Controlled Deposition of 3D Matrices to Direct Single Cell Functions

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Advances in engineered hydrogels reveal how cells sense and respond to 3D biophysical cues. However, most studies rely on interfacing a population of cells in a tissue-scale bulk hydrogel, an approach that overlooks the heterogeneity of local matrix deposition around individual cells. A droplet microfluidic technique to deposit a defined amount of 3D hydrogel matrices around single cells independently of material composition, elasticity, and stress relaxation times is developed. Mesenchymal stem cells (MSCs) undergo isotropic volume expansion more rapidly in thinner gels that present an Arg-Gly-Asp integrin ligand. Mathematical modeling and experiments show that MSCs experience higher membrane tension as they expand in thinner gels. Furthermore, thinner gels facilitate osteogenic differentiation of MSCs. By modulating ion channels, it is shown that isotropic volume expansion of single cells predicts intracellular tension and stem cell fate. The results suggest the utility of precise microscale gel deposition to control single cell functions.
Figure 1. Controlled local 3D deposition of hydrogel matrices around single cells. A) A droplet microfluidic approach to control the local microscale deposition of alginate gels around single cells. i) Scheme illustrating the microfluidic device fabrication method (see the Experimental Section). (1 and 2) An SU-8 mold with predefined patterns was fabricated by standard soft lithography with a digitally designed photomask. (3 and 4) The mold was used to create a PDMS device bonded to glass surface. ii) Overview of the droplet microfluidic device. The perspective view indicates the junction where the oil and the aqueous phases meet to form droplets. The aqueous phase consists of CaCO3-coated cells and alginate precursors (alg.) dissolved in the buffered medium, while the oil phase consists of fluorinated oil, surfactant, and acetic acid. H: height, W: width. iii) Representative 3D-reconstructed and 2D-projected (maximum intensity) confocal images show MSCs encapsulated in varied gel deposition (1 vs $6 \times 10^4 \mu m^3$ in volume) after encapsulation. Red: alginate gel (alginate-rhodamine), Green: cytoplasm (Calcein), Blue: nucleus (Hoechst) of MSCs. Scale bar = 20 µm. B) Local gel deposition can be precisely controlled by tuning device design, fluid flow parameters, and crosslinking, spanning over an order of magnitude in volume while maintaining tight control with coefficient of variation < 10% in all groups. Sample sizes for each device channel size (height, width in µm): (15h, 10w) n = 16, (15h, 20w) n = 23, (30h, 30w) n = 24, (30h, 50w) n = 25 from three independent experiments. Inset: representative images of gel droplets after generation. Scale bar = 50 µm. C) Young’s modulus (E) of the gel remains constant at ≈2 kPa regardless of varied deposition as measured by AFM (Mean ± standard deviation (S.D.) from n = 3 independent experiments, 20 gels per experiment). E of the bulk alginate gel from the same material composition is shown as comparison (Mean ± S.D. from n = 3 gel preparations, 10 measurements per gel).
gel deposition around cells (gel thickness: 2–15 \( \mu \text{m} \), gel volume: 2000–45 000 \( \mu \text{m}^3 \), total droplet size: 20–45 \( \mu \text{m} \)) (Figure 1B). The polymer concentration was kept at 1% w/v of \( \approx 240 \) kDa alginate, and Young’s modulus (\( E \)) was maintained at \( \approx 2 \) kPa[17,18] (Figure 1C). Furthermore, stress relaxation times are not altered by gel deposition (Figure S1, Supporting Information). [Ca\(^{2+}\)] in the medium remains physiological (\( \approx 2 \times 10^{-3} \) M) across the different experimental groups (Figure S2A, Supporting Information). Crosslinking of the polymer occurs simultaneously with droplet formation, which helps maintain cell viability after encapsulation in the gel with varied deposition (Figure S2B, Supporting Information). The estimated swelling ratio (\( Q_s \)) of gels remained the same (\( \approx 1.5 \)) regardless of their size (Figure S2C, Supporting Information), which is expected for a constant w/v % and polymer crosslinking.[15] Thus, this approach enables tunable local 3D gel deposition around single cells in a deterministic manner.

We sought to leverage the method to understand volume regulation of single cells as a function of local 3D matrix deposition, which remains an unaddressed fundamental question. MSCs were chosen as a model cell because they have been extensively investigated to understand cell–matrix interactions.[2–6,9,11,16,17] Clonally derived murine D1 MSCs were used, since they provide less cell-to-cell heterogeneity compared to primary cells.[3,5,8,9] Single MSCs were encapsulated with the alginate gel with varied deposition: 9.6 (thin), 20.0 (medium), or 57.0 (thick) \( \times 10^3 \) \( \mu \text{m}^3 \) in gel volume. The alginate gel-coated MSCs were subsequently embedded in collagen-I gel at a sparse density (5000 cells in 20 \( \mu \text{L} \)) followed by confocal imaging analysis of live cells to evaluate their volume change over time. Gel-coated MSCs were compared with MSCs encapsulated in a bulk alginate gel at the same cell density, composition, and \( E \) (Figure 1C). Molecular weight \( \approx 240 \) kDa alginate was chosen, since single cells encapsulated in this gel formulation do not proliferate but remain viable in culture.[16] Without any adhesion ligand, the volume of cytoplasm and nucleus is \( \approx 1000 \mu \text{m}^3 \) each, regardless of varied gel deposition (Figure S3A, Supporting Information). Uncoated MSCs embedded in collagen-I within \( \approx 2 \) h also show similar volume (Figure S3B, Supporting Information). Thus, we refer to 1000 \( \mu \text{m}^3 \) as the baseline volume (\( V_0 \)) of cytoplasm and nucleus.

MSCs were then encapsulated in the alginate gel conjugated to the Arg-Gly-Asp (RGD) ligand, which binds to \( \alpha_5\beta_1 \) and \( \alpha_\beta_3 \) integrins (alginate-RGD). The volume of gel deposition remains unchanged over 3 d in culture (Figure 2A). In contrast to the adhesion ligand-free gel, MSCs in the thin alginate-RGD gel rapidly (\( t_{1/2} \approx 1–2 \) h) undergo volume expansion to \( \approx 1500 \mu \text{m}^3 \) for both the cytoplasm (Figure 2B) and the nucleus (Figure 2C), while the rate of cell volume expansion becomes slower as local gel deposition increases. The rate of nuclear volume expansion is more sensitive to varied gel deposition than the rate of cytoplasmic volume expansion (Figure 2B,C insets). Similar effects were also observed with alginate conjugated to a CD44-binding peptide (A5G27)[20] (Figure S3C, Supporting Information), suggesting that the effects may be generalizable to other adhesion ligands. In contrast to cell volume expansion by cell spreading as observed in degradable[4] or fast stress relaxing[9] 3D gels, cell volume expansion in alginate-RGD gels is isotropic, as MSCs remain mostly spherical over time (Figure S3D, Supporting Information). Nearly all MSCs remain within the gel over 3 d (Figure S3E, Supporting Information). The location of gel-coated MSCs along the z-depth (0–450 \( \mu \text{m} \)) does not

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**Figure 2.** Local gel deposition tunes the rate of isotropic single cell volume expansion. A–C) Quantification of gel, cytoplasm, and nuclear volumes of MSCs over time in culture with varied gel deposition. For (A), the data were fit to a straight line with slope = 0, while for (B) and (C), the data were fit to a one-phase association equation: \( V = V_0 + (V_m - V_0)(1 - e^{-\frac{t}{t_{1/2}}}) \), where \( V_0 = 1000 \mu \text{m}^3 \) and \( V_m = 1500 \mu \text{m}^3 \). All data are shown as mean ± standard error of the mean (S.E.M) from \( n = 3 \) independent experiments, 15 cells per experiment. * p = 2.3 \times 10^{-6}, C) p = 6.4 \times 10^{-6} for column factor (varied gel deposition) via two-way ANOVA followed by Tukey’s multiple comparisons test. The graphs in the inset of (B) and (C) show half-maximum times for both the cytoplasm (Figure 2B) and the nucleus (Figure 2C), which remains an unaddressed fundamental question. MSCs were then encapsulated in the alginate gel conjugated to the Arg-Gly-Asp (RGD) ligand, which binds to \( \alpha_5\beta_1 \) and \( \alpha_\beta_3 \) integrins (alginate-RGD). The volume of gel deposition remains unchanged over 3 d in culture (Figure 2A). In contrast to the adhesion ligand-free gel, MSCs in the thin alginate-RGD gel rapidly (\( t_{1/2} \approx 1–2 \) h) undergo volume expansion to \( \approx 1500 \mu \text{m}^3 \) for both the cytoplasm (Figure 2B) and the nucleus (Figure 2C), while the rate of cell volume expansion becomes slower as local gel deposition increases. The rate of nuclear volume expansion is more sensitive to varied gel deposition than the rate of cytoplasmic volume expansion (Figure 2B,C insets). Similar effects were also observed with alginate conjugated to a CD44-binding peptide (A5G27)[20] (Figure S3C, Supporting Information), suggesting that the effects may be generalizable to other adhesion ligands. In contrast to cell volume expansion by cell spreading as observed in degradable[4] or fast stress relaxing[9] 3D gels, cell volume expansion in alginate-RGD gels is isotropic, as MSCs remain mostly spherical over time (Figure S3D, Supporting Information). Nearly all MSCs remain within the gel over 3 d (Figure S3E, Supporting Information). The location of gel-coated MSCs along the z-depth (0–450 \( \mu \text{m} \)) does not

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impact cytoplasmic or nuclear volume regardless of varied gel deposition (Figure S3F, Supporting Information). Thus, tunable local 3D gel deposition with an adhesion ligand can be used to control the rate of isotropic single cell volume expansion.

As cells expand in volume, they will likely stress the surrounding gel. Since cell volume expansion in engineered gel deposition is isotropic (Figure 2), it is possible to abstract the system into simple components and derive an analytical solution to calculate the stress on the inner gel surface ($\sigma_{gel}$) with a given $E$ when an encapsulated cell with the radius $r_0$ expands radially by $u_r$ in response to an adhesion ligand (Figure 3A i). The analytical solution of the corresponding linear elasticity problem (Supporting Information) suggests that when the gel is incompressible (Poisson’s ratio, $\nu = 0.5$), $\sigma_{gel}$ can be expressed as a function of the gel thickness ($d_{gel}$):

$$\sigma_{gel} = \frac{E u_r}{r_0} \left( \frac{4 r_0^2}{3 (r_0 + d_{gel})^2} \right)^{2/3}$$

Given the constant $E$, $r_0$, and $u_r$, $\sigma_{gel}$ is increased with thinner $d_{gel}$ (Figure 3A ii)—this trend is also observed if the gel is extendable (i.e., $0 \leq \nu \leq 0.5$). The analysis further shows that if the gel is extendable, the gel volume is expected to increase as a result of cell volume expansion, and hence the $E$ of the gel will likely decrease due to reduced polymer density (Supporting Information). However, the atomic force microscopy (AFM) analysis shows that $E$ of the outer gel surface remains unchanged when MSCs are encapsulated in the thin gel in the presence or absence of RGD—with or without cell volume expansion, respectively (Figure S4A, Supporting Information). Hence, the gel consisting of 1% w/v, 240 kDa alginate as used in this study is likely close to incompressible. Large strain finite element analysis yields similar results as the analytical solution, and also shows that the stress on the gel is the highest at the cell–matrix interface (Figure 3A iii; Supporting Information). Thus, the gel thickness is an important determinant of the gel stress exerted during isotropic cell volume expansion.

To test whether cells interpret differences in gel stress and subsequently tune their membrane tension as a function of varied gel deposition, we leveraged a fluorescent lipid tension reporter that changes fluorescent lifetime ($\tau$) upon molecular twisting in response to cell membrane tension,[21] as measured by two-photon fluorescence lifetime imaging (FLIM) (Figure S4B, Supporting Information). Since the reporter is a small molecule, it readily diffuses into alginate gels, which are $\approx 5$ nm in pore size,[22] and labels the membrane of encapsulated MSCs (Figure 3B i). As a positive control, the relationship between cortical tension measured by evaluating $E$ using AFM and membrane tension measured by $\tau$ after seeding cells on 2D poly(ethylene glycol)diacrylate (PEGDA) hydrogels conjugated to RGD with varied elasticity. As expected, both $E$ (Figure S4Ci, Supporting Information) and $\tau$ (Figure S4Cii, Supporting Information) of cells increase when substrate elasticity—plotting these two parameters shows that $E$ scales with $\tau$ with power law exponent ($a \approx 1.8$ (Figure S4Ciii, Supporting Information). Results show that MSCs in the thin alginate-RGD gel show significantly higher $\tau$ than MSCs in thicker gels (Figure 3B ii). Thus, tunable local 3D gel deposition with an adhesion ligand can be used to control the membrane tension of single cells independently of local matrix $E$.

Greater cell volume expansion[9] or intracellular tension[2,4,8] has been reported to skew commitment of MSCs toward osteogenic lineages. Since both phenotypes are observed with varied gel deposition, we tested whether tuning microscale gel deposition alone is sufficient to influence MSC differentiation. After culturing alginate-RGD gel-encapsulated MSCs for 7 d in the medium containing an osteogenesis-promoting cocktail, alkaline phosphatase (ALP) activity was measured to quantify early osteogenic commitment. Strikingly, ALP activity increases as gel deposition becomes thinner even when the gel $E$ remains at $\approx 2$ kPa (Figure 3C). To test whether these results reflect osteogenic commitment of multipotent MSCs, gel-coated MSCs were cultured for 10 d in the presence of both osteogenesis and adipogenesis-promoting cocktails. While MSCs in the thin gel show higher gene expression levels of osteogenic markers, including $alp$ and $runx2$, MSCs in the thick gel and the bulk gel show a higher level of an adipogenic marker, $pparg$ (Figure 3D). The diffusivity of small molecules that promote MSC differentiation is less likely impacted by varied gel deposition, since the diffusion kinetics of fluorescein (FITC)-dextran ($\approx 20$ kDa) into the gel remains unchanged (Figure S5, Supporting Information). Thus, varied local 3D gel deposition with an adhesion ligand impacts the lineage specification of single MSCs.

To establish the causality between isotropic cell volume expansion and membrane tension or osteogenic differentiation regulated by varied local gel deposition, we modulated the activity of mechanosensitive ion channels, including Piezo1 and transient receptor potential vanilloid 4 (TRPV4), since they play roles in cell volume regulation.[23] Activation of some ion channels is known to drive cell shrinkage by water efflux.[24] Treatment of MSCs in the thin alginate-RGD gel with GSK1016790A (GSK101, TRPV4-selective agonist) for 2 h after encapsulation reduces both cytoplasmic and nuclear volumes (Figure S6A, Supporting Information). In contrast, treatment of MSCs in the thick gel with GsMTx-4 (inhibitor of some mechanosensitive ion channels, including the Piezo family) or HC-067047 (selective TRPV4 inhibitor) increases both cytoplasmic and nuclear volumes (Figure S6B, Supporting Information). As expected, GSK101 reduces membrane tension in the thin gel (Figure S6C, Supporting Information), while GsMTx-4 or HC-067047 increases membrane tension in the thick gel (Figure S6D, Supporting Information). Consequently, membrane tension directly correlates with cell volume (Figure 3E).

To reduce any potential nonspecific effects by prolonged treatment of ion channel modulators during MSC differentiation, MSCs were treated with small interfering RNA (siRNA) against Piezo1 or TRPV4 prior to encapsulation, which leads to a $\approx 70\%$ decrease in target gene expression (Figure S7A, Supporting Information). The knockdown of either Piezo1 or TRPV4 prior to encapsulation, which leads to a $\approx 70\%$ decrease in target gene expression (Figure S7A, Supporting Information). The knockdown of either Piezo1 or TRPV4 prior to encapsulation, which leads to a $\approx 70\%$ decrease in target gene expression (Figure S7A, Supporting Information). The knockdown of either Piezo1 or TRPV4 prior to encapsulation, which leads to a $\approx 70\%$ decrease in target gene expression (Figure S7A, Supporting Information). The knockdown of either Piezo1 or TRPV4 prior to encapsulation, which leads to a $\approx 70\%$ decrease in target gene expression (Figure S7A, Supporting Information).
Figure 3. Isotropic volume expansion of single cells modulated by varied gel deposition predicts intracellular tension and stem cell differentiation. A) Calculation of the stress on the gel upon isotropic cell volume expansion. i) Schematic of a simple system where a cell with the radius $r_1$ expands by the length $u_0$ against the gel with varied thickness ($d_{gel}$), leading to the stress on the inner gel surface ($\sigma_{gel}$). External stress is set to zero ($\sigma_{rr} = 0$). ii) Analytical solution for $\sigma_{gel}$ when $E = 2000$ Pa, $r_1 = 7.82$ µm (radius of MSCs prior to volume expansion), and $u_0 = 1.13$ µm (cell volume expansion by 50%) (Supporting Information). iii) Finite element analysis showing a gradient of tension along the gel depth when a cell expands in volume by 50%. B) Measurement of membrane tension at 6 h after encapsulation of MSCs in alginate-RGD gels. Fluorescence lifetime imaging (FLIM) was used to evaluate decay lifetime ($\tau$) of a lipid tension reporter that binds to cell membrane. i) Representative images showing $\tau$ when MSCs are in the thin or thick gel. ii) $\tau$ values from MSCs in gels with varied deposition. $n = 15$ cells pooled from three independent experiments, and shown as mean ± S.D. *p = 1.5 × 10^{-9}$ via one-way Welch’s ANOVA followed by Dunnett T3 multiple comparisons test. C) Quantification of alkaline phosphatase (ALP) activity...
impact ALP activity in the thin gel (Figure S7D, Supporting Information). While Piezo1 siRNA fails to rescue ALP activity, TRPV4 siRNA rescues ALP activity in the thick gel (Figure S7E, Supporting Information), suggesting that isotropic volume expansion of both the cytoplasm and the nucleus will likely be required to promote osteogenic differentiation. The results collectively show that ALP activity scales with cell volume with the power low exponent \( a \approx 4.6 \) (Figure 3F).

Together, we describe a method to control the microscale deposition of engineered hydrogels around individual cells in a 3D space. We show that varied gel deposition alone has a profound impact on the rate of isotropic cell volume expansion in the presence of an adhesion ligand, which subsequently regulates membrane tension and stem cell differentiation in a predictable manner. Results from this study will help facilitate precision engineering of cell–material interactions for fundamental biological science and translational therapeutic applications. For example, the method described here can be readily expanded to elucidate downstream mechanisms behind how single cells respond to engineered local matrix deposition, and how gene expression governing cell fate decision and long-term lineage differentiation is subsequently altered in a distinct manner from elastic modulus and viscoelasticity. Our method can also be adapted to be combined with single cell sequencing technologies, in order to understand single cell heterogeneity in biophysical cell–matrix interactions. In engineering cells for regeneration of rigid tissues such as bone, our findings suggest a practical strategy to augment the osteogenic potential of donor MSCs by using a minimal amount of materials, potentially reducing the risk of foreign body reaction and the cost of materials.

**Experimental Section**

**Cell Culture:** Clonally derived D1 mouse MSCs were purchased from American Type Cell Culture (CRL-12424, ATCC). D1 MSCs were cultured in complete medium composed of high-glucose Dulbecco’s modified Eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin-streptomycin (P/S), and 1% GlutaMAX (Thermo Fisher Scientific). Cells were passaged when they reached ≈80% confluence by detaching with trypsin-EDTA (Thermo Fisher Scientific). D1 MSCs with passage number less than 13 were used in the study.

**Alginate Preparation:** Sodium alginate with ≈240 kDa molecular weight (LF200) was purchased from FMC Biopolymer. To enable cell adhesion to alginate, an integrin-binding peptide consisting of Arg-Gly-Asp (GGGGRGDSP; Peptide 2.0) or a CD44-binding peptide A5G27 (RLVSYNGIIFFLK; Peptide 2.0) was covalently conjugated to alginate by 1-l-lysine for 2 h. The slide was then placed in an MFP-3D system (Asynt) for patterning. Polydimethylsiloxane (PDMS) (Dow Corning) was then mixed with cross-linker at ratio 10:1, degassed, poured, and cured for at least 3 h at 65 °C. The cured PDMS was peeled off the wafer and bonded to a glass slide by oxygen-plasma treatment of both surfaces. Microfluidic channels were then treated with Aqualup (PPG Industries) and dried. Polyethylene tubing (inner diameter: 0.38 mm; outer diameter 1.09 mm) and 27G × 1/2 needles were used to connect microfluidic channels to syringes (Becton Dickinson). Aqueous and oil flow rates in syringes were controlled by syringe pumps (Harvard Apparatus).

**Tuning Alginate Gel Deposition around Single Cells:** CaCO\(_3\) nanoparticles (CaEssence; 900 nm diameter) were resuspended in complete medium and dispersed by sonication with Vibra Cell Sonicator at 75% amplitude for 1 min. The nanoparticles were then centrifuged at 50 g for 5 min to discard larger aggregates, followed by 1000 g for 5 min for collection. Purified CaCO\(_3\) nanoparticles were resuspended with serum-free DMEM medium—the concentration of CaCO\(_3\) was increased from 4.8 to 27.0 mg mL\(^{-1}\) with thicker alginate gel deposition. Cells were then incubated with CaCO\(_3\) by rotation at room temperature for 1 h. Excess CaCO\(_3\) nanoparticles were then washed out by centrifugation. The aqueous phase was prepared by resuspending CaCO\(_3\)-coated cells in the buffer consisting of DMEM with 50 × 10\(^{-3}\) M HEPES, 10% FBS, 1% P/S at pH 7.4, and mixing with 1% w/v alginate solution. The oil phase consisted of fluorinated oil (HFE-7500; 3M) with 1% perfluoropolyether (PFPE, Krytox; Miller Stephenson) as a surfactant and 0.03% acetic acid as an initiator of Ca\(^{2+}\) release from CaCO\(_3\). The aqueous and oil phases were injected into the microfluidic device. For thicker alginate gel deposition, channel dimensions of the microfluidic device and flow rates were increased as noted in Figure 1B. Emulsion was collected every 20 min followed by 40 min rotation at room temperature. Emulsion was then broken by the addition of 10% 1H, 1H, 2H, 2H-perfluorocotanol (Alfa Aesar). Gel-coated cells were washed twice with serum-free DMEM. Roughly 12 000 gel-coated cells were embedded in 50 μL of 1.25 mg mL\(^{-1}\) collagen-I matrix (Rat tail, Gibco/Thermo Fisher Scientific) on a 48-well glass bottom plate (P48G-1.5-6-F, MatTek Corporation), followed by culture at 37 °C in complete DMEM.

**Cell Encapsulation in Bulk Alginate Hydrogels:** Cells were resuspended in 1% w/v LF200 alginate in DMEM, and rapidly mixed with calcium sulfate by syringes. A final concentration of 10 × 10\(^{-3}\) M calcium sulfate was used to form the bulk hydrogel with E ≈ 2 kPa. The mixed solution was deposited between two glass plates with a 1 mm void thickness. After 1.5 h, the hydrogels were punched into discs and cultured in a 96-well glass bottom plate (P96C-1.5-5-S, MekTak) in complete medium.

**Mechanical Analysis of Gel Deposition around Cells:** Gel-coated cells were immobilized on a glass slide precoated with 0.1 mg mL\(^{-1}\) of poly-L-lysine for 2 h. The slide was then placed in an MFP-3D system (Asylum Research) to perform AFM with a silicon nitride cantilever with an 18° pyramid tip (MLCT, Bruker). A spring constant of the cantilever was determined from thermal fluctuations at room temperature (20–40 mN m\(^{-1}\)) before each analysis. A fluorescent microscope was used to bring the cantilever to the gel surface. Indentation was then performed under contact mode with force distance 500 nm and 1 μm s\(^{-1}\) velocity until the trigger cantilever deflection voltage (0.5 V) was reached, followed by retraction. To calculate Young’s modulus (E), force–indentation curves were fitted to the Hertzian model with a pyramid indenter[27,28] and Poisson’s ratio (ν) = 0.5.

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with varied gel deposition after 7 d culture of MSCs in the osteogenic medium. \( n = 3 \) independent experiments for each gel deposition and \( n = 4 \) for the bulk gel. D) Gene expression of i) alp, ii) runx2, and iii) pparg1 after 10 d culture in the mixed osteogenic and adipogenic medium. \( n = 3 \) independent experiments. For (C) and (D), mean ± S.E.M; *p < 0.0013, (D) i) p = 0.0034, ii) p = 0.0076, iii) p = 0.0071 via ordinary one-way ANOVA followed by Tukey’s multiple comparisons test. E) Correlation analyses of membrane tension (\( \tau \)) versus cell volume. Ctrl: control, HC: HC-067047, GS: GSKMTX-4, GSK: GSK1016790A. F) ALP activity versus cell volume. Scr: scrambled, Piezo1; Piezo1 siRNA, TRPV4; TRPV4 siRNA. For both (E) and (F), cell volume at 6 h after encapsulation is shown. The data were fit to a power-law equation and shown as mean ± S.E.M.
Stress relaxation times of gels with varied deposition were measured by AFM as previously described. The cantilever was brought toward the gel surface at a velocity of 1 µm s⁻¹. Once the trigger cantilever deflection voltage (0.5 V) was reached, z-height was increased to 100 nm, followed by a 9 s dwell time, and then the cantilever was retracted. The deflection of the tip during the dwell period was recorded under a constant load with the sampling rate = 120 Hz. Values for tip deflection over the dwell period were converted to force using the spring constant measured during AFM calibration. For all time t, force (F) was converted to stress (S) using the equation for pyramidal tip geometry:

\[
S(t) = \frac{F(t)}{\pi \sqrt{1 - \nu^2}} \left(1 - \nu^2\right)^{\frac{1}{2}}
\]

where ν is Poisson’s ratio (assumed to be 0.5 for hydrogels), \(v_0\) is the constant strain over the dwell period, and \(s\) is the pyramidal face angle. Stress curves over the dwell period were then fit to the equation:

\[
S(t) = E_x \left(1 + \frac{r_e - r_s}{r_s} - \frac{1}{e^{r_s}} \right)
\]

as described previously, where \(E_x\) is the relaxed modulus, \(r_e\) is the time of relaxation of load under constant load, and \(r_s\) is the time of relaxation of load under constant deformation. \(E_x\) can be calculated from Young’s modulus from force–indentation curves, since Young’s modulus \(E = 1.5E_x\).

Confocal Imaging and Image Analysis: Cells in gels containing alginate–rhodamine were incubated with 1 × 10⁻⁶ M of Hoechst 33342 and 2 × 10⁻⁶ M of calcein AM (both from Thermo Fisher Scientific) for 1 h to stain nucleus and cytoplasm, respectively. Samples were then washed with HBSS and maintained in Fluorobrite DMEM (Thermo Fisher Scientific) at 37 °C 5% CO₂ during confocal imaging in the Zeiss LSM 770 system with a motorized stage and the 20×/0.8 M27 Plan-Apochromat objective. To analyze cell volume, z-stacks were captured with 60–90 µm total depth with each image at 0.77 µm for 75–115 images per z-stack. The stacks were then analyzed in Imaris (Bitplane, version 7.7.2). 3D reconstruction of each stack was performed by the built-in algorithm. Voxels were generated for red (alginate–rhodamine), green (calcein), and blue (Hoechst) signals after automatic thresholding. Thresholding values varied less than 10% across all the images from different experiments. A gel-coated cell was considered an outlier and hence excluded from the analysis if it met one of the following criteria: 1) Blue voxels extend beyond the boundary of green voxels. 2) Green voxels extend beyond the boundary of red voxels. 3) Red voxels do not contain green or blue voxels inside. 4) Green and blue voxels are not within red voxels. The total voxels above the threshold were then calculated to quantify gel, cytoplasmic, and nuclear volumes of each gel-coated cell. Sphericity of gel, cell, and nucleus was analyzed from the same set of voxels and defined as \(v^{1/3}/(6A^{2/3})\), where V is volume and A is surface area.

Chemical Inhibitors: The following chemical inhibitors were purchased from Cayman Chemical: GSK1016790A (No. 17289) and HC-067047 (No. 20001066). Gene Expression Analysis: Cells were lysed with 1 mL of Trizol reagent (Thermo Fisher Scientific) for 10 min. Samples in Trizol were stored at −80 °C if not processed immediately up to one week. 200 µL of chloroform were added per mL Trizol for phase separation. Samples were centrifuged at 15 min for 12,500 rpm. 4 °C. The top layer containing RNA was collected into a new tube, and then precipitated with 250 µL isopropanol, 2 µL glycogen, and 2 µL 3 M NaOAc, pH 5.2. The resulting pellet was washed with 70% ethanol, air-dried, and resuspended in 20 µL deionized water for subsequent measurement.

Measurement of Membrane Tension by Fluorescent Lifetime Imaging Microscopy (FLIM): Cells in gels were incubated with 1 × 10⁻⁶ M of Flipper-TR lipid membrane tension probe (Cytoskeleton, Inc.) for 30 min. FLIM was performed in the Ultima Multiphoton Microscope System equipped with a Becker and Hickl time-correlated single-photon counting module (Bruker). The probe was excited at 920 nm by the Chameleon Ultra II Two-Photon laser operating at 80 MHz. The emission signal was collected through a bandpass 595/50 nm filter for 1 min. Signal decay time (τ) values were extracted by fitting the average photon count versus time graph to a two-phase exponential decay fit (Figure S4B, Supporting Information) in the data analysis software SPCimage (Becker & Hickl GmbH)—τ values correspond to the first component of the lifetime (τ₁) in the curve fit, since the second component accounts for a minority of the signal.

Retrieval of Cells from Gels: Cells in gels were retrieved by digesting with 2.5 mg mL⁻¹ collagenase P (Sigma), 4 mg mL⁻¹ alginase lyase (Sigma), and 0.125% trypsin-EDTA (Thermo Fisher Scientific) at 37 °C for 30 min. Samples were then centrifuged at 3000 rpm for 5 min and washed twice with HBSS, followed by downstream analyses.

Cell Viability Analysis by Flow Cytometry: Cells retrieved from gels were added to the stain buffer consisting of HBSS with 2 × 10⁻⁷ M of calcein AM (Biotium) and 2 × 10⁻⁶ M ethidium homodimer-1 (Thermo Fisher Scientific) for 30 min. Samples were then analyzed by flow cytometry using LSRFortessa (Becton Dickinson). An event threshold of 5000 in forward scatter was used to exclude debris. Percent cell viability was calculated by dividing the number of calcein⁺ ethidium⁺ events by the total event number. In some cases, APC beads (Calibrite; Becton Dickinson) with a known number were added in each sample to calculate an absolute number of viable and dead cells.

Diffusion Assay: To characterize the diffusion kinetics with varied gel deposition, small (≈25 µm in diameter) or large (≈45 µm in diameter) gels without cells were generated by mixing alginate with 4.8 mg mL⁻¹ CaCO₃ and running through the droplet microfluidic device by using the same parameters as single cell encapsulation (Figure 1A), followed by confirmation of Young’s modulus by AFM (E ≈ 2 kPa). Small, large, and bulk alginate gels were then incubated with fluorescein isothiocyanate-dextran (FITC-dextran) with average molecular weight ≈20 kDa (Sigma). The media were collected, and gels were digested after incubation by using the cell retrieval protocol at different time points: 30, 60, 120, and 1440 min. FITC-dextran in media and digested gels were then measured in a black 96-well plate at excitation/emission = 490/520 nm by using PHERAstar (Victor N.4).

MSC Differentiation and Alkaline Phosphatase Activity Assay: To evaluate the differentiation potential of MSCs in gels 1 d after encapsulation, they were cultured in medium supplemented with either an osteogenic chemical cocktail (No. CCM009) alone or both osteogenic and adipogenic (No. CCM011) cocktails for 7 or 10 d, respectively. All reagents for MSC differentiation were purchased from R&D Systems. One half of each sample was used to quantify an absolute number of viable cells by flow cytometry as described previously, while the other half was used to evaluate ALP activity. To quantify ALP activity, samples were lysed with 100 µL passive buffer (No. E1941, Promega) for at least 10 min at 4 °C. Each lysate was then added to a black 96-well plate preloaded with 100 µL 4-methylumbelliferyl phosphate (4-MUP) substrate (No. M3168, Sigma). Signals were acquired with excitation at 360 nm and emission at 450 nm using a plate reader. Recombinant mouse ALP protein (Novus Biologicals) was used to generate a standard curve for calibration. ALP activity of each sample was then normalized to the number of viable cells.
Primer sequences are described as follows:

RNA Interference: Small interfering RNAs (siRNAs) were purchased from Thermo Fisher Scientific as follows: piezo1 (Assay ID: 502463), runx2 (Assay ID: 182204), and scrambled (Silencer negative control no. 1 siRNA, from Thermo Fisher Scientific) as follows: piezo1 (NM_001289726.1)

F: CTTTGTCAAGCTCATTTCCTGG
R: ATTTGTCCATCTCCAGCCG

alp (NM_001146038.2)

F: CTCTGCAAGCTCAACACCAATG
R: AATTTCCTACTTCTCCGACCG

ncm2 (NM_00146038.2)

F: GCTATTAAGTGACAGTGACGGG
R: GCCCTACAGCAAGAACAATGAG

pparg (NM_001127330.2)

F: TCTATATGGCTGAAACTCTGGG
R: AGACGCTTCATCCAGAGTTG

Supporting Information

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

Conflict of Interest

The authors declare no conflict of interest.

Keywords

droplet microfluidics, hydrogels, precision cell engineering, single cell engineering, stem cells

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