Supporting Information

Individual cell-only bioink and photocurable supporting medium for 3D printing and generation of engineered tissues with complex geometries

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Materials and Methods

Synthesis of OMA

Oxidized alginate (OA) was prepared by reacting sodium alginate with sodium periodate.[1] Sodium alginate (10 g, Protanal LF 200S, FMC Biopolymer) was dissolved in ultrapure deionized water (diH_2O, 900 ml) overnight. Sodium periodate (0.1 g, Sigma) was dissolved in 100 ml diH_2O, added to alginate solution under stirring to achieve 1 % theoretical alginate oxidation, and allowed to react in the dark at room temperature for 24 hrs. Methacrylation (20% theoretical) was performed to obtain oxidized, methacrylated alginate (1OX20MA OMA) macromer by reacting OA with 2-aminoethyl methacrylate (AEMA). To synthesize OMA, 2-morpholinoethanesulfonic acid (MES, 19.52 g, Sigma) and NaCl (17.53 g) were directly added to an OA solution (1 L) and the pH was adjusted to 6.5. N-hydroxysuccinimide (NHS, 1.176 g, Sigma) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 3.888 g, Sigma) were added to the mixture under stirring to activate 20 % of the carboxylic acid groups of the alginate. After 5 min, AEMA (1.688 g, Polysciences) (molar ratio of NHS:EDC:AEMA = 1:2:1) was added to the solution, and the reaction was maintained in the dark at RT for 24 hrs. The reaction mixture was precipitated into excess of acetone, dried in a fume hood, and rehydrated to a 1 % w/v solution in diH_2O for further purification. The OMA was purified by dialysis against diH_2O using a dialysis membrane (MWCO 3500, Spectrum Laboratories Inc.) for 3 days, treated with activated charcoal (5 g/L, 50-200 mesh, Fisher) for 30 min, filtered (0.22 μm filter) and lyophilized. To determine the levels of alginate methacrylation, the OMA was dissolved in deuterium oxide (D_2O, 2 w/v %), and ^{1}H-NMR spectra were recorded on a Varian Unity-300 (300MHz) NMR spectrometer (Varian Inc.) using 3-(trimethylsilyl)propionic acid-d_4 sodium salt (0.05 w/v %) as an internal standard. To prepare the OMAs with 2 (2OX20MA), 5 (5OX20MA) and 10 (10OX20MA) % theoretical degrees of oxidation, 0.2, 0.5 and 1.0 g of sodium periodate solution (100 ml) were added to separate alginate solutions, and then the methacrylation reaction was performed as described above.

Fabrication of OMA microgel slurry

To fabricate smaller OMA microgels, OMA (1.5 g) was dissolved in low-glucose Dulbecco's Modified Eagle's Medium (DMEM-LG,100 ml) containing 0.05 % photoinitiator (PI, 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, 0.05 w/v %, Sigma), placed in a syringe, and then dispensed into a gelling bath containing an aqueous solution of $CaCl_2$ (1 L, 0.2 M) bath. After fully ionically crossliking the OMA fibers in the bath for 30 min, the resultant OMA fibers were collected, washed with DMEM-LG three times, and then blended using a consumer-grade blender (Osterizer MFG, at "pulse" speed) for 90 sec with 100 ml DMEM-LG. Then, the blended OMA slurry was loaded into 50 ml conical tubes and centrifuged at 2000×g for 5 min. The supernatant was removed and replaced with a sterile 70 % ethanol. The slurry was vortexed back into suspension and centrifuged again. After the supernatant was removed, the OMA microgel slurry was vortexed with sterile 70 % ethanol and then stored until use at 4 °C. To fabricate larger sized OMA micogels, OMA solution was loaded into a 3-ml syringe, and then the syringe was connected to a custom coaxial microdroplet generator designed in our laboratory (Fig. S10 in ESI†). The OMA solution was pumped at 0.5 ml/sec with an outer air flow rate of 10 L/min, and the droplets dripped into a collection bath containing an aqueous solution of $CaCl_2$ (0.2 M). After fully ionically crosslinking the microgels in the bath for 30 min, the resultant OMA microgels were collected and washed with DMEM-LG three times. The OMA microgels were suspened in sterile 70 % ethanol and stored until use at 4 °C.

To evaluate the morphology and measure the size of OMA microgels comprising the slurries, the slurries were centrifuged at 2000×g for 5 min. The supernatants were removed and replaced with DMEM-LG containing 0.05 % PI, and the microgels were vortexed back into suspension and then centrifuged again. This process was repeated five times and then the supernatants were removed. To visualize the OMA microgels, they were stained with Safranin O and then imaged using a microscope (Leitz Laborlus S, Leica) equipped with a digital camera (Coolpix 995, Nikon). To measure the mean diameter of the smaller OMA microgels (prepared using a blender), 1 ml of the OMA microgels were suspended in 10 ml DMEM-LG containing 0.05 % PI and measured at room temperature by dynamic light scattering using a particle size analyzer (90Plus, Brookhaven Instruments). The mean diameter of the larger OMA microgels (prepared via the coaxial microdroplet generator) was measured using ImageJ with the images of Safranin O stained OMA microgels.

Rheological properties of OMA microgel slurry

Dynamic rheological examination of the OMA microgel slurries was performed to evaluate shear-thinning, self-healing and mechanical properties with a Haake MARS III rotational rheometer (ThermoFisher Scientific). In oscillatory mode, a parallel plate (80 mm diameter) geometry measuring system was employed, and the gap was set to 1 mm. After each OMA microgel slurry was placed between the plates, all the tests were started at 37 ± 0.1 °C, and the plate tempearature was maintained at 37 °C. Oscillatory frequency sweep (0.01-1.3 Hz at 1 % strain) tests were performed to measure storage moduli

(G'), loss moduli (G") and viscosity. Oscillatory strain sweep (0.1-100 % strain at 1 Hz) tests were performed to show the shear-thinning characteristics of the OMA microgels and to determine the shear-yielding points at which the OMA microgel slurries behave fluid-like. To demonstrate the self-healing properties of OMA microgel slurries, cyclic deformation tests were performed at 100 % strain with recovery at 1 % strain, each for 1 min at 1 Hz.

Degradation of OMA microgel slurry

Smaller OMA microgel slurries with OMAs of various degrees of oxidation were prepared as described above. To photocrosslink the OMA slurries, the slurries were centrifuged at 2000×g for 5 min. The supernatants were removed and replaced with DMEM-LG containing 0.05 % PI, and the microgels were vortexed back into suspension and then centrifuged again. This process was repeated five times and then the supernatants were removed. The OMA microgel slurries were photocrosslinked under UV at 20 mW/cm^2 for 1 min and lyophilized. After dry weights were measured (W_i), dried microgel slurry samples were immersed in 10 ml of DMEM-LG and incubated at 37°C, and DMEM-LG was replaced every week. At predetermined time points, the DMEM-LG was removed and the samples were lyophilized and weighted (W_d). The percent mass loss was calculated by (Wi-Wd)/Wi × 100 (N=3 for each time point).

Preparation of cell-only bioinks

To isolate human bone marrow-derived mesenchymal stem cells (hMSCs)[2], bone marrow aspirates were obtained from the posterior iliac crest of a healthy twenty seven-year old male donor under a protocol approved by the University Hospitals of Cleveland Institutional Review Board. The aspirates were washed with growth medium comprised of DMEM-LG (Sigma) with 10 % prescreened fetal bovine serum (FBS, Gibco). Mononuclear cells were isolated by centrifugation in a Percoll (Sigma) density gradient and the isolated cells were plated at 1.8 x 10^5 cells/cm^2 in DMEM-LG containing 10 % FBS and 1 % penicillin/streptomycin (P/S, Thermo Fisher Scientific) in an incubator at 37 °C and 5 % CO_2. After 4 days of incubation, non-adherent cells were removed and adherent cell were maintained in DMEM-LG containing 10 % FBS, 1 % P/S and 10 ng/ml FGF-2 with media changes every 3 days. After 14 days of culture, the cells were passaged at a density of 5×10^3 cells/cm^2, cultured for an additional 14 days, and then stored in cryopreservation media in liquid nitrogen until use. To use hMSC as a bioink, hMSCs were expanded in growth media consisting of DMEM-LG with 10% FBS (Sigma), 1 % P/S and 10 ng/ml FGF-2. hMSCs (passage 3) were harvested with trypsin/EDTA (Thermo Fisher) and concentrated by centrifugation at 300 x g for 5 min. Following aspiration of the supernatant, pelleted hMSCs were loaded into a 2.5-ml syringe (Gastight Syringe, Hamilton Company) to use as a cell-only bioink.

Human adipose-derived stem cells (hASCs) were isolated from adipose tissue using a previously reported method.[3] Briefly, lipoaspirates were digested with 200 unit/mg collagenase type I (Worthington Biochemical Products) for 40 min at 37 °C. The stromal fraction was then isolated by density centrifugation and the stromal cells were plated at 3500 cell/cm^2 on tissue culture plastic in DMEM/F12 (Thermo fisher Scientific) with 10 % FBS and 1% P/S in an incubator at 37 °C and 5 % CO_2. hASCs (passage 3) were harvested with trypsin/EDTA and concentrated by centrifugation at 300 x g for 5 min. Following aspiration of the supernatant, pelleted hASCs were loaded into a 2.5-ml syringe to use as a cell-only bioink.

Human dermal fibroblasts (hDF, ATCC) were expanded in high-glucose DMEM (HG-DMEM, Sigma) with 10 % FBS and 1% P/S in an incubator at 37 °C and 5 % CO_2. hDFs were harvested with trypsin/EDTA and concentrated by centrifugation a 300×g for 5 min. Following aspiration of the supernatant, pelleted hDFs were loaded into a 2.5-ml syringe to use as a cell-only bioink.

To form hMSC aggregates, agarose microwells (2 w/v %, Denville Scientific Inc.) were casted using polydimethylsiloxane negative microwells, which were generously provided by Professor Kent Leach at the University of California, Davis, based on a previously described method.[4] hMSC suspensions (2000 cells/well) were added to the agarose microwells, centrifuged at 300 x g for 5 min, and cultured in an incubator at 37 °C and 5 % CO_2 for 48 hours. After harvesting the hMSC aggregates by gentle pipetting, the MSC aggregates centrifuged at 300 x g for 5 min. Upon removal of the supernatant, hMSC aggregates were loaded into a 2.5-ml syringe for 3D bioprinting.

Modification of 3D printer

hMSC, hASC and hMSC aggregate-only bioink 3D printing was performed using a 3D printer (PrintrBot Simple Metal 3D Printer, Printrbot) modified with a syringe-based extruder[5], and the hDF-only bioink was 3D printed using a commercial extrusion-based 3D bioprinter (Biobot Basic, Advanced Solutions Life Sciences). The stock thermoplastic extruder assembly was replaced with a custom-built syringe pump extruder. The syringe pump extruder was designed to use the NEMA-17 stepper motor from the original Printrbot thermoplastic extruder and mount directly in place of the extruder on the *x*-axis carriage. The syringe pump extruder was printed with polylactic acid using the thermoplastic extruder on the Printrbot before its removal. Using the same stepper motor, the syringe pump extruder was natively supported by the software that came with the printer. The design for the syringe pump extruder and the image file of the human femur were downloaded as STL files from the NIH 3D Print Exchange (http://3dprint.nih.gov) under open-source license. Digital image files of letters for 3D printing were generated from www.tinkercad.com. The file for the human ear was downloaded from www.thinkiver.com/thing:304657 under the terms of the Creative Commons Attribution license, which permits unrestricted use, reproduction and distribution in any medium.

3D printing of cell-only bioinks

Individual hMSC-only, individual hASC-only, or individual hMSC aggregate-only bioinks were loaded into syringes, connected to 0.5-inch 22G stainless steel needles (McMaster-Carr) and mounted into the syringe pump extruder on the 3D printer. A petri dish was filled with OMA microgel slurry at room temperature to serve as a supporting bath and placed on the building platform. The tip of each needle was positioned at the center and near the bottom of the dish, and the print instructions were sent to the printer using the host software (Cura Software, Ultimaker), which is an open source 3D printer host software. An hDF-loaded syringe was connected to a 0.5-inch 25 G stainless needle and mounted onto a commercial extrusion-based 3D bioprinter (Biobot Basic, Advanced Solutions Life Sciences), and cells were printed using TSIM host software (Advanced Solutions Life Sciences). After 3D printing of the cell-only bioinks, OMA microgel supporting mediums with 3D printed constructs were stabilized by photocrosslinking under UV at 20 mW/cm^2 for 1 min. After slurry photocrosslinking, 3D printed hMSC constructs in the photocrosslinked OMA microgel slurry were transferred into 6-well tissue culture plates with growth media, chondrogenic differentiation media or osteogenic differentiation media, and placed in a humidified incubator at 37 °C with 5 % CO_2. 3D printed hMSC aggregate constructs in the photocrosslinked microgel slurry were transferred into 6-well tissue culture plates and cultured in chondrogenic differentiation media.

Analysis of printed hMSC structures

Linear hMSC filaments were printed in the OMA microgel supporting baths with 22, 25 and 27 G needles, baths were photocrosslinked under UV light a 20 mW/cm^2 for 1 min, and then 5 ml culture media was added. The viability and morphology of 3D printed hMSC filaments were investigated using a Live/Dead staining comprised of fluorescein diacetate [FDA, 1.5 mg/ml in dimethyl sulfoxide (Research Organic Inc.), Sigma] and ethidium bromide (EB, 1 mg/ml in PBS, Thermo Fisher Scientific). The staining solution was freshly prepared by mixing 1 ml FDA solution and 0.5 ml EB solution with 0.3 ml PBS (pH 8). 100 µl of staining solution was added into each well and incubated for 10 min at room temperature, and then stained 3D printed hMSC filaments were imaged using a fluorescence microscope (ECLIPSE TE 300) equipped with a digital camera (Retiga-SRV). Diameters of the 3D printed hMSC filaments were measured at least 400 times for each group using ImageJ (National Institutes of Health)

Osteogenic and chondrogenic differentiation of the 3D printed hMSC constructs

3D printed hMSC constructs in the photocrosslinked OMA microgel supporting baths were differentiated by culture with osteogenic differentiation media [10 mM β-glycerophosphate (CalBiochem), 37.5 µg/ml ascorbic acid (Wako USA), 100 nM dexamethasone (MP Biomedicals), and 100 ng/ml BMP-2 in HG-DMEM] containing 10 % FBS and 1% P/S or chondrogenic differentiation media [1 % ITS+ Premix, 100 nM dexamethasone, 37.5 µg/ml l-ascorbic acid-2-phosphate, 1 mM

sodium pyruvate, 100 μM nonessential amino acids, and 10 ng/ml TGF-β_1 in HG-DMEM]. The osteogenic and chondrogenic media was changed twice a week. After 4 weeks of culture in osteogenic differentiation media, 3D printed hMSC constructs were harvested by simple agitation, fixed in 10 % neutral buffered formalin overnight at 4 °C and stained with Alizarin red S. Cryosectioned samples were also stained with Alizarin red S. After 3 weeks of culture in chondrogenic differentiation media and harvest by simple agitation, the viability of a 3D printed hMSC construct was investigated using a Live/Dead assay as described above. After other samples were again harvested by simple agitation, they were then fixed in 10 % neutral buffered formalin over night at 4 °C and stained with Toluidine blue O. Cryosectioned samples were also stained with Toluidine blue O. For quantification of alkaline phosphatase (ALP) activity, DNA content and calcium deposition, osteogenically differentiated 3D printed hMSC constructs were homogenized at 35,000 rpm for 30 s using a TH homogenizer (Omni International) in 1ml ALP lysis buffer (CelLytic™ M, Sigma). The homogenized solutions were centrifuged at 500 g with a Sorvall Legent RT Plus Centrifuge (Thermo Fisher Scientific). For ALP activity measurements, supernatant (100 μl) was treated with p-nitrophenylphosphate ALP substrate (pNPP, 100 μl, Sigma) at 37 °C for 30 min, and then 0.1 N NaOH (50 μl) was added to stop the reaction. The absorbance was measured at 405 nm using a plate reader (FMAX, Molecular Devices) (N=4). A standard curve was made using the known concentrations of 4-nitrophenol (Sigma). DNA content in supernatant (100 μl) was measured using a Quant-iT PicoGreen assay kit (Invitrogen) according to the manufacturer's instructions. Fluorescence intensity of the dye-conjugated DNA solution was measured using a plate reader (FMAX) set at 485 nm excitation and 538 nm emission (N=4). After an equal volume of 1.2 N HCl was added into each lysate solution, the mixed solutions were centrifuged at 500 g with a Sorvall Legent RT Plus Centrifuge. Calcium deposition of the constructs was quantified using a calcium assay kit (Pointe Scientific) according to the manufacturer's instructions. Supernatant (4 μl) was mixed with a color and buffer reagent mixture (250 μl) and the absorbance was read at 570 nm on a plate reader (FMAX, N=4). All ALP activity and calcium deposition measurements were normalized to DNA content. To measure GAG production, chondrogenically differentiated 3D printed hMSC constructs were digested in papain buffer (1 mL, pH 6.5)) containing papain (25 μg m/l, Sigma), l-cysteine (2×10^{-3}M, Sigma), sodium phosphate (50×10^{-3}M) and EDTA (2×10^{-3}M) at 65 °C overnight. GAG content (N=4) was quantified by a dimethylmethylene blue assay and DNA content (N=4) was measured using the PicoGreen assay as described above. GAG content was also normalized to DNA content. Quantitative data were expressed as mean ± standard deviation. Statistical analysis was performed with unpaired Student t-test using Graphpad Prism (GraphPad). A value of $p<0.05$ was considered statically significant.

Supplementary figures

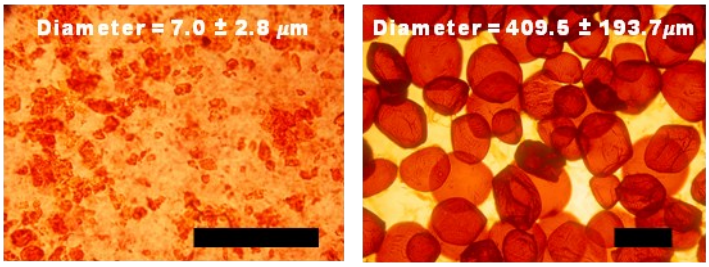

Fig. S1. Photomicrographs of Safranin-O stained smaller (left) and larger (right) OMA microgels. Scale bars indicate 200 μm.

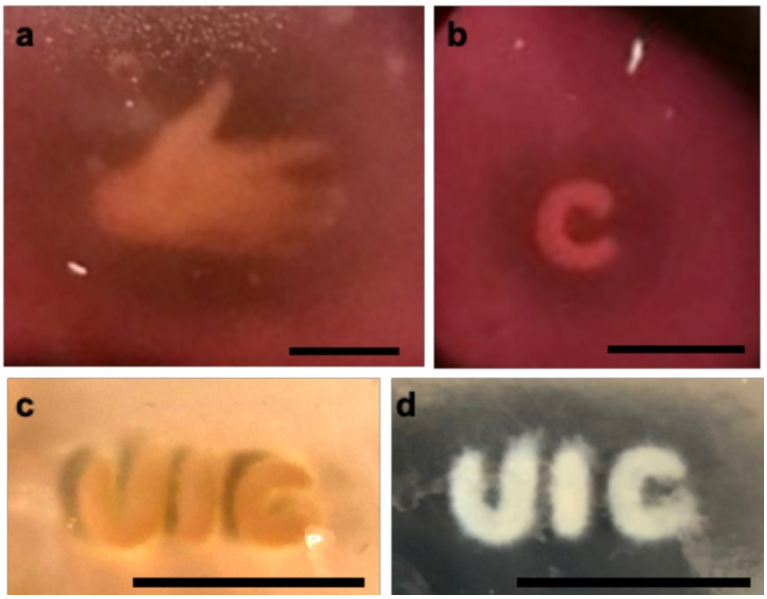

Fig. S2. Optical mages of 3D bioprinted structures of **(a)** a hand and **(b)** a letter "C" using individual cell-only hASC bioink and letters comprising the acronym "UIC" using individual cell-only human dermal fibroblast bioink in alginate microgel supporting medium **(c)** before and **(d)** after photocrosslinking. Scale bars indicate 5 mm.

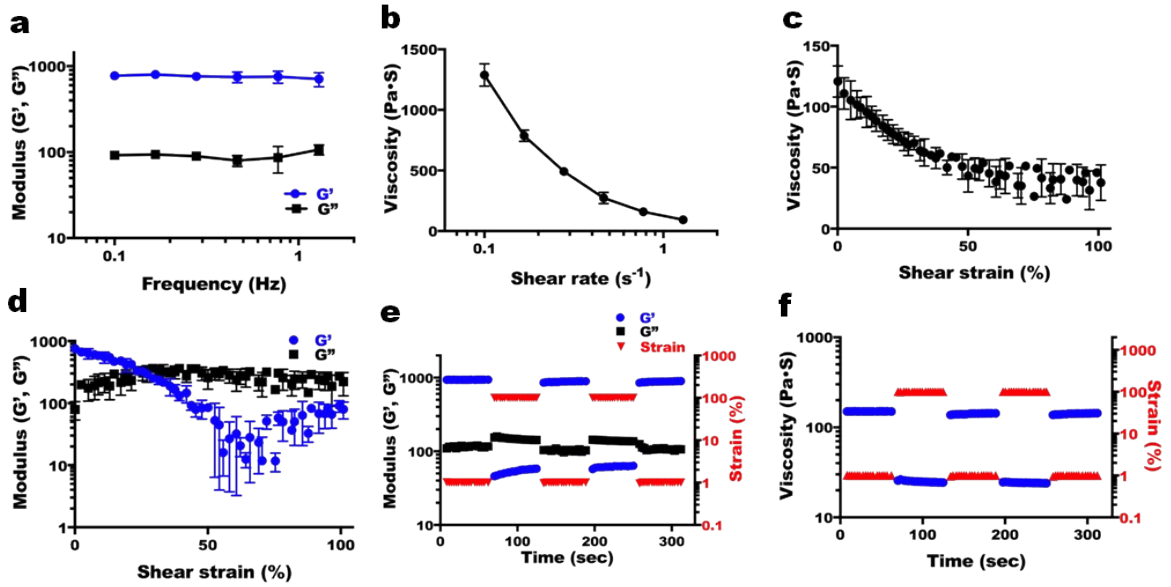

Fig. S3. (a) Storage (G') and loss (G") moduli of alginate microgel supporting medium (mean microgel diameter = 409.6 ± 193.7 μm) as a function of frequency. G' is larger than G" over the measured frequency range and both moduli exhibit frequency independence. Viscosity measurements of alginate microgel supporting medium as a function of **(b)** shear rate and **(c)** shear strain demonstrate its shear-thinning behavior. **(d)** G' and G" of the alginate microgel supporting medium as a function of shear strain exhibit its shear-yielding behavior and gel-to-sol transition at higher shear strain. **(e)** Shear moduli and **(f)** viscosity changes in dynamic strain tests of the alginate microgel supporting medium with alternating low (1%) and high (100%) strains at 1 Hz demonstrate its rapid recovery of strength and viscosity within seconds, which indicates "self-healing" or thixotropic properties.

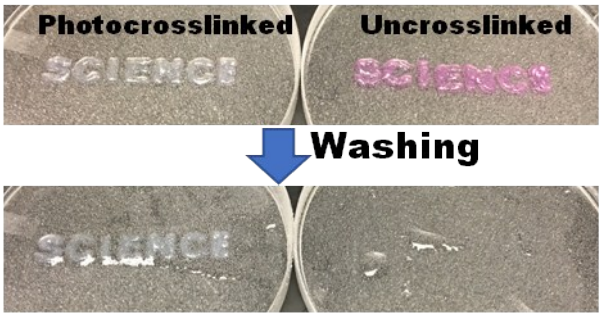

Fig. S4. Optical images of photocrosslinked and uncrosslinked microgels before and after washing process.

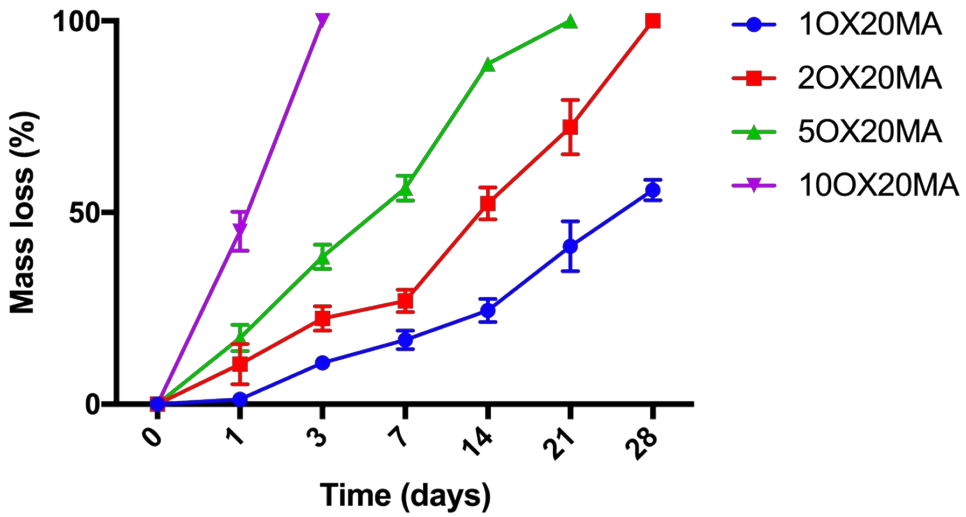

Fig. S5. Degradation profiles of the photocrosslinked microgel slurry baths.

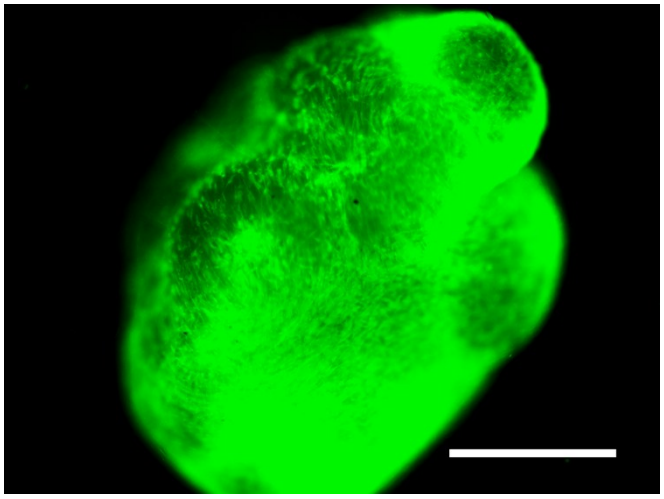

Fig. S6. Live/Dead staining of 3D printed construct using individual hMSC cell-only bioink after 3-week chondrogenic differentiation. The scale bar indicates 400 μm.

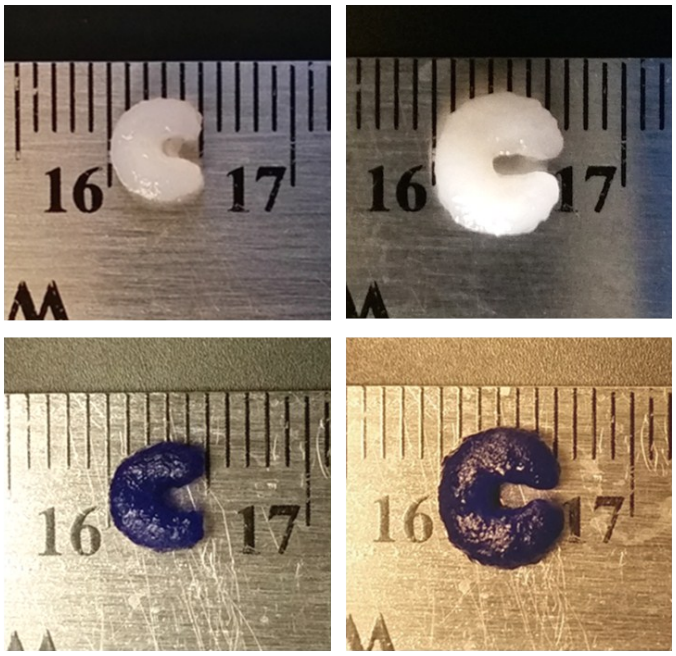

Fig. S7. Optical images of 3D printed chondrogenically differentiated hMSC constructs formed after 3 weeks using individual cell-only bioink before (top) and after (bottom) Toluidine blue O staining (letter "C").

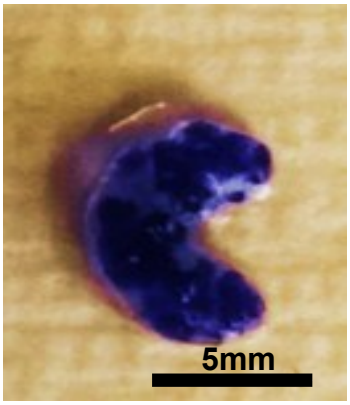

Fig. S8. Optical image of 3D printed hMSC aggregates that fused to form a tissue construct (letter "C") while being chondrogenically differentiated over the course of 3 weeks (Toluidine blue O staining).

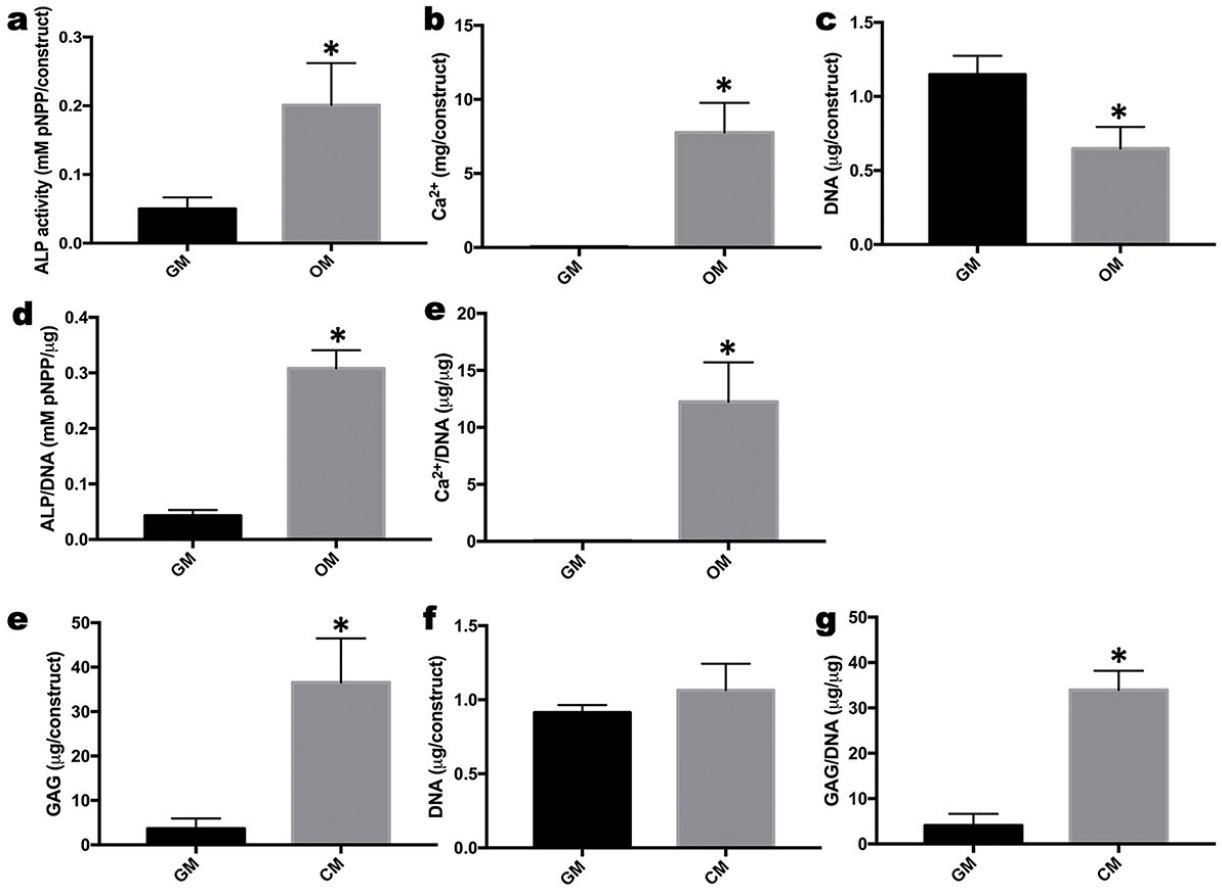

Fig. S9. Quantification of (a) ALP activity, (b) Ca^{2+} and (c) DNA content in the 3D printed hMSC constructs cultured in growth media (GM) and osteogenic media (OM) for 4 weeks. (d) ALP activity and (e) Ca^{2+} normalized by DNA. Quantification of (e) GAG production and (f) DNA content in the 3D printed hMSC constructs cultured in growth media (GM) and chondrogenic media (CM) for 3 weeks. (g) GAG content normalized by DNA. *p<0.05 compared to GM.

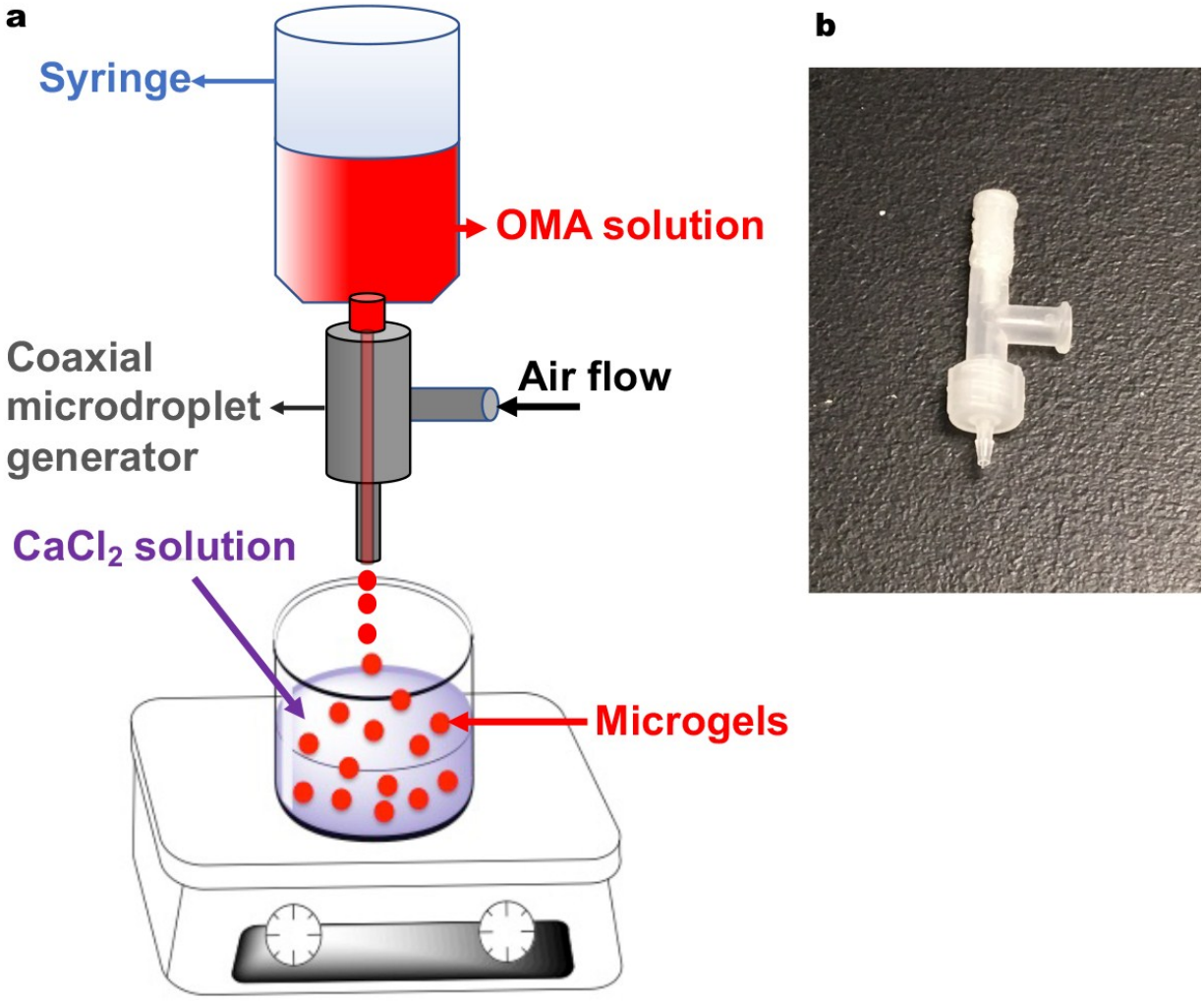

Fig. S10. (a) Schematic diagram of coaxial airflow-induced microgel generating system and (b) a photograph of the custom coaxial airflow-induced microdroplet generator.

Movie S1. Bioprinting of the letter "C" using a living stem cell-only bioink into an alginate microgel supporting medium.

Movie S2. Bioprinting of an ear using a living stem cell-only bioink into an alginate microgel supporting medium.

Movie S3. The printing needle can freely move within an alginate microgel supporting medium without creating crevasses.

Reference

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