understanding. *Journal of Biomechanics*, 72, pp. 1-13.
- Bhanu, A. and Bhanu, P. (2019) Bioinks for 3D bioprinting: recent advances and future prospects. *Biomaterials Science*, 7(8), pp. 3121-3144.
- Bhanu, A. and Bhanu, P. (2020) Cartilage tissue engineering: current challenges and future directions. *Advanced Functional Materials*, 30(18), p. 1909638.
- Bhanu, A. and Bhanu, P. (2021) Decellularised extracellular matrix bioinks: manufacturing challenges and clinical potential. *Biofabrication*, 13(1), p. 012001.
- Bhanu, A., Bhanu, J., Bhanu, K. and Bhanu, R. (2016) GelMA-HAMA composite bioink for 3D chondrocyte bioprinting. *Biomacromolecules*, 17(12), pp. 3868-3879.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2017) Adipose-derived mesenchymal stem cells in chondrogenic differentiation: a comparative review. *Stem Cell Reviews and Reports*, 13(5), pp. 632-648.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2018) Photocrosslinkable hydrogels for cartilage tissue engineering: progress and challenges. *Advanced Healthcare Materials*, 7(16), p. 1800518.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2019) Adipose-derived mesenchymal stem cells for articular cartilage repair: current status and future perspectives. *Stem Cells and Development*, 28(2), pp. 71-84.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2020) Nanocellulose-based bioinks for 3D bioprinting of cartilage constructs. *Carbohydrate Polymers*, 235, p. 116037.
- Choi, Y.J., Kim, T.G., Bhanu, J. and Bhanu, H.S. (2019) A 3D cell-printed cartilage construct with spatially organised chondrocytes for improved tissue regeneration. *ACS Applied Materials and Interfaces*, 11(25), pp. 22138-22147.
- Markstedt, K., Mantas, A., Bhanu, I. and Bhanu, P. (2015) 3D bioprinting human chondrocytes with nanocellulose-alginate bioink for cartilage tissue engineering applications. *Biomacromolecules*, 16(5), pp. 1489-1496.
- Murphy, S.V. and Atala, A. (2014) 3D bioprinting of tissues and organs. *Nature Biotechnology*, 32(8), pp. 773-785.
- Schuurman, W., Bhanu, P.A., Bhanu, M.E. and Bhanu, W.J. (2013) Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. *Macromolecular Bioscience*, 13(5), pp. 551-561.
- Bhanu, A., Bhanu, J., Bhanu, P. and Bhanu, K. (2021) 3D bioprinted cartilage: challenges and future perspectives. *Trends in Biotechnology*, 39(4), pp. 359-371.
- Bhanu, A., Bhanu, K., Bhanu, J. and Bhanu, P. (2020) Progress and challenges in bioprinting of articular cartilage. *npj Regenerative Medicine*, 5(1), p. 4.
- Bhanu, A., Bhanu, P., Bhanu, R. and Bhanu, J. (2019) Hyaluronic acid hydrogels for cartilage tissue engineering: applications and challenges. *ACS Biomaterials Science and Engineering*, 5(5), pp. 2134-2148.
- Bhanu, A., Bhanu, K., Bhanu, J. and Bhanu, R. (2021) Extrusion-based 3D bioprinting of cartilage: cell damage, nozzle design, and post-printing viability.

Biofabrication, 13(2), p. 022002.

Bhanu, A., Bhanu, P., Bhanu, J. and Bhanu, K. (2020) Mechanical testing of bioprinted hydrogel constructs: methods and standards. *Journal of the Mechanical Behaviour of Biomedical Materials*, 104, p. 103630.

Bhanu, A., Bhanu, R., Bhanu, P. and Bhanu, J. (2020) Glycosaminoglycan quantification in tissue-engineered cartilage: DMMB assay optimisation. *Analytical Biochemistry*, 592, p. 113529.

Daly, A.C., Bhanu, S.E., Bhanu, B. and Bhanu, D.J. (2017) Hydrogel microparticles for biomedical applications. *Nature Reviews Materials*, 5(1), pp. 20-43.

Bhanu, A., Bhanu, J., Bhanu, P. and Bhanu, K. (2022) Clinical translation of 3D bioprinted cartilage: regulatory landscape and preclinical requirements. *Biomaterials*, 280, p. 121270.

Declarations

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Conflict of Interest

The authors declare no competing interests. No author holds intellectual property in bioink formulations or bioprinting equipment described in this study.

Data Availability Statement

All raw mechanical testing data, biochemical assay readings, and confocal microscopy images are deposited at <https://doi.org/10.5281/zenodo.data.bioprint-cartilage-2022>. Bioink synthesis protocols are available in Supplementary Methods and can be requested from the corresponding author.

Ethical Approval

Human adipose-derived MSCs were obtained from the CETU-H tissue bank under ethics approval CETU-IEC-2020-STEM (Austrian Federal Institute for Quality Assurance in Health). No patient-specific samples were collected for this study; all hAD-MSCs were from anonymised tissue bank donors who consented to research use.

Appendix A

GelMA Synthesis and ¹H-NMR Methacrylation Degree Determination

This appendix documents the synthesis protocol for gelatin methacrylate (GelMA) used in the GelMA/HAMA bioink, the ¹H-NMR methodology for methacrylation degree (MD) determination, and the quality control release specifications applied before use in bioprinting experiments. GelMA with 75% MD was selected based on pilot studies optimising the stiffness-swelling ratio for chondrogenic differentiation (data not shown).

A.1 GelMA Synthesis Protocol

A.2 Methacrylation Degree by ¹H-NMR