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Modular microporous hydrogels formed from microgel beads with orthogonal thermo-chemical responsivity: Microfluidic fabrication and characterization

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A B S T R A C T

Despite the significant advances in designing injectable bulk hydrogels, the inability to control the pore interconnectivity and decoupling it from the matrix stiffness has tremendously limited the applicability of stiff, flowable hydrogels for 3D cellular engineering, e.g., in hard tissue engineering. To overcome this persistent challenge, here, we introduce a universal method to convert thermosensitive macromolecules with chemically-crosslinkable moieties into annealable building blocks, forming 3D microporous beaded scaffolds in a bottom-up approach. In particular, we show gelatin methacryloyl (GelMA), a widely used biomaterial in tissue engineering, may be converted into physically-crosslinked microbeads using a facile microfluidic approach, followed by flow of the microbead suspension and chemical crosslinking in situ to fabricate microporous beaded GelMA (B-GelMA) scaffolds with interconnected pores, promoting cell functionality and rapid (within minutes) 3D seeding in stiff scaffolds, which are otherwise impossible in the bulk gel counterparts. This novel approach may set the stage for the next generation modular hydrogels with orthogonal porosity and stiffness made up of a broad range of natural and synthetic biomaterials.

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2215-0161/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
• This method combines well-known flow focusing microfluidic devices with facile post-processing steps to fabricate microporous scaffolds.
• Temperature-driven physical crosslinking of the microbeads enables the facile purification of gel building blocks without further chemical reactions.
• This method provides a simple approach to fabricate microporous scaffolds, which overcomes some of the challenges of newly emerging beaded scaffolds, including oxygen-mediated impaired crosslinking.

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 specifications table

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| Name and reference of original method: | A. Sheikhi, J. de Rutte, R. Haghnaz, O. Akouissi, A. Sohrabi, D. Di Carlo, A. Khademhosseini, Microfluidic-enabled bottom-up hydrogels from annealable naturally-derived protein microbeads, Biomaterials. 192 (2019) 560–568. D.R. Griffin, W.M. Weaver, P.O. Scumpia, D. Di Carlo, T. Segura, Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks, Nat. Mater. 14 (2015) 737–744. Anna, S. L., Bontoux, N. & Stone, H. A. Formation of dispersions using ‘flow focusing’ in microchannels. Appl. Phys. Lett. 82, 364–366 (2003). |
| Resource availability: | Raw data are available upon request. |

Method details

Materials and methods

Materials

Silicon wafers (University Wafer, MA, USA), negative photoresist (KMPR 1050, MicroChem Corp., MA, USA), and polydimethylsiloxane (PDMS) base and the curing agent (SYLGARD™ 184 Elastomer Kit, Dow Corning, MI, USA) were used to construct the microfluidic chips. Tygon Flexible Plastic Tubing 0.02” ID x 0.06” OD (Saint-Gobain PPL Corp., CA, USA) and 1569-PEEK Tubing Orange 1/32” OD x .020” ID (IDEX Corp., IL, USA) were used with the microfluidic device. Aquapel® Glass Treatment was purchased from Pittsburgh Glass Works LLC (PA, USA). 3M™ Novec™ 7500 Engineered Fluid (Novec 7500 oil) was provided by 3 M (MN, USA). Type-A gelatin from porcine skin (300 bloom), methacrylic anhydride (MA, 94%), photoinitiator 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959), 1H,1H,2H,2H-Perfluoro-1-octanol 97%, and fluorescein isothiocyanate-dextran solution (500 kDa) were purchased from Sigma-Aldrich (MO, USA). Dialysis membrane with 12–14 kDa molecular weight cutoff (MWCO) was provided by Spectrum Lab Inc (CA, USA). Microscope glass cover slides (25 mm x 75 mm x 1 mm) were from Fisher Scientific (PA, USA), and cover slips (No. 1) were from VWR (PA, USA). Milli-Q water with an electrical resistivity ~ 18.2 MΩ cm at 25 °C was provided by Millipore Corporation. Pico-Surf™ 1 (5% (w/w) in Novec™ 7500) was purchased from Sphere Fluidics Inc (Cambridge, UK). Biopsy punch (diameter ~ 8 mm) was from Integra Miltex.
(NJ, USA). Cyanoacrylate-based adhesive was Krazy glue (Elmer’s Products, NC, USA). Cell-Tak tissue adhesive (Corning, NY, USA), AFM probes (spring constant $\sim 0.01$ N m$^{-1}$) with an affixed polystyrene bead (diameter $\sim 2.5$ μm), mounted on a silicon nitride (SN) cantilever (Novascan, IA, USA) were used for AFM indentation experiments. NIH/3T3 fibroblast cells and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, VA, USA). Fetal bovine serum (FBS), penicillin/streptomycin (P/S), Dulbecco’s modified Eagle medium (DMEM), Dulbecco’s phosphate-buffered saline (DPBS) solution (1X) and powder, trypsin-ethylenediaminetetraacetic acid (EDTA) (0.5%, 10X), and Hank’s Balanced Salt Solution (HBSS, 1X) were provided by Gibco (NY, USA). Endothelial cell growth medium 2 and SupplementMix were from PromoCell (Heidelberg, Germany). PrestoBlue™ cell viability reagent and LIVE/DEAD™ viability/cytotoxicity kit were from Invitrogen by ThermoFisher Scientific (OR, USA). Cell culture flasks (75 cm$^2$, Corning, NY, USA) and polystyrene 6-well tissue culture-treated plates (Falcon, NC, USA) were used to culture cells.

Methods

GelMA synthesis. GelMA with a high degree of methacyryloyl substitution was synthesized according to our previous publications [5,6]. Briefly, DPBS (100 mL) was heated to 50 °C to dissolve the gelatin (10 g). While stirring at 240 rpm, MA (8 mL) was added to the gelatin solution dropwise, resulting in a turbid mixture, which was stirred for 2 h at 50 °C. This condition has been particularly chosen to prevent the hydrolysis of protein [6,7]. To stop the reaction, excessive DPBS was added to the reaction mixture, followed by dialysis using 12–14 kDa molecular weight cutoff (MWCO) membranes for at least seven days at 40 °C to remove methacrylic acid and other impurities. A clear solution was obtained after dialysis, which was lyophilized to yield white solid GelMA.

Bulk GelMA fabrication. Freeze-dried GelMA was dissolved in DPBS, containing the photoinitiator (Irgacure 2959, 0.5% w/v) at 80 °C. The crosslinking setup consisted of a microscope glass cover slide with two stacks of cover slips on both sides to set the sample thickness $\sim 0.3–1$ mm, length $\sim 25$ mm, and width $\sim 10–20$ mm. A desired amount of the solution was pipetted in the gap between the cover slips and covered with a cover slip, followed by UV light (360–480 nm) exposure at an intensity $\sim 10$ mW cm$^{-2}$ (Omnicure, Excilitas, CA, USA) for 1, 2, or 3 min to yield bulk gels from which small samples were cut (for tensile tests) or punched (for compression experiments). Other samples were prepared similarly in smaller sizes for cell culture using the cover glasses and spacers.

Fabrication of the microfluidic device. We have modified a microfluidic water-in-oil emulsion method [1–3] to fabricate uniform-sized microbeads [4]. A flow focusing device was fabricated using soft lithography. Briefly, 4-inch mechanical grade silicon wafers were coated with 80 and 70 μm layers of negative photoresist (KMPR 1050) and patterned in sequence using standard photolithography techniques. PDMS base and the curing agent were mixed at a ratio of 10 to 1, poured onto the molds in petri dishes, degassed, and cured in an oven at 65 °C for $>4$ h. The PDMS device was peeled from the mold and punched with 0.8 mm holes at the inlets and outlets. Devices and glass slides were then activated via air plasma (Plasma Cleaner, Harrick Plasma, NY, USA) and bonded together to enclose the microchannels. The devices were then treated with Aquapel and subsequently washed with Novec 7500 oil to make channel surfaces fluorophilic.

GelMA bead fabrication. GelMA was dissolved in a desired medium, such as DPBS, including the photoinitiator (0.5% w/v, Irgacure 2959) to afford GelMA solutions upon heating at 80 °C for at least 20 min. These solutions were used as the aqueous, dispersed phase in the microfluidic device along with the pinching flows of Novec 7500 oil–surfactant (0.5 wt% PicoSurf) mixture, injected into the flow focusing device using syringe pumps (Harvard Apparatus PHD 2000, MA, USA) to form surfactant-stabilized micron-sized beads of GelMA in the oil (continuous) phase. The syringe and tubing were maintained at 37–40 °C using an electric space heater to prevent sol-gel transition and device blockage. The bead suspension in oil was collected in a microcentrifuge tube and stored at 4 °C overnight to create physically crosslinked GelMA beads.
Fabrication of beaded GelMA (B-GelMA) scaffolds. To prepare the microbeads for annealing, excess oil was removed from the suspension through pipetting, and a 20% perfluorooctanol solution in Novec 7500 oil was added to the bead suspension (1:1 vol ratio) to break down the emulsion and remove the surfactant at 4 °C. DPBS solution (4 °C) including the photoinitiator (0.5% (w/v) Irgacure 2959) was added to the suspension for dilution, and the microbeads were transferred to a separate container via pipetting. The suspension was pulse centrifuged (6300 rpm, 10 s, GmCLab mini centrifuge, Gilson, France) to pack the microbeads, followed by removing the supernatant. The sample temperature was always maintained at 4 °C using a cold-water bath. The concentrated microbead suspension was then pipetted on a microscope glass slide using a positive displacement pipette (MICROMAN® E, Gilson, WI, USA), and sandwiched similar to the bulk gels, followed by UV light exposure at an intensity ~ 10 mW cm$^{-2}$ for 1, 2, or 3 min to create microporous scaffolds composed of covalently crosslinked microbeads.

Pore size measurement. Samples were prepared as previously described using 20% (w/v) GelMA beads exposed to 10 mW cm$^{-2}$ UV light for 1, 2, and 3 min. Scaffolds were incubated in a fluorescein isothiocyanate-dextran solution (15 mM) to visualize the void space in the scaffolds. Confocal images of the scaffolds were taken using a Leica inverted SP5 confocal microscope (Germany) at the California NanoSystems Institute (CNSI). For each sample (3 per condition), 77 z-slices were captured, spanning a total distance of 100 μm. Void fraction and pore diameter were analyzed using a custom-developed Matlab code (Matlab, version 2016b). Briefly, adaptive thresholding was used to convert stacked images into discrete regions, and void fraction was calculated based on voxel volume of void space regions. Average pore diameter was calculated as previously described [8].

Mechanical analyses. For compression tests, GelMA samples were exposed to 10 mW cm$^{-2}$ UV light for 1, 2, or 3 min, punched in disks with diameter ~ 8 mm and height ~ 1 mm. The gels were removed from the cover glass and incubated in DPBS for 1 h at room temperature. Compression tests were conducted using an Instron mechanical tester (Instron 5542, Norwood, MA, USA) at a rate ~ 1 mm min$^{-1}$. The best fit to the linear stress-strain region at 0–10% strain was used to calculate the compression modulus (stress/strain). For tensile tests, crosslinked samples were cut (10 mm × 15 mm × 1 mm), glued to two pieces of paper from each end using Krazy glue and transferred to the Instron tensile test grips to perform the experiment at a rate ~ 10 mm min$^{-1}$. The slope of linear stress-strain region at strain <10% provided the tensile modulus.

Atomic force microscopy (AFM) indentation. Young’s modulus of microparticles and their corresponding bulk gels were measured using the BioScope Catalyst AFM (Bruker, CA, USA), equipped with the Zeiss LSM 5 confocal microscope (Germany). Samples were mounted on glass slides using Cell-Tak tissue adhesive, submerged in DPBS for measurements, and visualized using a Zeiss LSM 5 confocal microscope. Measurements were carried out through ~ 100 nm indentation of the sample surface. Compression moduli were calculated based on the Hertz contact mechanical model (suitable for spherical probes) [9] in the NanoScope Analysis software (version 1.8, Bruker). For the B-GelMA, 3 samples were analyzed (5 beads per sample, characterized 5 times each, total measurements per sample = 25), for the GelMA, 3 sample were analyzed (5 spots per sample, characterized 5 times each, total measurements per sample = 25).

Rheological analyses. Oscillatory shear rheology was conducted to characterize the rheological properties of the gels prepared in various forms using an MCR 302 Rheometer (Anton Paar, Graz, Austria). A parallel plate geometry (8 mm with a sandblasted measuring plate, PP08/S) was used to load the samples, following equilibration at room temperature. To register the viscoelastic moduli, oscillatory frequency sweep was performed at 0.1–100 rad s$^{-1}$ under a small oscillatory strain ~0.1% for all samples at 25 °C in the linear viscoelastic region. The gels were maintained hydrated during the experiments (total time ~ 20 min) in an enclosed chamber. The viscoelastic moduli versus angular frequency were registered.
Swelling analysis. The forced swelling of non-annealed GelMA beads (crosslinked for 120 s through the UV exposure at 10 mW cm\(^{-2}\)) was investigated by diluting DPBS (1X)-loaded beads with Milli-Q water (1:1 vol ratio) at 37°C. The shrinkage was conducted similarly by adding DPBS (5X). Similar studies were conducted with the physically-crosslinked beads at 4°C. The beads were imaged by brightfield microscopy at predefined time intervals, and their size was measured by analyzing the images using ImageJ (Version 1.52e, National Institute of Health, USA).

In vitro characterization of cellular function. This section includes cell culture, 3D cell encapsulation, 3D cell seeding, metabolic activity assessment, and live/dead assay.

Cell culture. A standard cell culture incubator (Thermo Fisher Scientific, PA, USA) was used to culture cells in the cell culture flasks under a 5% CO\(_2\) atmosphere at 37°C. NIH/3T3 fibroblasts were cultured in DMEM, supplemented with 10% FBS and 1% P/S, typically passaged twice a week. HUVECs were cultured in the endothelial growth medium 2, mixed with SupplementMix and 1% P/S. Every 2–3 days, the media was exchanged for both cell lines. To conduct in vitro characterization of cellular function, fibroblast cells and HUVECs were trypsinized using 0.5% trypsin-EDTA, followed by counting using a hemocytometer and resuspension either in the media (for 3D cell seeding tests), pre-gel solution (for bulk encapsulation), or physically-crosslinked bead suspension (for encapsulation in B-GelMA).

3D cell encapsulation. Cell-laden GelMA and B-GelMA samples were prepared by mixing 20 μL of NIH/3T3 fibroblast cell suspension (cell density \(\sim 1 \times 10^6\) in 1 mL DMEM) with 80 μL of GelMA (final concentration \(\sim 20\%\) (w/v)) and concentrated bead suspension (\(\sim 45 \times 10^4\) of beads per mL of DPBS + PI), respectively, followed by pipetting the mixture on a glass slide between two spacers to form a disc (height \(\sim 0.3\) mm, diameter \(\sim 10\) mm), flattened by a cover glass, and crosslinked using UV light exposure at 10 mW cm\(^{-2}\) for 2 min (2 \(\times 10^5\) cells per sample). The casted gels were gently washed with warm DPBS (37°C) to remove the unreacted materials, transferred to 6-well plates, and cultured in 2 mL media for up to 14 days with intermittent media exchange every 2 days.

3D cell seeding. HUVEC seeding inside the gel samples (discs with height \(\sim 0.3\) mm, diameter \(\sim 10\) mm) was assessed by gently pipetting 50 μL of the cell suspension (cell density \(\sim 1 \times 10^6\) in 1 mL media) on top of the crosslinked gels in a petri dish, immediately stained with the live/dead assay, and imaged. The infiltrated cells inside the gel samples were imaged at various heights using confocal microscopy.

Metabolic activity assessment. PrestoBlue \(\oplus\) assay was used to assess the cellular metabolic activity on days 1, 3, 7, and 14 based on the manufacturer’s protocol. The outcome (fluorescence intensity) was registered using a microplate reader (excitation \(\sim 530\) nm and emission \(\sim 590\) nm, BioTek UV/vis Synergy 2, VT, USA) and corrected with respect to the background signal of the PrestoBlue \(\oplus\)-containing cell-free media.

Live/Dead assay. A live/dead fluorescence assay was used to assess the cell viability within the hydrogel samples. Briefly, the hydrogels were incubated for 20 min with 1 mL of the staining solution, prepared by adding ethidium homodimer-1 (20 μL) and calcein AM (5 μL) to DPBS (10 mL), and imaged using fluorescence microscope (Axio Observer 5, Zeiss, Germany) at excitation/emission wavelengths \(\sim 494/515\) nm for calcein and 528/617 nm for ethidium homodimer-1.

Statistical analysis. Measurements were conducted at least in triplicate. The data were reported as mean values ± standard deviation. The one-way analysis of variance (ANOVA) was carried out followed by Tukey’s multiple comparisons, and statistically significant differences were identified when p-values were lower than 0.05 (\(^*p < 0.05\)), 0.01 (\(^{**}p < 0.01\)), 0.001 (\(^{***}p < 0.001\)), and 0.0001 (\(^{****}p < 0.0001\)).
**Method validation.** Physical and biological properties of fabricated beaded hydrogels were thoroughly characterized [10]. Furthermore, the results were compared to the bulk hydrogel counterparts [10].

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