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3D-PRINTED GELATIN-BASED BIOINKS: A REVIEW OF L929 FIBROBLAST VIABILITY AND IN VITRO SCRATCH ASSAY DYNAMICS

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ABSTRACT

Gelatin-based bioinks have become widely used in 3D bioprinting because they support cell growth and can be easily modified for printing. However, the way these inks are prepared—particularly through crosslinking and extrusion—can either help or harm the cells inside them. This review brings together findings from two common tests: the L929 cytotoxicity assay, which checks if the material is toxic to cells, and the scratch assay, which measures how well cells can move and close a wound-like gap. Our analysis shows that while stiffer gels hold their shape better during printing, they often slow down cell movement, and that high shear stress during extrusion can temporarily damage cells. We also highlight major challenges, including poor reporting standards and the limited relevance of mouse-derived cell lines to human biology. To move the field forward, we recommend adopting standardized reporting guidelines, using artificial intelligence to optimize printing conditions, and transitioning to human primary cells for more meaningful results.

KEYWORDS: 3D Bioprinting, Gelatin Methacryloyl (GelMA), Bioinks, L929 Fibroblasts, Cytotoxicity Assay, In Vitro Scratch Assay, Extrusion-Based Printing, Cell Viability, Wound Healing Dynamics, Tissue Engineering.

INTRODUCTION

Three-dimensional (3D) bioprinting has emerged as one of the more transformative approaches within tissue engineering, offering the ability to deposit cells and biomaterials in

precisely controlled, layer-by-layer architectures that mimic native tissue organization.[1,2] Central to this technology is the bioink — the printable, often cell-laden, hydrogel formulation that must simultaneously satisfy two demanding and, at times, competing requirements: it must flow readily enough to be extruded through a printing nozzle, yet retain sufficient structural integrity once deposited to preserve the intended geometry.[3,4] The choice of bioink material therefore dictates, to a considerable extent, both the fidelity of the printed construct and its eventual biological performance.

Among the wide range of natural and synthetic biomaterials explored for this purpose, gelatin — and its photocrosslinkable derivative, gelatin methacryloyl (GelMA) — has steadily established itself as one of the most extensively investigated bioink candidates.[5] This preference is not incidental. Gelatin is derived from the partial hydrolysis of collagen, the principal structural protein of the extracellular matrix, and consequently retains cell-adhesive and enzymatically degradable motifs that support cell attachment, spreading, and proliferation more readily than many purely synthetic alternatives.[5] Its low cost, ready availability, and tunable mechanical and degradation profiles — particularly following methacrylation and photocrosslinking — have made it an attractive scaffold material across a range of tissue engineering applications. Yet these very processing steps, including UV exposure, photoinitiator selection, and chemical or enzymatic crosslinking, introduce variables that can meaningfully alter both the biological safety and the functional performance of the resulting construct.

Evaluating a gelatin-based bioink cannot, therefore, rest on printability and mechanical characterization alone; it requires evidence that the printed material is both biologically safe and functionally supportive of the cellular processes it is intended to facilitate. Two in vitro assays have become particularly prominent in addressing these related but distinct questions. The L929 murine fibroblast cytotoxicity assay, performed in accordance with the ISO 10993-5 standard[6], has become the de facto benchmark for determining whether a biomaterial — or the residues of its fabrication process — compromises basic cell viability. Complementing this, the in vitro scratch (wound-healing) assay provides a functional readout, capturing whether a material supports or impairs the migratory behaviour of fibroblasts as they close an induced gap in a confluent monolayer, a process that closely parallels early-stage wound repair in vivo.[7] Taken together, these two assays offer a more complete picture than either can provide alone: one establishes that a material does no harm, while the other suggests that it may actively contribute to tissue-repair processes.

This review seeks to address that gap. Specifically, it aims to (i) summarize the compositional and processing variables reported across the gelatin bioink literature, (ii) synthesize reported trends in L929 fibroblast viability and their association with formulation and printing parameters, (iii) synthesize reported trends in scratch-assay migration outcomes for gelatin-based constructs, and (iv) identify points of convergence or divergence between viability and migration data that may inform future bioink design.

Gelatin-Based Bioinks: Composition and Crosslinking

Native, unmodified gelatin is a poor candidate for standalone 3D printing: it dissolves near physiological temperature and lacks the mechanical robustness needed to hold a printed shape once deposited. This limitation is what drove the development of gelatin methacryloyl (GelMA), produced by reacting gelatin with methacrylic anhydride to introduce methacryloyl groups onto lysine and hydroxylysine residues along the protein backbone. The resulting material can be locked into a stable, covalently crosslinked network through UV or visible-light photocrosslinking, while still retaining the arginine-glycine-aspartic acid (RGD) cell-adhesive motifs and matrix metalloproteinase-cleavable sites inherited from its parent collagen.[8] Notably, this modification does not merely enable printability — the concentration of GelMA used directly tunes the mechanical stiffness of the final construct and, in turn, measurably shapes how seeded fibroblasts spread, proliferate, and remodel their surrounding matrix.[8]

Gelatin is rarely used in isolation, however, and is frequently blended with other biopolymers to offset its individual weaknesses. Gelatin-alginate composites are among the most common, pairing gelatin's inherent bioactivity with alginate's ability to ionically crosslink almost instantly, which improves post-printing shape fidelity and structural stability.[4] Gelatin-hyaluronic acid blends, often crosslinked with genipin, aim to better mimic the native extracellular matrix and have been shown to support high cell density and strong short-term viability gains in printed constructs.[9] Gelatin-nanocellulose composites, meanwhile, use cellulose nanofibrils to improve shear-thinning behavior and structural integrity during extrusion without compromising the biocompatibility gelatin already offers.[10]

Whichever formulation is chosen, some form of crosslinking is ultimately required to keep the printed construct stable at physiological temperature — and the method selected has a direct bearing on the biological response of cells in contact with it, a relationship that becomes central to the viability data discussed later in this review. UV or visible-light

photocrosslinking, the dominant approach for GelMA, relies on a photoinitiator to trigger the reaction; the choice and residual concentration of that photoinitiator, along with light dose and exposure time, can themselves influence cytotoxicity if not carefully optimized. Among chemical crosslinkers, EDC/NHS is a popular "zero-length" agent that forms amide bonds directly between gelatin's carboxyl and amine groups, though at higher crosslinking densities it has been shown to reduce integrin-specific cell attachment in fibroblast-seeded collagen-based constructs — a close structural analog of gelatin, though not identical to it.[13] Genipin, a plant-derived crosslinker, is generally regarded as comparably or more cytocompatible than EDC/NHS[13], while glutaraldehyde, though highly efficient, is consistently associated with reduced cell viability across the literature, largely attributed to residual unreacted aldehyde groups.[12] Enzymatic crosslinking with transglutaminase offers a gentler alternative that avoids harsh chemical residues altogether, though its reaction kinetics must be carefully tuned, since prolonged or premature crosslinking can itself interact with extrusion-related shear stress to affect cell survival during printing.[11] These same compositional and crosslinking variables reappear throughout Sections 4 and 5 as the primary determinants of both L929 viability and scratch-assay migration outcomes reported in the literature.

Printing Techniques and Process Parameters

Extrusion-Based Printing

Extrusion-based bioprinting remains the dominant methodology for fabricating three-dimensional gelatin and GelMA constructs owing to its scalability, cost-effectiveness, and capability to deposit high-viscosity hydrogels into thick, multi-layered scaffolds (Günther, 2025). The technique relies on pneumatic, piston, or screw-driven systems to continuously dispense bioink filaments through a microfluidic nozzle onto a collector stage (Dobrisan et al., 2024). Successful execution requires a careful rheological profile; the bioink must exhibit strong shear-thinning characteristics—where viscosity drops under high shear rates inside the needle to ease flow—while immediately recovering its structural integrity once deposited to maintain shape fidelity (Cavallo et al., 2025).

Alternative Bioprinting Modalities

While extrusion leads the field, alternative modalities are selectively utilized:

- **Inkjet Bioprinting:** Utilizes thermal or piezoelectric pulses to eject picoliter droplets, offering high printing speeds and mild cellular environments, though restricted to very low-viscosity bioinks (Dobrisan et al., 2024).

- **Digital Light Processing (DLP) / Stereolithography:** Employs patterned light projection to rapidly photopolymerize entire layers concurrently, achieving exceptional structural resolution without mechanical nozzle shear (Xu et al., 2022).
- **Laser-Assisted Bioprinting:** Employs a pulsed laser beam focused onto an absorbing ribbon layer to propel micro-droplets of bioink toward a substrate, completely avoiding nozzle-induced clogging at the expense of lower throughput.

Key Parameters Affecting Cell Fate

Cell survival during printing is dictated by an intricate interplay of mechanical and thermal inputs. **Nozzle geometry and gauge** directly influence internal fluid dynamics; smaller internal diameters generate steep velocity gradients that elevate local shear stress, triggering membrane disruption and apoptosis (Cidonio et al., 2019). **Shear stress** thresholds generally indicate that forces below 5 kPa preserve high viability (>95%), whereas exposures exceeding 10 kPa cause sharp declines in cell survival (Cidonio et al., 2019). Finally, **temperature control** is critical for gelatin-based matrices, as operating too close to thermal gelation points causes premature clogging, while elevated temperatures can induce thermal shock before crosslinking occurs (Yusuf Olatunji et al., 2024).

L929 Fibroblast Viability: Methods and Findings

Why L929 and ISO 10993-5

L929 murine subcutaneous connective tissue fibroblasts serve as the golden-standard immortalized cell line for preliminary biomaterial screening. Governed by ISO 10993-5 standards, they offer exceptional reproducibility, rapid doubling times, and high sensitivity to toxic leachates, making them ideal for high-throughput cytotoxicity evaluations prior to primary human cell culture.

Common Assay Methods

Assessment of bioprinted constructs predominantly employs the **MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay** to quantify metabolic activity via mitochondrial dehydrogenase function, paired alongside **Live/Dead fluorescent staining** (calcein-AM/propidium iodide) to visualize spatial cell distribution and membrane integrity under confocal microscopy.

Main Synthesis: Literature Trends on Viability

- **Effect of Polymer Concentration:** Higher gelatin or GelMA concentrations (e.g., 15% to 20%) substantially enhance structural stiffness and shape definition, but over-dense macromolecular networks impede vital nutrient diffusion and restrict cellular spreading, frequently lowering long-term metabolic viability (Cavallo et al., 2025).
- **Effect of Crosslinker Choice and Dose:** Aggressive chemical crosslinkers (such as glutaraldehyde) or prolonged UV-photoinitiator exposure severely compromise cell viability due to unreacted aldehyde residues and cytotoxic radical generation. In contrast, enzymatic or mild photo-click systems yield significantly superior post-printing survival rates (Yao & Molotnikov, 2022).
- **Effect of Shear Stress and Printing Parameters:** Increased dispensing pressures and reduced needle calibers exponentially increase fluid shear stress, establishing an inverse relationship between printing resolution and overall cell viability (Dobrisan et al., 2024).

Limitations of the L929 Model

Despite their utility, L929 fibroblasts are transformed murine cells that fail to replicate the complex phenotypes, extracellular matrix remodeling rates, or specific metabolic responses of primary human cells, necessitating secondary validation in target-specific tissue models.

In Vitro Scratch Assay: Methods and Findings

Assay Principle

The in vitro scratch assay is a straightforward, cost-effective method used to evaluate directional cell migration and wound closure by creating a cell-free gap in a confluent monolayer and monitoring the rate at which cells repopulate the cleared zone.[21]

Common Variants

- **Manual Scratch:** Utilizes a standard pipette tip to create a straight-line defect, offering high accessibility but suffering from minor variations in scratch width across replicates.[22]
- **Standardized Devices:** Employs custom stamps, stencils, or automated insertion tools to ensure highly reproducible, uniform gap geometry and standardized initial wound areas.[23,24]

Main Synthesis: Migration and Wound-Closure Trends

- **Effect of Composition and Crosslinking:** Modulating hydrogel stiffness and matrix density plays a pivotal role in dictating cellular motility; highly dense or overly rigid crosslinked networks physically impede pseudopodia extension and slow down migration rates, whereas moderately compliant gelatin matrices promote optimal traction and faster wound closure.(25)
- **Effect of Printing Parameters:** High shear stress experienced during extrusion can temporarily damage the cytoskeletal machinery of fibroblasts, leading to a transient lag phase in early cell migration before recovery and subsequent closure commence.

Known Confounders

Researchers must carefully distinguish between true cellular migration and cell proliferation, as an increase in gap closure over time can sometimes be heavily driven by rapid cell division rather than directional movement alone.(26)

Correlating Viability and Migration Data & Applications

Cross-Study Pattern: Viability vs. Migration

Higher post-printing cell viability generally correlates with accelerated wound closure, as a larger pool of healthy, metabolically active cells is available to drive collective migration. However, excessive crosslinking that maximizes cell survival can sometimes overly restrict matrix pore size, creating an inverse trade-off where structural rigidity inhibits the physical space required for rapid cell translocation.

Comparative Literature Overview

Author, Year	Gelatin Type	Cross linker	L929 Viability (%)	Scratch Closure (%)	Key Finding
Cavallo et al., 2025	Gelatin-Alginate	$CaCl_2$/ Ionic	~85-90%	~75%	Balanced ratios support high early fibroblast viability and progressive wound closure.
Yao & Molotnikov, 2022	GelMA	UV / Photo-click	~80-90%	~82%	Optimized photo-crosslinking maintains structural integrity without sacrificing migratory capacity.
Song & Ren, 2021	Gelatin Microgel	Transglutaminase	~88-93%	~85%	Enzymatic crosslinking yields exceptional cytocompatibility and enhanced cell motility.
Shie & Lee, 2020	GelMA	UV / LAP	~75-85%	~68%	Higher macromer concentrations improve shape fidelity but slightly reduce

					migration rates due to increased density.

Application Context

Because gelatin naturally mimics extracellular matrix components and contains RGD binding motifs, these bioink formulations are exceptionally well-suited for skin tissue engineering and accelerated wound dressing applications, promoting rapid dermal cell infiltration and tissue regeneration.

Challenges and Future Perspectives

Lack of Standardized Reporting

The most prominent hurdle in current bioprinting literature is the extreme heterogeneity in experimental design—such as varying nozzle geometries, inconsistent shear rate calculations, and differing assay timepoints—making direct cross-study comparisons difficult.[23]

Translatability of Murine L929 Data

While L929 fibroblasts provide a robust and reproducible baseline for initial screening, their immortalized murine nature limits direct translation to human clinical outcomes, as they fail to replicate the complex inflammatory and phenotypic nuances of primary human dermal cells.[27]

Forward-Looking Horizons

- **AI-Assisted Optimization:** Machine learning models are increasingly deployed to predict ideal rheological parameters and minimize trial-and-error in bioink formulation.[24]
- **Standardized Reporting Checklists:** The implementation of universal minimum information guidelines for bioprinting experiments will significantly improve reproducibility across laboratories.[28]
- **Alternative Cell Models:** Transitioning toward primary human dermal fibroblasts or co-culture systems will bridge the gap between preliminary cytotoxicity screens and true physiological relevance.[29]

CONCLUSION

Current literature demonstrates that gelatin-based bioinks offer a remarkably versatile, biocompatible platform for tissue engineering when balanced carefully with appropriate crosslinking and printing parameters. The primary practical takeaway for researchers is that

optimizing bioink formulations requires balancing structural printability with the biological needs of the cells, avoiding overly dense networks or harsh crosslinkers that hinder viability and migration.

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