Layman summary

Unveiling the Inner Workings of Hematopoietic Stem Cells Using Cutting-Edge Techniques

Hematopoietic stem cells (HSCs) are the unsung heroes of our bloodstream, responsible for generating various blood cell types like red blood cells, white blood cells, and platelets. They reside in the bone marrow, where a special environment called the hematopoietic stem cell niche controls their destiny.

But the HSC niche can be quite sensitive and vulnerable to issues like malignancies. To better understand and protect these vital cells, we are developing a nifty model of the bone marrow. This model aims to mimic the bone marrow environment and might be a game-changer.

The way it works,

First, we use 3D printing, print a structure with a special gel that's really good at holding water. This structure mimics the 3D environment our cells live in within our bodies, which is way different from flat 2D cultures in a petri dish.

Then, we simulate blood flow with a technique called perfusion. It's like mimicking a tiny river inside the bone marrow to keep things fresh. Nutrients go in, waste goes out, and the cells stay happy.

To follow up. We take it a step further by introducing mesenchymal stem cells (MSC) and endothelial colony-forming cells (ECFC) into the mix. These cells are known to exist in the vascular bone marrow niche and interact with HSCs, playing a crucial role in HSC self-renewal and differentiation.

In a nutshell, this new approach isn't just about 3D printing – it's about understanding our body's inner workings, protecting our precious blood cells, and discovering new ways to fight diseases.

Merging perfusion, volumetric bioprinting and porous hydrogels to biofabricate a bone marrow model

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\<Abstract\>

Hematopoietic stem cells (HSCs) hold a distinct and pivotal role as the progenitors of diverse blood cell lineages throughout an individual's lifespan. The sustenance, regulation, and functionality of these vital cells are intricately intertwined with the bone marrow niche.

However, the homeostatic equilibrium of the hematopoietic stem cell (HSC) niche is vulnerable to perturbation due to factors such as malignancies or other disruptive influences.

The amalgamation of hydrogel matrices and cutting-edge bioprinting techniques represents an advanced strategy in the realm of bone marrow simulation. In our pursuit, we endeavor to establish a more refined in vitro HSC model. This pursuit involves meticulous adjustments to hydrogel solidification conditions and bioprinting parameters, aiming to attain a tailored platform that closely emulates the intricate HSC microenvironment.

\<Introduction\>

The Significance of Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells (HSCs) hold a paramount role as unique and indispensable entities responsible for the perpetual generation of diverse blood cell types, encompassing red blood cells, white blood cells, and platelets. [1] Anchored within the bone marrow niche, a specialized microenvironment within the marrow, HSCs are intricately nurtured and regulated to orchestrate their function, maintenance, and equilibrium. [2] The bone marrow niche bestows a vital shield, safeguarding HSCs from external stressors, radiation, and harmful agents. Comprising an assembly of cellular constituents such as stromal cells, osteoblasts, endothelial cells, adipocytes, and the intricate extracellular matrix (ECM), the niche orchestrates a dynamic interplay with HSCs through both physical proximity and the orchestrated release of signaling molecules, including cytokines and growth factors. A well-orchestrated bone marrow niche furnishes an environment that effectively governs the behavior of HSCs. This governance extends to the precise modulation of their self-renewal capacity, dictating their aptitude to perpetuate stem cell numbers, as well as steering their differentiation trajectory, a pivotal process culminating in the generation of

mature blood cells. By meticulously preserving the 'stemness' of HSCs, the niche ensures the perpetuation of their distinct properties across time, thus forming the cornerstone for the perpetual replenishment of the body's array of blood cells over an individual's lifetime[3]

The Dynamic Hematopoietic Stem Cell (HSC) Niche and Its Susceptibility to Pathological Disruptions

The homeostatic equilibrium of the hematopoietic stem cell (HSC) niche is inherently vulnerable to perturbations instigated by malignancies. In light of this vulnerability, the development of a robust and versatile bone marrow model emerges as a promising avenue for addressing this concern. An intricately designed bone marrow model presents the prospect of cultivating a meticulously regulated and reproducible microenvironment. This, in turn, lays the groundwork for comprehensive exploration into a spectrum of disorders afflicting the bone marrow, encompassing leukemia, lymphoma, and various anemias.

This construct facilitates the emulation of intricate pathological bone marrow microenvironments, thereby affording a nuanced understanding of disease progression and the systematic evaluation of potential therapeutic interventions. Specifically, this model could enable the dissection of disease trajectories, unraveling the intricate interplay between malignant cells and the native marrow milieu. Moreover, it provides a platform for the meticulous assessment of novel therapeutic agents targeting these malignancies.[4]

In the realm of bone marrow transplantation, these engineered models hold substantial promise. They offer a controlled environment in which transplantation protocols can be meticulously refined.[5] By systematically evaluating conditions and factors influencing engraftment efficiency while concurrently mitigating potential complications, the transplantation process could be significantly optimized.

A pivotal dimension of these advancements lies in their potential to curtail the dependence on animal experimentation within drug development and disease investigation. The establishment of functional bone marrow models within laboratory settings aligns profoundly with ethical considerations while concurrently enhancing the fidelity of insights garnered regarding human responses. This transition not only embraces cutting-edge scientific innovation but also underscores a conscientious commitment to refining research practices."

bone marrow models and their advantages and disadvantages

"In the realm of traditional culture techniques, hematopoietic stem cells (HSCs) have long been cultivated and expanded atop flat polystyrene surfaces. This process typically entails the addition of cytokines, small molecules, or feeder cells to facilitate growth. Yet, researchers have transcended these conventional approaches by pushing the boundaries of surface capabilities and modifying their physical attributes, such as rigidity.[2, 6] This deliberate manipulation aims to mimic the intricate characteristics of the extracellular matrix (ECM) within meticulously controlled experiments.

A transformative shift to three-dimensional (3D) environments, exemplified by techniques like spheroid formation, embedding within polymer matrices, or confinement within scaffold pores, has fundamentally revolutionized the cultivation of HSCs[7-9]. The bone marrow stroma itself embodies a dynamic, partially solid tissue architecture. Cells dwelling within this marrow milieu inevitably encounter an array of mechanical forces, ranging from fluid shear stress (FSS) to tensile strain and hydrostatic pressure. These mechanical cues exert a profound influence on the developmental trajectories of niche cells, directing them toward specific differentiation pathways.

The introduction of bioreactors and microfluidic platforms has introduced yet another layer of refinement to these culture systems. These platforms optimize the provision of nutrients while seamlessly integrating additional dimensions of the natural environment. This encompasses an assortment of substrates, varying levels of rigidity, distinct cellular constituents, soluble signaling molecules, and even intricately modeled structures resembling the intricate vascular systems found within the body. Collectively, these innovations stand as a testament to the ongoing evolution of HSC cultivation techniques, aligning progressively closer with the intricacies of the native bone marrow microenvironment."

The Importance of Hydrogels in Three-Dimensional Tissue Engineering

Hydrogels are pivotal in diverse tissue engineering applications, offering tunable properties and high water content that mimic physiological conditions. [10] Local mechanical cues can wield significance comparable to established biochemical signals in shaping cellular behaviors. Hydrogels emulating the natural extracellular

matrix deliver such cues to enclosed cells, enabling the investigation of their effects on cellular responses. [2, 11, 12]Their role is particularly pronounced in constructing three-dimensional (3D) tissue models, with specific relevance in stem cell bioprinting. Ideal hydrogels for such applications must exhibit biocompatibility, mechanical resilience, and bioactive cues akin to the extracellular matrix.[7]

Advancements in bioprinting have accentuated hydrogels' importance. Photopatterning involves using light to inscribe patterns on materials. Given GelMA's photocrosslinkable nature, using photopatterning to shape GelMA-based scaffolds intuitively imparts topographies and 3D architectures. Employing photomasks facilitates convenient creation of micropatterned GelMA scaffolds, enhancing applications in tissue engineering and beyond.[13] In stem cell bioprinting, hydrogels must be printable, provide structural support, and offer bioactivity for cell adhesion, proliferation, and differentiation. Additionally, they can guide stem cell fate through controlled release of growth factors or microenvironment replication.

In essence, hydrogels provide versatile platforms for intricate 3D tissue engineering.[14] Their tunability and mimetic qualities are vital in replicating native tissue environments. As bioprinting and regenerative medicine progress, hydrogels will play a central role in generating functional tissue constructs for research and therapeutic applications.[7, 13]

The Significance of Microfluidics

Establishing a conducive 3D cellular environment that enables self-assembly solely through diffusion within hydrogels proves inadequate for sustaining cell viability and facilitating nutrient exchange and waste removal.[7] The absence of efficient nutrient transport and waste clearance can lead to central necrosis, limiting its potential as a model. Thus, the integration of microfluidics not only facilitates nutrient exchange and waste clearance but also offers precise flow control, creating a controlled platform. This advantage is instrumental in simulating the intricate cellular microenvironment within biological organs, aiding in self-assembly and differentiation simulations.[15] Among microfluidic materials, polydimethylsiloxane (PDMS) stands out due to its affordability, biocompatibility, breathability, optical transparency, and facilitation of cell adhesion, rendering it a suitable microfluidic framework.[16]

Purpose of the experiment

By tuning parameters such as material stiffness, porosity, and media flow, we can

closely simulate the bone marrow microenvironment, particularly its vascular niches. Collaborating with 3D printing technology enables the construction of organ-like structures, affording spatial control over material-cell distribution and facilitating niche formation. Through a combined approach involving 3D printing, hydrogel systems, and fluid perfusion, we aspire to closely replicate the bone marrow's vascular niches. Co-culturing mesenchymal stem cells (MSCs) and endothelial colony-forming cells (ECFCs) aims to foster robust vasculature, enhancing vascularization post-self-assembly. By incorporating essential factors, we seek to establish an artificial HSC niche, forming a long-term in vitro model for expansion, differentiation, and regeneration.

<Material Method>

Hydrogel fabrication

Soak all the tools and mold we need in alchohol for sterile purpose for 30 minutes and dry in the hood. Gelatin powder warming up for 15 minutes after taking it from -20 C, measure the proper weight. we add PBS inside and to make a 10% or 20% stock and store it in incubator to avoid it curing. Initiator of Ruthenium and Sodiumphaste powder after add some PBS till proper concentration. Rutheum need to avoid the light and both of them need to put in 4 C fridge for temporate store purpose. Mix proper quantity MSCs inside. Mix them carefully and throrughly, ensuring a homogenous mixture. Pour it into PDMS mold and curing it with floodlight for 5 minutes. Take it out and put it in petri dish with culture medium and go into incubator.

PDMS mold making

-Fabrication of an Inverse-Installing Die Mold using DLP 3D Printing for PDMS Mold Creation

Design of Inverse-Installing Die Mold: Utilizing Tinkercad, an inverse-installing die mold design was created. File was transferred to the computer connected to the DLP 3D printer. A suitable combination of RO5 resin and the 60mm lens size was determined using software (RP 3.1.1540). The DLP 3D printer was then used to fabricate the inverse-installing die mold layer by layer. Carefully removed from the printer, ultrasonic cleaning for 15 minutes, repeated three times.

-Preparation of PDMS Mold using Inverse-Installing Die

The predetermined ratio of PDMS to elastomer was strictly adhered to (10:1). The PDMS material was carefully added to the mixing container, followed by the elastomer. Using stirring rods, the PDMS and elastomer were mixed thoroughly for 5 minutes, ensuring a homogenous mixture. The blended PDMS-elastomer mixture was poured into the DLP inverse-installing die mold, allowing it to conform to the mold's intricate 3D structure.To eliminate any air bubbles within the PDMS mixture, the mold was placed within a vacuum pump chamber for 1.5 hours. The vacuum treatment effectively removed trapped air, guaranteeing a bubble-free mold. Subsequent to the vacuum treatment, the mold containing the PDMS mixture was transferred to an oven set to 50 degrees Celsius. The curing process took place over a duration of four hours, during which the PDMS solidified, capturing the intricate details of the original mold.

A rectangular prism with dimensions of 15 mm (length), 8 mm (width), and 5 mm (height) was constructed. A central cavity measuring 4 mm (length), 10 mm (width), and 2 mm (height) was excavated within the prism to accommodate the hydrogel. Two orifices were incorporated at the bottom as inlet and outlet ports for fluid flow. The PDMS mold was positioned with the cavity facing upward. Following hydrogel infusion, the model's periphery was coated with UV glue and sealed with glass, thereby immobilizing the hydrogel within. Polymerization of the hydrogel occurred under UV light exposure. Subsequently, water-filled tubing was inserted into the designated model apertures, enabling experimentation through applied pressure. Two distinct liquid perfusion techniques were employed, one utilizing gravity-driven force and the other involving manual pressure application. This configuration serves as the basis for ensuing fluidic investigations.

Coating Enhancement for PDMS Devices:

To mitigate the inherent high surface hydrophobicity of PDMS devices, two distinct coatings were employed: fibronectin and poly-d-lysine. The fibronectin solution, at 30 µg/ml concentration, and poly-d-lysine at 1 mg/ml, were employed. Devices were initially loaded with the coating solution and incubated, requiring 1 hour for fibronectin and 4 hours for poly-d-lysine. Subsequently, the solution was removed; for fibronectin, triple washing with PBS followed by thorough aspiration was executed, while poly-d-lysine-coated devices were washed thrice with sterile deionized water and similarly aspirated. The coated devices were then placed in a sterile dish and subjected to an oven at 80°C for 24 to 72 hours, rendering them

hydrophilic and ready for use.

Incorporating PEO increases the porosity within the hydrogel

Polyethylene oxide (PEO) was incorporated into the hydrogel formulation to enhance scaffold porosity due to its physical incompatibility with the hydrogel matrix. Given its insolubility within the hydrogel, PEO introduced increased pore density. After thorough and uniform mixing with the hydrogel, visible light was employed for crosslinking. Subsequent to solidification, the hydrogel was subjected to repetitive washing with phosphate-buffered saline (PBS) for a consistent duration of 30 minutes. This washing process effectively removed PEO, resulting in the formation of pores within the hydrogel scaffold.

A PEO (Poly(ethylene oxide)) solution was meticulously prepared to attain a concentration of 1.5%. In this procedure, 75mg of PEO was introduced into 5ml of PBS, initiating dissolution. The mixture was subjected to incubation with rotation overnight to facilitate complete dissolution. Subsequently, a vortex followed by a brief centrifugation were performed, after which the solution was gently heated to reach 50°C. To ensure the solution was meticulously filtered through 0.22μm syringe filters.

Cell metabolic test

MSCs were cultured in 5% GelMA within cylindrical constructs, complemented by 0.5mM Ruthenium and 5mM SPS (sodium persulfate). The culture medium employed was MEM, with a cell density of 5 million cells per milliliter (5M/mL).

To initiate the metabolic assessment, the following steps were meticulously executed: The MEM medium and 10X alamarBlue stock solution were gently warmed to room temperature over a 30-minute duration in dark. Subsequently, the alamarBlue stock solution was diluted with the medium. The existing medium in the culture plate was aspirated and replaced with the prepared alamarBlue working solution, with each condition featuring three repetitions. To prevent light interference, the plate was wrapped in aluminum foil and placed within an incubator for a four-hour incubation period to facilitate the reaction. Upon completion of the incubation, the alamarBlue solution was retrieved for subsequent metabolic testing in the machine.

Cell Viability assay

The Live/DEAD Viability/Cytotoxicity Kit from ThermoFisher Scientific was employed for assessing cell viability. Calcein (Cat#: C3100MP, Lot#) and ethidium homodimer (Ref# E1169, Lot#) iodide were retrieved and allowed to warm for 5 minutes within the hood, all the while minimizing exposure to light. Dilution: Calcein stock, with a concentration of 4mM, and ethidium homodimer stock at 2mM, were each diluted 1000 times to achieve the working concentration. The culture medium was aspirated from the sample plate, and a single wash with PBS was performed. GelMA samples were subsequently transferred to a 96-well plate, and each gel was treated with 40 μl of the dye solution. The plate was covered with aluminum foil to shield it from light and placed within an incubator for 30 minutes in darkness. Following this incubation period, the samples underwent three consecutive 5-minute PBS washes.

For visualization, confocal imaging was conducted, capturing the stained cells. The images were subsequently analyzed using ImageJ software to determine the live-to-dead cell ratio.

Sol fraction

Uniformity in shape and volume was established among samples. Initial mass measurements were taken before subjecting the samples to overnight freeze-drying. Post-freeze-drying mass assessment followed. Subsequently, the samples were immersed in PBS in a plate, placed in a 37°C incubator overnight, and their mass was recorded after incubation. Another round of freeze-drying occurred overnight, culminating in the final mass evaluation. These steps collectively yielded sol fraction data, contributing to comprehensive sample analysis.

Volumetric 3D bioprinting

For volumetric 3D bioprinting, GelMA stock was stored at 37°C, while Ru/SPS were kept on ice in a dark environment. A colleague's design was uploaded, adjusted, and printed within a chosen vial configuration. Tubes were pre-cooled on ice for 15 minutes, then filled with GelMA, Ru/SPS, and PBS mixture, solidifying on ice for another 15 minutes. Following water removal, tubes were placed in the printer, light settings calibrated, and printing initiated. After printing, a brief incubation occurred, and the tube was placed in a 37°C water bath, washed with warm PBS, and the printed hydrogel cured with floodlight in the same concentration of photoinitiator for 1 minute. This method yielded intricately structured 3D bioprinted constructs.

<Result>

Development of a Fluidic Chip for Testing Fluid Flow in GelMA Hydrogel:

a. Initial Perfusion Fluidic Chip Fabrication: The fluidity of liquids within hydrogels holds critical relevance for their utility as biomaterials. To assess fluid permeation in GelMA hydrogels, a PDMS-based fluidic test setup was devised. Initial efforts unveiled challenges: liquid flow tended to escape the hydrogel edges instead of permeating. This could be attributed to suboptimal UV glue adhesion between the glass and PDMS container. We surmised this leakage could result from poor adhesion or a misalignment of liquid flow direction. To counter this, we adopted Kunststoff superglue for enhanced adhesion. This adaptation effectively resolved the edge leakage issue.

b. Refining the Primary Fluidic Chip: While addressing edge leakage, we encountered the need for excessive pressure to drive fluid flow, risking hydrogel rupture. This excessive pressure might stem from direct contact between edge adhesive and the hydrogel or mismatched fluid flow directions. To address this, our model was iteratively improved. Shallow gully were added on PDMS surfaces to channel excess adhesive, preventing direct contact with the hydrogel.[fig1] Additionally, inlet and outlet positions were repositioned. Although fluid still struggled to permeate the hydrogel, the refined model mitigated leakage and excessive pressure issues.
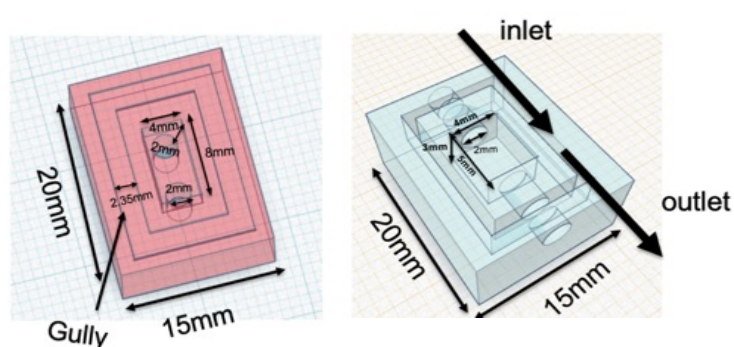

Figure 1. PDMS preliminary design drawing
(A) shallow gully. (B) reposition the inlet and out let

Despite curbing edge leakage, a gap persisted between PDMS walls and the hydrogel, causing fluid to migrate towards this gap. This was attributed to the inherent hydrophobicity of PDMS surfaces. To rectify this, PDMS underwent specialized

surface modification to enhance hydrogel adhesion. Two methods were compared: extracellular matrix protein coating and charged molecule modification. [fig2] Experimental outcomes demonstrated that Fibronectin surface coating yielded superior hydrogel-PDMS adhesion compared to poly-d-lysine, effectively inhibiting fluid migration.
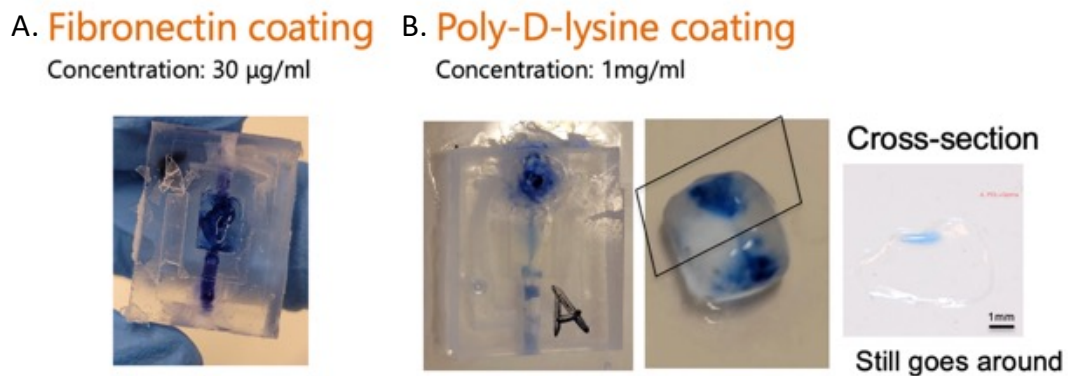

Figure 2. To reduce the inherent high surface hydrophobicity of PDMS devices by coating different materials
(A)fibronectin coating (B) Poly-d-lysine coating

c. Refinement of GelMA Hydrogel Structure in the Chip:

In preceding experiments, we observed the GelMA hydrogel structure to be densely bonded with nearly nanoscale porosity. While existing studies proposed that vascular endothelial cells can invade hydrogels with pore sizes of 25-50 μm, the range of 50-150 μm promotes the formation of more mature vascular tissue within hydrogels, facilitating cell adhesion and vascular tissue development (Chiu, Cheng et al. 2011). To foster an environment conducive to angiogenesis and controlled perfusion rates, we aimed to artificially create larger hydrogel pores. By experimenting with varying PEO concentrations (0%, 0.1%, 0.25%, 0.5%), we successfully generated pore sizes of
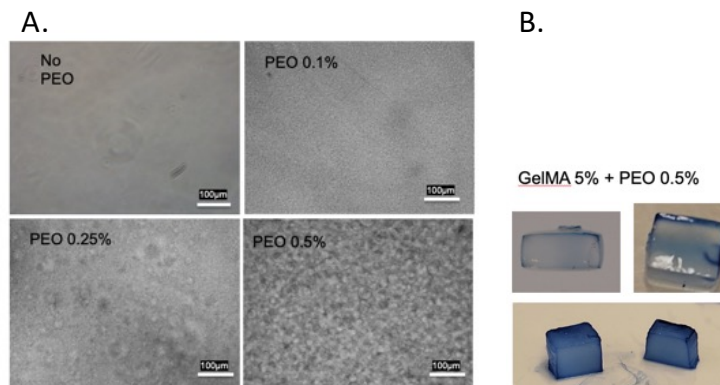

Figure 3. To augment the pore dimensions within the hydrogel, we employed supplemental polyethylene oxide (PEO).
(A) Hydrogel structures of varying pore sizes were fabricated by employing different ratios of PEO (B) Rapid diffusion of the liquid

0 µm, 5-7 µm, 15-27 µm, and 10-40 µm, respectively.[fig3] Integrating these findings with our refined PDMS model, we observed that, in combination with 5% GelMA and 0.5% PEO, the Fibronectin surface-coated fluidic chip enabled successful fluid flow, fostering uniform and rapid liquid diffusion within the hydrogel.[fig3]

Biocompatibility Assessment of the Chips

a. Cell Metabolism Evaluation:

GelMA hydrogels, renowned for their high water content, polymer flexibility, and cell-friendly characteristics, are commonly considered optimal for 3D cell cultivation. While PEO's non-toxicity and compatibility with cells have been established, its influence on cell growth within GelMA warrants investigation. Alamar Blue assays were employed to assess cell metabolism. Cells were cultured within hydrogels with varying PEO concentrations (0%, 0.1%, 0.25%, and 0.5%) for Days 1, 4, and 7. Notably, on Day 1, no significant differences emerged among groups. However, by Day 4 and 7, the metabolic rate of cells within the no PEO group exceeded that of the 0.5% PEO group, and the metabolic rates of both PEO 0.25% and PEO 0.5% groups were markedly lower than the control group. Based on these findings, it was determined that PEO in the hydrogel might adversely impact cell physiological metabolism.[Fig4]
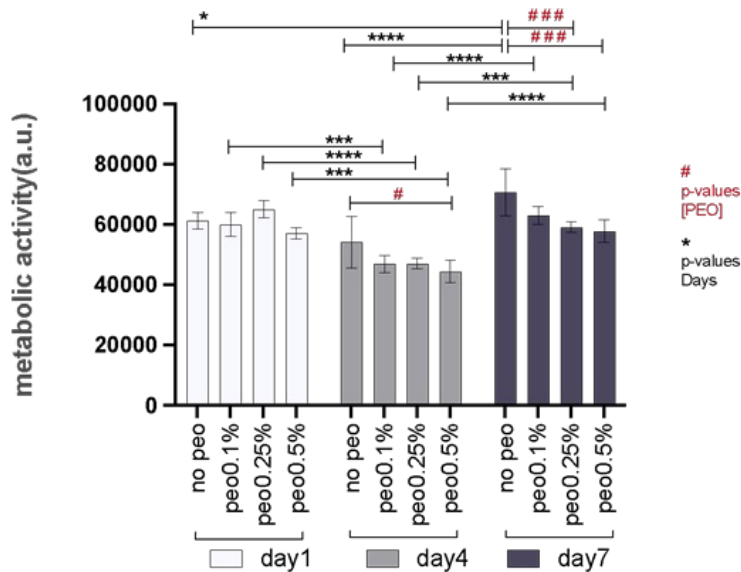

Figure 4. To evaluate cellular metabolic activity within the hydrogel culture.

b. Cell Viability Assessment:

In addition to metabolic evaluations, live/dead staining was performed. The results indicated that cells containing PEO exhibited significantly lower viability compared to the PEO-free control group. After seven day of culture, cell viability rates for PEO 0%, PEO 0.1%, and PEO 0.25% were 72.8%, 30.5%, and 36.8% respectively. Overall, these

findings underscore the potentially detrimental impact of PEO on cell viability.[Fig5]
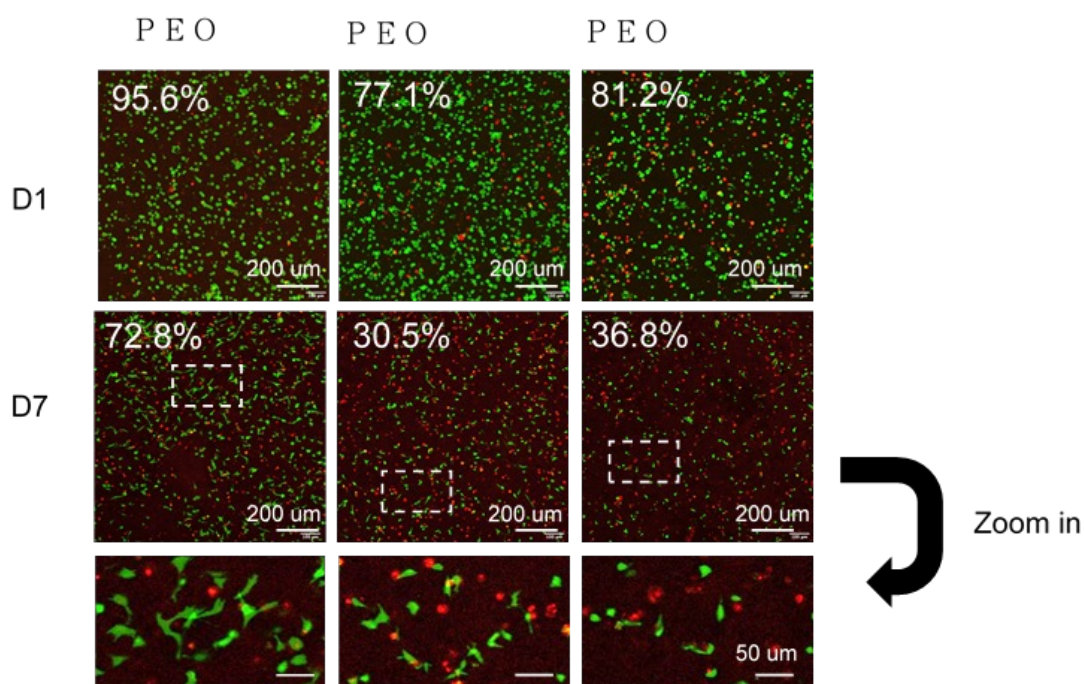

Figure 5. To evaluate cell viability within the hydrogel culture.

Enhancing Biocompatibility through Hydrogel Fabrication Optimization:
In pursuit of avoiding detrimental effects on cells caused by hydrogel additives, we opted to refine the hydrogel fabrication process using an additive-free approach. Volumetric 3D bioprinting, a cutting-edge technique widely employed in biomaterial fabrication, offers rapid generation of intricate centimeter-scale structures, minimizing cellular damage by streamlining the 3D printing process. [17]This method uses GelMA, and during its curing process, Ru/SPS serves as a preferred photoinitiator for enhanced cell microenvironment and improved light penetration. However, the optimal combination of Ru and SPS concentrations is contingent on the structure of the printed product.

To achieve our initial goal of generating larger-pored and complex porous hydrogel configurations, we began by designing the 3D structure using Tinkercad software. By inputting these parameters into the printer, we attempted to manufacture hydrogel constructs that aligned with our expectations. Experimental results demonstrated that under light dose 400/Ru 0.02mM/SPS 10mM, the shape fidelity was 88.02%, whereas under light dose 550/Ru 0.01mM/SPS 15mM, the shape fidelity was 43.26%.[Fig6] Although we obtained the desired hydrogel structure, the low Ru/SPS concentrations used raised concerns relative to related literature. To address this, we

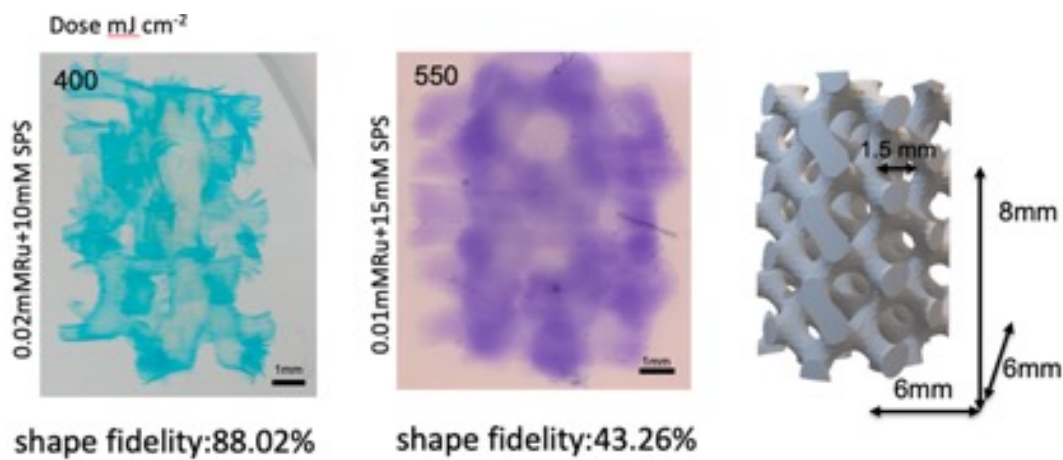conducted tests on hydrogel mass loss and swelling ratio to ensure crosslinking stability.

Figure 6. Output following 3D volumetric bioprinting

These tests revealed that, at equivalent SPS concentrations, lower Ru concentrations yielded greater mass loss and swelling ratios. Notably, low concentrations of Ru/SPS did induce crosslinking instability.[Fig7] Therefore, the need to recalibrate the printing parameters emerged, considering that the balance of Ru and SPS concentrations is pivotal for achieving the intended crosslinking stability.
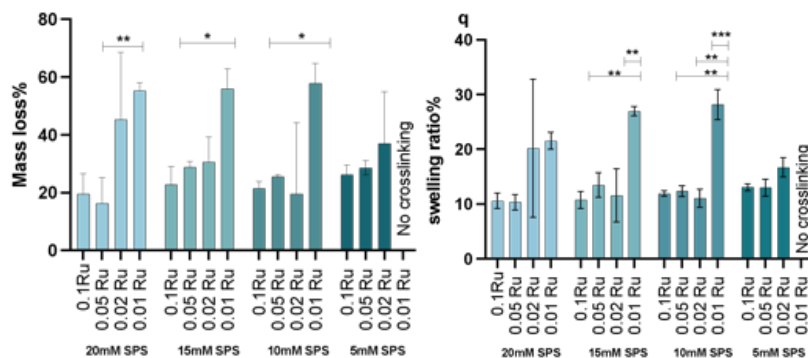

Figure 7. To appraise the mass reduction and swelling ratio of the resultant 3D volumetric bioprinted construct.

<Discussion>

The cell metabolic test in this study reveals dynamic trends within GelMA hydrogel constructs. The initial drop in metabolic activity from Day 1 to Day 4 could arise from cells adapting to the new matrix, experiencing altered substrate availability and

mechanical cues. Limited nutrient and oxygen diffusion to the hydrogel core might contribute to decreased activity. As the culture progressed to Day 7, increased metabolic activity suggests improved adaptation and potential cell proliferation. Cells may have established communication and better nutrient utilization, enhancing metabolic state. The consistency of trends across groups underscores the robustness of findings.

These patterns likely reflect GelMA's influence on cell behavior. Future investigations could probe specific metabolic markers and live-cell imaging for deeper insights. Overall, the test unveils the intricate interaction between cells and the GelMA microenvironment, guided by factors like adaptation, nutrient diffusion, and potential proliferation. Such insights are pivotal for optimizing cellular responses within 3D hydrogel systems.

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