

Thermoresponsive BrushGel Microcarriers for Efficient Cell Expansion and Enzyme-Reduced Harvesting

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Scaling up cell therapy requires efficient expansion of high-quality cells. Microcarrier(MC)-based systems offer high surface-to-volume ratios and reduce culture media usage. In this study, we developed BrushGel, a temperature-responsive MC composed of gelatin methacryloyl (GelMA) hydrogel particles coated with poly(N-isopropyl acrylamide) (PNIPAM) polymer brushes via covalent grafting. BrushGel was fabricated using a flow-focusing droplet microfluidic device and functionalized using carbodiimide chemistry (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-N-Hydroxysuccinimide, EDC-NHS). The degree of PNIPAM coating was tuned by varying the degree of methacrylation (DOM) of GelMA and the concentration of PNIPAM. Human dermal fibroblast (HNDf) cells cultured on the BrushGel under dynamic conditions showed a 4.9 fold increase in cell density, 12-fold upregulation in COL1A1 gene expression and elevated procollagen protein secretion compared to static culture. Low temperature detachment (4 °C) yielded up to 65% detachment efficiency with >95% post-detachment viability. Clinical grade human bone marrow-derived mesenchymal stromal/stem cells (MSCs) expanded 5.3 fold over five days on BrushGel with 69% detachment efficiency and 80% post-harvest viability using 10-fold less enzyme. BrushGel supported over 10 days of culture in spinner flasks, enabling enzyme-minimized, scalable cell expansion. These findings position BrushGel as a promising platform for dynamic cell culture systems in regenerative medicine.

1. Introduction

Manufacturing of therapeutic cells serves as a key component across different biotechnological industries, with important applications demonstrated in cell therapy^[1] tissue engineering^[2] and biopharmaceutical (biopharma) products.^[3] Most significantly, mammalian cells have become important drivers in the biopharma industry, contributing to almost 70% of this sector.^[4] The explosion of biotechnology companies will lead to a growing demand for biologics consisting of cells and cell-based products over the next few years. Furthermore, considerable advancements into cellular biology, primarily with stem cells^[5] and fibroblast cells^[6] have allowed for compelling clinical applications in regenerative medicine and even in cultivated meat progress. These advancements necessitate effective culture methodologies and scalable biomanufacturing practices that need to be established for these cell types.

MCs, the primary element of dynamic culture of adherent cells, offer significantly greater surface-area-to-volume ratios compared to conventional static culture

systems (flask and plate-based cell culture). Consequently, MCs facilitate production of higher cell densities and reduced volumetric footprints.^[7,8] Their adjustable sub-millimeter size offers an increased surface area for enhanced cell expansion. Also materials such as gelatin, cellulose, dextran, polystyrene, and poly(vinyl alcohol) have been commonly used to produce MCs.^[9] Numerous studies including our group, have shown that the size of MCs affects cell expansion, gene expression, protein secretion, and cell yield.^[10]

Batch emulsion methods yield polydisperse MCs, necessitating post-processing steps to achieve high monodispersed MCs.^[11] Conversely, droplet microfluidic approaches are renowned for their precise regulation of particle size and production of monodisperse particles.^[12] Regarding MC's composition, synthetic polymer-based MCs suffer from drawbacks like low initial cell attachment and inefficient cell detachment at high cell confluency, necessitating protein and extracellular matrix biomolecule coating.^[13] Conversely, natural polymer-based MCs exhibit superior biocompatibility and tunable physico-chemical properties. GelMA, in particular, is widely used in

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DOI: 10.1002/adhm.202404538

this field for its rapid and uniform crosslinking and robust cell adhesion.^[14,15] Monodispersed GelMA MCs fabricated using droplet microfluidics have enhanced cell attachment and proliferation, aligning with key requirements for high-density cell expansion. However, they require enzymatic dissociation for cell detachment, which adds to the cost and complexity of the process and can negatively impact cell viability and function.^[14]

Current cell manufacturing workflow relies on proteolytic enzymes such as trypsin or animal origin-free trypsin-like enzymes such as TrypLE.^[16] However, high concentrations of enzymes can potentially damage cell membranes and result in loss of function.^[17,18] Enzymes can also disrupt the cytoskeletal structure of cells, compromise cell-to-cell connections, and degrade extracellular matrix (ECM) proteins, potentially reducing the quality of harvested cells.^[19] Mechanical detachment methods have also been used for various cell types, particularly in pluripotent stem cell monolayer culture.^[20] Ultrasonic vibration has been explored for detaching cells from conventional tissue culture flasks without using enzymes, showing promising efficiency at a small scale, albeit with drawbacks such as limited feasibility for large-scale manufacturing and suspension culture, as well as decreased cell viability due to membrane damage.^[21]

Thermoresponsive coatings for enzyme-free cell harvesting have been employed in various applications, including smart flask. Cultured cells can be noninvasively harvested from “smart” PNIPAM surfaces as tissue-like cell sheets by simply reducing the temperature from 37 °C to 20 °C. This cell manipulation technique eliminates the need for enzymes, addressing a significant limitation of traditional cell manufacturing.^[15,17,22–24] Notably, PNIPAM has been coated on the surface of polystyrene MCs to achieve enzyme-free cell detachment.^[25,26] However, because of high stiffness, plastic substrates often do not mimic the natural pliability of soft tissues, which can negatively impact cell behavior, including proliferation, differentiation, and maturation.^[7,27] To address the challenges of using high concentrations of enzymes for cell dissociation, gelatin MCs with PNIPAM coating have recently been reported for thermal cell harvesting.^[28,29] However, gelatin has low mechanical properties and low stability, resulting in the formation of premature MC degradation and fragmented particulates in the final product, hampering their applications in dynamic bioreactor tanks.^[30] To address this issue and improve the mechanical properties of hydrogel-based MCs, we fabricated MCs from an interpenetrating network of PNIPAM and GelMA,^[15,24] showing improved mechanical properties of MCs and supporting cell expansion in the stirrer tank bioreactors. However, the amount of PNIPAM interpenetrated in the MCs could potentially create safety concerns for therapeutic cell production.^[22]

In this study, we introduced GelMA MCs with a PNIPAM coating as a potential alternative to gelatin MCs. GelMA enables light-induced crosslinking, producing stable hydrogels with customizable mechanical properties. The primary amine groups in GelMA facilitate the grafting of PNIPAM onto the MC's surface.^[30] These reactive groups readily interact with other molecules, simplifying the process compared to modifying pure gelatin and providing a more robust binding strategy for PNIPAM. Moreover, the degree of crosslinking can be controlled

with light exposure, enabling precise tailoring of mechanical properties (stiffness, elasticity) to suit various cell types and applications.

Here, we employed a 3D printed microfluidic droplet generation technique to generate GelMA MCs, followed by an EDC-NHS carbodiimide chemistry step, to form PNIPAM brush-grafted MCs (BrushGel) (**Figure 1**). Unlike alternative grafting methods such as electron beam irradiation,^[31] atom transfer radical polymerization,^[32] and electrostatic interactions,^[31] which entail high-intensity irradiation, toxic solvents, labor-intensive procedures, and weak interactions, respectively, the EDC linker facilitates the linking of COOH functional groups to primary NH₂ groups in an aqueous and environmentally friendly manner, rendering the process more sustainable and accessible.^[33,34] Since targeting the carboxylic acid groups of GelMA during the EDC-NHS grafting process has been shown to alter the mechanical properties, swelling behavior, and bioactivity of the final hydrogel,^[35] we utilized the amine groups retained during the controlled GelMA synthesis process. First, we demonstrated, using 1H-NMR and the 2,4,6-trinitrobenzene sulfonic acid assay (TNBS) assay, that a controlled percentage of primary amine groups in gelatin could be preserved during the methacrylation process. This controlled availability of amine reaction sites for PNIPAM-COOH grafting provided an additional level of control over the grafting process, which is otherwise unfeasible with gelatin due to the inability to regulate the number of reaction sites.^[34] Then, we optimized MC composition focusing on improving attachment and temperature-induced detachment efficiencies by evaluating cell viability, proliferation, gene expression, and protein secretion across various MC compositions. We further demonstrated the cell expansion and detachment of clinical-grade MSCs on BrushGel. MSCs are multipotent cells that have gained therapeutic relevance due to their ability to induce immunomodulation, the potential to promote tissue regeneration, and their anti-inflammatory effects.^[36] MSCs are being examined in a wide range of therapeutic contexts, including treating severe infections, immune disorders, and tissue injuries due to their ability to reduce inflammation, promote healing, and induce tissue repair.^[37,38] MSCs for clinical applications require high cell doses to treat patients; therefore, scalable culture methods such as MC systems are required to efficiently expand the necessary cell numbers.

2. Experimental Section

2.1. Materials

Gelatin (type A, porcine skin), methacrylic anhydride (MA), carboxylic acid terminated (PNIPAM-COOH, average Mn 5000), EDC, NHS, 2-morpholinoethanesulfonic acid monohydrate (MES), lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP), Span80, TNBS and Agarose from Sigma were procured, employing these materials without further purification. Additionally, clear photocurable resin for the droplet generator device fabrication was obtained from Anycubic, while Tygon plastic tubing was used for outlet tubing passing through crosslinking parts and connecting lines between syringes and devices. Fisher Chemical supplied the heavy mineral oil (CAS#8042-

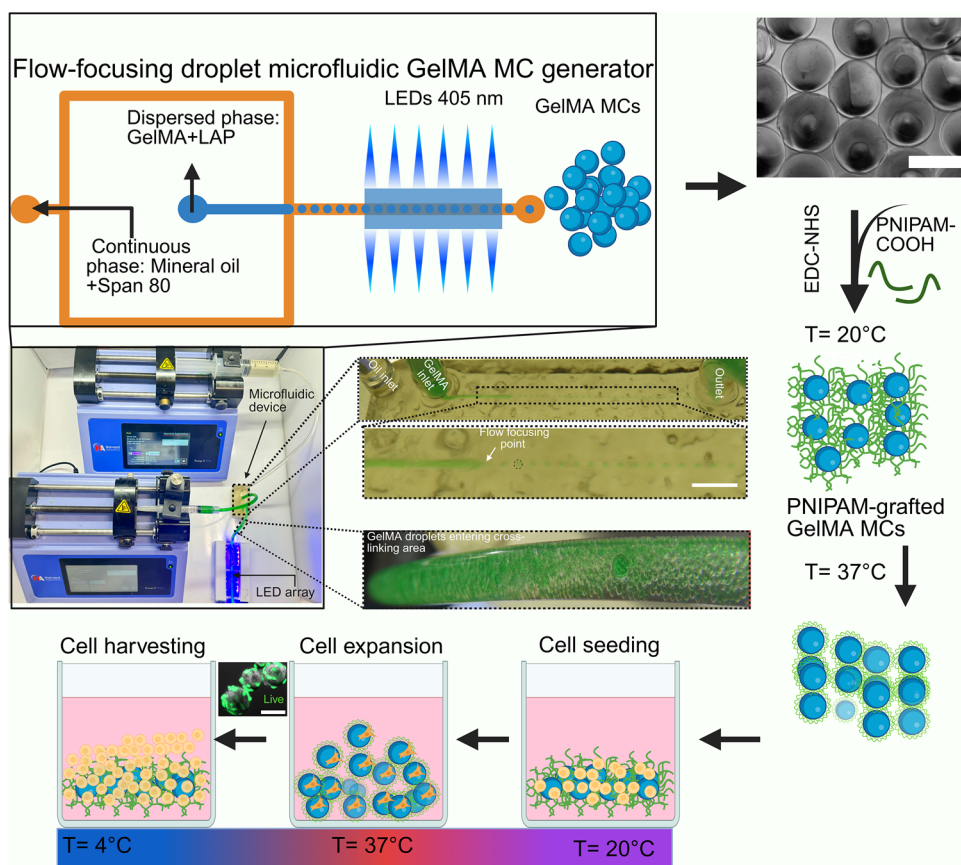


Figure 1. Schematic showing the fabrication process and use of the surface-modified GelMA MCs for cell growth and harvesting. i) GelMA MCs are made in a flow-focusing droplet microfluidic system using the dispersed phase (GelMA + Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) with the continuous phase (mineral oil + span 80), and then cross-linked with LEDs at 405 nm to prepare GelMA MCs (scale bar: 1 mm). ii) GelMA MCs are then surface-modified with PNIPAM-COOH using EDC-NHS chemistry for thermo-responsive properties that allows for cell expansion and harvesting (scale bar: 500 μ m). iii) Cells are seeded onto the surface-modified GelMA MCs and growing at 37 °C (cell expansion phase), then harvested at 4 °C via thermo-responsive PNIPAM brushes (scale bar: 200 μ m). Figure was created using BioRender.

47-5) used in this study, and Fisher Scientific provided the dialysis membrane (Spectro/Por molecular porous membrane, MWCO 12–14 kDa). Light-emitting diodes (LEDs, 405 nm wavelength) were purchased from Mouser Electronics. For cell culture purposes, we acquired Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), or NutriStem hPSC XF medium (FroggaBio), PLT Gold Human Platelet Lysate (Sartorius), and Trypsin from Gibco (ThermoFisher Scientific). Cell viability and metabolic activity were assessed using a live/dead staining kit and PrestoBlue from invitrogen (ThermoFisher Scientific).

2.2. 3D Printing of Flow-Focusing Droplet Generator Device

The design of microfluidic chip was completed utilizing the SolidWorks software (Blender 4.2), followed by its production with the manufacturer's Anycubic Photon Mono 4K 3D Printer. The flow-focusing droplet generator was manufactured using Mono 4K 3D printer with clear resin. Full inspections of the printer and resin vat prior to each print cycle were conducted to check for contamination and if present, eliminated. The printer

was dusted off with compressed air and a pipette was used to remove contaminants in the resin vat. The design and dimensions are provided in Figure S1 (Supporting Information). The CAD file was processed and sliced using CHITUBOX Basic V2. The layer height was set to 20 μ m, with each layer cured for 5 s under full-intensity illumination, while the first layer received an extended exposure of 30 s. Then the printed microfluidic devices were further cleaned to remove any residual uncured resin using isopropanol before being dried using compressed air. The parts were air-dried using compressed air and subsequently post-cured for 30 minutes in a dedicated chamber.

2.3. GelMA Synthesis

The process of synthesizing GelMA was performed using slightly modified methods from a previous report.^[24] First, ten grams of porcine skin gelatin were dissolved in 200 mL of phosphate buffer saline (PBS) pre-warmed to 60 °C to prepare a 5% (w/v) gelatin mixture. Second, 2, 1, or 0.5 mL of MA was added dropwise to the gelatin solution while stirring at 300 rpm for 3 h at 60 °C, to achieve high, medium, and low DOM, respectively. To

halt the solution, 300 mL of PBS was added. The resulting mixture was placed in dialysis bags with a 12–14 kDa cut-off, and incubated for 2 weeks at 44 °C.^[15,24,39] To ensure elimination of unreacted MA and potential impurities, the dialysis bags were immersed in distilled water under stirring at 50 rpm. The distilled water was replaced twice each day, while reversing if necessary. Finally, the GelMA solution was filtered (Whatman Grade 1 Qualitative Filter Papers, Cytiva) and lyophilized to yield GelMA in solid form.

2.4. GelMA MCs Fabrication

To fabricate the MCs, an aqueous solution comprising GelMA (10% w/v) and LAP (0.3% w/v) served as the dispersed phase. Three distinct DOMs of GelMA (High, Medium, and Low) were employed individually as the dispersed phase to fabricate MCs with specific chemistries. The continuous phase was composed of heavy mineral oil, mixed with 10% v/v Span-80, a nonionic surfactant, to support droplet formation. Tygon tubing was used to link plastic syringes containing each phase to the inlets of the microfluidic chip. Syringe pumps (Harvard Apparatus PHD 2000, USA) equipped with 10 mL plastic syringes were used to introduce both phases into the chip. At the flow-focusing region of the chip, droplets were generated by maintaining a flow rate ratio of 1:12 between dispersed and continuous phase (Continuous phase flow rate: 120 $\mu\text{L min}^{-1}$ and dispersed phase flow rate: 10 $\mu\text{L min}^{-1}$), traversing through a crosslinking LEDs exposed to visible light (405 nm), inducing in situ photocrosslinking of the MCs, Figure 1. The MCs were denoted as GH, GM, and GL, where G, H, M, and L represent GelMA, high, medium, and low DOM, respectively. Subsequently, to remove residual oil and contaminants from the crosslinked MCs, centrifugation (300 g, 5 min) was employed, followed by three rinses using distilled water containing 1% dish detergent, followed by washes in 50% ethanol, and multiple. After rehydration in PBS, the MCs were kept at 4 °C for storage.

2.5. PNIPAM Brush-Grafting on the Surface of GelMA MCs

The EDC-NHS carbodiimide chemistry was utilized to covalently graft PNIPAM-COOH chains to the surface of GelMA MCs using an established method with modifications.^[34] First, EDC (20 mg) and NHS (10 mg) solution were prepared in 5 mL of 0.1 M MES buffer (pH = 5.5 $\pm$ 0.2) to prepare activation buffers, and vortexed to ensure complete dissolution. Additionally, 2x solutions of PNIPAM-COOH at 1 $\times 10^{-3}$, 5 $\times 10^{-3}$, and 20 $\times 10^{-3}$ M in 0.1 M MES (pH = 5.5 $\pm$ 0.2) buffer were prepared. Then, the PNIPAM-COOH solution was mixed with activation buffer (1:1 v/v) and allowed to react for 4 h at 4 °C. Prior to carrying out the experiment, 1 mL of swollen concentrated MCs which corresponds to 0.065 mg mass of dry hydrogel were dispersed in 0.1 M MES buffer (10 mL, pH = 6.5 $\pm$ 0.2) and were washed 3 times. All sampling was performed in triplicate ($n = 3$) and the results of the experiment, evaluated by the TNBS assay (Section 2.9), were reported as mean $\pm$ SD. Since the reaction was conducted under continuous stirring and all reagents were carefully weighed, any variation in sampling could increase the SD. To ensure uniform

Table 1. Composition and labeling of BrushGel MCs used in this study.

		PNIPAM-COOH concentration [mM]			
		0	1	5	20
DOM of GelMA	Low (GL)	BGLP0	BGLP1	BGLP5	BGLP20
	Medium (GM)	-	-	BGMP5	-
	High (GH)	-	-	BGHP5	-

sampling, any result with an SD greater than 10 was considered nonuniform. The MCs were then centrifuged at 2000 rpm for 5 min before discarding the supernatant. Following, 5 mL of activated PNIPAM solution was added to MC tube and allowed to react at 4 °C with stirring at 150 rpm for 24 h to ensure even reaction of PNIPAM to the MCs' amine groups. In the next step, the MCs were washed multiple times with PBS to remove excess, unreacted reagents and were then stored in a refrigerator for further characterizations. The MCs were then labeled using the formula BGXPY where X is the degree of DOM (H, M and L) and Y is the concentration of PNIPAM-COOH (1, 5 and 20 mM) used in the coupling reaction. Table 1 shows the labeling of all MC formulations used in this study.

2.6. ¹H-NMR Analysis

To directly verify the DOM of GelMA, ¹H-NMR experiments were conducted using the Bruker Avance NEO at 500 MHz and data was analyzed using Topspin 4.1.3 (Bruker). Spectrometer. Each lyophilized GelMA sample (50 mg) was reconstituted in 1 mL of deuterium oxide (D₂O) and incubated at 40 °C. In the resulting spectra, the peak area of the aromatic acids in the GelMA samples served as a reference. The calculation of the DOM was based on the peak area of the lysine methylene protons, which appeared at $\approx$ 2.8 ppm.^[40,41] The DOM was determined using the following formula:

$$\text{DOM (\%)} = (1 - [\text{area of lysine methylene in GelMA}] / [\text{area of lysine methylene in gelatin}]) \times 100.$$

2.7. MC Size Characterizations

The particle size distribution of the prepared MCs was assessed by measuring their size with a Zeiss Axio Observer microscope (Germany). To evaluate the MC size, a small volume of MC suspension was dispensed onto a coverslip and gently overlaid by a second coverslip to facilitate bright-field imaging. Afterward, the size of the MCs was quantified by imaging at least 200 MCs using an inverted microscope and analyzing the images by ImageJ software. MC size distributions were displayed in 10 μm bin intervals and monodispersity was defined using the Polydispersity Index (PDI) as shown in Equation (1) with σ and D_m denoting the standard deviation and the mean diameter of MCs, respectively

$$PDI = \frac{\sigma}{D_m} \quad (1)$$

2.8. Scanning Electron Microscopy (SEM)

MC surface morphology was analyzed using SEM imaging to evaluate changes in morphology and surface properties before and after grafting of PNIPAM brushes. The MCs were dehydrated through a series of ethanol dilutions, starting with solutions of 30%, 50%, 70%, 90%, and finally 100%. Briefly, the MCs were centrifuged at $1000 \times g$ for 3 min, and the supernatant was discarded. A 30% v/v ethanol solution was then added, and the MCs were resuspended in the solution and incubated at room temperature for 5 min before being centrifuged at $500 \times g$ for 3 min. The dehydration process involved gradually increasing the ethanol concentration to replace the aqueous phase, with a 5-min incubation period at each step. At the end of the process, the MCs were left under a chemical cabinet to allow the ethanol to evaporate. Finally, the dried MCs were gold sputtered and imaged using a Hitachi S-4800 FE SEM microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 1 kV.

2.9. TNBS Assay for Primary Amine Quantification

The TNBS is a colorimetric assay to quantify primary amines in proteins, peptides, and other biological and biomaterials.^[40] TNBS reacts specifically with the free primary amine moieties to create a chromogenic complex, and the subsequent color change can be measured using a spectrophotometer. The TNBS assay was performed following the previously described methodology.^[46] All sample materials were first dispersed in the buffered solution of 0.1 M sodium bicarbonate adjusted to pH 8.5, and the pH was checked to ensure it was at pH 8.5 across all sample solutions. Next, a volume of 250 μL of 0.01% v/v TNBS prepared in buffer was mixed with 500 μL of the various test sample solutions at 0.5 mg mL⁻¹ in buffer, and incubated at 37 °C for 3 h, followed by quenching the reaction with 250 μL of 10% wt sodium dodecyl sulfate and 125 μL of 1 M HCl. For PNIPAM-grafted GelMA samples, the difference in primary amine concentration before and after grafting was calculated and reported as the reacted primary amine percentage. Glycine in buffer at a standard curve concentration of 167×10^{-6} , 83×10^{-6} , 62×10^{-6} , 42×10^{-6} , 21×10^{-6} , and 0×10^{-6} M was used to determine the amount of primary amines in the samples. The chromogenic product of the reaction was evaluated via absorbance measurement at 405 nm.

2.10. U251 and HNDF Cell Culture

U251 (Creative Bioarray, CSC-6321 W) and HNDF (ATCC, Cedarlane labs, PCS-201-012) were cultured in T-25 flasks (Corning) within a humidified incubator (ThermoFisher, PA, USA) maintained at 37 °C and 5% CO₂. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin with media changes performed every other day. When the cells reached 80–90% confluency, they were subcultured by treating with 0.25% trypsin-EDTA for 5 min to promote detachment. The trypsin-EDTA was then deactivated and the detached cells were collected by centrifuging at $300 \times g$ for 5 min. Afterward, the cells were resuspended, counted, and seeded in the plates.

2.11. MSCs Culture

Bone marrow from one donor was purchased through a commercial supplier (25 mL from AllCells), MSCs were isolated and cultured in NutriStem XF complete media (05-200-1A-KT from Froggabio) and cryopreserved in NutriFreeze D10 (05-713-1E from Froggabio). All experiments used MSCs at passage 3. The capacity of these MSCs to differentiate into adipocytes, osteocytes, and chondrocytes was previously demonstrated, with immune-phenotype of MSCs evaluated using flow cytometry detecting surface marker expression of CD73, CD90, and CD105; and lack of CD14, CD19, CD34, CD45, and HLA-DR (BD Biosciences) expression, according manufacturer's instructions.^[38] Previously frozen MSCs were thawed and allowed to recover in T-175 flask prior to seeding onto BrushGel MCs. MCs were hydrated using complete growth medium supplemented with 5% Platelet Lysate (Sartorius, USA), and plated onto an ultra-low attachment 6-well culture plate (Corning, USA) creating a suspension of 10 mg dried weight in 1 mL of growth medium per well. To clarify our process, we first weighed out a bulk amount of dried-weight MCs for the experiment. We then reconstituted the MCs using the appropriate volume of cell culture media to produce a solution with a concentration of 10 mg of MCs per mL. Next, 1 mL of working solution was added per well to achieve 10 mg of MCs per well. MSCs were added to MC suspension at 100 000 cells per well (unless otherwise stated), carefully mixed by pipetting, and cultured for the indicated culture period. MSCs were harvested as described in the previous section. We assessed MSC function using an established assay by co-culturing MSCs with activated T cells to evaluate their capacity to suppress T cell expansion.^[42] Peripheral blood mononuclear cells (PBMCs) were initially labeled with carboxyfluorescein succinimidyl ester (CFSE) and then activated with Human T-Activator beads CD3/CD28 (Gibco). MSCs from each condition were counted, seeded and co-cultured with the activated PBMCs for 5 days then measured the levels of T cell proliferation (and the inhibition of proliferation) by flow cytometry (Attune NXT, ThermoFisher, USA).

2.12. Cell Attachment Studies on the Surface MCs

Prior to cell seeding, the 96-well plates were coated with 1 wt% agarose to make each well noncell adhesive. The plates were then exposed to ultraviolet (0.8 mW cm^{-2}) for a period of 3 h. Meanwhile, the MCs, that had previously rehydrated, were sterilized by incubating them in 70% v/v ethanol overnight at 4 °C, followed by three successive PBS washes before being placed at 37 °C to allow complete ethanol evaporation. After the sterilization process was complete, the MCs were placed in the FBS-free DMEM solution for 24 h then centrifuged at $2000 \times g$ for 5 min to eliminate residual DMEM. Finally, 70 μL of the rehydrated MC suspension (~ 3000 MCs) was carefully pipetted into each well, followed by pipetting 30 μL of the cell suspension containing 50×10^4 cells on the top of the MC suspension. Optimal mixing of the cells and MCs was achieved by pipetting several times to assure that all of the MCs' surfaces were exposed to the cells. After 2 h of incubation, 150 μL of cell culture media was added to each well, with an additional 24 h of incubation. Thereafter, the media within each well was changed to fresh, warmed (37 °C) media three times

to remove unattached cells. To quantify the number of cells attached to the MCs, culture media was collected by centrifugation at $300 \times g$ for 5 minutes. After collecting the media, 300 μL of 0.25% trypsin-EDTA was applied to each well and incubated for 10 minutes. Trypsin was neutralized, and the cells were resuspended in 1 mL cell culture media before being counted using a hemocytometer for both U251 and HDF. The number of attached cells was determined 24 h post-seeding by counting the number of cells that detached by the enzyme; cell attachment efficiency was calculated as shown in Equations (2):

$$\text{Attachment efficiency (\%)} = \frac{\# \text{ number of attached cells}}{\text{Initial seeding density}} \times 100 \quad (2)$$

2.13. Cell Harvesting

To thermally detach the cells, we added 1 mL of 4°C DPBS to the MC suspension instead of trypsin and incubated for 120 min in a fridge. At predefined time points, the detached cells present in the cell culture medium were quantified using a hemocytometer. In the following step to improve efficiency for low-temperature treatment for cell detachment, we diluted concentrations of trypsin was performed. BGLP5 was the optimized formulation based on the highest cell attachment and thermal detachment efficiency. To boost cell detachment, we added trypsin to the cells at 0.005%, 0.025%, and 0.05% v/v concentrations. Detachment effectiveness was determined using Equation (3) and cell viability was examined for all groups using: trypan blue assay for U251 and HDF cells, or, for MSCs, with an NC-200 NucleoCounter (Chemometec) cell counting assay.^[43]

$$\text{Detachment efficiency (\%)} = \frac{\# \text{ number of detached cells}}{\# \text{ attached cells}} \times 100 \quad (3)$$

At each time point, cells were trypsinized and detached from MCs to calculate the number of attached cells.

2.14. Stirred Bioreactor Culture of HNDF Cells on MCs

A custom bioreactor was constructed for pilot dynamic culture (Figure S2, Supporting Information). The system consisted of a steel support frame, a glass vessel, a Teflon stirrer with a shaft, a machined cap for the vessel, a coupler, DC motors, and a power and control module powered by an external power supply. The glass vessel was 51.2 mm in height, 19 mm in width, and 1 mm in glass thickness, providing a maximum volume capacity of 14.5 mL and a usable volume of ≈ 10 mL. A Teflon blade stirrer, machined for precision, was employed to ensure effective mixing. The blade dimensions were 9 mm in length, 6 mm in width, and 2 mm in thickness. The stirring mechanism was driven by a DC motor capable of operating within a stirring rate range of ≈ 20 –167 rpm. The motor was coupled to the stirrer shaft using a machined coupler to ensure stable operation. The system's stirring speed was adjustable via the power and control module, allowing fine control over mixing conditions. The glass vessel was secured to the system using a machined cap, ensuring a leak-proof and stable configuration during operation. Dynamic culture was conducted at a maximum working volume of 10 mL.

The system components were assembled and aligned to minimize mechanical friction and ensure uniform stirring.

In the stirred bioreactor culture, 1 mL of the final optimized (BGLP5) MC formulation containing $42, 500 \pm 50$ particles together with a 2×10^5 cell suspension in 500 μL was added to vessel. The vessels were then mounted to the frame for incubation in a 37 °C cell culture incubator with stirring at 30 rpm in 6 cycles of agitation for 5 min followed by 25 min of static incubation. Afterward, the spinner flasks were incubated statically overnight. Next, 80–90 % of the medium was withdrawn and replaced with a fresh warm cell culture medium. This phase was done twice to eliminate nonadherent cells. After this step, the media was increased to 9 mL for the expansion phase, then the vessels were placed in the incubator at 37 °C and agitated at (30, 60, and 120) rpm. For cell counting and staining, 200 μL samples containing at least 900 MCs in triplicate were taken from each vessel daily. In summary, the cells were completely dissociated by incubating the cell-containing MCs with trypsin (0.25%) and subsequently counted using a hemocytometer. The cell count was subsequently normalized to the surface area provided by MCs during the sampling process.

2.15. Cell Metabolic Activity and Expansion Rate

Cell proliferation on the MC surface began by seeding 2×10^5 cells onto the 1 mL of MCs suspension in 24-well plates coated with agarose (1 wt% in PBS) under static conditions. The same ratio of cell/MC was utilized in the dynamic culture environment, and cells were grown on the three different stirring rates, including 30 ± 3 , 60 ± 4 , and 120 ± 3 rpm. Cellular metabolic activity at each time point was assessed using a PrestoBlue assay following manufacture's protocol. Fluorescence intensity data was normalized against day 1 values for each respective group and reported as fold of increase in metabolic activity.

2.16. Live-Dead Staining

Viability of U251 and HNDF cells was examined using a Live/Dead fluorescence-based staining method on the MCs, in which each was incubated for 30 minutes at room temperature in 1 mL of the fluorescence dye solution. The staining mix was made by combining 2 μL ethidium homodimer-1 (stock concentration) and 0.5 μL calcein-AM (stock concentration) with 1 mL of DPBS. The MCs were subsequently washed with DPBS prior to capturing images using the Zeiss Axio Observer imaging system. For MSC experiments, viability and cell size were measured using the NC-200 NucleoCounter viability assay following the manufacturer's instructions.

2.17. Surface Marker Studies

Surface markers presented on the cells in the various experiments were analyzed using flow cytometry. Cell pellets were assembled from each group at the same starting cell counts and resuspended in 50 μL PBS + 2 % BSA as a blocking buffer. Cells were stained with 200 $\mu\text{g mL}^{-1}$ fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 and PE-conjugated anti-human

CD90 at 4 °C for 30 min for incubation. All samples received isotype control antibodies to determine off-target binding of the antibodies. Finally, each sample was diluted in PBS and analyzed using the Attune NxT flow cytometer. Propidium iodide (PI) staining was assessed separately from the antibodies. The PI staining was performed immediately after cell detachment with the PI solution and 5 min incubation at room temperature. Data was processed using FlowJo software.

2.18. Gene Expression

Primer sequences for type I collagen (COL1A1, NM_000660) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, NM_008084, internal reference) were generated using Gene Runner and Allele ID software (V.7). Following each intervention, the cell pellets were dissolved in TRIZOL (Gibco, USA), and total mRNA was extracted from the cultured HNDF samples following the protocol provided by the manufacturer. Complementary DNA was produced using a synthesis kit from ThermoFisher (USA). The relative expression fold change was calculated based on the cycle threshold (Ct) values obtained from the Bio-Rad machine after performing real-time polymerase chain reaction (PCR) using the SYBR Green method. This analysis was conducted for COL1 with GAPDH serving as the housekeeping gene.

2.19. Pro COL-1A Enzyme-Linked Immunosorbent Assay (ELISA)

The ability of collagen to be produced by the HNDF cells grown on the surface of BrushGel compared to bare GelMA MCs and culture flask under dynamic and static culture conditions was measured according to the manufacturer's instructions by using Human pro-collagen α -1 Duo set ELISA assays (R&D Systems, Minneapolis, MN, USA). Briefly, HNDF cells were cultured on MCs and flasks to achieve an approximate confluence of 80%. The supernatant was collected, and the concentration of collagen was measured. ELISA data was adjusted according to the number of the cells on the surface of each MCs or the number of cells per volume of media.

2.20. Statistical Analysis

Each experiment included at least three replicates, with results expressed as mean $\pm$ standard error of the mean (SEM). Mean values were compared by using one-way ANOVA by Tukey's multiple comparisons with GraphPad Prism 10. Statistical significance follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

3. Result and Discussion

3.1. Tailoring Thermoresponsive Properties on GelMA MCs through Precision PNIPAM Grafting

GelMA offers advantages such as rapid photo-crosslinking kinetics, excellent cell adhesion properties, and the ability to con-

trol stiffness and surface chemistry through multistep synthesis and crosslinking processes, rendering it a promising candidate for cell expansion applications.^[30] However, GelMA displays thermoresponsive properties solely in its physically crosslinked state because of the gelatin backbone; once covalently crosslinked with light, it transforms into a stiff gel, and changes in temperature no longer affect its properties.^[30] Additionally, incorporating thermoresponsive polymers into GelMA through interpenetrating networks requires large quantities of the polymer and may alter the mechanical properties of the resulting hydrogel.^[15] Notably, the thermoresponsive properties of these hydrogels often fall short of expectations due to microscale aggregation and phase separation within the hydrogel core.^[44] Moreover, since the reaction of primary amine groups in gelatin with methacrylate anhydride during GelMA synthesis consumes these groups, the DOM of GelMA can influence the subsequent grafting of COOH-terminated materials onto its surface. Therefore, we hypothesize that grafting PNIPAM-COOH chains onto the surface of GelMA MCs can impart thermoresponsive properties, but that the sensitivity of this response may depend on the DOM of the GelMA MCs.

Understanding the chemical interaction between MA and gelatin chains during GelMA synthesis allowed us to target the production of GelMA with different DOM, ensuring that some amine groups remain available for secondary reactions (Figure 2A). Three different degrees of DOM of GelMA were synthesized by modifying the methacrylation process and utilizing varying concentrations of MA. 1H-NMR results indicated that DOM decreased with a decrease in MA concentration, particularly below 0.1 mL of MA per gram of gelatin. This trend was also observed in experimental results obtained from the TNBS assay (Figure 2C), supporting the presence of available amine groups on the GelMA structure. The group with the lowest DOM ($32.43 \pm 1.5\%$) was achieved at an MA (mL)/gelatin (g) ratio of 0.05/1. Our findings align with previous reports regarding the impact of MA on the concentration of primary amines of gelatin and DOM.^[40]

We employed a flow-focusing microfluidic droplet generator device to produce monodisperse MCs (Figure 2D) by adjusting the flow rate ratios (Figure 2B). Subsequently, the MCs were crosslinked in situ by exposing the exit channel of the device to localized 405 nm LED light. The bright-field image of the MCs utilized in this study and their size distribution is depicted in Figures 2E,F. In our previous study, we optimized the effect of MC size on HNDF proliferation, gene expression, and protein secretion. We found that MCs around 300 μ m provided the best outcomes in terms of enhanced cell proliferation, procollagen secretion, and HNDF-related gene expression. Based on these findings, we used MCs with a median size of 310 ± 33 μ m in this study.^[24] PNIPAM-COOH was selected as the thermoresponsive polymer due to its lower critical solution temperature (LCST) of ≈ 32 °C and available functional groups at the end of each chain, facilitating covalent grafting onto the surface of GelMA MCs.^[34] GelMA contains adjustable amine (NH₂) terminal groups, enabling covalent attachment with the carboxyl functional group (–COOH) of PNIPAM. Grafting was facilitated through the use of the carbodiimide compounds, EDC, and NHS. EDC functions as a zero-length linker, leaving no residue in the final compound after the reaction concludes. Initially, EDC reacted

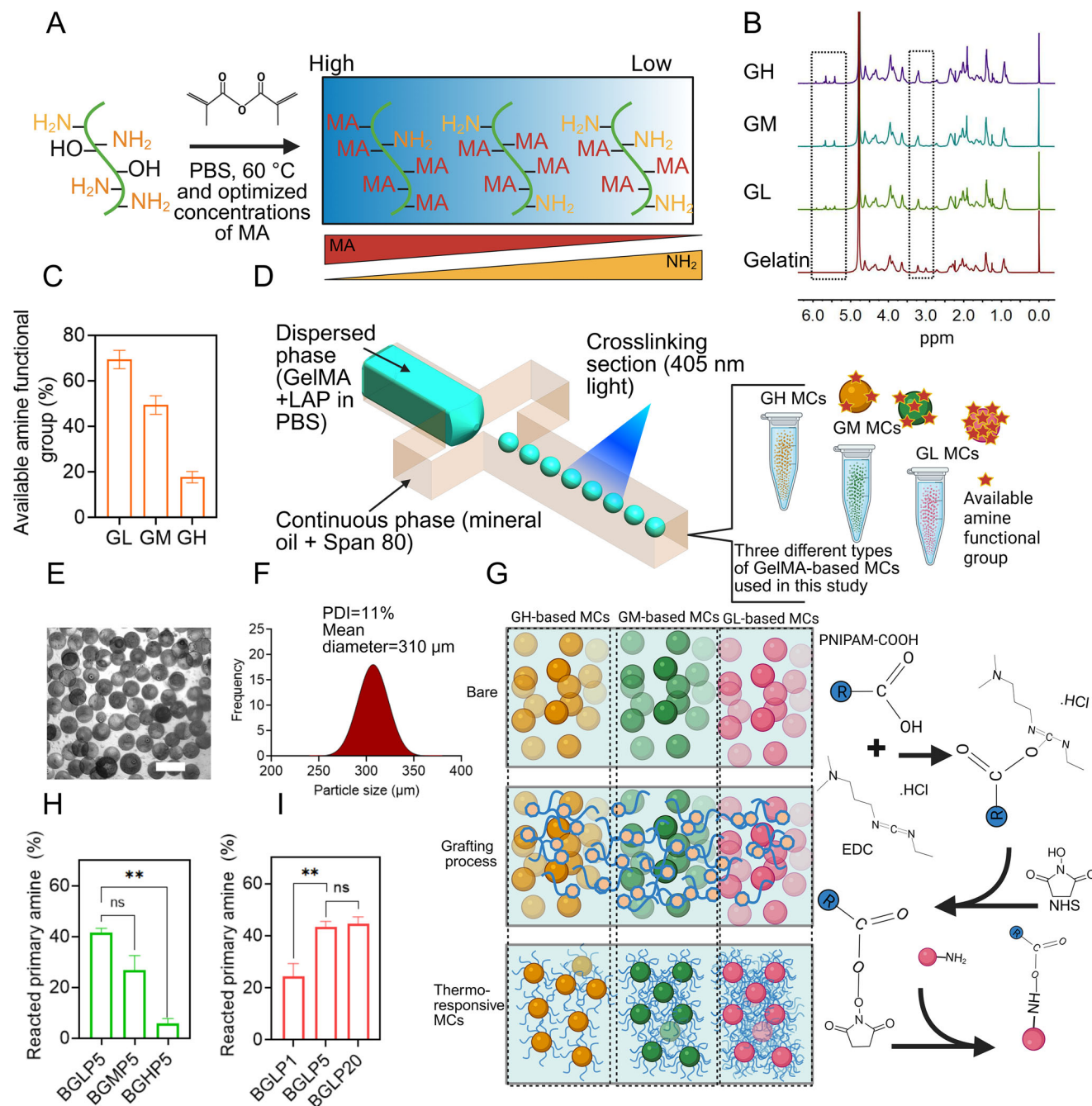


Figure 2. Workflow depicting the fabrication process of PNIPAM-grafted GelMA MCs with adjustable PNIPAM density on the surface of GelMA MCs. A) Schematic representation of GelMA synthesis, where amine groups on gelatin were modified with MA to yield GelMA with varying DOM and residual primary amine functional groups. B) Quantification of DOM (%) using 1H-NMR spectra, showcasing chemical shifts indicative of the presence of acrylic protons from methacrylate groups (5.35 ppm and 5.6 ppm) and reduction in peak intensity of lysine methylene protons (≈2.9 ppm) with increasing DOM. C) Percentage of free amine remaining determined through TNBS assay ($n = 5$). D) Generation of three distinct batches of MCs utilizing a Flow-focusing droplet generator. E,F) Bright-field microscopic image and size distribution of MCs fabricated using the microfluidic device respectively. G) Schematic representation of the reaction between the COOH group of PNIPAM and the NH₂ group of GelMA employing EDC as a zero-length linker, along with the PNIPAM-grafted MCs fabrication process. In Table 1 the labeling scheme of MCs is described. H) Percentage of primary amine groups in MCs ($n = 5$) when the feeding concentration of PNIPAM-COOH was 5×10^{-3} M. I) Influence of different feeding concentrations of PNIPAM-COOH on the percentage of reacted primary amine groups of MCs after the reaction ($n = 5$). Data is expressed as the mean $\pm$ SEM. P -values were computed using one-way ANOVA followed by Tukey's post hoc test. N represents the number of independent samples ($n = 5$). Error bars represent the SEM. A p -value < 0.05 was considered significant and statistical significance follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Figure was created using BioRender.

with the carboxylic acid to generate an unstable *o*-Acylisourea intermediate.^[34] Upon addition of NHS, this unstable intermediate transformed into a stable NHS ester, capable of effectively binding to the free amines present in GelMA. An outline of carbodiimide grafting is illustrated in Figure 2G. The density of PNIPAM chains on the surface of MCs significantly influenced their thermosensitive properties.^[25] Two key factors were investigated to fabricate MCs with high-density PNIPAM polymer chains: i) primary amine groups available on the GelMA structure and ii) initial feeding concentration of PNIPAM polymer.

To further optimize the PNIPAM grafting conditions, we investigated the effects of a 5×10^{-3} M PNIPAM concentration on the initial GelMA DOM adjustment step (Figure 2H). We conducted experiments with varying GelMA MC DOMs (High, Medium, and Low) to determine the optimal GelMA DOM condition for achieving the highest percentage of reacted primary amine groups and the highest grafting efficiency. The reaction of primary amine groups with carboxylic group of PNIPAM, as shown in Figure 2H, revealed that compared to the bare groups, BGHP5, BGMP5 and BGLP5 consumed $6.1\% \pm 1.5$, $25 \pm 5\%$, and $41 \pm 1\%$ of amine groups to react with PNIPAM chains respectively. Correspondingly, the reacted primary amine resulted in Figure 2H indicated that transitioning the GelMA of MCs formulation to low DOM resulted in a higher consumption of primary amines compared to reactions conducted with other DOM above this value. This suggests that the reactions of amine groups at GH with PNIPAM-COOH chains might be impeded by the lower number of free amino groups available because of MA groups density as reaction sites for the attachment of PNIPAM chains.

We investigated the dependence of PNIPAM grafting on the PNIPAM-COOH feeding concentration to identify the range for controllability of grafting density. The feed ratio ranged from 1×10^{-3} to 20×10^{-3} M of PNIPAM-COOH to study the impact of molar ratio of reagents. Figure 2I summarizes the results of the reacted primary amine percentage based on different feed ratios. The results indicate that the percentage of reacted primary amine groups for different PNIPAM-COOH feeding concentrations significantly increased from 1×10^{-3} to 5×10^{-3} M. In particular, it was observed that the reaction of amine groups approached saturation at around 5×10^{-3} M feeding (BGLP5) concentration of PNIPAM-COOH, and there was no statistically significant difference between the 5×10^{-3} M (BGLP5) and 20×10^{-3} M (BGLP20) concentrations. Compared to previous reports that targeted the carboxylic acid groups of GelMA for covalent bonding of a molecule using the EDC-NHS chemistry approach, our method utilized the available amine groups in the GelMA structure, leaving the carboxylic acid groups available for secondary applications.^[35,45] The impact of PNIPAM grafting on the surface topography and morphology of MCs was analyzed using SEM (Figure S3, Supporting Information). No significant changes in the porosity or texture of the MCs were observed before and after grafting. This could be attributed to the fact that the MCs were already photo-crosslinked prior to grafting, and the EDC-NHS grafting process did not induce additional crosslinking. Furthermore, due to the nanoscale size of the PNIPAM chains, their presence is not easily detectable by SEM imaging. These findings align with previously reported data in the literature.^[46]

3.2. Enhanced Cellular Attachment and Sustained Proliferation on BrushGel MCs: Impact of Matrix and PNIPAM Grafting

The influence of PNIPAM chains grafted to the GelMA MCs on cell adhesion properties was examined using U251 and HNDF cells representing cancerous and healthy tissue cells, respectively. First, we studied the cell attachment on the MCs with varying formulations and then cell's proliferation on the surface of MCs was assessed (Figure 3A). Figure 3B illustrates the number of attached U251 cells on MCs. The presence of arginine, glycine, aspartic acid (RGD) peptide cell-binding motifs on the MCs attachment, thanks to the GelMA matrix, facilitated U251 cell attachment with up to 50% attachment observed on the BGLP0 MCs, allowing cells to proliferate over their surface. Grafting PNIPAM chains onto the MCs' surface enhanced cell attachment efficiency by up to 80% for BGLP5, with no significant difference observed in cell attachment efficiency between BGLP5 and BGMP5 (Figure 3B). Interestingly, increasing the concentration of PNIPAM-COOH in the fixed DOM of GelMA enhanced U251 cellular attachment, with the significantly highest cell attachment achieved by BGLP20 (Figure 3C). The significant enhancement of cellular attachment on PNIPAM-grafted MCs may be resulted from the hydrophobic nature of PNIPAM chains at 37 °C. This hydrophobicity likely promoted interactions between the MC surface and proteins or growth factors in the cell culture medium. The adsorption of these proteins could, in turn, facilitate cellular attachment. Figure 3D displays Live/Dead fluorescence microscope images taken on day three post-seeding of U251 cells on the MCs, revealing the intensity of green phase (live cells) spread across all MC surfaces with minimal dead cells (red). We found that low DOM of GelMA coated with varying concentrations of PNIPAM could provide supportive environment for cells to attached. For the rest of the study, we used low-DOM GelMA as the core material of MCs, with varying PNIPAM chains grafted onto their surface. To investigate whether PNIPAM-grafted MCs can promote healthy cell attachment, we analyzed the attachment of HNDF cells. Initially, at a fixed cell seeding density, we studied cell attachment on MCs with varying concentrations of PNIPAM grafted to them. As shown in Figure 3E and Figure S4 (Supporting Information), increasing the PNIPAM-COOH concentration up to 5×10^{-3} M (BGLP5) led to an increase in the number of attached cells. Beyond this concentration, there were no significant differences between BGLP5 and BGLP20. It has been shown that grafting PNIPAM chains onto the surface of polymeric MCs or films can influence fibronectin adsorption.^[25,47] Since fibronectin plays a key role in cell adhesion, its adsorption level directly correlates with the number of adhering cells. We hypothesize that PNIPAM grafting may reduce the hydrophilicity of the GelMA surface, potentially enhancing the binding of fibronectin and other proteins involved in cell attachment. Further studies are needed to investigate how PNIPAM-induced surface chemistry changes affect the adsorption of ECM-related proteins.

Next, we evaluated the effect of cell seeding density on the attachment of HNDF cells in the BGLP5 group. As illustrated in Figure 3F and Figure S5 (Supporting Information), increasing the cell seeding density from 5×10^3 per well to 10×10^3 per well improved the attachment efficiency for BGLP5, but beyond that, no significant differences were observed. The increase in seeding density enhanced attachment efficiency by 10 % on BGLP5 by

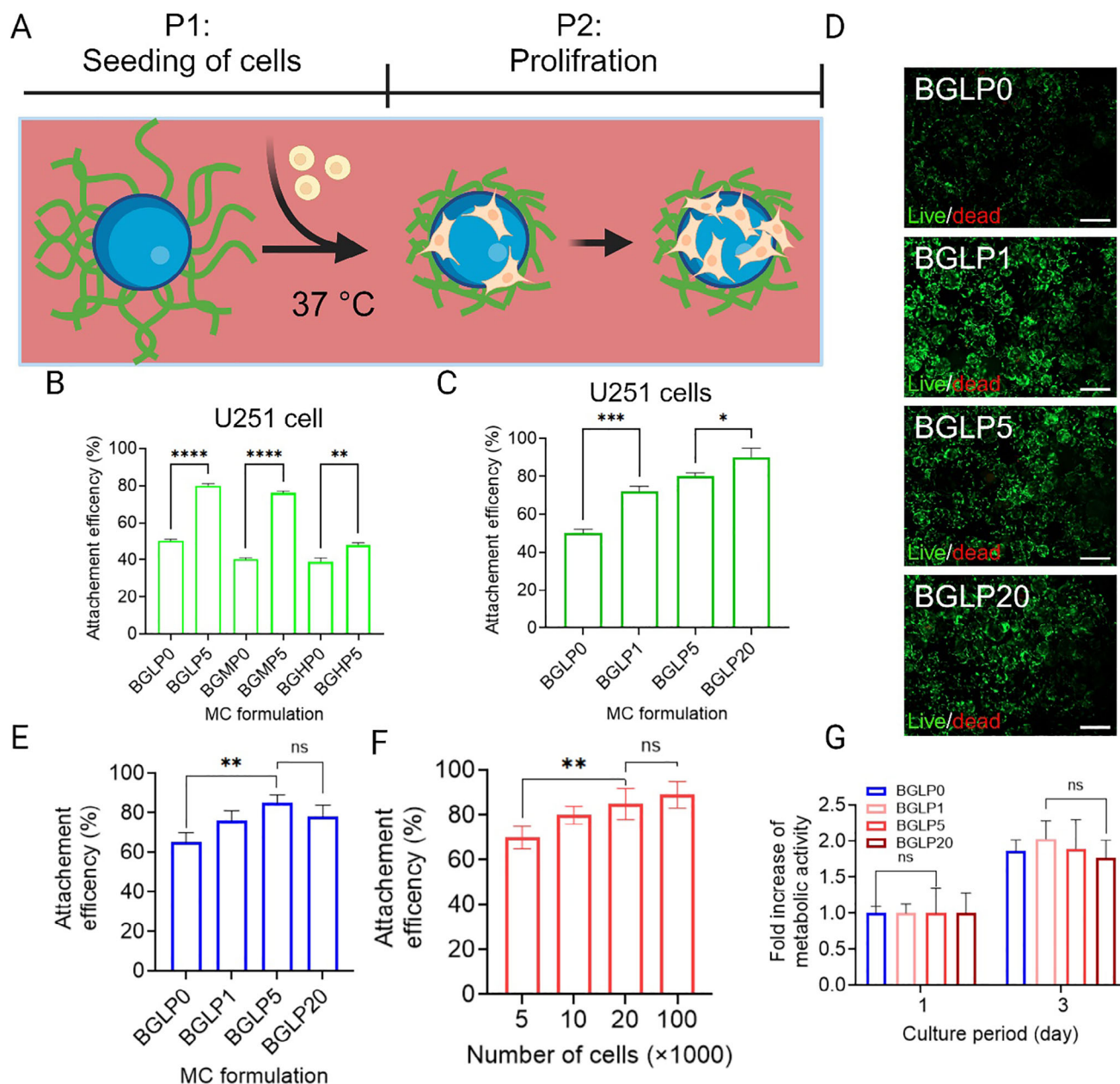


Figure 3. Cellular adhesion and proliferation on PNIPAM-grafted GelMA MCs. **A)** Schematic depicting cell attachment and proliferation as distinct phases in the cellular expansion process. **B)** U251 cell attachment efficiency for an initial cell seeding number of 50000 cells per well ($n = 3$) for MC formulations. **C)** Effect of PNIPAM-COOH feeding concentration on U251 cell attachment efficiency on the surface of MCs ($n = 3$). **D)** Viability analysis of U251 cells on the surface of MCs. Cells stained with calcein-AM (green) and ethidium homodimer (red) three days after seeding (Scale bar = 500 μ m). **E)** HNDF cell attachment efficiency on the surface of MCs ($n = 3$). **F)** Influence of initial cell seeding number on HNDF cell attachment efficiency on the surface of BGLP5 MCs. **G)** Metabolic activity of HNDF cells on the surface of MCs over 3 days. Data are expressed as the mean $\pm$ SEM. P-values were computed using one-way ANOVA followed by Tukey's post hoc test. N represents the number of independent samples ($n = 3$). Error bars represent the SEM. A p-value < 0.05 was considered significant and statistical significance follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Figure was created using BioRender.

promoting cell-cell interactions and optimizing the use of available binding sites. However, excessively high densities (10×10^4 cells per well) led to reduced cell viability likely due to over confluency on the surface of MCs which could result in nutrient depletion and waste accumulation. The proliferation of HNDF cells was consistent across all formulations (BGLP0, BGLP1, BGLP5,

and BGLP20), over the three-day culture period. There were no significant differences in the proliferation rates among these formulations (Figure 3G). These results suggest that variations in PNIPAM-COOH concentration did not substantially affect the metabolic activity of cells over time. The steady metabolic activity across formulations suggests that while initial attachment effi-

ciency may differ, as seen in previous panels, cell viability and proliferation capability remain unaffected by these differences in the short-term. This could imply that once attached, the cells could continue proliferating at a similar rate regardless of the MC formulation, likely due to the availability of sufficient binding sites and nutrients in the culture medium. However, long-term effects of PNIPAM-COOH concentration grafted to MCs on cell behavior might require further investigation to assess any potential influences on differentiation, viability, or other cellular functions.

3.3. BrushGel MCs Preserve HNDF Phenotype while Significantly Boosting COL1A1 Expression and ECM Production

CD90(+) HNDF cells represent primary fibroblasts, which have the potential to differentiate into myofibroblasts, a critical step in wound healing.^[48] Maintaining the CD90(+) phenotype without inducing differentiation into the myofibroblast state is particularly important for tissue regeneration such as those aiming at collagen replacement.^[49] To evaluate whether the MCs affect the phenotype of HNDF cells during culture, the expression of CD90 on cells was quantified using flow cytometry. The density dot plots in **Figure 4A,B** reveal that over 95% of HNDF cells cultured on MCs formulations retain the CD90(+)/CD45(-) phenotype, suggesting that neither the coating nor the mechanical properties of the MCs induce any unwanted differentiation.

Fibroblasts are crucial in synthesizing ECM proteins such as collagen (particularly types I and III) and fibronectin.^[50] Type I collagen (Col1) is the most abundant fibrillar collagen in mammals, mainly produced by fibroblasts, and is vital for providing structural strength to ECM-rich tissues like skin. To investigate whether the presence of PNIPAM influenced COL1A1 gene expression in HNDF cells, we examined BGLP0, BGLP1, BGLP5, and BGLP20 formulations. As seen in **Figure 4C**, cells cultured on BGLP20 displayed the highest expression of the COL1A1 gene, significantly surpassing the levels seen in cells cultured on BGLP0, BGLP1, and BGLP5. This suggests that PNIPAM grafting on MC surfaces can enhance COL1A1 expression, possibly due to nanoscale modifications in surface stiffness or roughness.^[51]

Additionally, BGLP5 and BGLP20 exhibited significantly higher pro-collagen secretion compared to BGLP0 and BGLP1, indicating these formulations better support ECM production (**Figure 4D**). The low pro-collagen secretion in BGLP0 was consistent with its lower COL1A1 gene expression, suggesting this formulation hindered collagen production. Overall, BGLP5 and BGLP20 demonstrated superior support for collagen formation, making them ideal candidates for applications that require enhanced collagen production. This also aligns with the maintained fibroblast phenotype and the lack of differentiation into a myofibroblast phenotype, essential for tissue regeneration therapies.

3.4. Efficient Temperature-Triggered Cell Release from BrushGel MC Surfaces: Minimizing Enzyme Use for Optimal Harvesting

Detaching cells from MC's surfaces before complete coverage is essential to prevent several issues such as cell mortality, differ-

entiation, contact inhibition of growth, and other negative effects related to over-confluency. The goal of grafting PNIPAM polymer chains onto GelMA MCs is to reduce the reliance on enzymatic treatments during cell detachment. The harvesting protocol depicted in **Figure 5A** involved placing cell-laden MCs in cold media with diluted trypsin solutions to facilitate the complete detachment of adhered cells. Since the activation kinetics of PNIPAM brushes below their LCST is dependent on brush density and the time of incubation, we explored the effect of different PNIPAM concentrations and varying incubation times at 4 °C.

Figure 5B,C and **Figure S6** show that the effectiveness of PNIPAM brushes for detachment primarily depends on PNIPAM concentration and incubation time below LCST. Consistent with earlier studies, higher densities of PNIPAM brushes did not significantly enhance the detachment of cells from MC surfaces. In **Figure S6**, we observed the detachment efficiency of HNDF cells from MC surfaces with different PNIPAM concentrations across four incubation time points. After 15 min incubation at 4 °C, the BGLP5 and BGLP20 groups showed the highest detachment efficiency ($10 \pm 1.5\%$). By 30 min, the detachment efficiency increased to $21 \pm 2.1\%$ for both BGLP5 and BGLP20. After 120 min at 4 °C, the detachment efficiency peaked at $60 \pm 4.8\%$ for BGLP5, which showed the best performance in temperature-mediated detachment and was subsequently chosen for further experimentation. The thermoresponsive behavior of PNIPAM is governed by conformational changes in its polymer chains. Increasing the brush density can significantly reduce the extent of these conformational changes, thereby affecting its responsiveness. As BGLP1, BGLP5, and BGLP20 MCs contain low, medium, and high amounts of PNIPAM chains, respectively, their conformational changes (rate and intensity) in response to temperature alterations are expected to follow this trend: rapid but small (BGLP1), slow but intense (BGLP5), and slow but small (BGLP20). This trend supports our detachment results and is consistent with previous findings.^[25,47] The extent of cell coverage on MC surfaces played a significant role in determining the detachment efficiency of PNIPAM chains. We hypothesized that cell coverage could impact cell detachment efficiency, as high coverage may hinder the conformational change of PNIPAM. To test this, we cultured cells for up to 5 days on MCs with a fixed surface area, using four different seeding numbers ranging from low (5×10^3) to high (1×10^5). **Figure S7** shows the detachment efficiency of HNDF cells over time when cultured on BGLP5 MCs at different seeding numbers. The kinetics of cell detachment were also analyzed in this experiment. On day 1, increasing the cell seeding number by 20X reduced detachment efficiency by half across almost all incubation times, except for the 120-min incubation, where the reduction was slightly less than half. For the 5×10^3 and 1×10^4 groups, increasing the culture time to 5 days slightly reduced detachment efficiency at a given incubation time. However, for the higher seeding numbers (1×10^4 and 1×10^5), increasing the culture time resulted in a more decrease in detachment efficiency. As seeding number correlated with cell confluency on the MC surface given our attachment efficiency data, we observed that when cell coverage was below 50% (seeding densities of 5×10^3 and 10×10^3 on BGLP5), over 70% of cells detached within 60 min of incubation at 4 °C. However, when cell coverage exceeded 50% such as at a seeding density of 2×10^4 cells (95% confluency at 1×10^5 cells) detachment efficiency dropped to 41

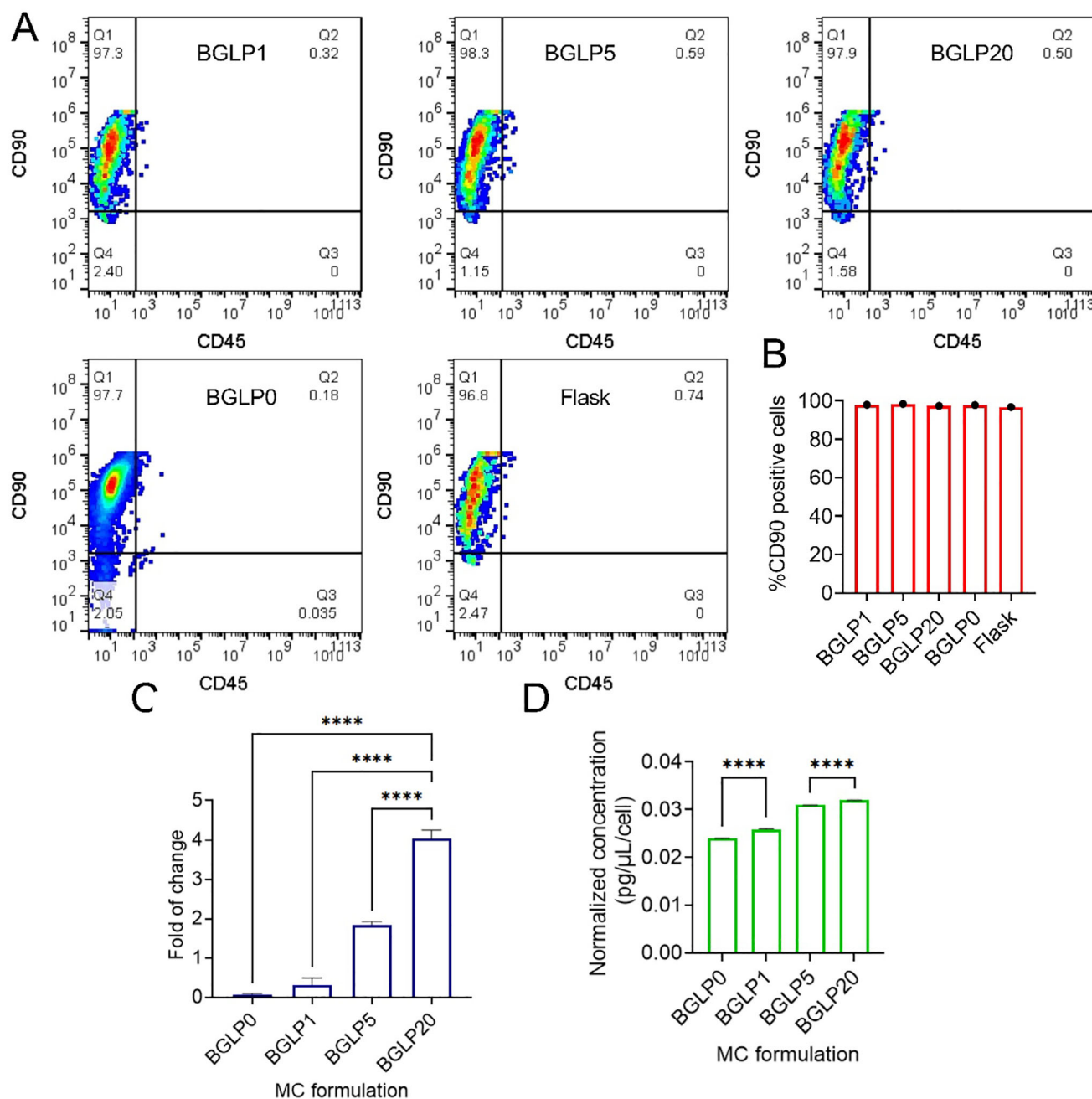


Figure 4. Characterization of HNDF cell phenotype, gene expression, and protein secretion when cultured on BrushGel MCs with different formulations. A) Visualization of CD90+/CD45- HNDF cells cultured on the surface of MCs. B) Representation of HNDF cell percentage through CD90+ expression on MCs. C) Analysis of COL1A1 gene expression in HNDF cells cultured on MCs with different PNIPAM polymer concentrations on their surfaces. D) Quantification of procollagen protein secretion by HNDF cells cultured on MCs with varying surface chemistries. Data are expressed as the mean $\pm$ SEM. P-values were computed using one-way ANOVA followed by Tukey's post hoc test. N represents the number of independent samples ($n = 3$). Error bars represent the SEM. A p-value < 0.05 was considered significant and statistical significance follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

$\pm 2.5\%$ and $30 \pm 3.1\%$, respectively. This decrease in detachment efficiency could be attributed to the formation of tight junctions between cells and increased ECM production, which may interfere with PNIPAM chain swelling and conformational changes. Overall, these results demonstrate that cell coverage on MCs is a critical factor influencing cell harvesting efficiency.

For efficient cell expansion, it is crucial to detach nearly all cells from MC surfaces. To achieve this, we treated HNDF cells on BGLP5 MCs with media containing 0.05%, 0.025%, and 0.005% trypsin for up to 120 min, combining low temperature with diluted enzyme to enhance detachment (Figure 5E). The 0.05% trypsin solution detached all cells within 15 min. As the

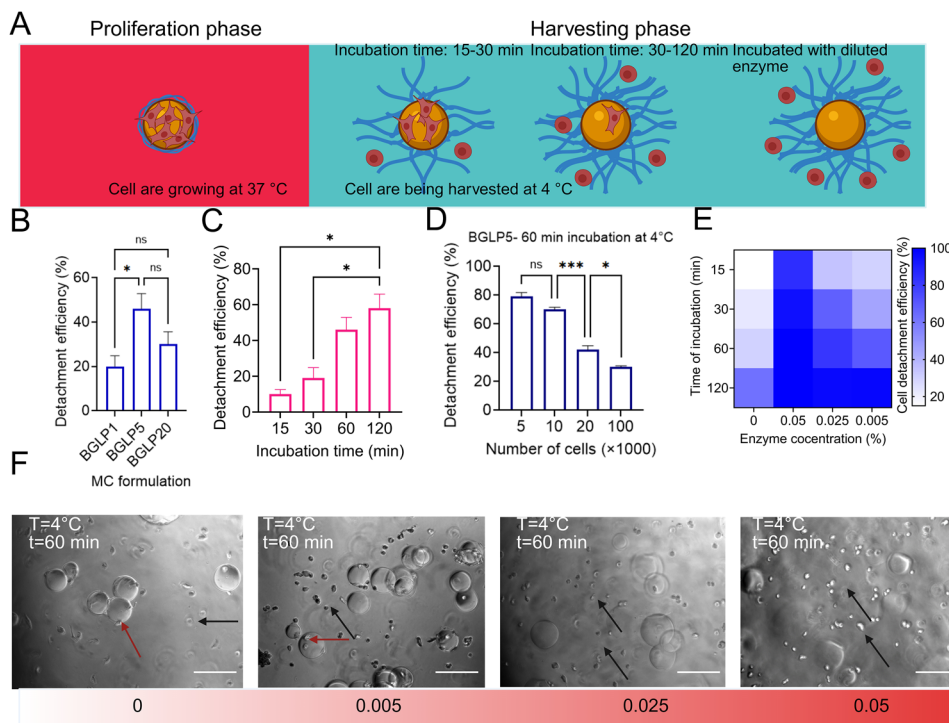


Figure 5. Harvesting HNDF cells from BrushGel MCs. A) Schematic representation illustrating the proliferation and harvesting phases in this study. Cells were detached using low-temperature treatment to assess the functionality of PNIPAM brushes, while treatment with diluted trypsin solutions was also employed to enhance detachment efficiency. B) Low-temperature detachment efficiency of HNDF cells from the surface of MCs at 120 min incubation time C) Evaluation of the impact of low-temperature incubation duration on the detachment efficiency of HNDF cells from BGLP5 MCs. D) Detachment efficiency of HNDF cells cultured on the surface of BGLP5 at a low-temperature incubation duration of 60 min for varying cell coverage percentages. E) Cluster heat map illustrating the synergistic impact of low-temperature and enzyme treatment on cell detachment with the seeding number of 5×10^3 . Rows represent incubation time, while columns represent enzyme concentration, with each cell color-coded based on detachment efficiency percentage. F) Bright-field microscopy images depicting cell detachment from MC surfaces using low temperature and combinations of enzymes with different concentrations (red arrows show the cells on the surface of MCs, black arrows show the detached cells). Data is expressed as the mean $\pm$ SEM. *P*-values were computed using one-way ANOVA followed by Tukey's post-hoc test. *N* represents the number of independent samples ($n = 3$). Error bars represent the SEM. A *p*-value < 0.05 was considered significant and statistical significance follows: **p* < 0.05 , ***p* < 0.01 , ****p* < 0.001 , *****p* < 0.0001 . Figure was created using BioRender.

enzyme concentration decreased, detachment efficiency gradually declined, but even with 0.025% trypsin, more than 90% of cells were detached after 60 min of incubation at 4 °C. Similarly, 90% of cells detached with 0.005% enzyme after 120 min of incubation.

Figure S8 shows that more than 95% of the detached cells remained viable (trypan blue assay) after 60 min of incubation at 4 °C, with no statistically significant differences between the groups treated with enzyme and those cooled without enzyme treatment and with no significant difference compared to cells maintained at 37 °C and cultured on BGLP5 MCs (Figure S8). These results indicate that short-term cold incubation with low-concentration enzyme does not significantly compromise HNDF viability. Brightfield images in Figures 5F and S9 demonstrate the detachment process, with some cells remaining attached to the MCs without enzyme treatment. As enzyme concentration increased, most cells were detached, leaving MC surfaces largely free of cells. Importantly, previous results indicated that cell phenotype remained stable when cultured on MCs. To confirm that the detached cells retained a consistent phenotype, we analyzed CD90 and CD45 marker expression. Figure S9 shows that both detached and attached cells maintained their surface

marker expression, suggesting that differentiation was not responsible for hindering detachment. One challenge associated with using enzymes for cell harvesting from soft gelatin-based MCs is the potential dissolution of the MCs themselves, as seen with the 0.05% enzyme group after 60 min (Figure 5F). It also has been shown that GelMA-based hydrogels could be fully degraded when they are treated with enzyme.^[52] This raises concerns about residual biomaterial traces left behind after enzymatic harvesting, which could pose regulatory challenges in clinical applications. We did not observe significant cell death after detachment (Figure S8). However, to mitigate concerns about residual enzymes or biomaterial traces, additional washing steps may be necessary, although this could increase labor and costs.

3.5. Temperature-Mediated Cellular Detachment Extends to Culture of Clinical Grade Human MSCs

To examine the harvest dynamics of clinical grade MSCs from BrushGel MCs (BGLP5 group), we employed a similar harvesting approach as described in Figure 5A, this time determining

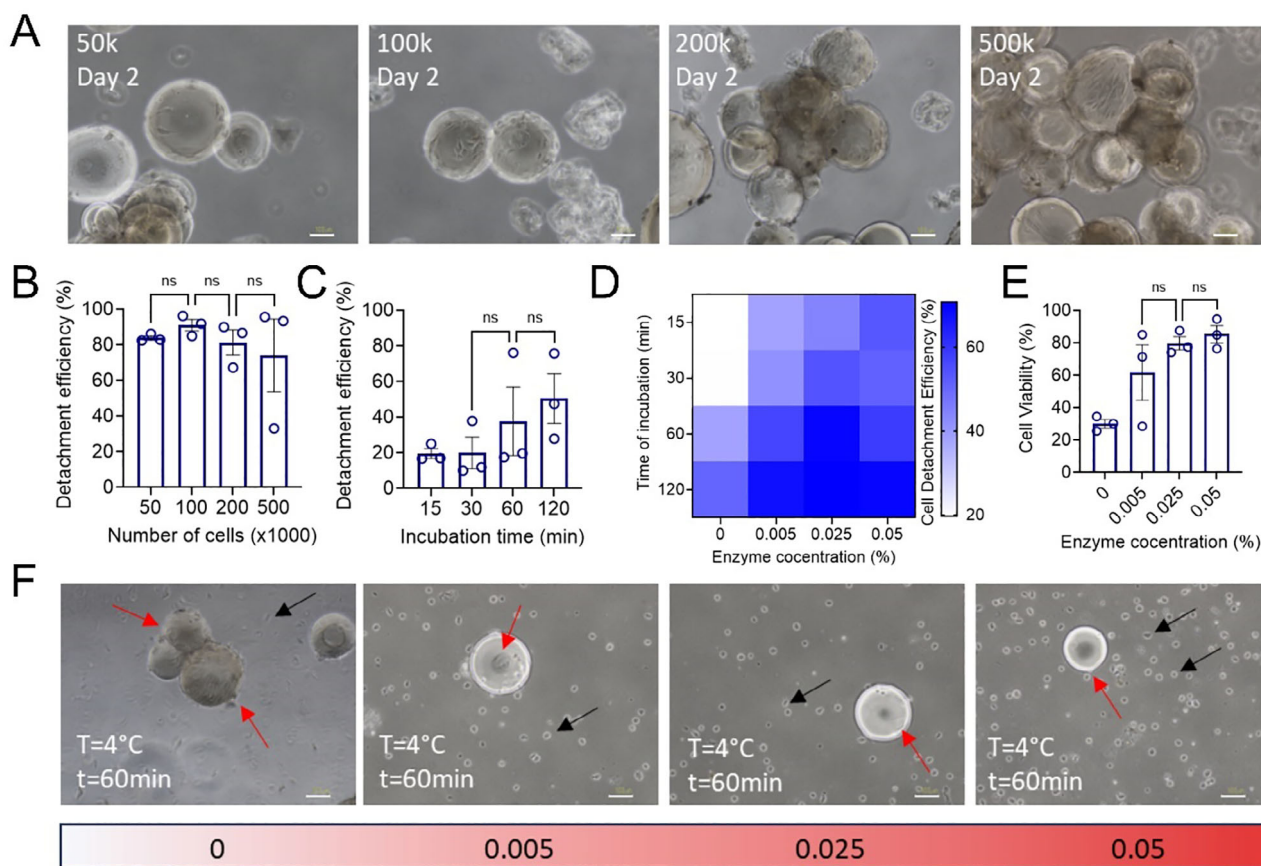


Figure 6. Harvesting MSCs from BrushGel MCs. A) Brightfield images depicting MSC attachment to BrushGel MCs after 2 days in culture after seeding with a range of cell numbers (indicated top left). B) Evaluation of impact of MSC seeding density on detachment efficiency upon 60-min low temperature incubation C) Evaluation of 4 °C incubation duration on enzyme-free MSC detachment efficiency from MCs. D) Cluster heat map showing the effects of low temperature incubation time and enzyme concentration on MSC detachment efficiency. Rows represent incubation time, columns show enzyme concentration, and colors indicate MSC detachment efficiency. E) MSC viability upon 4 °C harvest from MCs upon incubation with varying enzyme concentrations. F) Brightfield microscopy images depicting MSC detachment from the MCs upon 60-min 4 °C incubation using different enzyme concentrations. Red arrows indicate examples of cells that remained associated with MCs after the 60-min low temperature exposure period, while black arrows show detached cells. Data is expressed as the mean $\pm$ SEM. P-values were computed using one-way ANOVA followed by Tukey's post-hoc test. N represents the number of independent samples ($n = 3$). Error bars represent the SEM. A p-value < 0.05 was considered significant and statistical significance follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

how MSCs cultured on the MCs respond to varying seeding densities, combinations of 4 °C incubation times and enzyme concentrations.

To investigate whether MSCs could attach to the BrushGel MC surface, different numbers of MSCs were seeded on MCs, ranging from 5×10^4 to 5×10^5 cells. Brightfield microscopy images showed cell attachment in all conditions after 2 days in culture (Figure 6A), at which time MSCs were harvested by enzyme-free 60-min low temperature incubation. Notably, harvest of 5×10^4 and 10×10^4 and 20×10^4 cell seeded conditions yielded over 80% cell dissociation after 60-min enzyme-free incubation at 4 °C, while the higher 5×10^5 cell seeding density yielded 74% of attached cells (Figure 6B). Variation in MSC detachment efficiency increased at higher seeding densities, which, in combination with highest average detachment deficiency in the 10×10^4 -cell seeding group, led to the decision to proceed with that seeding number in subsequent experiments. Next, detachment efficiency of MSCs from BrushGel MCs by 4 °C low temperature

exposure after a standard 5-day culture was evaluated in enzyme-free conditions (Figure 6C). Detachment efficiency was lowest after the shortest incubation times, with 15- and 30-min 4 °C exposure both yielding 20% cell dissociation. Detachment efficiency increased to 38% after 60 min, with the highest detachment efficiency, 51%, being observed after 120-min 4 °C incubation (Figure 6C). When total cell numbers harvested on day 5 from the MCs were tallied and compared to total cells seeded, MSCs were found to have expanded an average of 5.3-fold over the 5-day culture. In a similar experimental design as HNDF cells, we aimed to enhance harvest efficiency by testing the impact of up to 50 $\times$ dilution of trypsin solution on cell detachment efficiency by subjecting typical 5-day MC-MSC cultures to 0.005%, 0.025%, and 0.05% trypsin solutions for up to 120 min (Figure 6D). Notably, as little as 0.005% enzyme treatment released up to 68% of the attached cells by 120 min of incubation, while increasing the enzyme concentration to 0.025% and 0.05% delivered diminishing returns of 71% and 70% cell detachment, respectively. Taken

together with the enzyme-free harvest data, these results suggest that MSC dissociation from BrushGel MCs can be achieved after low temperature incubation and boosted to achieve high MSC harvest yields by adding highly diluted dissociation enzyme solution.

Cell viability of detached cells by 60-min 4 °C incubation after 5-day culture appeared to be affected by enzyme concentration. Enzyme-free detached cells displayed a viability of 30%, while cells harvested with trypsin concentrations of 0.005%, 0.025% and 0.05% displayed viability of 62%, 80% and 86%, respectively (Figure 6E). Brightfield microscopy images visually show attached and detached cells after 60-min low temperature incubation with varying enzyme concentrations, with examples of detached cells denoted by black arrows, and cells remaining attached to MCs by red arrows (Figure 6F). These images corroborate earlier findings, with the most cells visually observed attached to MCs after enzyme-free incubation, and the number of attached cells decreasing in conditions with higher enzyme concentration. Interestingly, viability appeared to increase along with increasing concentrations of enzyme. This may be due to high levels of cell-cell support mediated by ECM secreted by the MSCs after 5 full days in culture on the MCs. While enzyme-free 4 °C incubation was able to detach cells on culture day 2, this incubation alone was likely insufficient to disrupt a more established ECM, with MSCs on culture day 5 being primarily released by mechanical force, thus damaging the cells and resulting in low detached cell viability. With the increasing concentrations of trypsin during the harvest incubation, more of the ECM could be digested, resulting in increased viability in these conditions. In a pilot study, we assessed MSC function using an established assay by co-culturing MSCs with activated T cells to evaluate their ability to inhibit T cell proliferation.^[42] We observed a 10% inhibition of T cells when MSCs were harvested without enzyme, compared to a 34% inhibition with cells harvested using 0.025% enzyme. In our previous study, we reported a 10%-45% range of MSC's inhibitory effect on T cells. Therefore, the current data is within the normal range we see from our MSCs.^[42] Additionally, we observed a lower cell viability in the complete absence of enzyme (52.8% viability with 0% enzyme versus 97.8% viability with 0.025% enzyme). Notably, without enzyme, detachment efficiency was low (37%), requiring mechanical stress to detach cells. This may have contributed to the lower viability and to the reduced functional capacity of these cells observed in the no-enzyme condition. While our findings indicate that combining low enzyme concentration with cold temperature enhances both post-harvest MSC viability and immunomodulatory function after culture on Brush MCs, further optimization is needed to improve cell viability, detachment efficiency, and functional performance of MSCs when harvested without any enzymatic treatment.

3.6. Optimizing BrushGel MCs for Stirred Bioreactor Cultures: Enhancing Cell Viability and ECM Production through Controlled Agitation

The efficacy of MCs was evaluated in a dynamic environment using stirring bioreactors. Stirring within the bioreactor reduces MC aggregation, thereby enhancing cell viability and expansion

rates.^[9] The workflow of dynamic culture studies is depicted in Figure 7A. BGLP5 MCs were loaded into the bioreactor, followed by seeding HNDf cells and adhering to the dynamic culture protocol. One crucial factor in dynamic culture is the stirring rate, which is directly related to the shear stress experienced within the vessel. Higher shear rates have been reported to potentially damage cell membranes, increase cellular differentiation or promote cell death.^[53] Given that our MC formulation had not been previously reported in dynamic culture literature, we investigated cell proliferation in a dynamic culture environment at three stirring rates: 30, 60, and 120 rpm. This range of stirring rates is commonly used for MSC expansion in dynamic culture platforms.^[54]

Figure 7B illustrates the cell count at specific predetermined time points for the three stirring rates. At the highest stirring rate (120 rpm), cell numbers reached a plateau after 1 week of culture, suggesting that MCs became confluent. Conversely, at lower stirring rates (30 rpm), cell numbers continued to increase over time, with no observed plateau region. The fold increase in cell density for the three stirring rates at the end of culture (day 10) is presented in Figure 7C. Culturing cells at a lower stirring rate (30 rpm) resulted in a 4.8-fold increase in cell density, compared to 3.3-fold increase at 120 rpm. The results show that cell density is highest at 30 RPM, indicating that lower agitation rates provide a more favorable environment for cell density by reducing shear stress. In contrast, at 120 RPM, the cell density is significantly lower, likely due to increased shear forces that disrupt the ability of cells to adhere to the MCs.

We determined that a stirring rate of 30 rpm was the optimal stirring speed for dynamic cell expansion. Figure 7D compares the fold increase in metabolic activity between static and dynamic cultures at a stirring rate of 30 RPM over 10 days. The dynamic culture (30 RPM) shows a significantly higher increase in metabolic activity compared to the static culture, particularly by day 7, where the metabolic activity peaks. The dynamic culture experiences a rapid rise in metabolic activity from day 1 to day 7, while the static culture has a more gradual increase. By day 10, both conditions plateaued, but the dynamic culture maintained a higher overall metabolic activity compared to the static condition. In Figure 7D, the viability of cells is shown on the secondary y-axis (red line), with cell viability percentage displayed for both static and dynamic cultures at 30 RPM. The cell viability in the dynamic culture (30 RPM) is consistently higher than in the static culture throughout the 10-day period. Around day 7, the viability in the dynamic culture reaches close to 100%, while the static culture shows lower viability. By day 10, the viability in the dynamic condition remained high, demonstrating that the mechanical stimulation at 30 RPM supported both increased metabolic activity and cell viability compared to static culture conditions.

Figure 7E presents flow cytometry histograms of PI staining, which is used to identify dead cells (PI-positive) in cultures at three different stirring rates (30, 60, and 120 RPM) compared to static conditions. The data indicate that the percentage of dead cells was lowest in the 30 RPM dynamic culture (7.49%) and increased with higher stirring rates (13.1% at 120 RPM), while the static culture had the highest percentage of dead cells (32.2%). Figure 7F quantifies these results, showing that the static culture exhibited the most significant cell death, while the 30 RPM condition promoted the highest cell viability, with much fewer PI-positive cells. The data suggest that moderate stirring (30 RPM)

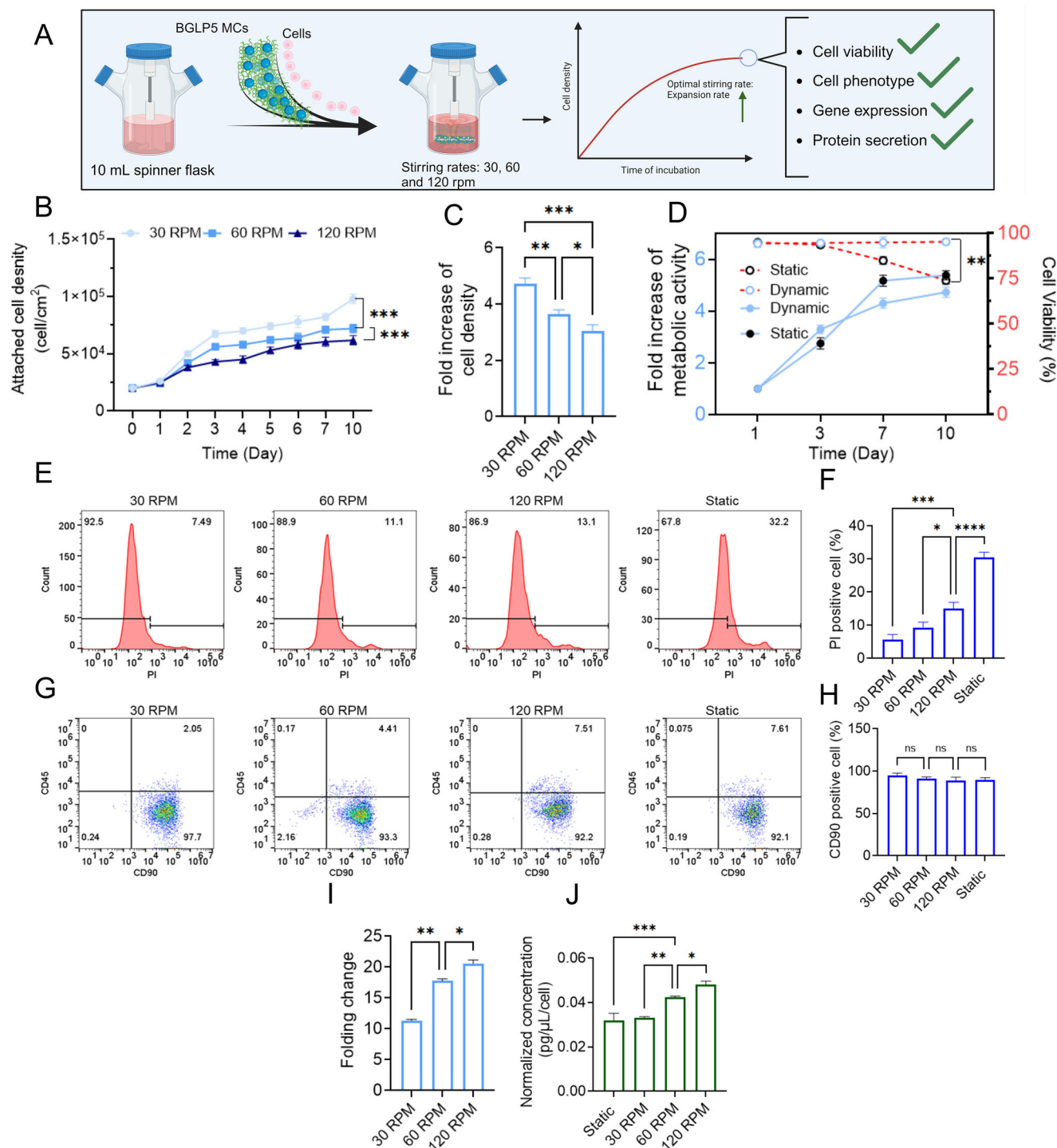


Figure 7. BrushGel MCs in a dynamic culture environment with HNF cells. A) Schematic illustration of the workflow for cell culturing in the dynamic environment. B) Density of attached HNF cells at various time points during culture under different stirring rates. C) Fold increase in cell density under different stirring rates on day 10. D) Right Y-axis: Metabolic activity of HNF cells cultured in the dynamic environment (stirring rate: 30 rpm) compared to the static environment (no stirring) at different time points. Left Y-axis: Cell viability of HNF cells at predetermined time points in dynamic and static environments. E and F) Histograms and bar charts depicting the population of PI+ HNF cells at day 10 under different stirring rates and static conditions. G,H) Representative dot plots of HNF cells and bar charts showing CD90+/CD45- HNF cell populations grown under different stirring rates and in static culture. I) Expression of COL1A1 gene in HNF cells cultured in a dynamic environment with varying stirring rates. J) Secretion of pro-collagen protein by HNF cells cultured dynamically at different rates and in static culture. Data are expressed as the mean $\pm$ SEM. P-values were computed using one-way ANOVA followed by Tukey's post-hoc test. N represents the number of independent samples ($n = 3$). Error bars represent the SEM. A p-value < 0.05 was considered significant and statistical significance follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Figure was created using BioRender.

provides an optimal environment for cell survival, likely due to improved nutrient and oxygen diffusion, more effective waste removal, and mild mechanical stimulation that enhances cell viability. In contrast, higher stirring rates, such as 120 RPM, introduced excessive shear stress, which slightly increases cell death. Overall, these results demonstrate that dynamic culture at 30 RPM created a favorable environment for maintaining cell viability compared to static conditions or more intense agitation.

Figure 7G,H shows flow cytometry dot plots that assess the expression of CD90 and CD45 surface markers on cells cultured under different conditions (30 RPM, 60 RPM, 120 RPM, and static). The majority of cells across all condition's expressed high levels of CD90 and low levels of CD45, indicating that the HNDF cells retained their mesenchymal stem cell phenotype across both dynamic (30, 60, 120 RPM) and static cultures. Notably, the expression of CD90 slightly decreased at 120 RPM, indicating that higher stirring rates affected the phenotype stability. Together, Figure 7G,H demonstrates that dynamic culture at 30 and 60 RPM preserved the HNDF phenotype (high CD90 expression and low CD45 expression) just as well as static conditions.

Figure 7I presents the fold change in gene expression of COL1A1 in HNDF cells cultured on the BGLP5 MCs under different stirring rates (30 RPM, 60 RPM, 120 RPM). The data show that COL1A1 expression was significantly upregulated in dynamic cultures, with the highest fold change occurring at 60 RPM, indicating that moderate mechanical stimulation enhanced the production of collagen by HNDFs. This is beneficial in tissue engineering and wound healing contexts, as collagen plays a critical role in skin structure and repair. This suggests that dynamic culture, particularly at 60 RPM, promoted beneficial extracellular matrix production (COL1A1) in HNDFs, maintaining a healthy fibroblast phenotype. Next, we quantified the pro-collagen protein secretion by HNDF cells cultured on BGLP5 MCs in a dynamic culture setting using the ELISA assay. Figure 7J shows the results of an ELISA assay measuring the normalized concentration of pro-collagen secretion by HNDF cells cultured on BGLP5 MCs under different conditions: static, 30 RPM, 60 RPM, and 120 RPM stirring rates. The data indicate that pro-collagen secretion increased as the stirring rate increased, with the highest levels observed at 120 RPM. In static conditions, the pro-collagen secretion is relatively low compared to dynamic conditions. At 30 RPM, there is a noticeable increase, with further enhancement at 60 RPM and the highest secretion at 120 RPM. This suggests that mechanical stimulation in dynamic culture systems promoted the secretion of pro-collagen by HNDFs, likely due to the enhanced cellular interaction with the extracellular matrix and improved nutrient diffusion under agitation. Although higher stirring rates led to increased pro-collagen production, it's important to balance this with the potential impact on cell viability and phenotype, as seen in other panels. Nevertheless, these results demonstrated that dynamic conditions, especially at 60 and 120 RPM, significantly improved pro-collagen secretion, which is crucial for applications in tissue regeneration and wound healing where collagen synthesis is vital. Shear stress induced by stirring promotes pro-collagen secretion in fibroblasts by activating mechanotransduction pathways, such as integrin and TGF- β signaling, which upregulate collagen gene expression. The mechanical stimulation improves nutrient diffusion and waste removal, creating a healthier microenvironment that supports enhanced

extracellular matrix production.^[55] Additionally, the cytoskeletal reorganization triggered by shear stress further stimulates collagen secretion as cells adapt to the mechanical forces, mimicking physiological conditions where fibroblasts naturally produce collagen in response to mechanical cues.^[56]

4. Discussion

Our results demonstrate that grafted thermoresponsive polymer brushes on the surface of soft GelMA MCs facilitated cell detachment and reduced the need for enzyme use during epithelial cell manufacturing. This work builds upon and extends the elegant study by Tamura et al.,^[25] who used a grafting-to approach to attach thermoresponsive polymer chains to polystyrene MCs. They demonstrated that a medium brush density was optimal for temperature-mediated detachment of Chinese hamster ovary (CHO-K1) cells. In our study, we observed that when thermoresponsive polymer chains were grafted onto MCs with a surface chemistry distinct from polystyrene, they enhanced cell attachment in a PNIPAM concentration-dependent manner. This enhancement likely stemmed from the combined effects of PNIPAM chains and GelMA chemistry, which might promote the adsorption of fibronectin or other proteins essential for cell adhesion while preserving GelMA's natural cell-attachment properties.

Our approach offers significant advantages, including reduced enzyme consumption and improved cell detachment efficiency from the surface of MCs. Unlike prior studies that relied on large amounts of thermoresponsive polymers to achieve bulk temperature-sensitive properties or concentrated enzymes, our findings indicate that short PNIPAM chains grafted to the MC surface can achieve similar functionality.^[15,22,24,28] This reduces both the synthetic material required and the enzyme concentration needed to achieve nearly 100% cell harvesting efficiency. As a result, BrushGel MCs present a promising solution for Good Manufacturing Practices (GMP)-grade cell manufacturing by lowering costs and minimizing risks associated with synthetic materials and enzyme use.

As cell manufacturing and cell therapy research expands beyond MSCs to include diverse cell types ranging from cosmetic applications to disease modeling there is a growing need for functional MCs that can accommodate different cell attachment and detachment behaviors. We tested our MCs with three cell types (cancer, normal, and stem cells) and found that temperature-induced detachment efficiency slightly decreased as cell types changed from cancer to normal to stem cells. This may be attributed to variations in cell-cell and cell-matrix interactions among the cell types, which could influence the functionality of PNIPAM brush chains.^[57]

While the decoupling enzymes from harvesting of cells during the cell expansion represents a critical step forward in improving cell manufacturing efficiency, further work is necessary to evaluate the function of cell post harvesting in compared with enzyme-treated in primary cells. Different cell types, such as induced pluripotent stem cells (iPSCs)^[58] and patient-derived stem cells, may exhibit distinct behaviors on surface of MCs, as suggested by our findings on cell type dependency. Examining the impact of low-temperature treatment on their stemness, function, and therapeutic index is crucial for translating MCs from the research lab to clinical applications. A promising avenue for

future research involves integrating machine learning tools to design MC properties for the target cell that is needed to be expand, maximizing yield of process. Additionally, scaling this platform for use in large-scale bioreactors, where low-speed continuous agitation and low-temperature detachment can be combined, represents an important goal for advancing cell expansion technologies. In large-scale bioreactors, soft hydrogel-based MCs may be susceptible to shear forces, which could impact shape fidelity and cell viability. Further studies are needed to address these concerns, and strategies such as low-shear impellers or wave bioreactors could be explored to mitigate shear-related damage. Since BrushGel MCs rely on low-temperature treatments for cell harvesting, optimizing uniform temperature transitions will be critical to ensure efficient detachment while minimizing cellular stress in large-scale bioreactors.^[59] Additionally, large-scale fabrication of hydrogel-based MCs must be optimized to ensure batch-to-batch consistency, reproducible surface chemistry, and uniform grafting efficiency, all of which are crucial for industrial applications.

5. Conclusion

This study focused on developing temperature-responsive polymer-grafted hydrogel-based MCs to lower the cost of enzymatic dissociation. By combining low-temperature treatment with diluted enzyme harvesting, the approach successfully reduced enzyme consumption by two orders of magnitude and improved cell harvesting efficiency. The MCs, named BrushGel, were fabricated from GelMA MCs using a flow-focusing microfluidic droplet generator, ensuring uniform size and crosslinking density key factors for consistent cell cultures. This was followed by an EDC-NHS grafting technique in order to immobilize the PNIPAM chains. Surface chemistry adjustments on the MCs influenced cellular behavior, including attachment, gene expression, and detachment, enabling optimized formulations. Results from the TNBS assay demonstrated that the PNIPAM brush density on GelMA surfaces could be fine-tuned through polymer concentration or the DOM of GelMA, resulting in temperature-responsive cell detachment. The optimized MCs achieved higher cell attachment, excellent viability, and temperature-controlled cell release for three cell types, underscoring their potential for efficient cell harvesting. For HNDf cells, no significant differences in viability were observed before and after detachment either with temperature or with diluted enzymes. In a pilot study, we assessed the post-detachment function of MSCs harvested using two approaches: temperature-mediated detachment without enzyme, and low-concentration enzyme treatment combined with cold incubation. The immunomodulatory function of the harvested MSCs was evaluated by their ability to inhibit T cell proliferation. Both groups exhibited immunosuppressive activity within the expected range for donor-derived MSCs, with the low-enzyme group showing higher inhibition. However, due to time constraints, we were unable to conduct more extensive functional analyses. Further studies are needed to fully understand how different harvesting conditions impact MSC functionality. Cell coverage density and varying enzyme concentrations were studied to achieve maximum harvesting efficiency in 60 min incubation at 4 °C. Additionally, these MCs performed well in stirring bioreactors, utilizing minimal

media volumes, making them suitable for the large-scale culture of anchorage-dependent cells. This technology represents a significant advancement by minimizing enzyme usage and providing a safer, more cost-effective method for industrial cell-based therapies and regenerative medicine.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to acknowledge the Mathematics of Information Technology and Complex Systems (Mitacs) Foundation, Replicel Life Sciences, Natural Sciences and Engineering Research Council of Canada (NSERC), Canada Foundation for Innovation (CFI) and BC Knowledge Development Foundation. The authors acknowledge the support of the Advanced Microscopy Facility at the University of Victoria for electron microscopy imaging.

Conflict of Interest

The authors declare no conflict of interest.

CRediT Authorship Contribution Statement

E.A.: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. M.S.B.: Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. A.S.: Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. M.R.: Methodology, Investigation. J.N.: Methodology, Investigation. A.B.P.M.: Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. M.S.: Visualization, Validation, Formal analysis, Data curation. Y.T.: Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. S.H.J.M.: Writing – review & editing, Validation, Supervision, Resources. M.A.: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Use of AI tools

Grammarly and ChatGPT (OpenAI) were used solely to improve grammar and clarity. No scientific content, data analysis, or figure generation involved AI assistance. The authors take full responsibility for the manuscript's content.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

cell expansion, dynamic culture, mesenchymal stem cell, microcarrier, temperature-responsive

Received: December 17, 2024
Revised: May 21, 2025
Published online: July 14, 2025

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