

Original Article

Diffusion-Mediated Post-Gelation Microstructural Tuning of GelMA Hydrogels for Cell-Permissive Tissue Engineering

Karthika Muthuramalingam¹  and Hyun Jong Lee^{1,*} ¹School of Chemical, Biological and Battery Engineering, Gachon University, 13120 Seongnam-si, Gyeonggi-do, Republic of Korea

Abstract

Background: Engineering hydrogel scaffolds for tissue engineering requires simultaneous control of mechanical stability and microstructural permissiveness. However, in conventional bulk hydrogels, these properties are strongly coupled because the network architecture is largely defined during gelation. Here, we report a diffusion-mediated strategy that temporally separates covalent network formation from subsequent microstructural evolution in photocrosslinked gelatin methacryloyl (GelMA) hydrogels using physically incorporated Poloxamer 407 (Pluronic F127, PF127) as a transient additive. **Methods:** GelMA hydrogels containing physically incorporated PF127 were photocrosslinked to establish covalent networks, followed by aqueous equilibration to promote PF127 diffusion and post-gelation microstructural evolution. Structural changes were characterized using scanning electron microscopy (SEM), hydrated-state brightfield microscopy, Fourier-transform infrared spectroscopy (FTIR), and dry mass loss analysis. Oscillatory rheology evaluated viscoelastic behavior, while encapsulated C2C12 myoblast viability, proliferation, and cytoskeletal organization were assessed to determine cytocompatibility and cellular permissiveness. **Results:** Semi-quantitative FTIR analysis, supported by dry mass loss measurements, demonstrated substantial PF127 depletion after 24 h equilibration in 5% GelMA formulations, while preserving GelMA amide bands. SEM revealed increased apparent porosity following equilibration, whereas rheology confirmed retention of elastic-dominant viscoelastic behavior across all formulations. Remodeling was attenuated in 10% GelMA formulations, reflecting diffusion constraints imposed by higher network density. Encapsulated C2C12 myoblasts maintained high viability and exhibited increased metabolic activity over time, with G5P5 showing the greatest filamentous actin-positive cytoskeletal coverage, indicating enhanced cellular permissiveness. **Conclusions:** These findings establish diffusion-mediated post-gelation microstructural tuning as a compositionally adjustable strategy for enhancing hydrogel scaffold permissiveness while preserving bulk mechanical integrity, highlighting its potential for tissue engineering applications.

Keywords: GelMA hydrogels, post-gelation microstructural tuning, Poloxamer 407, diffusion-mediated remodeling, viscoelastic stability, cell permissiveness.

***Address for correspondence:** Hyun Jong Lee, School of Chemical, Biological and Battery Engineering, Gachon University, 13120 Seongnam-si, Gyeonggi-do, Republic of Korea. E-mail: hjlee2@gachon.ac.kr.

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Introduction

Engineering hydrogel scaffolds for tissue engineering requires simultaneous control of mechanical stability and microstructural permissiveness [1–3]. Hydrogels must maintain sufficient structural integrity to withstand fabrication, handling, and *in vitro* culture, while providing a three-dimensional microenvironment that permits cell spreading, cytoskeletal organization, and matrix interaction. In conventional bulk hydrogels, however, these two requirements are governed by the same structural variables. Polymer concentration and crosslink density collectively determine network stiffness, mesh size, free volume, and solute transport capacity, such that increasing network density improves mechanical stability at the cost of microstructural permis-

siveness, and reducing it achieves the opposite [3–5]. This intrinsic coupling between structure and mechanics represents a persistent design challenge for hydrogel-based scaffolds [6].

Gelatin methacryloyl (GelMA) exemplifies both the promise and the limitation of this design paradigm. Its photocrosslinkability, biocompatibility, biodegradability, and intrinsic cell-adhesive motifs make it one of the most widely used platforms for cell-laden hydrogel fabrication [7,8]. Yet, as in most chemically crosslinked hydrogel systems, the microstructure and mechanical properties of GelMA hydrogels are established simultaneously at the point of network formation and remain largely fixed thereafter. Strategies developed to introduce porosity into GelMA-based ma-

trices, including granular hydrogel scaffolds, cryogelation, sacrificial porogen templating using polyethylene oxide or Pluronic derivatives, and aqueous emulsion systems, have demonstrated meaningful improvements in void fraction and cell infiltration [9–14]. However, these approaches share a common feature: pore architecture is defined during or prior to gelation, through pre-formed microgel packing, ice crystal templating, or porogen-induced phase separation concurrent with photopolymerization. Once the network is formed, the resulting microstructure is largely static. Furthermore, these methods can involve trade-offs including reduced mechanical integrity after pore generation, incomplete porogen removal, or fabrication complexity that limits practical implementation.

An alternative, less explored strategy is to design microstructural evolution after network formation, within an already-established covalent hydrogel framework. While sacrificial-template systems, including aqueous two-phase GelMA/poly(ethylene oxide) (PEO) emulsions, have demonstrated that transient aqueous domains can be removed after hydrogel crosslinking to generate porous GelMA scaffolds [15], these approaches generally rely on the formation and preservation of templating domains prior to gelation and have primarily focused on the resulting porous architecture and biological performance, with comparatively less emphasis on the physicochemical processes governing structural evolution during and after transient component removal. In contrast, the use of physically incorporated transient additives that undergo diffusion-mediated removal during aqueous equilibration provides a potential route to induce local microstructural reorganization within an already-crosslinked hydrogel network. This temporal separation between network formation and microstructural reorganization could, in principle, allow porosity to increase while preserving the bulk viscoelastic behavior and mechanical integrity provided by the covalent hydrogel framework.

Poloxamer 407 (Pluronic F127, PF127), an amphiphilic poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymer capable of aqueous self-assembly and non-covalent association through its relatively hydrophobic segments, represents a suitable transient additive for this strategy. Its physical incorporation into GelMA precursor solutions may generate local compositional heterogeneity during photocrosslinking, while its subsequent diffusion-mediated removal during physiological aqueous equilibration may promote further microstructural reorganization within the pre-formed GelMA network. In this study, we investigate this diffusion-mediated post-gelation tuning concept using GelMA/PF127 composite hydrogels. GelMA/PF127 precursors were photocrosslinked to form composite hydrogels across a range of GelMA concentrations and PF127 contents, and the resulting constructs were systematically characterized by temporal mass-change analysis, Fourier-

transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and oscillatory rheology before and after aqueous equilibration. The biological relevance of the remodeled microenvironment was evaluated using encapsulated C2C12 myoblasts, with emphasis on cell viability and cytoskeletal organization as indicators of matrix permissiveness.

Materials and Methods

Hydrogel Preparation

GelMA (degree of methacrylation >90%; derived from type B porcine gelatin, 300 Bloom; CleCell, Seongnam, South Korea) was used to fabricate photocrosslinkable hydrogels. A concentrated GelMA stock solution of 20% w/v was first prepared by dissolving GelMA in phosphate-buffered saline (PBS) under continuous magnetic stirring at 60 °C until a clear and homogeneous solution was obtained. The stock solution was subsequently diluted with PBS to obtain the desired GelMA concentrations for hydrogel preparation. The photoinitiator 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959; Cat. No. 410896, Sigma-Aldrich, St. Louis, MO, USA) was incorporated at a final concentration of 0.2% w/v in all formulations, followed by thorough mixing to ensure homogeneous distribution. The precursor solution was then cooled to 25–30 °C to maintain GelMA in a fluid state while preventing premature physical gelation. An ice-cold solution of PF127 (Cat. No. P2443, Sigma-Aldrich, St. Louis, MO, USA) was prepared separately and added at the required concentrations with gentle mixing to obtain homogeneous precursor formulations.

Using this approach, five hydrogel groups were prepared based on the final polymer composition: G5, G5P2.5, G5P5, G10, and G10P5. These correspond to 5% w/v GelMA, 5% w/v GelMA + 2.5% w/v PF127, 5% w/v GelMA + 5% w/v PF127, 10% w/v GelMA, and 10% w/v GelMA + 5% w/v PF127, respectively. The precursor solutions were transferred into molds and exposed to 365 nm ultraviolet irradiation (Altis UVcure, Incheon, Republic of Korea) for 40 s to initiate GelMA photopolymerization, generating crosslinked GelMA networks with physically incorporated PF127.

In Vitro Mass Change Analysis Under Physiological Conditions

To evaluate temporal mass variation under physiological conditions, GelMA-based hydrogels with different compositions were freshly prepared as described above. Immediately after crosslinking, excess surface moisture was gently removed using lint-free tissue, and the initial wet weight of each hydrogel was recorded as W_0 . Each formulation was prepared in triplicate. Individual samples were immersed in Dulbecco's phosphate-buffered saline (DPBS) and incubated at 37 °C under static conditions. At predetermined time points of Days 1, 2, 3, 6, 8, 10, and 14, hydro-

gels were carefully removed from the incubation medium, and excess surface liquid was gently blotted without applying compressive force. The wet weight at each time point was recorded as W_t . Following weighing, the samples were returned to fresh DPBS to maintain consistent aqueous conditions throughout the experiment.

The percentage mass change was calculated using the following equation (Eqn. 1):

$$\text{Mass Change (\%)} = \frac{W_0 - W_t}{W_0} \times 100 \quad (1)$$

where W_0 represents the initial wet weight at Day 0 and W_t represents the wet weight at each time point. In this definition, positive values indicate net mass loss, whereas negative values indicate net mass gain. This value reflects the combined effects of water uptake, release of loosely associated components, diffusion of physically incorporated PF127, and early network equilibration. Because PF127 is physically incorporated and may diffuse from the hydrogel matrix during incubation, mass change was calculated relative to the initial wet weight to capture the coupled contribution of hydration and diffusion-mediated mass variation.

Morphological and Chemical Characterization

Field-emission scanning electron microscopy (FE-SEM) and FTIR were performed to investigate the morphological and compositional characteristics of GelMA-based hydrogels before and after short-term aqueous equilibration. Hydrogels with different formulations were prepared as described above and divided into two experimental conditions: freshly crosslinked and DPBS-equilibrated. For the freshly crosslinked condition, hydrogels were immediately quenched in liquid nitrogen after crosslinking and subsequently lyophilized for 48 h to obtain dried specimens. For the aqueous-equilibrated condition, hydrogels were immersed in DPBS and incubated at 37 °C for 24 h under static conditions. After incubation, samples were removed from DPBS, gently blotted to remove excess surface liquid, rapidly frozen in liquid nitrogen, and lyophilized for 24 h.

The dried samples were mounted on aluminum stubs, sputter-coated with a thin layer of platinum, and observed using FE-SEM (SU8600, Hitachi, Tokyo, Japan; Smart Materials Research Center for IoT supported by the Korea Basic Science Institute, NFEC-2023-02-285654) to examine the apparent dried-state internal morphology of the hydrogels. Apparent porosity was quantified from thresholded SEM images using ImageJ (National Institutes of Health, Bethesda, MD, USA) and expressed as the area fraction of void space. For each hydrogel formulation, porosity values were obtained from SEM images of freshly prepared and 24 h DPBS-equilibrated samples and used for comparative analysis of microstructural openness. In addition, freshly prepared hydrogels and hydrogels incubated

in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C for 48 h were imaged under brightfield conditions using an EVOS M5000 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) at 20× magnification to qualitatively evaluate formulation-dependent microstructural features before and after aqueous equilibration.

FTIR spectra of the dried samples were recorded over the range of 400–4000 cm^{-1} using a Fourier transform infrared spectrometer (Nicolet iS50, Thermo Fisher Scientific, Madison, WI, USA) to identify characteristic functional group vibrations corresponding to GelMA and PF127 components. Spectral comparisons between freshly prepared and DPBS-equilibrated samples were performed to assess diffusion-mediated compositional changes and to verify preservation of the GelMA backbone following aqueous exposure. To semi-quantitatively evaluate PF127-associated signal attenuation, the PF127-associated C–O–C stretching band was integrated over 1050–1150 cm^{-1} and normalized to the GelMA amide I band integrated over 1600–1700 cm^{-1} after baseline correction. The normalized C–O–C/amide I peak-area ratio was used as an indicator of PF127-associated signal retention within the hydrogel matrix. The percentage decrease in this ratio after 24 h DPBS equilibration was calculated relative to the freshly prepared state using the following equation (Eqn. 2):

$$\text{Decrease (\%)} = \left(1 - \frac{R_{24h}}{R_{\text{fresh}}} \right) \times 100 \quad (2)$$

where R_{fresh} and R_{24h} represent the normalized C–O–C/amide I peak-area ratios before and after 24 h DPBS equilibration, respectively. GelMA-only hydrogels were excluded from this calculation because they did not contain PF127 and therefore did not provide a meaningful PF127-associated C–O–C/amide I ratio. The normalized C–O–C/amide I peak-area ratio was determined from three independently prepared hydrogel samples ($n = 3$), and data are presented as mean $\pm$ standard deviation.

To further evaluate diffusion-mediated compositional changes, dry mass loss and equilibrium swelling analyses were performed. Freshly prepared hydrogels ($n = 3$) were first lyophilized to determine the initial dry mass (W_0). The samples were then immersed in PBS at 37 °C, and the swollen mass was recorded after 1, 3, 5, 24, and 48 h. After 48 h, the hydrogels were re-lyophilized to obtain the final dry mass (W_f). Dry mass loss was calculated using the following equation (Eqn. 3):

$$\text{Dry Mass Loss (\%)} = \frac{W_0 - W_f}{W_0} \times 100 \quad (3)$$

The equilibrium swelling ratio was calculated using the swollen mass at equilibrium (24 h) normalized to the corresponding final dry mass after re-lyophilization.

$$\text{Equilibrium Swelling Ratio} = \frac{W_s - W_f}{W_f} \quad (4)$$

Where W_s represents the equilibrium swollen mass, and W_f represents the final dry mass after re-lyophilization. To assess longer-term compositional stability, an additional set of hydrogels was immersed in PBS for 7 days, after which dry mass loss was determined using the same procedure.

Mechanical Characterization of Hydrogels

Hydrogels with varying formulations were prepared as described above. For rheological measurements, 200 μL of each precursor formulation was cast and photocrosslinked to form hydrogel discs. The samples were evaluated under two conditions: freshly crosslinked and aqueous-equilibrated. Rheological measurements were performed using a modular compact rheometer (MCR92, Anton Paar, Graz, Austria) equipped with a parallel-plate geometry at 37 °C. Prior to frequency sweep measurements, strain sweep tests were performed to identify the linear viscoelastic region, and all frequency sweeps were conducted at a fixed strain within this region. Oscillatory frequency sweep tests were performed over a frequency range of 0.1–10 Hz to determine the storage modulus and loss modulus of the hydrogels. To evaluate the stability of the viscoelastic properties following prolonged aqueous exposure, additional frequency sweep measurements were performed after 10 days of incubation. Acellular hydrogels were incubated in PBS at 37 °C, whereas cell-laden hydrogels were cultured in DMEM under standard culture conditions for 10 days prior to rheological testing.

Compression testing was performed using a universal testing machine (34SC-1, Instron, Norwood, MA, USA) in an unconfined configuration at a crosshead speed of 5 mm min^{-1} using cylindrical hydrogel specimens ($n = 3$). Representative stress–strain curves were recorded, and the compressive Young's modulus was determined from the initial linear region of the stress–strain curve.

Cell Culture and Encapsulation

Murine myoblast C2C12 cells (CRL-1772TM, American Type Culture Collection, Manassas, VA, USA) at passages below 14 were used for all encapsulation studies. Cells were suspended in hydrogel precursor solutions at defined densities prior to photocrosslinking. The cell–polymer mixtures were cast into molds and subjected to UV irradiation as described above to obtain cell-laden hydrogels.

For viability studies, cells were encapsulated at a density of 75,000 cells per hydrogel within a total hydrogel volume of 100 μL . For cytoskeletal analysis, cells were encapsulated in all hydrogel formulations at a den-

sity of 7.5×10^5 cells/mL, with each hydrogel prepared at a volume of 50 μL . Following encapsulation, constructs were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin under standard culture conditions. This growth medium was used for viability and metabolic activity assessments. For cytoskeletal analysis, constructs were subsequently transferred to differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin–streptomycin). For cell experiments, cell-laden hydrogels were transferred to culture medium after photocrosslinking.

Cell Viability, Metabolic Activity, and Cytoskeletal Analysis

Cell viability within the hydrogels was assessed after 48 h and during extended culture (7 and 10 days) using an InvitrogenTM Live/DeadTM Viability/Cytotoxicity kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were washed with DPBS and incubated in a staining solution containing calcein-AM and ethidium homodimer-1 prepared in DPBS for 1 h at 37 °C. Following staining, hydrogels were gently washed to remove excess dye and imaged using the EVOS M5000 Imaging System. Live cells were identified by green fluorescence, whereas dead cells exhibited red fluorescence.

Cell metabolic activity was evaluated using a Cell Counting Kit-8 (CCK-8, GlpBio, Montclair, CA, USA) assay after 3 and 7 days of culture. At each point, the culture medium was replaced with fresh medium containing 10% (v/v) CCK-8 reagent and incubated for 2 h at 37 °C. Subsequently, 100 μL of the reaction solution from each well was transferred to a 96-well plate, and absorbance was measured at 450 nm using a microplate reader. Blank wells containing culture medium and CCK-8 reagent without hydrogels were used for background subtraction. To evaluate potential interference from the hydrogel matrices, acellular hydrogels of each formulation were also incubated with CCK-8 reagent under identical conditions. No significant differences in background absorbance were observed among the hydrogel formulations. Relative cell proliferation was calculated as the fold increase in absorbance at Day 7 normalized to the corresponding Day 3 value. Data are presented as mean $\pm$ standard deviation ($n = 3$).

For cytoskeletal analysis, cell-laden hydrogels were initially cultured in growth medium for 3 days, after which the medium was replaced with differentiation medium. The constructs were maintained under these conditions for 14 days with regular media changes. At the end of the culture period, hydrogels were fixed, permeabilized, and washed with PBS. Filamentous actin (F-actin) was stained by incubating samples with a red fluorescent phalloidin conjugate (CytoPainter, ab112127, Abcam, Cambridge, UK) for 2 h at room temperature, followed by nuclear counterstaining for 10 min. The stained samples were then imaged using the

same imaging system to evaluate cytoskeletal organization and cell morphology within the hydrogel matrices. F-actin-positive area per field was quantified using ImageJ under identical threshold settings. For each formulation, images were obtained from three independently prepared hydrogel constructs, and the data are presented as mean $\pm$ standard deviation ($n = 3$ independent hydrogels per group).

Statistical Analysis

Statistical significance was analyzed using ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, with $p < 0.05$ considered statistically significant. Quantitative data are presented as mean $\pm$ standard deviation from three independently prepared hydrogel samples ($n = 3$) unless otherwise stated.

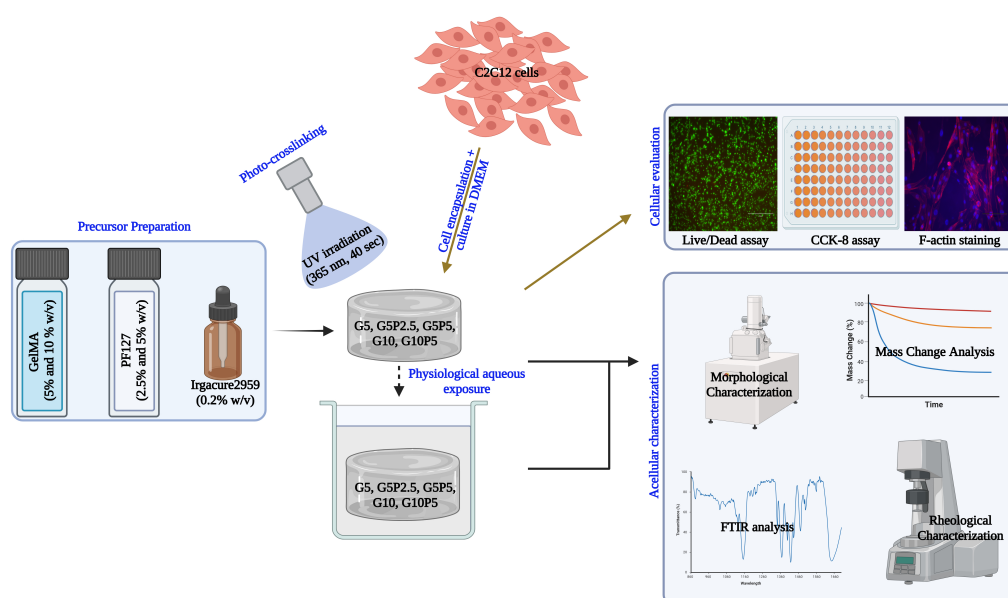
Results

The overall fabrication and characterization workflow for the GelMA/PF127 hydrogels is shown in Scheme 1. GelMA/PF127 precursor solutions were photocrosslinked to form covalent GelMA hydrogels containing physically incorporated PF127. Acellular hydrogels were exposed to DPBS to examine aqueous diffusion-mediated PF127 release and post-gelation microstructural evolution, whereas cell-laden hydrogels were directly cultured in medium to evaluate cell viability and cytoskeletal organization.

Microstructural Characteristics of Fresh and Aqueous-Equilibrated Hydrogels

Optical imaging, hydrated-state brightfield microscopy, and SEM were used to examine the macroscopic appearance and microstructural characteristics of GelMA-based hydrogels before and after aqueous equilibration. Freshly crosslinked hydrogels formed uniform disk-shaped constructs with smooth surfaces (Fig. 1a). GelMA-only hydrogels appeared translucent, whereas PF127-containing hydrogels showed increased opacity that was more pronounced at higher PF127 content. This composition-dependent optical difference is consistent with the presence of PF127-associated domains within the GelMA network that scatter visible light, suggesting that PF127 influences local network organization at the point of gelation.

SEM images of freshly crosslinked hydrogels revealed formulation-dependent morphological differences (Fig. 1b,d). G5 showed a relatively open apparent morphology with irregular void features and thin polymer walls, whereas G10 exhibited a denser matrix with reduced apparent void space, reflecting its higher crosslink density. In PF127-containing formulations, apparent porosity was higher than in the corresponding GelMA-only controls even before aqueous equilibration, ranging from 30.80% in G5P2.5 to 39.38% in G5P5 compared with 18.64% in G5. Among fresh samples, G5P5 showed the most open apparent morphology, while G10P5 (17.64%) remained comparatively compact despite increased apparent porosity relative to G10 (5.78%). Supporting these observations, hydrated-



Scheme 1. Schematic illustration of GelMA/PF127 hydrogel fabrication, aqueous diffusion-mediated remodeling, and characterization workflow. Created in BioRender. Lee, H. J. (2026) <https://BioRender.com/d9m2uji>.

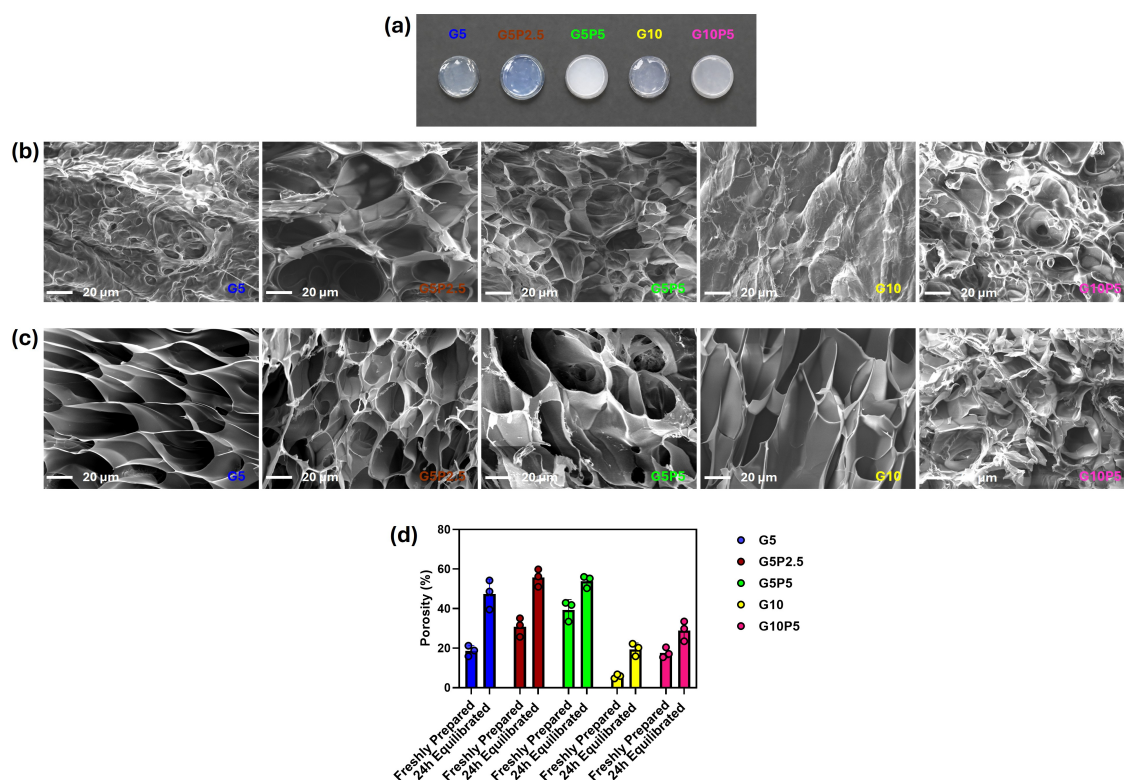


Fig. 1. Structural and microstructural characterization of GelMA/PF127 hydrogels. (a) Optical images of freshly crosslinked hydrogels showing macroscopic appearance and transparency differences across formulations. (b) SEM images of freeze-dried hydrogels immediately after crosslinking. (c) SEM images of hydrogels following 24 h equilibration in DPBS and subsequent freeze-drying, showing comparative apparent dried-state morphology associated with post-gelation microstructural reorganization. Scale bar: 20 μm . (d) Apparent dried-state porosity quantified from thresholded SEM images using ImageJ and expressed as the area fraction of void space. This figure was generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

state brightfield microscopy of freshly prepared hydrogels (**Supplementary Fig. 1a**) revealed formulation-dependent microstructural heterogeneity in PF127-containing formulations, particularly G5P5 and G10P5, prior to lyophilization. These observations suggest that PF127 influences local GelMA network organization during photocrosslinking, generating local structural heterogeneity that is already reflected in the dried-state morphology of freshly prepared hydrogels.

After 24 h DPBS equilibration, all formulations showed further increases in apparent pore openness (Fig. 1c, d). Apparent porosity increased to 53.85% in G5P5 and 55.68% in G5P2.5 after equilibration, representing increases of 14.47 and 24.88 percentage points relative to their respective fresh-state values. GelMA-only hydrogels also showed increased apparent porosity after equilibration, with G5 increasing from 18.64% to 47.44% and G10 increasing from 5.78% to 19.43%, suggesting that network relaxation during hydration contributes independently of PF127 content. G10P5 showed an increase from 17.64% to 28.95% after equilibration, although the overall morphology remained denser than the 5% GelMA-based formulations, reflecting the dominant influ-

ence of GelMA concentration on structural rearrangement. Hydrated-state brightfield microscopy following 48 h immersion (**Supplementary Fig. 1b**) showed formulation-dependent structural features that paralleled the trends observed in the corresponding SEM images, indicating that the observed microstructural differences are not solely attributable to freeze-drying artifacts. Taken together, the two-stage morphological progression observed in PF127-containing hydrogels, with compositional effects present in the fresh state and further structural development after equilibration, is consistent with the proposed mechanism in which PF127 influences the initial network organization and subsequently promotes post-gelation microstructural remodeling following diffusion-mediated removal.

Aqueous Equilibration Behavior Under Physiological Conditions

Temporal mass change was monitored over 14 days to characterize the aqueous equilibration behavior of GelMA-based hydrogels under physiological conditions (Fig. 2). All formulations showed rapid mass variation within the first 24 h, followed by progressively smaller changes over the remaining incubation period, indicating that the primary

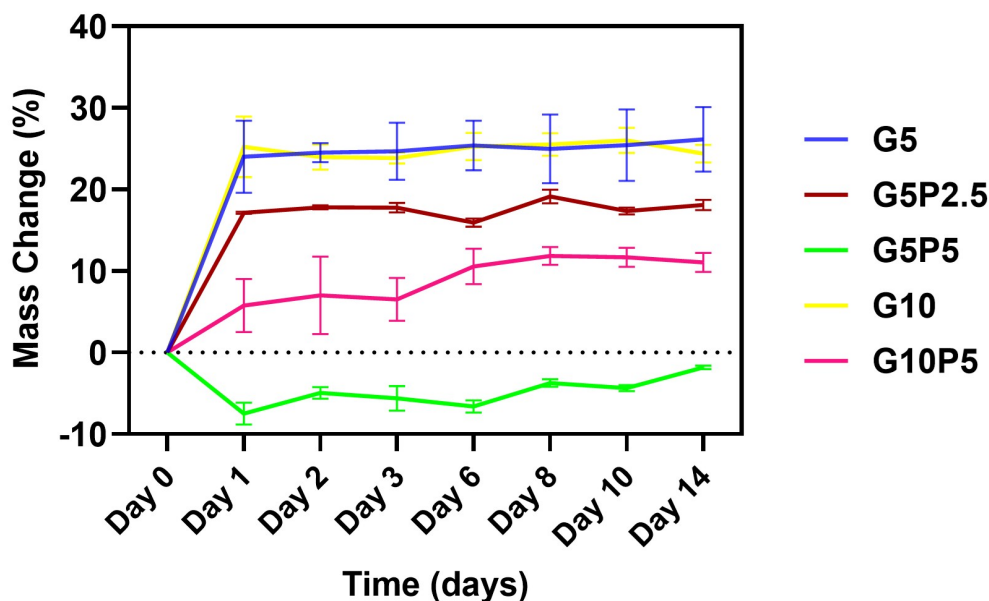


Fig. 2. Temporal mass change behavior under physiological conditions. Net mass change relative to initial wet weight of GelMA-based hydrogels during incubation in DPBS at 37 °C over 14 days. Mass change was calculated as $(W_0 - W_t)/W_0 \times 100$. Positive values indicate net mass loss, whereas negative values indicate net mass gain. Data are presented as mean $\pm$ standard deviation. This figure was generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

physicochemical reorganization occurred during early equilibration.

GelMA-only hydrogels showed sustained positive mass change throughout the experiment, with G5 and G10 reaching approximately 24–25% by day 1 and remaining within a similar range thereafter. This behavior likely reflects the release of loosely associated or uncrosslinked GelMA components during initial hydration, followed by stabilization of the swollen covalent network.

PF127-containing hydrogels exhibited composition-dependent mass-change profiles that reflect the competing contributions of water uptake and diffusion-mediated PF127 loss. G5P2.5 showed moderate positive mass change of approximately 16–19% over 14 days, lower than that of G5, suggesting that partial PF127 loss offset water uptake relative to the GelMA-only control. G5P5, in contrast, showed negative mass change during early incubation and remained below the initial wet weight for most of the observation period. This apparent net mass gain indicates that water uptake substantially exceeded diffusible component loss, consistent with the more open apparent morphology observed in G5P5 by SEM, where increased void space would be expected to accommodate greater water absorption. By day 14, the mass change of G5P5 approached the baseline, suggesting completion of the primary equilibration process. G10P5 showed moderate positive mass change reaching approximately 10–12% after prolonged incubation, lower than G10 but consistently positive, indicating that PF127 loss occurred but was insufficient to off-

set water uptake to the same extent as in the 5% GelMA-based formulations. The attenuated mass-change response of G10P5 relative to G5P5 is consistent with restricted diffusive transport within the denser 10% GelMA network.

To further distinguish the relative contributions of water uptake and diffusion-mediated material loss during aqueous equilibration, dry mass loss and equilibrium swelling were evaluated after 48 h immersion (**Supplementary Fig. 2**). PF127-containing hydrogels exhibited greater dry mass loss compared with the corresponding GelMA-only controls, with G5P5 showing the highest mass reduction among all formulations (**Supplementary Fig. 2a**). In contrast, equilibrium swelling showed only modest formulation-dependent differences (**Supplementary Fig. 2b**). Although G5P5 exhibited a moderately increased swelling ratio compared with G5, the relatively small change in water uptake could not account for the pronounced increase in dry mass loss. These findings suggest that the increased mass reduction in PF127-containing hydrogels primarily arises from diffusion-mediated loss of physically incorporated PF127 during aqueous equilibration rather than from differences in water absorption alone.

To evaluate whether further material loss occurred after the initial equilibration period, dry mass loss was also measured following 7 days of PBS immersion (**Supplementary Fig. 3**). Dry mass loss after 7 days remained comparable to that measured after 48 h, indicating that most diffusible components were removed during the

initial equilibration period and that the hydrogel formulations subsequently reached a relatively stable state.

Together, the temporal mass change, dry mass loss, and equilibrium swelling analyses indicate that the major physicochemical remodeling occurs during the early equilibration period and is associated with diffusion-mediated PF127 depletion rather than swelling alone. These findings are further supported by the compositional changes revealed by FTIR analysis in the following section.

FTIR Analysis of Fresh and Aqueous-Equilibrated Hydrogels

FTIR spectroscopy was used to examine compositional changes in GelMA-based hydrogels before and after aqueous equilibration (Fig. 3a,b). Freshly prepared hydrogels exhibited characteristic GelMA-associated amide bands [16–18] derived mainly from the gelatin backbone, with amide I around 1650 cm^{-1} and amide II around 1540 cm^{-1} assigned to C=O stretching and N–H bending vibrations, respectively. Additional bands near 3300 cm^{-1} and $1240\text{--}1270\text{ cm}^{-1}$ were assigned to N–H/O–H stretching and amide III vibrations.

PF127-containing formulations showed additional bands in the $1050\text{--}1150\text{ cm}^{-1}$ region, corresponding to C–O–C stretching vibrations of the PEO and PPO segments of PF127 [19,20]. This indicates that PF127 was physically present in the freshly crosslinked hydrogels before aqueous equilibration. After 24 h immersion in DPBS, the PF127-associated bands markedly decreased in all PF127-containing formulations. In contrast, the GelMA-associated amide bands remained clearly detectable after equilibration.

To semi-quantitatively evaluate PF127-associated signal changes, the C–O–C band area at $1050\text{--}1150\text{ cm}^{-1}$ was normalized to the GelMA amide I band area at $1600\text{--}1700\text{ cm}^{-1}$ (Fig. 3c). GelMA-only G5 and G10 hydrogels were excluded from this analysis because they did not contain PF127. The normalized C–O–C/amide I ratio decreased by approximately 98.9%, 97.7%, and 81.7% for G5P2.5, G5P5, and G10P5, respectively, after 24 h DPBS equilibration. The near-complete reduction in the C–O–C signal for G5-based formulations confirms that PF127 was largely removed from the GelMA network within the equilibration period. The comparatively lower reduction observed in G10P5 is in line with the more restricted diffusive transport expected within a higher-density crosslinked network, corroborating the attenuated mass-change behavior reported in Section (Aqueous Equilibration Behavior Under Physiological Conditions) and indicating that GelMA concentration is a determinant of the extent to which PF127 can be removed under physiological aqueous conditions.

Mechanical Properties Before and After Aqueous Equilibration

Oscillatory rheology was performed to evaluate the bulk viscoelastic behavior of GelMA-based hydrogels before and after aqueous equilibration (Fig. 4). All formulations exhibited elastic-dominant behavior, with storage modulus (G') consistently exceeding loss modulus (G'') across the tested frequency range, confirming the formation of stable crosslinked hydrogel networks.

For freshly crosslinked hydrogels, G' increased with GelMA concentration. G10-based formulations showed substantially higher G' values than G5-based formulations (on the order of 10^3 Pa versus 10^2 Pa), as expected from the higher polymer content and greater network density of the 10% GelMA hydrogels. Within the same GelMA concentration, PF127-containing hydrogels showed higher G' values than the corresponding GelMA-only controls. This observation suggests that physically incorporated PF127 may contribute to network stiffness in the fresh state, possibly through transient physical interactions within the GelMA matrix, polymer entanglement, or local compositional heterogeneity, rather than acting as a simple diluent.

After 24 h incubation in DPBS, all hydrogels retained elastic-dominant behavior with limited frequency dependence. G' was maintained or slightly increased relative to fresh-state values across all formulations, with no substantial reduction observed in any group. This mechanical stability after equilibration is notable given the concurrent microstructural changes documented in Section (Microstructural Characteristics of Fresh and Aqueous-Equilibrated Hydrogels), where PF127-containing hydrogels showed increased apparent porosity following the same equilibration procedure. The preservation of bulk viscoelastic integrity alongside increased apparent void fraction suggests that PF127 removal reorganizes local free volume within the covalent GelMA framework without compromising the load-bearing capacity of the crosslinked network.

To further evaluate the mechanical response under large deformation, unconfined compression testing was performed on freshly prepared hydrogels (**Supplementary Fig. 4**). Representative stress-strain curves exhibited progressive strain stiffening in all formulations. The compressive Young's modulus increased markedly with GelMA concentration, from $0.0259 \pm 0.0055\text{ MPa}$ for G5 to $0.2390 \pm 0.0490\text{ MPa}$ for G10. Incorporation of PF127 further enhanced compressive stiffness within each GelMA concentration, with G5P2.5 and G5P5 exhibiting Young's moduli of 0.0354 ± 0.0043 and $0.0542 \pm 0.0047\text{ MPa}$, respectively, while G10P5 displayed the highest modulus ($0.8786 \pm 0.1911\text{ MPa}$), approximately 3.7-fold greater than G10. These results mirror the trends observed in the oscillatory rheological measurements, which likewise showed increased mechanical stiffness with increasing GelMA concentration and upon PF127 incorporation. Together, these findings indicate that PF127 incorporation preserves—and

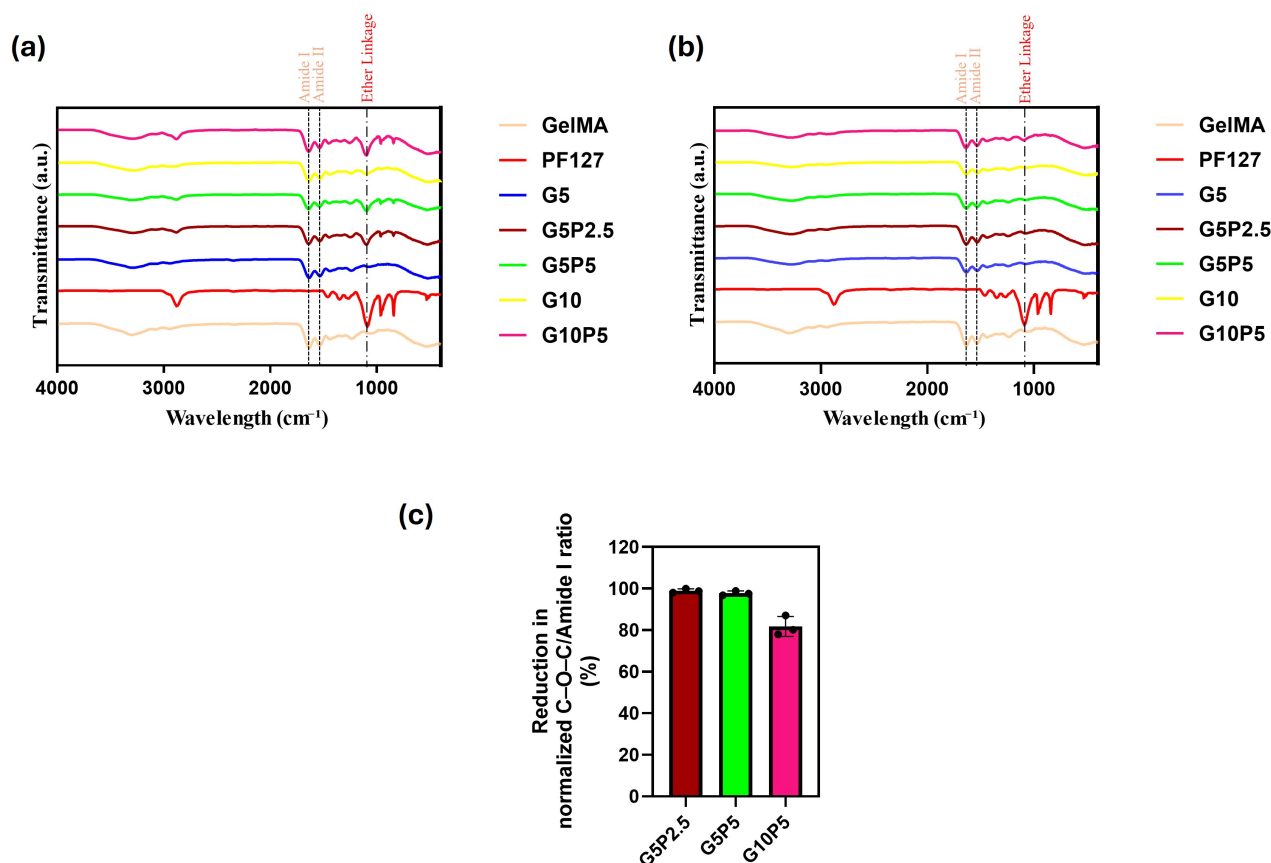


Fig. 3. FTIR analysis of compositional changes before and after equilibration. FTIR spectra of GelMA-based hydrogels under (a) freshly prepared and (b) 24 h DPBS-equilibrated conditions. (c) Semi-quantitative analysis of the normalized C–O–C/amide I peak-area ratio. Spectra were baseline-corrected prior to integration, and peak areas were integrated over $1050\text{--}1150\text{ cm}^{-1}$ (C–O–C) and $1600\text{--}1700\text{ cm}^{-1}$ (amide I). Data are presented as mean $\pm$ standard deviation ($n = 3$). GelMA-only G5 and G10 hydrogels were excluded from this analysis because they did not contain PF127. The marked attenuation of the normalized C–O–C/amide I ratio indicates substantial reduction of the PF127-associated FTIR signal, while preservation of GelMA amide bands supports the chemical stability of the GelMA network. This figure was generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

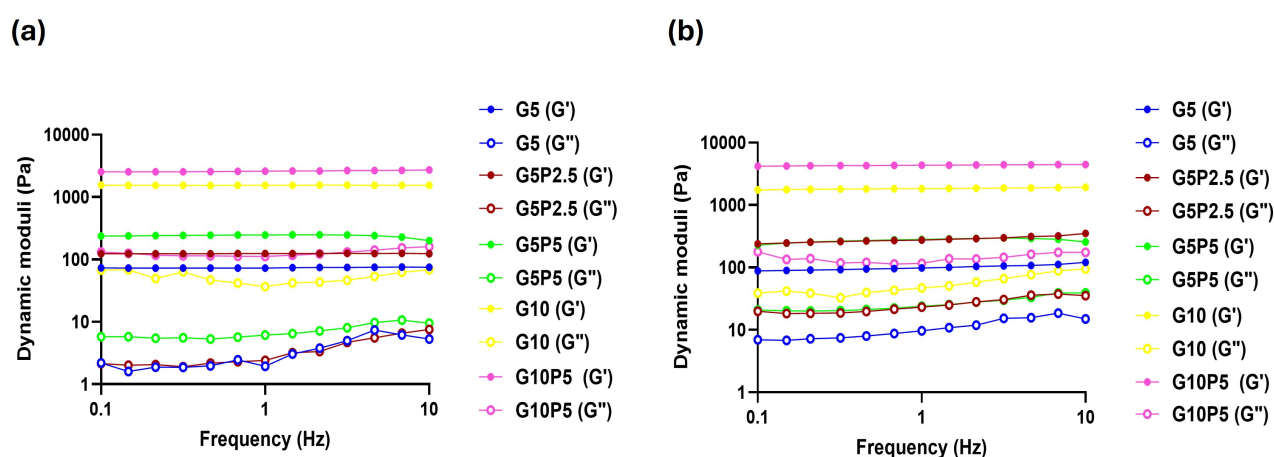


Fig. 4. Viscoelastic properties of hydrogels before and after equilibration. Oscillatory rheological analysis showing storage modulus and loss modulus as a function of frequency for (a) freshly crosslinked and (b) 24 h DPBS-equilibrated hydrogels. All formulations exhibited elastic-dominant behavior, with storage modulus higher than loss modulus across the tested frequency range. This figure was generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

in the present formulations, enhances—the compressive mechanical properties of freshly crosslinked GelMA hydrogels.

To examine the long-term mechanical stability of the hydrogels, oscillatory rheological measurements were performed after 10 days of incubation under both acellular and cell-laden conditions (**Supplementary Fig. 5**). In the acellular hydrogels following 10 days of PBS immersion (**Supplementary Fig. 5a**), all formulations retained predominantly elastic behavior, with storage modulus (G') exceeding loss modulus (G'') across most of the tested frequency range. A similar trend was observed for C2C12-laden hydrogels after 10 days of culture in DMEM (**Supplementary Fig. 5b**), where the relative mechanical ranking among formulations remained unchanged ($G10P5 > G10 > G5P5 > G5P2.5 > G5$). Notably, the G5 formulation exhibited a crossover of G' and G'' at the highest frequencies under both acellular and cell-laden conditions, indicating a transition toward viscous-dominant behavior under rapid oscillatory deformation. In contrast, all PF127-containing formulations and the G10 hydrogel maintained $G' > G''$ throughout the frequency sweep, indicating maintenance of an elastic-dominant response under oscillatory loading. This sustained mechanical behavior was further corroborated by the retained macroscopic structural integrity of all hydrogel formulations after 10 days of PBS immersion (**Supplementary Fig. 6**), collectively demonstrating that PF127-mediated pore formation does not compromise the long-term structural or mechanical stability of the GelMA network.

Biological Evaluation of GelMA/PF127 Hydrogels

C2C12 cell viability was evaluated after 48 h using Live/Dead staining (Fig. 5a). All formulations showed predominant green fluorescence with minimal red fluorescence, indicating high cell viability across all groups. The absence of cytotoxic effects in PF127-containing formulations suggests that residual PF127 after exposure to culture medium, if present, did not adversely affect cell survival under the applied crosslinking and culture conditions.

To further assess cellular metabolic activity during early culture, CCK-8 assays were performed after 3 and 7 days of culture (Fig. 5b). All hydrogel formulations exhibited increased metabolic activity from Day 3 to Day 7, indicating continued cell survival and proliferation within the hydrogel matrices. Relative proliferation analysis, calculated by normalizing Day 7 metabolic activity to the corresponding Day 3 values for each formulation (Fig. 5c), demonstrated formulation-dependent differences in proliferative response. Among the 5% GelMA formulations, G5P5 exhibited the greatest fold increase in metabolic activity, followed by G5P2.5, whereas the GelMA-only control showed the lowest relative proliferation. In contrast, the higher GelMA formulations exhibited comparatively smaller increases in relative metabolic activity despite their

greater mechanical stiffness. These findings suggest that PF127-mediated post-gelation microstructural remodeling preferentially enhances cellular proliferation in the more compliant 5% GelMA matrices.

Long-term cell viability was further evaluated by Live/Dead staining after 7 and 10 days of culture (**Supplementary Fig. 7**). All formulations maintained predominantly viable cell populations throughout the culture period, confirming sustained cytocompatibility of the hydrogels. Notably, the PF127-containing 5% GelMA formulations, particularly G5P5, exhibited increased cell spreading and elongated cellular morphology after 10 days compared with the corresponding GelMA-only controls and the denser 10% GelMA formulations, consistent with the enhanced proliferative response observed in the CCK-8 assay.

Cytoskeletal organization was evaluated after 14 days of culture under serum-reduced conditions. Cells in all formulations exhibited detectable F-actin structures, indicating successful cytoskeletal development within the hydrogel matrices. Quantification of F-actin-positive area per field (Fig. 5d) revealed formulation-dependent differences in cytoskeletal coverage. G5P5 showed the highest F-actin-positive area among all groups, followed by G5P2.5, both of which were significantly higher than the GelMA-only controls. In contrast, G10P5 exhibited the lowest F-actin-positive area, reflecting the more constrained microenvironment of the higher-density GelMA matrix. Representative F-actin fluorescence images (Fig. 5e) illustrate these quantitative findings, with PF127-containing 5% GelMA hydrogels, particularly G5P5, exhibiting more extensive actin filament organization and elongated cell morphology, whereas G10P5 showed more restricted cytoskeletal development, mirroring the lower apparent porosity and denser morphology observed after equilibration. Together, these findings indicate that PF127-mediated post-gelation remodeling produces a more permissive microenvironment for cell spreading and cytoskeletal organization in compliant GelMA matrices.

Discussion

PF127-Mediated Post-Gelation Remodeling: Compositional and Microstructural Evidence

The convergent evidence from mass-change analysis, dry mass loss and equilibrium swelling measurements, FTIR spectroscopy, hydrated-state brightfield microscopy, and SEM establishes that physically incorporated PF127 undergoes diffusion-mediated removal from the GelMA network during physiological aqueous equilibration, and that this removal is associated with progressive microstructural reorganization within an intact covalent framework. The key mechanistic implication is that structural change in this system is temporally decoupled from network formation: the GelMA crosslinked network is first established by photopolymerization, and microstructural evolution then

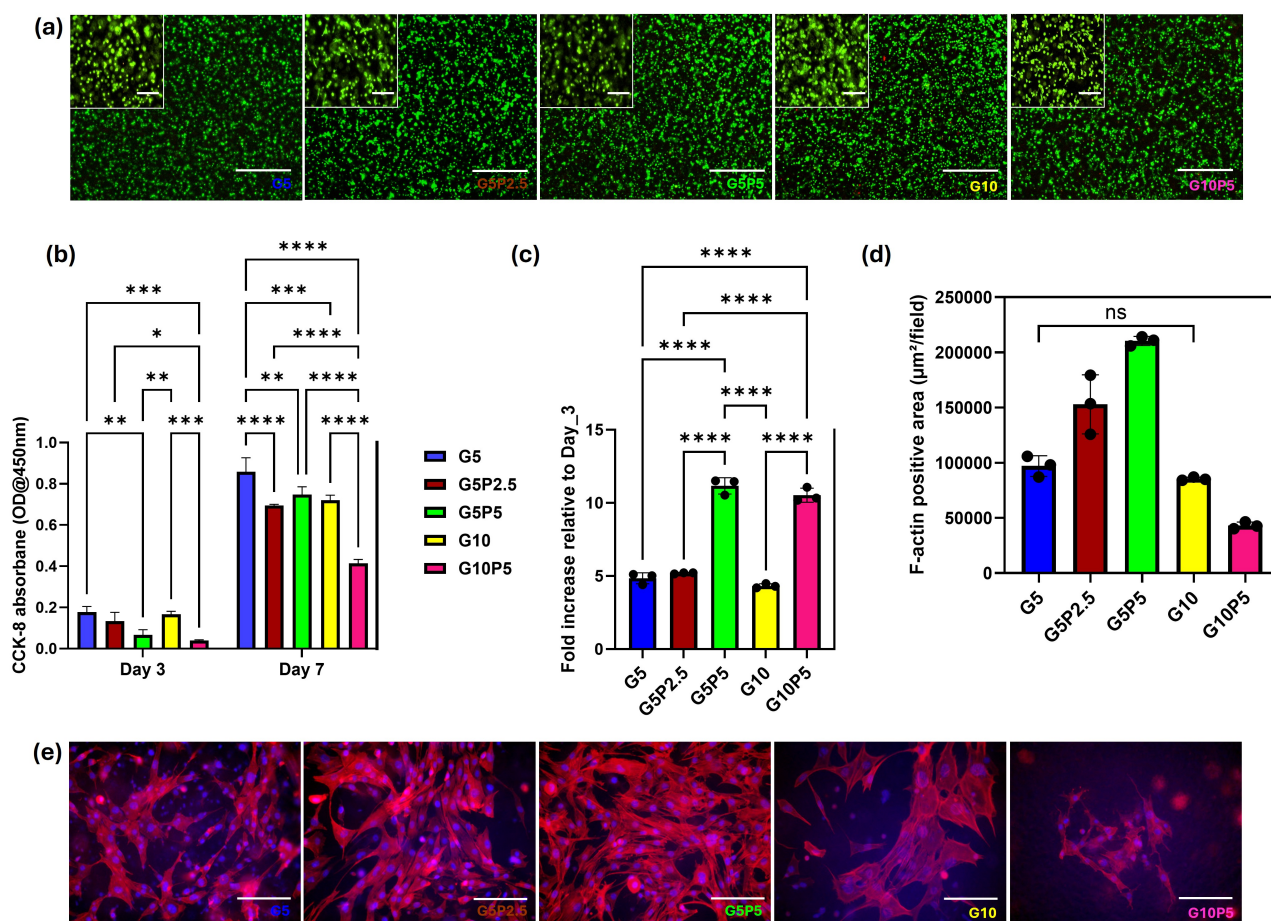


Fig. 5. Biological evaluation of C2C12-laden GelMA/PF127 hydrogels. (a) Live/Dead staining of encapsulated C2C12 myoblasts after 48 h of culture, demonstrating high cell viability across all formulations. Live cells appear green and dead cells appear red. Scale bar: 750 μm ; inset scale bar: 300 μm . (b) CCK-8 assay showing metabolic activity of encapsulated C2C12 cells after 3 and 7 days of culture. (c) Relative cell proliferation expressed as the fold increase in CCK-8 absorbance at Day 7 normalized to the corresponding Day 3 values for each hydrogel formulation. Data are presented as mean $\pm$ standard deviation ($n = 3$). Statistical significance is denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. (d) Quantification of F-actin-positive area per field from red-channel images using ImageJ. Images were obtained from three independently prepared hydrogels per group, and data are presented as mean $\pm$ standard deviation ($n = 3$). Statistical significance was analyzed using ordinary one-way ANOVA followed by Tukey's multiple comparisons test. ns indicates no significant difference between G5 and G10. (e) Representative F-actin staining images after 14 days of culture under serum-reduced culture conditions, showing cytoskeletal organization and cell morphology within the hydrogel matrices. Scale bar: 150 μm . This figure was generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

proceeds as PF127 exits the matrix under aqueous conditions. This sequence distinguishes the present approach from conventional porogen-based strategies, where pore formation and gelation occur concurrently, and the resulting architecture is fixed at the point of network formation.

This behavior suggests that PF127 acted as a transient, physically incorporated additive rather than a covalently integrated network component. The decreases in the normalized C–O–C/amide I ratio, 98.9%, 97.7%, and 81.7% for G5P2.5, G5P5, and G10P5, respectively, represent attenuation of the PF127-associated FTIR signal rather than absolute PF127 mass removal. The higher residual signal in G10P5 is consistent with restricted diffusion within the denser 10% GelMA network, high-

lighting the role of polymer concentration in controlling additive transport and post-gelation structural rearrangement. The dry mass loss measurements further support this interpretation, demonstrating greater loss of diffusible material in PF127-containing formulations, particularly G5P5, whereas equilibrium swelling exhibited comparatively modest formulation-dependent differences. Together, these findings indicate that the observed structural remodeling is primarily associated with PF127 depletion rather than water uptake alone.

SEM analysis further supports a PF127-mediated morphological effect. Importantly, hydrated-state brightfield microscopy demonstrated formulation-dependent structural heterogeneity both immediately after fabrication and fol-

lowing aqueous equilibration, indicating that the observed microstructural differences are not solely artifacts of freeze-drying. Fresh PF127-containing hydrogels displayed more open apparent dried-state morphologies despite retaining PF127-associated FTIR bands, indicating that the initial morphological differences were not caused by pre-equilibration additive extraction. Instead, PF127 likely generated local physical heterogeneity during photocrosslinking through PF127-rich or water-rich domains that influenced GelMA chain organization. After DPBS equilibration, PF127-associated signal loss and hydration-induced network relaxation were associated with more spatially continuous apparent porous structures, especially in G5P5.

Together, the mass-change, dry mass loss, equilibrium swelling, FTIR, hydrated-state imaging, SEM, and rheological results indicate that PF127-mediated post-gelation equilibration can increase apparent microstructural openness while bulk viscoelastic behavior is retained. This outcome is not equivalent to independent control of structure and mechanics, as both remain influenced by GelMA concentration. Rather, it reflects the fact that PF127 removal reorganizes local free volume within an already-formed covalent network, a process that alters apparent dried-state morphology without requiring further disruption of the load-bearing GelMA framework. The degree to which this reorganization occurs is governed by GelMA network density, as demonstrated by the more restricted structural change and lower PF127 signal attenuation in G10P5 relative to the 5% GelMA-based formulations.

Compositional Dependence of Cell Permissiveness: Cytoskeletal Responses to Post-Gelation Microstructural Remodeling

The biological results demonstrate that GelMA/PF127 hydrogels support C2C12 viability and cytoskeletal development in a composition-dependent manner. High cell viability across all formulations at 48 h confirms that GelMA concentration, PF127 incorporation, and the applied photocrosslinking conditions did not induce cytotoxic effects under the selected fabrication parameters, in agreement with the established cytocompatibility of both GelMA and Pluronic-based additives reported in prior literature [21–23]. Furthermore, the CCK-8 assays demonstrated sustained increases in metabolic activity between Days 3 and 7 across all formulations, while long-term Live/Dead staining confirmed maintenance of viable cell populations through 10 days of culture. Together, these findings indicate that PF127 incorporation supports not only short-term cytocompatibility but also continued cellular adaptation within the remodeled hydrogel microenvironment.

The formulation-dependent variation in cell proliferation and cytoskeletal coverage after 14 days closely recapitulates the microstructural hierarchy established by physicochemical characterization. Notably, G5P5 also exhibited

the greatest relative increase in metabolic activity between Days 3 and 7, suggesting that the enhanced microstructural permissiveness produced by PF127-mediated remodeling supports both cellular proliferation and cytoskeletal organization within compliant GelMA matrices. Conversely, G10P5, which retained a denser morphology due to restricted PF127 diffusion within the higher-density GelMA network, showed the lowest cytoskeletal coverage among all tested groups. This parallel between apparent microstructural openness and cellular cytoskeletal organization suggests that the post-gelation remodeling produced by PF127 removal translates into a functionally more permissive matrix environment, at least with respect to cell spreading and cytoskeletal development. Although the contribution of residual PF127 or differentiation medium-induced effects on cytoskeletal organization cannot be completely excluded, their influence is expected to be minimal due to substantial PF127 depletion during equilibration and identical culture conditions across all formulations. Therefore, the observed differences are interpreted to primarily reflect formulation-dependent microstructural environments and cell–matrix interactions.

The finding that PF127 incorporation improved cytoskeletal coverage in 5% GelMA hydrogels but not in the 10% GelMA system underlines the conditional nature of this strategy. In G10P5, the dominant physical confinement imposed by the denser crosslinked network appears to override the relatively modest microstructural changes introduced by PF127, suggesting that a threshold of matrix compliance may be required for PF127-mediated remodeling to produce biologically meaningful differences. This interpretation is supported by the rheological data, which showed higher G' values for G10-based formulations and supports the view that bulk mechanical confinement, rather than apparent microstructural openness alone, is a primary determinant of cytoskeletal response in this system. Together, these results indicate that the diffusion-mediated post-gelation tuning strategy investigated here can produce hydrogel matrices with compositionally adjustable permissiveness for cell proliferation, spreading, and morphological development, within the range of GelMA concentrations and PF127 contents examined.

Post-Gelation Tuning as a Design Strategy for Cell-Permissive GelMA Scaffolds

The central design principle investigated in this study is the temporal separation of network formation and microstructural evolution. In conventional hydrogel fabrication strategies, including sacrificial porogen templating, cryogelation, and emulsion-based approaches, pore architecture is established concurrently with or prior to gelation, such that the final microstructure is largely determined at the point of network formation. The present approach differs in that a covalently crosslinked GelMA network is first established by photopolymerization, and structural reorga-

nization then proceeds autonomously during aqueous equilibration as PF127 diffuses from the matrix. This sequence offers a practical advantage: the mechanical integrity of the scaffold is set by the GelMA network at the point of fabrication, while microstructural permissiveness develops subsequently without requiring additional processing steps.

From a fabrication standpoint, the method is experimentally straightforward. PF127 is incorporated by simple mixing into the GelMA precursor solution prior to photocrosslinking, and post-gelation remodeling occurs spontaneously under physiological aqueous conditions without the need for harsh extraction solvents, elevated temperatures, or specialized equipment. This simplicity distinguishes it from approaches that require prefabricated microgel assembly, controlled freeze-thaw cycling, or emulsion stabilization protocols. The cytocompatibility data further indicate that cell encapsulation is feasible during the fabrication step, suggesting that post-gelation remodeling and early cell adaptation can occur concurrently within the same construct. The sustained cell viability observed after 10 days of culture together with the preserved mechanical properties following prolonged aqueous incubation further demonstrate that this remodeling process proceeds without compromising either hydrogel integrity or the viability of encapsulated cells.

The tunability of this system is governed primarily by GelMA concentration and PF127 content. Increasing PF127 content within a 5% GelMA matrix produced progressive increases in apparent porosity, relative cellular proliferation, and cytoskeletal coverage, while increasing GelMA concentration to 10% attenuated both PF127 removal and the resulting biological response. This concentration-dependent behavior provides a practical handle for adjusting scaffold permissiveness without fundamentally changing the fabrication protocol, though it also highlights that the strategy operates within boundaries set by network density. The results suggest that this approach is most effective in mechanically compliant matrix formulations and may be less suitable for applications requiring high crosslink density.

Considered in the broader context of GelMA scaffold design, the post-gelation tuning concept represents a complementary rather than competing strategy. It does not replace methods capable of generating well-defined macroporous architectures, but it offers an operationally simple route to increasing microstructural openness in photocrosslinked GelMA matrices where fabrication simplicity, cytocompatibility during encapsulation, long-term mechanical stability, and viscoelastic retention are priorities. The extent to which this approach can be generalized to other physically incorporated additives or hydrogel chemistries remains an open question and represents a direction for further investigation.

Limitations and Future Directions

Several limitations of the present study should be considered. Although hydrated-state brightfield microscopy supported the formulation-dependent structural trends observed by SEM, the detailed microarchitecture was primarily evaluated using freeze-dried samples. Consequently, the SEM images represent apparent dried-state morphology rather than the native hydrated pore architecture. Freeze-drying is known to introduce structural artifacts through ice crystal formation and network collapse [24]. Future studies employing higher-resolution hydrated-state imaging techniques, such as cryogenic scanning electron microscopy or confocal microscopy, would provide more direct characterization of the three-dimensional network.

Although FTIR analysis was complemented by dry mass loss measurements supporting PF127 depletion during aqueous equilibration, PF127 release was not directly quantified. Quantitative techniques such as solution-state ^1H NMR would enable rigorous determination of PF127 release kinetics and mass balance [23], while direct visualization of PF127 distribution during gelation and equilibration would further clarify the proposed post-gelation remodeling mechanism.

The mechanical and biological evaluations demonstrated sustained scaffold integrity, cytocompatibility, and composition-dependent cell responses over the investigated culture period. However, the hydrogels were not evaluated under cyclic loading, fatigue, stress-relaxation, or creep conditions that more closely resemble the dynamic mechanical environment encountered *in vivo* [25]. Furthermore, the present study did not investigate whether the enhanced microstructural permissiveness translates into tissue-specific maturation. Therefore, additional studies examining long-term degradation profiling, including enzymatic degradation, released product characterization, dynamic mechanical behavior under physiologically relevant loading, and tissue-specific functional outcomes, such as myogenic differentiation and matrix remodeling, will be important for further assessing the translational potential of this strategy.

Finally, the present study investigated a limited range of GelMA and PF127 compositions under a single equilibration protocol. Whether diffusion-mediated post-gelation remodeling can be generalized to other GelMA formulations, degrees of methacrylation, Pluronic variants, polymer concentrations, or different equilibration conditions remains to be established. Systematic investigation of these variables will help define the broader design space and applicability of this strategy across different hydrogel systems.

Conclusions

This study demonstrates that physically incorporated PF127 enables diffusion-mediated post-gelation microstructural tuning of photocrosslinked GelMA hydro-

gels, wherein structural reorganization occurs within an already-established covalent network rather than concurrently with gelation. Semi-quantitative FTIR analysis, supported by dry mass loss measurements, indicated substantial PF127 depletion during aqueous equilibration in 5% GelMA-based formulations, and this process was associated with increased apparent porosity without substantial loss of elastic-dominant viscoelastic behavior. The degree of remodeling was attenuated in the 10% GelMA system, reflecting diffusion constraints imposed by higher network density. The remodeled hydrogels maintained cytocompatibility and exhibited formulation-dependent differences in C2C12 proliferation and cytoskeletal organization, with G5P5 showing the most permissive cellular response. Collectively, these findings provide proof-of-concept that diffusion-mediated post-gelation microstructural tuning can improve microstructural permissiveness while preserving bulk mechanical integrity within the formulation space explored in this study. Although further validation through hydrated-state structural characterization and direct quantification of PF127 release is required, the combined physicochemical and biological findings highlight the potential of this approach for future tissue engineering applications.

List of Abbreviations

ANOVA, analysis of variance; C2C12, mouse myoblast cell line; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco's Modified Eagle Medium; DPBS, Dulbecco's phosphate-buffered saline; F-actin, filamentous actin; FE-SEM, field-emission scanning electron microscopy; FBS, fetal bovine serum; FTIR, Fourier-transform infrared spectroscopy; GelMA, gelatin methacryloyl; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PF127, Poloxamer 407/Pluronic F127; SEM, scanning electron microscopy; PEO, poly(ethylene oxide); PPO, poly(propylene oxide).

Availability of Data and Materials

Data will be made available upon request from the corresponding author.

Author Contributions

KM and HJL conceptualized and designed the work. KM performed the experiments, analyzed the data, and drafted the manuscript. HJL validated the results, provided resources, supervised the project, acquired funding, and critically revised the manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.22203/eCM.v059a08>.

References

- [1] Xu F, Dawson C, Lamb M, Mueller E, Stefanek E, Akbari M, *et al.* Hydrogels for Tissue Engineering: Addressing Key Design Needs Toward Clinical Translation. *Frontiers in Bioengineering and Biotechnology*. 2022; 10: 849831. <https://doi.org/10.3389/fbioe.2022.849831>.
- [2] Goswami AK, Giduturi VK, Yerramilli SN, Chauhan VS, Yadav N. Tissue engineering: Hydrogel scaffolds and mechanical properties as key design parameters. *Advances in Colloid and Interface Science*. 2026; 347: 103691. <https://doi.org/10.1016/j.cis.2025.103691>.
- [3] Feliciano AJ, van Blitterswijk C, Moroni L, Baker MB. Realizing tissue integration with supramolecular hydrogels. *Acta Biomaterialia*. 2021; 124: 1–14. <https://doi.org/10.1016/j.actbio.2021.01.034>.
- [4] Ghosh RN, Thomas J, B. R V, N. G D, Janardanan A, Namboothiri PK, *et al.* An insight into synthesis, properties and applications of gelatin methacryloyl hydrogel for 3D bioprinting. *Materials Advances*. 2023; 4: 5496–5529. <https://doi.org/10.1039/d3ma00715d>.
- [5] Richbourg NR, Peppas NA. The swollen polymer network hypothesis: Quantitative models of hydrogel swelling, stiffness, and solute transport. *Progress in Polymer Science*. 2020; 105: 101243. <https://doi.org/10.1016/j.progpolymsci.2020.101243>.
- [6] Foudazi R, Zowada R, Manas-Zloczower I, Fekke DL. Porous Hydrogels: Present Challenges and Future Opportunities. *Langmuir*. 2023; 39: 2092–2111. <https://doi.org/10.1021/acs.langmuir.2c02253>.
- [7] Kyeong J, Jeong D, Bae H. Temperature-independent and photocrosslinkable hydrolyzed GelMA with tunable mechanics for biofabrication. *Materials Today Bio*. 2025; 35: 102402. <https://doi.org/10.1016/j.mtbio.2025.102402>.
- [8] Das S, Jegadeesan JT, Basu B. Gelatin Methacryloyl (GelMA)-Based Biomaterial Inks: Process Science for 3D/4D Printing and Current Status. *Biomacromolecules*. 2024; 25: 2156–2221. <https://doi.org/10.1021/acs.biomac.3c01271>.
- [9] Kedzierski A, Kheirabadi S, Jaber A, Ataie Z, Mojazza CL, Williamson ML, *et al.* Engineering the Hierarchical Porosity of Granular Hydrogel Scaffolds Using Porous Microgels to Improve Cell Recruitment and Tissue Integration. *Advanced Func-*

- tional Materials. 2025; 35: 2417704. <https://doi.org/10.1002/adfm.202417704>.
- [10] Ying GL, Jiang N, Maharjan S, Yin YX, Chai RR, Cao X, *et al.* Aqueous Two-Phase Emulsion Bioink-Enabled 3D Bioprinting of Porous Hydrogels. *Advanced Materials*. 2018; 30: e1805460. <https://doi.org/10.1002/adma.201805460>.
- [11] Liu Z, Xuan L, Hou Y, Xie T, Li J, Cai J, *et al.* Microgel-Based Hierarchical Porous Hydrogel Patch with Adhesion and Resilience for Myocardial Infarction. *Advanced science (Weinheim, Baden-Württemberg, Germany)*. 2026; 13: e18646. <https://doi.org/10.1002/advs.202518646>.
- [12] Shi M, Xu Q, Ding L, Xia Y, Zhang C, Lai H, *et al.* Cell Infiltrative Inner Connected Porous Hydrogel Improves Neural Stem Cell Migration and Differentiation for Functional Repair of Spinal Cord Injury. *ACS Biomaterials Science & Engineering*. 2022; 8: 5307–5318. <https://doi.org/10.1021/acsbiomaterials.2c01127>.
- [13] Lu X, Song W, Sun X, Liu J, Huang Y, Shen J, *et al.* Porous hydrogel constructs based on methacrylated gelatin/polyethylene oxide for corneal stromal regeneration. *Materials Today Communications*. 2022; 32: 104071. <https://doi.org/10.1016/j.mtcomm.2022.104071>.
- [14] Walejewska E, Melchels FPW, Paradiso A, McCormack A, Szlazak K, Olszewska A, *et al.* Tuning Physical Properties of GelMA Hydrogels through Microarchitecture for Engineering Osteoid Tissue. *Biomacromolecules*. 2024; 25: 188–199. <https://doi.org/10.1021/acs.biomac.3c00909>.
- [15] Ying G, Manriquez J, Wu D, Zhang J, Jiang N, Maharjan S, *et al.* An open-source handheld extruder loaded with pore-forming bioink for in situ wound dressing. *Materials Today Bio*. 2020; 8: 100074. <https://doi.org/10.1016/j.mtbio.2020.100074>.
- [16] Moradian A, Behzadnasab M, Bagher Z, Pezeshki-Modaress M, Zandi M, Tabatabaei MH. Photo- and thermal-crosslinked GelMA/chitosan hydrogels: A novel approach to enhanced mechanical and biological properties. *Carbohydrate Polymer Technologies and Applications*. 2025; 10: 100834. <https://doi.org/10.1016/j.carpta.2025.100834>.
- [17] Yilmaz H, Gursay S, Calik H, Kazancioglu Y, Yildirim R, Kadir R, *et al.* Comparative studies of GelMA hydrogels: investigating the effect of different source on mechanical, physical and biological properties. *Materials Research Express*. 2024; 11: 075307. <https://doi.org/10.1088/2053-1591/ad62be>.
- [18] Yan Y, Cao Y, Cheng R, Shen Z, Zhao Y, Zhang Y, *et al.* Preparation and In Vitro Characterization of Gelatin Methacrylate for Corneal Tissue Engineering. *Tissue Engineering and Regenerative Medicine*. 2022; 19: 59–72. <https://doi.org/10.1007/s13770-021-00393-6>.
- [19] Suhail M, Hung MC, Chiu IH, Vu QL, Wu PC. Preparation and in-vitro characterization of 5-aminosalicylic acid loaded hydrogels for colon specific delivery. *Journal of Materials Research and Technology*. 2022; 21: 339–352. <https://doi.org/10.1016/j.jmrt.2022.09.031>.
- [20] Suhail M, Chiu IH, Lai YR, Khan A, Al-Sowayan NS, Ullah H, *et al.* Xanthan-Gum/Pluronic-F-127-Based-Drug-Loaded Polymeric Hydrogels Synthesized by Free Radical Polymerization Technique for Management of Attention-Deficit/Hyperactivity Disorder. *Gels*. 2023; 9: 640. <https://doi.org/10.3390/gels9080640>.
- [21] Luo L, Zhu Q, Li Y, Hu F, Yu J, Liao X, *et al.* Application of thermosensitive-hydrogel combined with dental pulp stem cells on the injured fallopian tube mucosa in an animal model. *Frontiers in Bioengineering and Biotechnology*. 2023; 10: 1062646. <https://doi.org/10.3389/fbioe.2022.1062646>.
- [22] Muthuramalingam K, Lee HJ. Effect of GelMA Hydrogel Properties on Long-Term Encapsulation and Myogenic Differentiation of C₂C₁₂ Spheroids. *Gels*. 2023; 9: 925. <https://doi.org/10.3390/gels9120925>.
- [23] Torre M, Giannitelli SM, Mauri E, Gori M, Bucciarelli A, Mozetic P, *et al.* Printability assessment workflow of a thermosensitive photocurable biomaterial ink for microextrusion bioprinting. *Bioprinting*. 2023; 30: e00262. <https://doi.org/10.1016/j.bprint.2023.e00262>.
- [24] Song X, Philpott MA, Best SM, Cameron RE. Controlling the Architecture of Freeze-Dried Collagen Scaffolds with Ultrasound-Induced Nucleation. *Polymers*. 2024; 16: 213. <https://doi.org/10.3390/polym16020213>.
- [25] Richardson BM, Walker CJ, Maples MM, Randolph MA, Bryant SJ, Anseth KS. Mechanobiological Interactions between Dynamic Compressive Loading and Viscoelasticity on Chondrocytes in Hydrated Covalent Adaptable Networks for Cartilage Tissue Engineering. *Advanced Healthcare Materials*. 2021; 10: e2002030. <https://doi.org/10.1002/adhm.202002030>.

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