ADVANCED MATERIALS

Supporting Information

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Buoyancy-Driven Gradients for Biomaterial Fabrication and Tissue Engineering

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Supplementary Information

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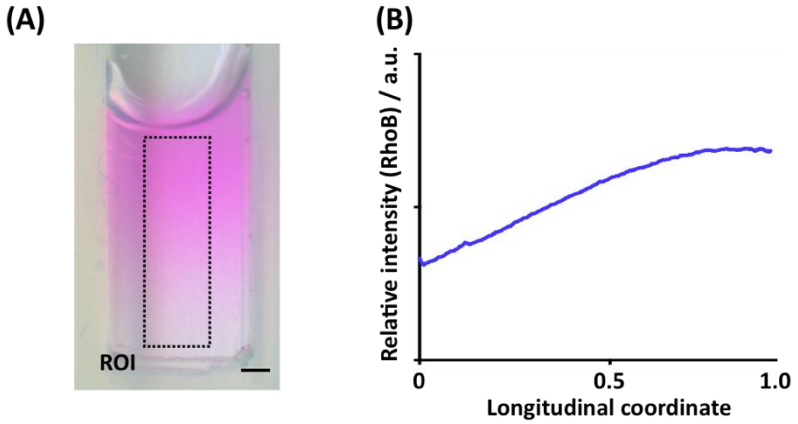
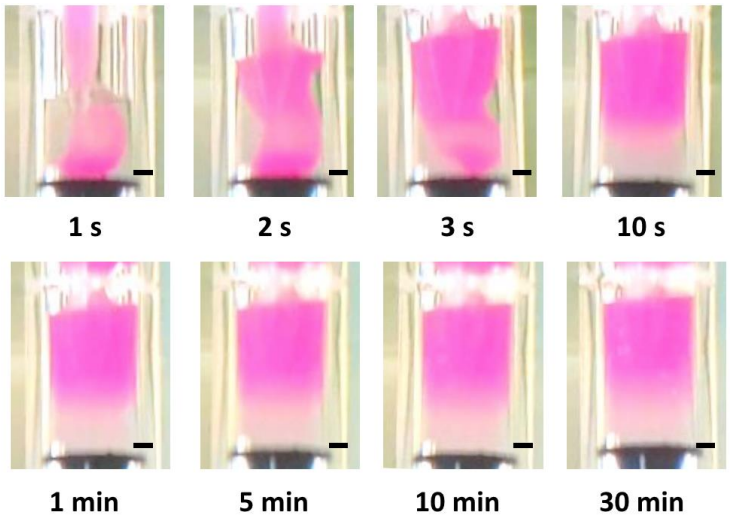
Supplementary Figures



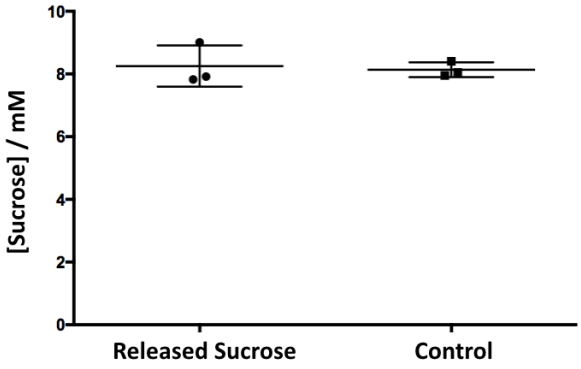
Supplementary Figure 1.

Example of buoyancy-driven gradient formation. 0.2% (w/v) agarose labelled with rhodamine B and 1% (w/v) agarose were used for the injection phase and base layer, respectively, with an injection rate of 17 µL s^{-1}. (A) Scan of a hydrogel showing a clear gradient of agarose labelled with rhodamine B (pink). Scale bar = 1 mm. (B) The relative intensity of rhodamine B signal along the hydrogel confirmed that a smooth gradient was formed under these conditions.



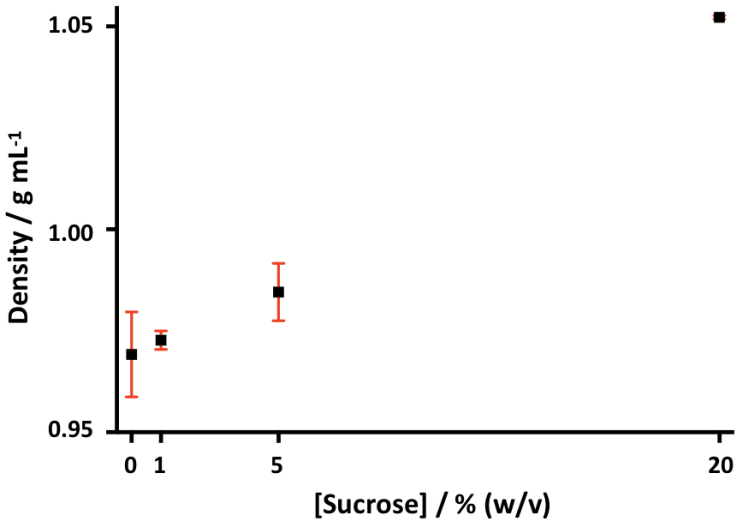
Supplementary Figure 2.

The rate of gradient formation and post-injection stability. The gradient formation process was demonstrated using 1% (w/v) agarose supplemented with 5% (w/v) sucrose as the static phase and 1% (w/v) agarose labelled with Rhodamine B as the injected phase, with an injection rate of 20 µL s^{-1} at 37 °C. The gradient formation process in liquid agarose was completed within 1 min, with the gradient retained for 30 min without gelation. For a video of this process, see Supplementary Video 1. Scale bars = 1 mm.



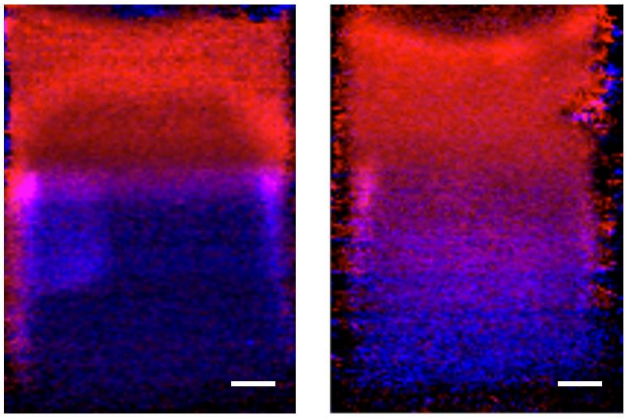
Supplementary Figure 3.

Sucrose release. A sucrose assay was used to calculate the amount of sucrose released from a 1% (w/v) agarose hydrogel, after immersion in PBS for 72 h. The measured value was similar to a control of PBS containing the initial loading concentration of sucrose. (n = 3, mean ± S.D.).



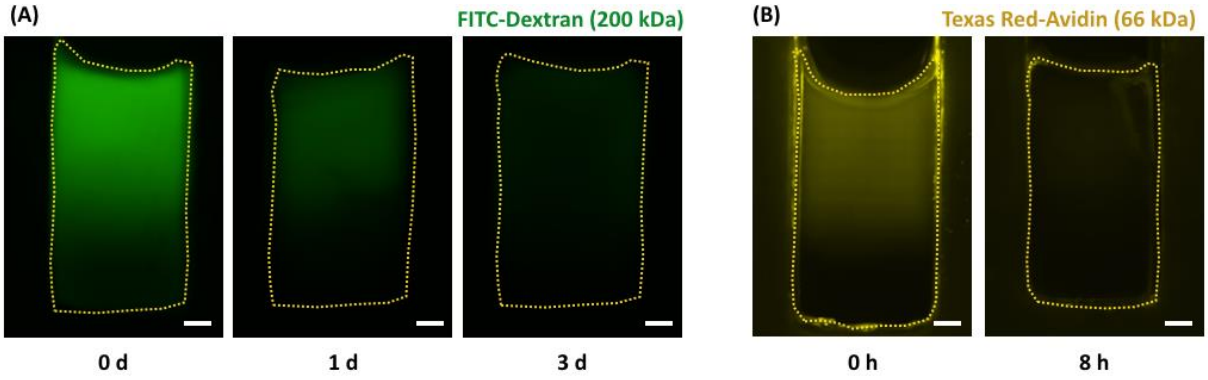
Supplementary Figure 4.

Density measurements. The density of 1% (w/v) agarose doped with different quantities of sucrose was measured. The mixtures were equilibrated at 37 °C and then the mass of a known volume was measured. (n = 3, mean ± S.D.).



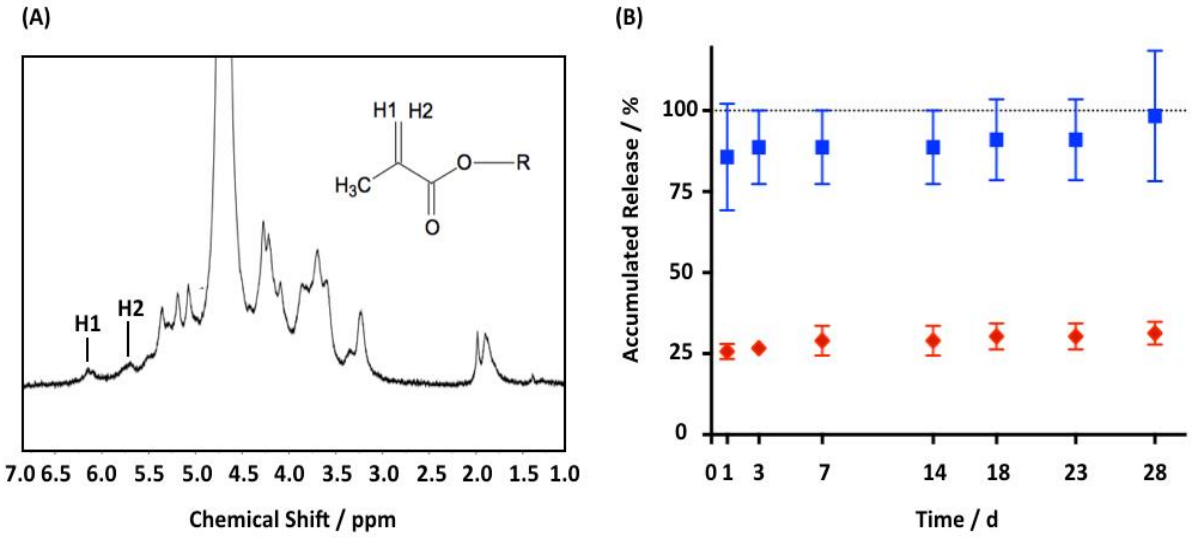
Supplementary Figure 5.

Raman spectroscopy of polymer gradient. *N*,*N*-dimethylacrylamide (ρ = 0.96 g mL^{-1}) and lauryl methacrylate (ρ = 0.87 g mL^{-1}) were used as the base and injection phases, respectively. Raman spectroscopic mapping was used to visualize the chemical composition of the polymers cast with a sharp transition (left image) and a gradient (right image). Red: (ν_s CH_2 stretching mode at 2850 cm^{-1} in lauryl methacrylate), blue: (ν_{as} CH_2 stretching mode at 2930 cm^{-1} in lauryl methacrylate and ν_s CH_3 stretching mode at 2933 cm^{-1} *N*,*N*-dimethylacrylamide). Scale bars = 1 mm.



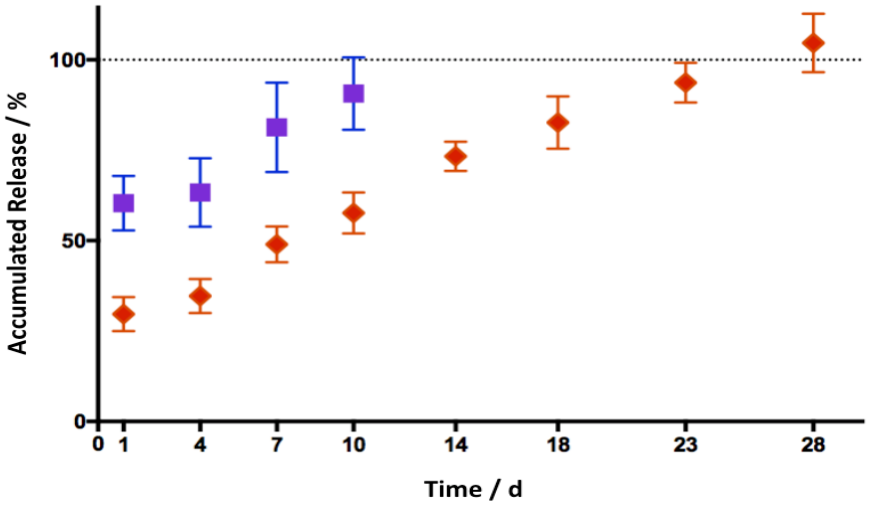
Supplementary Figure 6.

Stability of dextran and avidin gradients. Agarose hydrogels were cast as gradients of (A) 200 kDa dextran labelled with FITC (green) and (B) avidin labelled with Texas Red (yellow). The hydrogels were incubated with PBS in the dark at room temperature. Wide field fluorescence micrographs showing loss of fluorescence over time, which was attributed to the cargo leaching out of the hydrogel. Separate hydrogels were imaged for each timepoint to account for any potential photobleaching that may occur during imaging. Scale bars = 1 mm.



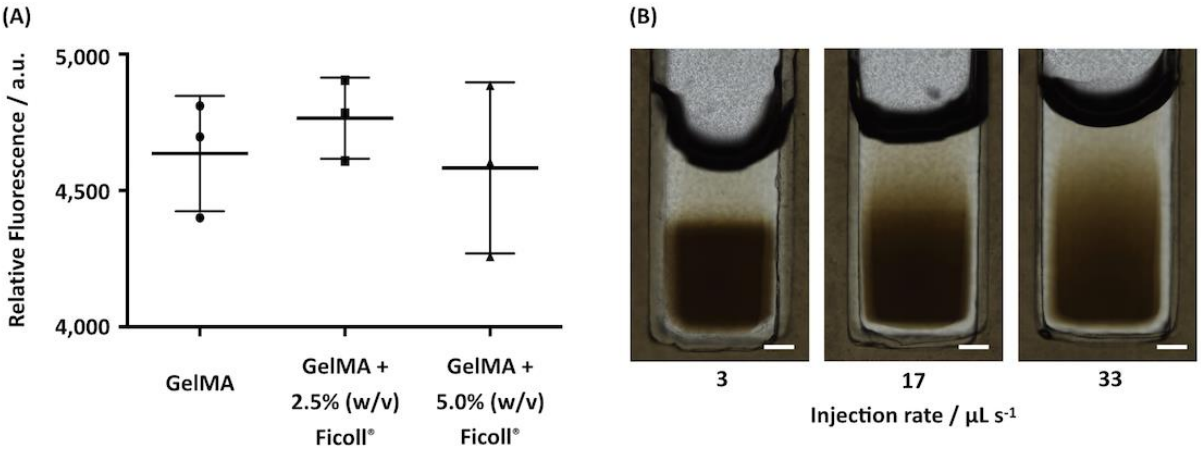
Supplementary Figure 7.

Characterization of heparin methacryloyl (HepMA). (A) Representative NMR spectrum of HepMA, showing conjugation of the methacrylate group (H1 and H2) on heparin. (B) Release profiles of heparin (blue markers) and HepMA (red markers) from GelMA hydrogels, indicating that HepMA could be retained to a greater degree than unmodified heparin. (n = 3, mean ± S.D.).



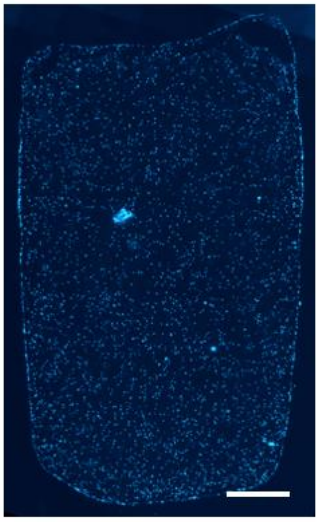
Supplementary Figure 8.

BMP-2 release profile. BMP-2 was mixed with either heparin (blue markers) or HepMA (red markers) and then cast in a 10% (w/v) GelMA hydrogel. The hydrogels were immersed in PBS with aliquots collected at various time points and analyzed using an enzyme-linked immunosorbent assay (ELISA) for BMP-2. This study showed a prolonged release of BMP-2 from the HepMA-GelMA constructs. (n = 3, mean ± S.D.).



Supplementary Figure 9.

Ficoll® as a density modifier. (A) An alamarBlue assay performed on hMSCs in GelMA supplemented with different concentrations of Ficoll®. No significant difference in metabolic activity was measured between samples (n = 3, mean ± S.D.). Statistical comparison was made using a Wilcoxon matched-pairs signed rank test. (B) Optical images of 5% GelMA hydrogels using 5% (w/v) Ficoll® in the base phase. Different injection rates could be used to generate sharp transitions or gradients, with the Ficoll® clearly visible in bright field microscope images (dark brown). Scale bars = 1 mm.



Supplementary Figure 10.

Cell distribution after 28 d of osteochondral tissue engineering. DAPI staining (nuclei, blue) performed on the osteochondral tissue constructs showed an even distribution of hMSCs throughout the tissue construct, with no visible margin or interface. Scale bar = 1 mm.

Supplementary Tables

Supplementary Table 1. Parameters used to form different gradients.

Figure	Injection Phase Components	Base Layer Components	Injection Rate / µL s^{-1}		Polymerization or Gelation Parameters
			Sharp Transition	Gradient Transition	
1B / 1C	1% (w/v) Rhodamine B conjugated agarose	1% (w/v) agarose, 5% (w/v) sucrose	-	17	Thermal setting 4 °C, 10 min
2A Polymer	Lauryl methacrylate, 2% (v/v) PEG dimethacrylate, 4 mg mL^{-1} 2,2-dimethoxy-2-phenylacetophenone	*N*,*N*-dimethylacrylamide, 2% (v/v) PEG dimethacrylate 2.75 mg mL^{-1} Irgacure 2959	3	20	UV irradiation 365 nm, 6 mW cm^{-2}, 20 min
2B Gelzan	1.0% (w/v) Gelzan, 0.03% (w/v) $CaCl_2$	1.5% (w/v) Gelzan, 0.03% (w/v) $CaCl_2$, 5.0% (w/v) sucrose	5	20	Thermal setting 4 °C, 10 min
2C Nanoparticles	1.0% (w/v) type VII agarose, gold nanoparticle solution (OD_{530} = 2.04)	1.0% (w/v) type VII agarose, 5.0% (w/v) sucrose	5	20	Thermal setting 4 °C, 10 min
2C Liposomes	1.0% (w/v) type VII agarose, 5 mg/mL Rhodamine B-liposome	1.0% (w/v) type VII agarose, 5.0% (w/v) sucrose	3	17	Thermal setting 4 °C, 10 min
2C Extracellular Vesicles	1.0% (w/v) type VII agarose, 2 x 10^{12} RFP-EVs / mL	1.0% (w/v) type VII agarose, 5.0% (w/v) sucrose	3	17	Thermal setting 4 °C, 10 min
2D Dextran	1.0% (w/v) type VII agarose, 0.5 mg/mL 200 kDa FITC-Dextran	1.0% (w/v) type VII agarose, 5.0% (w/v) sucrose	3	17	Thermal setting 4 °C, 10 min
2E Avidin	5.0% (w/v) GelMA, 1 mg mL^{-1} HepMA, 50 µg mL^{-1} Texas Red® avidin	5.0% (w/v) GelMA, 5% (w/v) Ficoll	3	17	Thermal setting 4 °C, 10 min *
3 Osteochondral Tissue Engineering	12.5 µg mL^{-1} BMP-2, 10 µg mL^{-1} HepMA, 10% (w/v) GelMA, 2.5 mg mL^{-1} Irgacure 2959 and 9 x 10^6 hMSCs mL^{-1}	5% (w/v) Ficoll-400, 10% (w/v) GelMA, 2.5 mg mL^{-1} Irgacure 2959, and 9 x 10^6 hMSCs mL^{-1}	-	17	UV irradiation 365 nm, 6 mW cm^{-2}, 20 min

*The hydrogel was imaged cold to prevent fluorescence loss from UV exposure.

Supplementary Experimental Section

Unless stated otherwise, all chemicals used were from Sigma Aldrich.

Synthesis of Agarose Conjugated with Rhodamine B

Rhodamine-labelled agarose was prepared following a method described in the literature.[1] In brief, 50 mg of type VII agarose was dissolved in 5 mL of anhydrous dimethyl sulfoxide at 70 °C. 50 mg of sodium hydrogen carbonate, 20 µL of dibutyltin dilaurate and 20 mg rhodamine B isothiocyanate was added to this solution. The mixture was stirred at 70 °C for 16 h, then diluted in 50 mL deionized water and precipitated in 100% ethanol. The sample was then vacuum dried and stored at -20 °C.

Synthesis of GelMA

GelMA was synthesized following a method described in the literature.[2] In brief, methacrylic acid was added dropwise to a 10% (w/v) solution of gelatin in deionized water (0.6 g of methacrylic acid per 1.0 g of gelatin). After 3 h of reaction, the solution was diluted with deionized water and dialysis was performed with a 12-14 kDa membrane (Spectrum Laboratories) against deionized water at 40 °C for 7 d. The sample was then lyophilized and stored at -20 °C.

Synthesis of HepMA

HepMA was synthesized following a method described in the literature.[3] In brief, methacrylic acid was added dropwise to a 2% (w/v) solution of heparin in deionized water (5.0 g of methacrylic acid per 1.0 g of heparin). The pH was adjusted to 8.5 using 5 M NaOH and stirred overnight at room temperature. The solution was precipitated in 100% ethanol and

then dialysis was performed with 1 kDa membrane (Spectrum Laboratories) against deionized water at room temperature for 7 d. The sample was then lyophilized and stored at -20 °C.

Synthesis of Gold Nanoparticles

79 mg of $HAuCl_4 \cdot 3H_2O$ was dissolved in in 185 mL of deionized water and then added to a 250 mL round-bottom flask and refluxed in an oil bath. The solution was stirred gently while 10 mL of 2% (w/v) trisodium citrate dihydrate was rapidly injected. After 5 min of fast stirring, the flask was removed from the oil bath and allowed to cool to room temperature. The nanoparticles were stored at 4 °C.

Preparation of Liposomes Labelled with Rhodamine B

2.5 mg of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 0.025 mg of Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE) in chloroform were added to a glass vial and the solvent removed by nitrogen flow. The resulting lipid film was hydrated with 0.5 mL of water, vortexed for 30 s and then extruded through a 50 nm polycarbonate membrane using an Avanti Mini Extruder (Avanti Polar Lipids).

Preparation of Exosomes Labelled with RFP

MDA-MB-231 cells expressing LAMP-RFP were cultured until confluent in high glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% (v/v) foetal bovine serum (Gibco) and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific). The cells were then cultured in the same medium, but without serum, for 3 d. The resulting conditioned media was clarified through a 0.45 μm filter and concentrated to a final volume of 500 μL using Amicon 100 kDa MWCO filters. The concentrated conditioned

media was separated into 1 mL fractions by size exclusion chromatography using a Sepharose CL-2B matrix. Extracellular vesicles were eluted in fractions 8-10, and stored at -80°C.

Characterization of Rhodamine-Agarose and Sucrose Gradients

An agarose-rhodamine gradient was generated using sucrose (see Supplementary Table 1) and the resulting hydrogel was equally dissected into 4 transverse sections. These sections were heated above the melting temperature of agarose, along with 1 mL of water to prevent gelation upon cooling. After the samples were cooled to room temperature and the agarose completely dissolved, the fluorescence intensity was measured using a microplate reader at an excitation of 543 nm and an emission of 580 nm. An identical experiment was performed using agarose instead of rhodamine-agarose (see Supplementary Table 1) with the dissolved sections measured using a sucrose assay kit (Abcam), following the manufacturer's protocol. In brief, the sucrose in the sample was converted into glucose using invertase and then measured using the reagent and enzyme provided in the kit. The absorbance at 570 nm was measured using a SpectraMax M5 plate reader.

Indentation Mapping

Stiffness gradients were characterized using Hertzian indentation to determine the bulk contact modulus. To prevent slippage during indentation, samples were adhered to the bottom of 35 mm diameter tissue culture dish using a thin layer of Loctite 406 (Loctite). 50 µl of PBS was added to the free surface of the hydrogels. A 3 mm diameter stainless steel ball bearing was indented and retracted at 0.5 volts s^{-1} (~1.4 mm s^{-1}) with a 0 s dwell time to a target displacement of 1 mm. Indentations were performed on a TA Electroforce 3200 test frame with a 250 g tension-compression load cell. Samples were indented every 0.5 mm along their gradient coordinate in order to map the local mechanical properties. Raw indentation (force-displacement) files were post processed in MatLab® to quantify the contact modulus as a

function of position. Briefly, the analysis code detected contact between the probe and sample, accounted for substrate effects, and fit the model,[4-5] which is based on Hertz's solution for contact between a sphere and an elastic half-space.[6]

Contact was determined through a piecewise fit to the force-displacement data. Using this method, we assume that prior to contact ($x<x_c$) $\frac{\Delta F}{\Delta x} = 0$; where x is the displacement, x_c is the point of contact, and F is the normal force. For $x>x_c$, we assume $F = \frac{4}{3} \cdot E' \cdot R'^{\frac{1}{2}} \cdot \delta^{\frac{3}{2}}$; where E' is the effective contact modulus, R' is the effective radius of curvature, and δ is the indentation depth: $x - x_c$. Under the assumptions that the sample was flat (infinite radius of curvature) and that the stainless-steel probe was infinitely stiff compared to the sample being indented, then E' is the contact modulus of the sample and R' is the radius of curvature of the indenter (1.5 mm).[6] However, to account for the finite thickness of these samples, a substrate correction model was necessary to properly quantify the contact modulus.[4-5]

Heparin / HepMA Release Assay

200 µg mL^{-1} solutions of heparin or HepMA were mixed with equivalent volume of solution containing 20% (w/v) GelMA and 5 mg mL^{-1} Irgacure 2959. The resulting solutions were cast into 100 µL hydrogels using UV irradiation (365 nm, 6 mW cm^{-2}, 5 min). The hydrogels were incubated for 28 d with 500 µL PBS supplemented with 0.02% (w/v) sodium azide (pH 7.4) at 37 °C. 250 µL of PBS was removed at intervals and frozen at -20 °C, with 250 µL of fresh PBS added to the hydrogel to ensure sink conditions were respected at all times. The concentration of released heparin or HepMA was quantified and measured using a dimethylmethylene blue colorimetric assay, following a method described in the literature.[7] In brief, 10 µL of sample or heparin standard was mixed with 90 µL of DMMB assay buffer (16 mg DMMB in 1 L water containing 3.04 g glycine, 2.37 g NaCl and 9.5 mL 0.1 M HCl), with the absorbance measured at 530 nm using a SpectraMax M5 plate reader.

alamarBlue® Assay

The cytotoxicity of Ficoll in GelMA hydrogels was assessed using an alamarBlue® assay (Thermo Fisher Scientific). hMSCs were encapsulated at 5 x 10^5 cells mL^{-1} in 7.5% (w/v) GelMA hydrogels containing 0, 2.5 or 5% (w/v) Ficoll and 2.5 mg mL^{-1} Irgacure 2959. 25 µL hydrogels were cast using UV irradiation (365 nm, 6 mW cm^{-2}, 5 min) and then cultured for 72 h in MesenPro RS (Thermo Fisher Scientific). The metabolic activity was measured using an alamarBlue assay, as per the manufacturer instructions (incubation time of 3 h). The fluorescence was measured using a microplate reader with an excitation of 570 nm and emission of 585 nm.

BMP-2 Release Assay

12.5 µg mL^{-1} of BMP-2 was mixed for 4 h with an equivalent volume of either 40 µg mL^{-1} heparin or 40 µg mL^{-1} HepMA. These solutions were then mixed with an equivalent volume of 20% (w/v) GelMA with 5 mg mL^{-1} Irgacure 2959. 100 µL of the final mixture were cast using UV irradiation (365 nm, 6 mW cm^{-2}, 5 min) and then incubated for 28 d in PBS with 0.02% (w/v) sodium azide (pH 7.4) at 37 °C. 500 µL of PBS was removed at intervals and frozen at -20 °C, with 500 µL of fresh PBS added to the hydrogel to ensure that sink conditions were respected at all times. The concentration of released BMP-2 was quantified using a BMP-2 ELISA kit (R&D Systems) as per the manufacturer's protocol, with the absorbance measured at 450 nm using a SpectraMax M5 plate reader.

Osteochondral Tissue Characterization

Osteochondral tissue constructs were harvested and fixed in 4% (v/v) paraformaldehyde for 2 h at room temperature and then embedded in paraffin wax. 5 µm thick sections were obtained, deparaffinized and hydrated. Consecutive sections were stained for 2 min with 2% (w/v)

Alizarin Red S (Abcam) at pH 4.3, or Alcian Blue for 30 min with 1% (w/v) Alcian Blue at pH 2.5. The latter was counterstained for 10 min with hematoxylin, and all sections were mounted in Histomount (National Diagnostics). For immunofluorescence staining, antigen retrieval was performed with proteinase K for 2 min (Dako), and then blocking for 1 h with 10% (v/v) donkey or goat serum. The sections were incubated overnight with goat anti type II collagen (Southern Biotech) at 1/20 dilution, rabbit anti osteopontin (Abcam) at 1/100 dilution, or negative controls of goat or rabbit IgG (Abcam). Excess primary antibody was removed with three 10 min PBS washes and sections were incubated with anti-goat or anti-rabbit secondary antibodies labelled with AlexaFluor 555 dye (Thermo Fisher Scientific). Nuclei were counterstained using DAPI (Thermo Fisher Scientific) and sections were mounted with Vectashield Antifade Mounting Medium (Vector Lab). All sections were imaged using a Zeiss Axio Observer Inverted Widefield Microscope.

Raman Spectroscopic Mapping

Raman spectroscopic mapping was performed using a confocal Raman microscope (alpha300R+, WITec) equipped with a 532 nm laser and either a x 10/0.25 NA or x 20/0.4 NA objective lens (EC Epiplan, Zeiss). Scattered light was coupled to the spectrometer through a 100 μm fiber, which further served as the confocal pinhole. The 600 lines per mm grating spectrograph (UHTS 300, WITec) and thermoelectrically-cooled back-illuminated CCD camera (Newton DU970N-BV-353, Andor), which yielded a spectral resolution of ~10 cm^{-1} (defined at full-width-at-half-maximum of mercury argon emission lines), were used to record spectra with 37 mW laser power focused through the objective to the sample. Full cross-sectional images with a 50 x 50 μm step-size were acquired with 0.5 s integration time for the polymer sample (x 10/0.25 NA objective, imaged through glass slides to control the sample interface), while higher spatial resolution maps were recorded with 20 x 20 μm step size for full constructs, or 2 x 2 μm step size for small regions of interest in the mineralized

cap. Raman spectra were corrected for the instrument response of the system using a traceable Raman standard (STM-2242, National Institute of Standards and Technology). Engineered osteochondral tissue samples were prepared as previously described.[8] After paraffin embedding and sectioning to 15 μm thickness on Superfrost Plus microscope slides, a standard dewaxing procedure was performed to remove confounding spectral signatures from the paraffin. Substrate signal was apparent in the acquired spectra in regions of low mineralization but did not affect the determination of mineral signatures. Full cross-sectional mineral images were calculated by integrating the measured HAP and TCP intensities (940–990 cm^{-1}) at each position in the construct map. Due to intensity differences in signal strength for different mineral components, each position in the high spatial resolution maps was fitted with a three component Gaussian mixture model *via* expectation maximization[9] and the relative feature weights for TCP (951 cm^{-1} feature) and HAP (961 cm^{-1} feature) were extracted. Each map position was first cropped to the mineral spectral region previously utilized (940–990 cm^{-1}) and then linearly detrended to account for substrate interference when no mineral was present. A threshold was then applied to mitigate the erroneous fitting of noise and the calculated center position of each Gaussian was assigned to a feature if it had a position that was within 2 standard deviations of the peak center for the Gaussian fit of the pure component spectrum mineral peak. Maps corresponded to HAP (ν_1 PO_4 at 962 cm^{-1}) and β-TCP (ν_1 HPO_4^{2-} at 951 cm^{-1}) which were experimentally validated in agreement with literature.[10]

References

[1] A. N. de Belder, K. O. Wik, *Carbohydr Res* **1975**, *44*, 251.

[2] D. Loessner, C. Meinert, E. Kaemmerer, L. C. Martine, K. Yue, P. A. Levett, T. J. Klein, F. P. Melchels, A. Khademhosseini, D. W. Hutmacher, *Nat Protoc* **2016**, *11*, 727.

[3] D. S. Benoit, K. S. Anseth, *Acta Biomater* **2005**, *1*, 461.

[4] A. C. Moore, J. F. DeLucca, D. M. Elliott, D. L. Burris, *J Tribol* **2016**, *138*, 0414051.

[5] M. Stevanovic, M. M. Yovanovich, J. R. Culham, *IEEE Trans Compon Packag Technol* **2001**, *24*, 207.

[6] H. Hertz, *J. Reine Angew Math.* **1881**, *92*, 156.

[7] D. M. Templeton, *Connect Tissue Res* **1988**, *17*, 23.

[8] C. Li, J. P. K. Armstrong, I. J. Pence, W. Kit-Anan, J. L. Puetzer, S. Correia Carreira, A. C. Moore, M. M. Stevens, *Biomaterials* **2018**, *176*, 24.

[9] R. O. Duda, P. E. Hart, D. G. Stork, *Pattern Classification (2nd Edition), Wiley-Interscience*, **2000**.

[10] S. Koutsopoulos, *J Biomed Mater Res* **2002**, *62*, 600.