



Aqueous two-phase printing of cell-containing contractile collagen microgels



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ABSTRACT

This work describes the use of aqueous two-phase systems to print cell-containing contractile collagen microdroplets. The fully aqueous conditions enable convenient formation of sub-microliter ‘microgels’ that are much smaller than otherwise possible to fabricate while maintaining high cell viability. The produced microgels contract over several days, mimicking the behavior of macroscale contraction assays, which have been valued as an important biological readout for over three decades. Use of microgels not only reduces reagent consumption and increases throughput of the assay, but also improves transport of molecules into and out of the collagen matrix, thereby enabling efficient and more precise studies of timed stimulation profiles. Utility of the technology is demonstrated by analyzing the effects of TGF- β 1 on gel contraction, and we demonstrate that brief ‘burst’ stimulation profiles in microgels prompt contraction of the matrix, a feature not observed in the conventional macroscale assay. The fully aqueous process also enables the integration of contractile collagen microgels within existing cell culture systems, and we demonstrate proof-of-principle experiments in which a contractile collagen droplet is fabricated in situ on an existing epithelial monolayer. The simplicity, versatility and ability to robustly produce collagen microgels should allow effective translation of this microengineering technology into a variety of research environments.

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1. Introduction

The ability for a cell to remodel the surrounding extracellular matrix (ECM) is a critical feature in development, homeostasis and disease progression. In particular, cell-mediated contraction of collagen matrices occurs in a variety of situations including embryo development [1–3], wound healing [4–7], and the formation of fibrotic scars and lesions [8]. In vitro collagen contraction assays have provided important insights into biological processes, by enabling the production of tissue-like structures [9] that may serve as engineered replacements [10–13] or as model systems to investigate disease pathologies [14–17]. Since the collagen contraction assay is simple to perform, captures relevant physiological details, and is broadly applicable in a variety of processes, it has remained an important research tool for over three decades. However, the complex mechanism driving the matrix contraction

process [18] is combinatorially influenced by multiple environmental factors, including the structure of the collagen matrix [19], the presence of soluble cytokines [20–23], and the type and density of cells driving the process [7,24,25]. Given the number of potential combinations of biological variations that influence contraction, there exists a need for technologies amenable to increasing throughput of this assay, particularly for applications in drug screening and personalized medicine.

Several limitations prevent the translation of this assay to a high-throughput format. Each assay requires a substantial volume of collagen material (from 100 μ L in a 96 well plate [26] up to 2.5 mL in a 35 mm dish [27]) to enable facile handling. This volume of material requires a correspondingly large number of cells to maintain a sufficient cell density to contract the collagen matrix [28], limiting the use of this assay to relatively low-throughput systems for which a sufficiently large supply of cells is easily available. This makes it challenging to use this assay for applications involving primary cell culture, or for personalized medicine, in which large scale expansion of isolated cells may cause significant phenotypic drift [29]. Furthermore, the relatively large volume of collagen gels limit diffusive transport, particularly of high

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molecular weight growth factors [30], known to play a significant role in influencing collagen matrix contraction [23]. While pore sizes in biomaterials may be engineered [31] to minimize this effect, these approaches require expensive equipment and careful characterization. Hence, despite the established role of timing in the application of signaling molecules to cells in the body [32,33], particularly in embryonic development [34,35] and regenerative medicine [36], the large sample volume of conventional collagen assays prevents uniform activation of cells within the matrix by the application of burst or pulsed biochemical stimuli.

Reducing the initial size of the collagen gels may address these concerns, but fabricating ‘microgels’ can pose significant technical challenges. While gel microdroplets are achievable in a variety of materials [37,38], most processes depend on rapid polymerization of the droplets. Collagen hydrogels are conventionally not amenable to such schemes, as the polymerization mechanism is pH-sensitive, thermally driven and relatively slow [39]. Once neutralized, collagen solutions require time at elevated temperatures to undergo complete gelation, with different protocols recommending gelation times from 30 min to 12 h [40]. While this time frame presents few concerns in polymerizing large volumes, small droplets are susceptible to a significantly greater degree of evaporation, which can impact cell viability and functionality. Careful control over processing conditions are therefore required to address these issues [41]. While evaporation may be mediated by polymerizing the collagen in an oil-water system [42–44], the thorough wash steps required adds time, complexity and uncertainty to the assay. Alternatively, small volumes of collagen have been patterned using microfluidic flow systems [45], or via a microfabricated template to produce small gels within confined boundaries [30,46,47]. Each of these techniques require substantial investment in microfabrication-related tools, peripherals and expertise, and are therefore challenging to translate into conventional wet-labs.

To simplify the formation of collagen microgels, we demonstrate an approach to maintain collagen in a distinct droplet during the polymerization process, within an aqueous environment. We utilize an aqueous two-phase system (ATPS), in which soluble polymers

thermodynamically drive aqueous systems to form two distinct phases. Our research group [48] and others [49] have demonstrated that biomolecules can selectively partition to one of the phases, and we have previously used this phenomenon to position and maintain biomolecules on living cells [48]. In the present work, we demonstrate that collagen matrix components selectively partition to the interface of a poly(ethylene) glycol/dextran aqueous two-phase system (Fig. 1C); and exploit this finding to successfully polymerize sub-microliter volumes of cell-laden collagen microdroplets, within an aqueous environment (Fig. 1A, B). We demonstrate this technology as a simple, versatile and easily managed approach to simplify, increase throughput and extend the capabilities of the widely used collagen matrix contraction assay. While this system is also compatible with more advanced technologies such as high-throughput bioprinters [41], automated liquid handlers [50], and microfluidic systems [51], a simple manual pipette is sufficient for most applications. Hence, we envision this aqueous two-phase approach to collagen printing as being broadly applicable in a variety of contexts.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma–Aldrich, fluorescent dyes from Invitrogen and all other equipment and materials from Fisher Scientific.

2.1. Cell culture

Four cell types were used in these experiments: HEK 293 embryonic kidney cells; NIH 3T3 murine fibroblasts; MC 3T3 murine osteoblast precursors cell line; and MCF10A epithelial cells. HEK 293 and NIH 3T3 cells were cultured in fully supplemented Dulbecco's Modified Eagle Medium (DMEM, with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% antibiotics-antimycotics). MC 3T3 cells were cultured in fully supplemented Alpha Minimum Essential Media (α MEM with 15% FBS, 1% antibiotics-antimycotics) in an incubator (37 °C, 5% CO₂). MCF10A cells were cultured in Mammary Epithelial Growth Medium (MEGM; Lonza). Cells were not allowed to reach confluence, and sub-cultured in 1:10 ratios by trypsinization. When at desired confluence, cells were washed with phosphate buffered saline (PBS) and 0.25% trypsin solution was added to the flask. Cells were incubated for 5 min, and then harvested and centrifuged (1000 RPM, 5 min) in a conical tube. The supernatant was aspirated and the cell pellet was re-suspended in fully supplemented culture

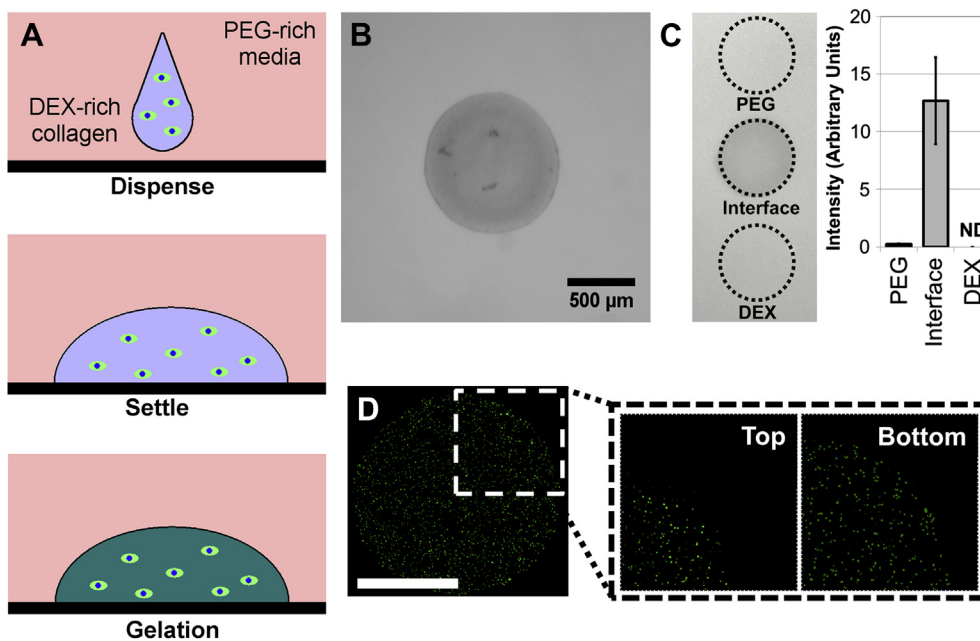


Fig. 1. Aqueous two phase patterning of collagen droplets. (A) Process schematic in which a droplet of DEX-rich collagen is dispensed into a PEG-rich media, allowed to settle, and undergo thermal gelation. (B) A 1 μ L collagen droplet after gelation in an ATPS, scale bar = 500 μ m. (C) Dot blot experiments indicate that collagen strongly partitions to the interface of a 5%PEG-3%DEX aqueous two phase system ($K_{part} = 0.011 \pm 0.005$; $n = 3$). (D) Confocal imaging of fluorescently labeled cells embedded within the microgel confirms that cells are uniformly distributed through the thickness of the hemispherical collagen microgel, scale bar = 1 mm.

medium. For ATPS-collagen formulations, cells were re-suspended at 13.6x the final desired concentration.

2.2. Preparation of aqueous two-phase system components

Stock solutions of DEX (20% w/w dextran T500; Pharmacosmos) were prepared in PBS on a rocker overnight, and centrifuged at 3000 RPM for 15 min to remove any undissolved particulates. A stock solution of PEG (20% w/w, MW: 35k, Fluka) was prepared in fully supplemented culture media, and centrifuged. Both stock supernatants were removed and passed through a 0.22 μm sterilizing syringe filter. PEG stock solutions were then diluted to working concentrations in fully supplemented culture medium, and balanced to 300 mOsm/kg by the addition of sterile deionized water, as measured with an osmometer (Vapro 5520 Vapor Pressure Osmometer; Wescor). PEG working solutions were stored for up to 2 weeks at 4 °C. Collagen-DEX solutions were prepared by diluting Type I bovine collagen (BD Biosciences) to 2 mg/mL in a sterile solution of 10% v/v 10x DMEM, 1% v/v 3 M NaOH, 15% v/v DEX stock solution (to a final concentration of 3% dextran) and 7.3% v/v additional buffer. Depending on the experiment performed, the buffer solution was either DMEM or a suspension of cells in fully supplemented DMEM. Neutralized collagen-DEX solutions were stored on ice for a maximum of 30 min before use.

2.3. Production of ATPS-collagen microdroplets

Working solutions of PEG were warmed to 37 °C and pipetted into a 48-, 96- or 384- well plate depending on the assay being performed. For manual production of droplets, collagen-DEX solutions were maintained on ice and pipetted directly into the PEG-enriched media. We experimentally found that rapid expulsion of the droplet from the pipette tip led to more uniform collagen droplets. Automated production of droplets was performed using a CybiWell automated liquid handler system (CyBi-Well, CyBio). A 96-well plate was chilled and loaded with collagen-DEX solution, and kept cold during the dispensing process using an ice-pack. The tip magazine was loaded with 96 sterile pipette tips (25 μL , CyBio), programmed to aspirate and dispense a desired volume into the target plate from a height of 1 mm above the plate surface. Following dispensation of the collagen-DEX solution, the plates were placed in an ambient air incubator at 37 °C for 30 min to allow the collagen to crosslink fully. The PEG-enriched medium was then carefully aspirated, and the droplets were washed with PBS and maintained under fully-supplemented culture medium with or without additional soluble factors. When applicable, medium was supplemented with varied concentrations of transforming growth factor (TGF)- β 1 (R&D Systems). As a final step prior to incubation, the cell culture media was pipetted rapidly to dislodge the collagen droplet from the underlying surface, before culture for up to 15 days.

2.4. Fluorescent labeling, imaging and analysis

Collagen microgels were imaged at selected timepoints using a standard inverted microscope (TE300, Nikon) and a 4 \times objective. Measurement of collagen droplet area was performed by analysis of binary threshold images, using a semi-automated script in ImageJ (NIH). For viability assays, cells were encapsulated in collagen droplets, and at the appropriate time point, the surrounding media was replaced with 4 μM Calcein-AM and 2 μM Ethidium Homodimer (EthD) in PBS for 30 min. The media was washed away and droplets were fluorescently imaged to assess viability. Percent viability was calculated as the number of cells labeled with Calcein AM, divided by the total number of cells identified with Calcein AM and EthD. To distinguish between cell populations, CellTracker dyes were used to label cells as per the manufacturer's protocol. To label and fix cells within microgels, the collagen droplets were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% triton-X for 10 min, and fluorescently stained for actin structures using FITC-phalloidin. Fluorescently labeled cells were imaged with a confocal microscope to confirm even distribution through the hemispherical gel.

2.5. Statistical analysis

All statistics are reported as means \pm standard deviation. ANOVA tests were performed using a commercially-available software package (SigmaStat 3.5; Systat Software Inc., San Jose, CA), using the Tukey test for post-hoc pairwise comparisons.

2.6. Scanning electron microscopy

Droplets of collagen were prepared in ATPS as described, and allowed to polymerize for 30 min on a glass coverslip. The droplets were gently washed in PBS three times, and then rinsed and stored in DI water. Samples were snap-frozen in liquid nitrogen, lyophilized, and coated with 10 nm of gold using a plasma-induced sputter deposition system. Samples were loaded into an XL30 FEG scanning electron microscope (Philips), and imaged at 5000 \times , with a beam voltage of 5 kV, and spot size of 3. Images were analyzed for fiber sizes using ImageJ, and expressed as a mean \pm standard deviation ($n = 3$).

2.7. Determining collagen partitioning behavior

Partitioning of collagen within the aqueous two phase system was determined by a dot blot assay. 500 $\mu\text{g}/\text{mL}$ of collagen was prepared in a solution containing the appropriate total quantities of PEG and dextran. Acidic collagen solution was prepared by mixing 20 μL 10X PBS, 33.3 μL of collagen stock solution, 30 μL of 20 wt% DEX, 116.2 μL 0.02 N acetic acid, and 200 μL 10% PEG in an Eppendorf tube. After centrifuging at 400 rcf for 7 min, the PEG, interface and DEX phases were separated and 20 μL of each phase was diluted in 20 μL of 0.02 N acetic acid. Small strips of polyvinylidene fluoride (PVDF) membranes (Bioexpress) were placed on the surface of methanol for 15 s. Then membranes were washed in distilled water for 2 min and PBS for 5 min. 0.2 μL samples of the diluted PEG, interface and DEX phases were spotted onto the wet PVDF membrane. The membrane was rinsed in distilled water and then transferred to solution containing Coomassie Blue stain for 10 min (0.2% (w/v) Coomassie Blue, 40% (w/v) methanol, 10% (w/v) acetic acid). The membrane was then de-stained for 3 min in 80% (w/v) methanol/10% (w/v) acetic acid. Finally, the membrane was placed in a second de-staining solution for 1 h (45% (w/v) methanol/10% (w/v) acetic acid). Brightfield images showing Coomassie-stained collagen were analyzed based on intensity levels and reported as arbitrary units.

2.8. Mass transport simulations

A commercially available finite element package (COMSOL v4.3; Burlington, MA) was used to simulate diffusion in ATPS-generated collagen droplets and in the conventional collagen assay, similar to previous work by Raghavan et al. [30]. The geometry of a 5 μL droplet was experimentally determined and modeled as an axisymmetric hemisphere with a 1.75 mm diameter. The conventional assay for collagen contraction was modeled as a cylinder with diameter 6.38 mm and depth 3.125 mm. The diffusion coefficient for TGF- β 1 in collagen was estimated as $1.36 \times 10^{-10} \text{ m}^2/\text{s}$ based on values reported in the literature for bovine serum albumin (MW 65 kDa) [30], and the Stokes–Einstein equation for diffusivity of particles. A boundary concentration was applied to the outside of the droplet, and a time-stepped transport of diluted species analysis was conducted to determine concentrations of TGF- β 1 in the center or bottom of the collagen droplet or cylinder.

3. Results

3.1. Characterization of collagen microgels

Dot blot tests reveal that collagen selectively partitions to the interface of a 3% dextran T-500 (DEX)/5% polyethylene glycol 35k (PEG) system, with a partition coefficient of 0.011 ± 0.005 ($n = 3$; Fig. 1). The partition coefficient is defined as the fraction of collagen that remains in the PEG phase, compared to the DEX and interfacial regions. As the DEX-enriched phase is denser than the PEG phase, it sinks to the bottom of the two-phase system. Although these measurements indicate that collagen selectively partitions to the interface, when the DEX droplet becomes sufficiently small, the accumulated collagen at the interface forms a hemispherical dome. The formation of a complete hemispherical dome was verified by visual inspection, and through cross-sectional views generated by confocal microscopy of fluorescently labeled embedded cells (Fig. 1D). Scanning electron microscopy (SEM) of the collagen fibers indicates consistently uniform fiber sizes ($70 \pm 24 \text{ nm}$; $n = 3$; Fig. 2A), consistent with collagen fiber diameters produced in conventional collagen gelation schemes. Introducing a low concentration of mouse fibroblast cells into the collagen droplet appears to locally increase pore size and increase collagen fibril diameter around the cells after one day in culture (Fig. 2B), consistent with the accepted mechanism by which cells compact collagen matrices [7,52].

ATPS-collagen droplets dispensed into well-plates demonstrate that the resulting collagen microgel dimensions depend on the volume of dispensed DEX/collagen solutions. The lateral microgel dimensions are similar to those expected for ideal hemispheres (Fig. 2C), with similar results if collagen droplets are formed on bare tissue-culture plastic (TCP) surfaces, or on a monolayer of viable HEK 293 cells. Our previous work indicates that the presence of cells on the substrate alters droplet pinning angles at the liquid/substrate interface, prompting differences in droplet spreading [48]; but this appears to be abrogated in the collagen ATPS. Though

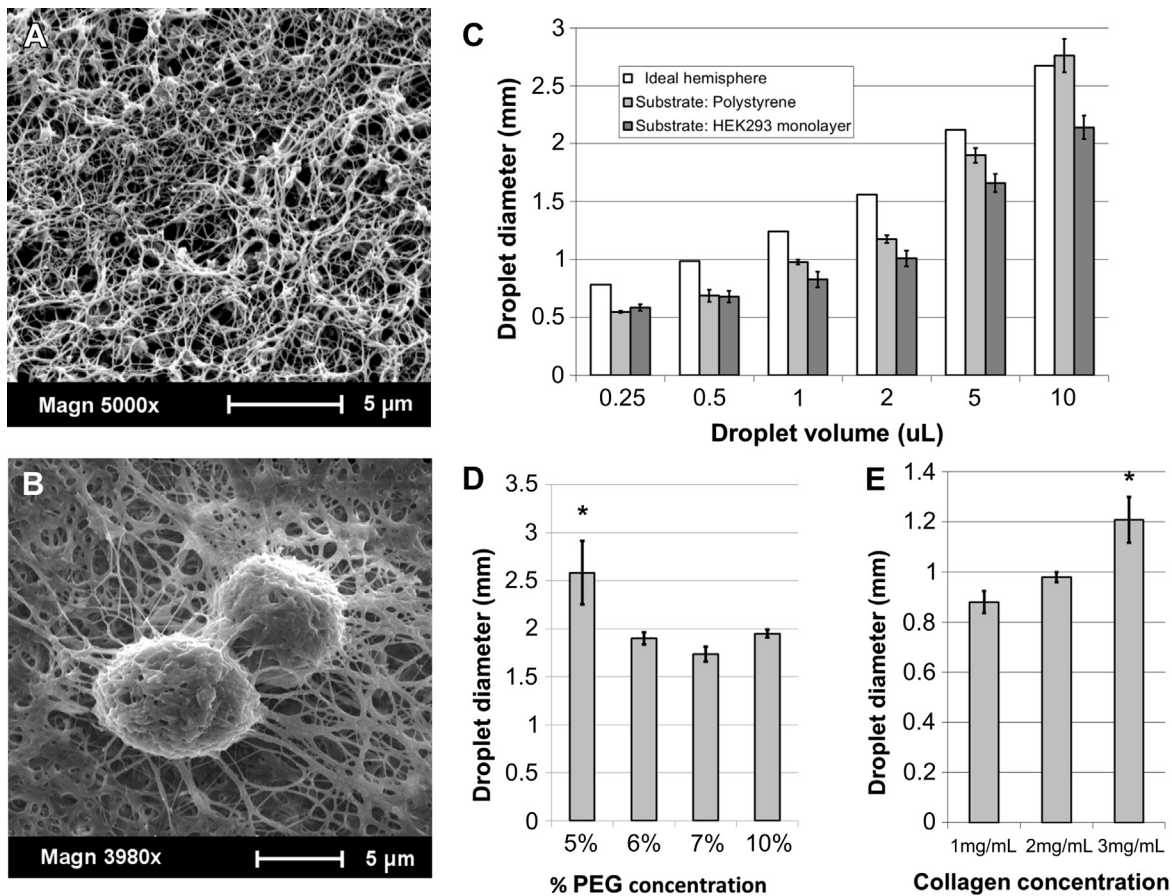


Fig. 2. Characterization of aqueously-processed DEX-rich collagen. (A) Scanning electron micrograph reveals that ATPS-collagen has a fiber size of 70 ± 24 nm, consistent with standard collagen fiber sizes. (B) MC 3T3 cells actively remodel the collagen matrix. (C) Droplet diameter scales with droplet volume producing near-hemispherical domes on either tissue culture polystyrene or a monolayer of HEK 293 cells as a substrate (produced in 6% PEG solution). Droplet diameter is also influenced by (D) concentration of PEG in the surrounding media (5 μ L droplets), and (E) concentration of collagen in the microdroplet (1 μ L droplets). All values reported as means \pm standard deviation ($n = 5$; $*p < 0.001$ compared to all other droplets of the same volume).

differences do exist between droplets plated on TCP and on cells, the difference is minimal, particularly at smaller droplet volumes.

Microgel dimensions are also affected by the concentration of PEG in the media. At concentrations less than 5%, droplets do not form, and droplets appear to take a more hemispherical shape at concentrations greater than 6% (Fig. 2D). While the possibility of long-chain PEG molecules interfering with cell function is low due to the excellent resistance of PEG to non-specific binding [53], all further experiments were conducted at the lowest robustly stable concentration to minimize any potential interactions with cells. Microgel dimensions are also influenced by concentration of collagen in the droplet (Fig. 2E), suggesting that collagen may play a role in thermodynamically driving separation in the ATPS.

3.2. Cell viability and contraction assays

NIH 3T3 cells were used as a model cell line to evaluate viability of cells encapsulated in ATPS-collagen microgel production. Two initial potential polymerization schemes were tested and evaluated for cell viability. In the first, the acidic collagen-DEX component was neutralized before the addition of cells. In the second, cells were mixed with the acidic collagen-DEX component and immediately dispensed into the PEG-enriched medium, where the droplet would neutralize because of the greater volume of the surrounding media. While this approach would eliminate the possibility that the collagen-DEX would polymerize before being

dispensed, viability assays indicate that cell viability is significantly impacted using the acidic method ($p < 0.001$ after 24 h, $n = 5$; Fig. 3C).

To compare the ATPS-mediated approach with conventional collagen gelation protocols in fabricating low-volume gels, viability of cells in 1 μ L droplets was tested 8 h after droplets were made using each method. When 1 μ L droplets were placed on the surface of a Petri dish (without an aqueous environment) and polymerized at 37 $^{\circ}$ C for 30 min, viability of cells significantly decreased as compared to those droplets allowed to polymerize within an ATPS ($p < 0.001$, $n = 10$; Fig. 3D). This is likely due to evaporation of water from the droplets and a consequent increase in osmolality during the time necessary for polymerization.

Sufficiently large concentrations of cells within collagen microgels cause contraction of the matrix over several days (Fig. 4A, B). Immediately after polymerization, the collagen microgels were washed to remove ATPS components which may alter contraction rates (Fig. S1). Microgels were then easily detached from the surface by pipetting a directed flow of medium at the droplet during the final washing step. Contraction rates are strongly dependent on cell type, with HEK 293 cells at 1 million cells mL^{-1} providing the smallest contraction over 2 days, and MC 3T3 pre-osteoblasts at the same density forcing the largest matrix contraction (Fig. 4C). As MC 3T3 pre-osteoblasts provided the greatest contraction, all further experiments were conducted using this cell type. These differences demonstrate that the ATPS-mediated production of collagen

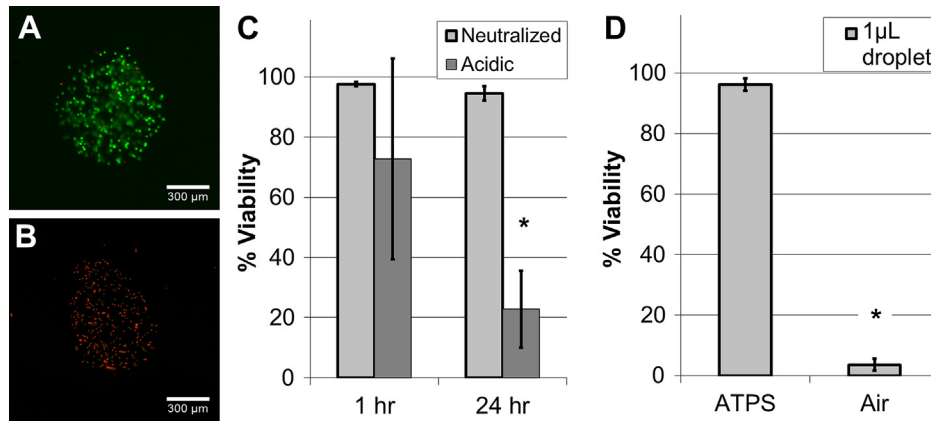


Fig. 3. Viability of cells within ATPS-collagen microdroplets, as measured by (A, B) Live/dead viability assays. (C) Cell viability under neutralized and acidic conditions after 1 and 24 h. Viability is maintained when the collagen-DEX droplet has been neutralized ($n = 5$). (D) Viability of cells encapsulated in small droplets formed under aqueous-two phase and air conditions demonstrate that viability is greatly improved under aqueous processing conditions, most likely due to rapid evaporation of liquid in the small droplets during the gelation time period (values reported as means \pm standard deviation, $n = 10$; * $p < 0.001$, compared within the same time point). Scale bars = 300 μm .

microgels maintains sensitivity of the assay in measuring contraction of the collagen matrix.

Macroscale collagen contraction assays were performed to compare the collagen microgel contraction assay with the existing standard technique. Following conventional protocols, macroscale assays were performed with 100 μL of cell-laden collagen in a single well of a 96-well plate [26]. Immediately after polymerization, additional culture media was introduced and the gels were gently released from the tissue culture plastic with a pipette tip. Comparison between this assay and with contraction of various droplet sizes fabricated using the ATPS production method, indicates that assay results are not significantly affected by the size of the collagen construct ($p = 0.124$; Fig. 5A), demonstrating that scaling down the assay volume still provides readouts comparable with the conventional technique.

As expected, cell density plays a key role in mediating collagen contraction (Fig. 5B). Collagen droplets expanded slightly when cultured without cells, while a low concentration of 50 cells/ μL maintained droplet size over 4 days. Greater cell concentrations caused rapid contraction of the droplets, consistent with reports in conventional assays [28].

Cell-mediated contraction of the collagen matrix is also influenced by the presence of soluble growth factors, including transforming growth factor $\beta 1$ (TGF- $\beta 1$). Concentrations as low as 1 ng/mL were sufficient to prompt a rapid increase in contraction of the collagen microgels. Concentrations greater than 1 ng/mL had no statistically significant effect upon contraction rate (Fig. 5C). These data demonstrate that fabricating collagen microgels via an aqueous two-phase production method produces similar responses as compared to the conventional assay.

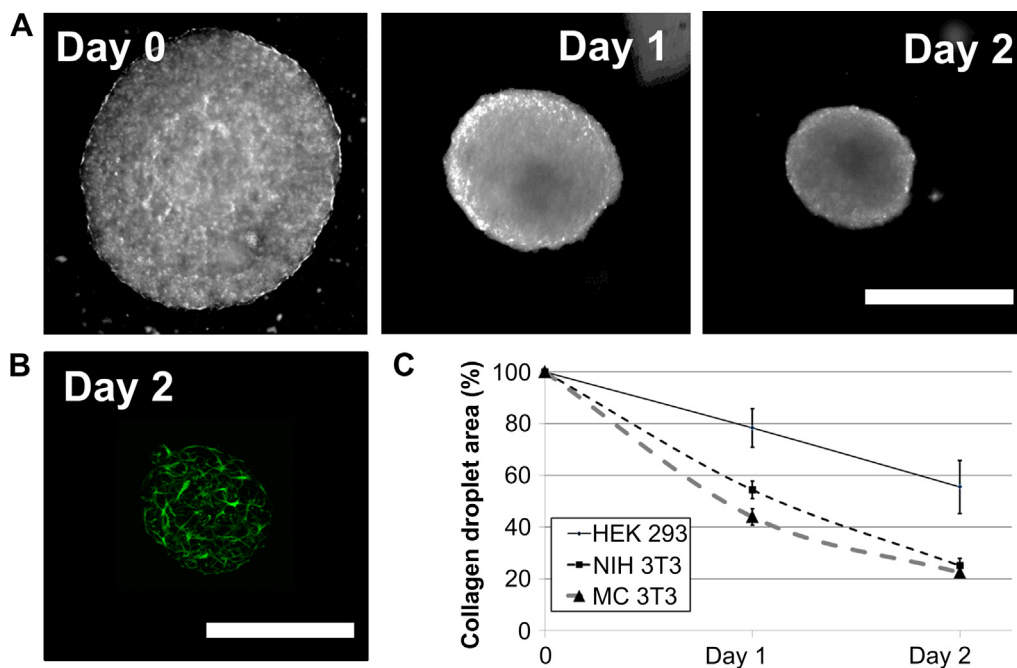


Fig. 4. Contraction of collagen microdroplets. (A) Representative brightfield images of MC 3T3 cells contracting the collagen microdroplet over two days in culture. (B) Confocal image showing cell spreading within the collagen matrix after two days. Scale bar = 1 mm. (C) Human embryonic kidney (HEK293), mouse fibroblast (NIH 3T3) and mouse preosteoblast (MC 3T3) at densities of $1 \times 10^6 \text{ mL}^{-1}$ reduce the droplet area of collagen at distinctly different rates ($n = 3$; HEK 293 significantly different ($p < 0.003$) compared with other cell types).

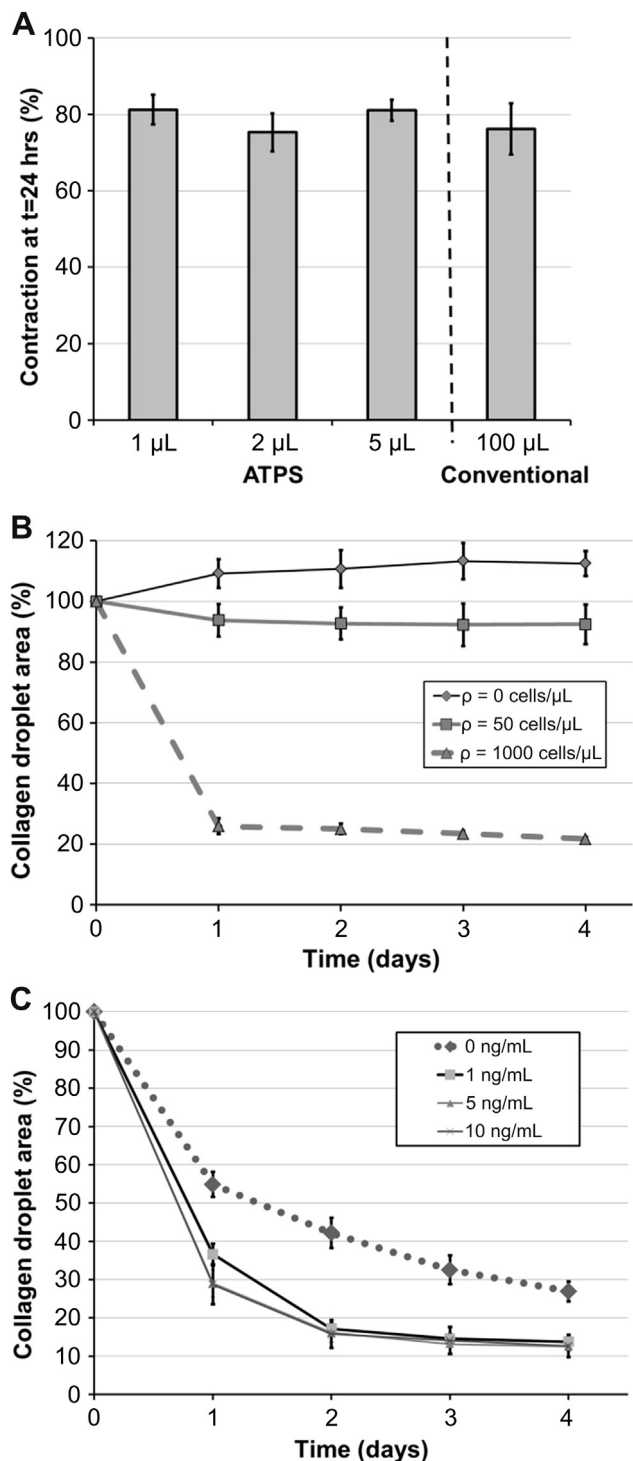


Fig. 5. Characterization of contraction in collagen microdroplets. (A) Comparison of MC 3T3 contraction of ATPS-collagen microdroplets against the conventional collagen contraction assay reveals that baseline percent contraction is independent of droplet or assay volume ($n = 7-8$ for ATPS; $n = 3$ for conventional assays). (B) Collagen droplet contraction rates depend on cell density and (C) presence of soluble factors such as TGF- β 1 (at cell density = 500 cells/ μ L). 1, 5 and 10 ng/mL of TGF- β 1 produced similar increases in contraction of the droplets ($n = 7-8$).

3.3. Exploiting transport properties of microgels

To demonstrate the potential for microscale systems in improving transport of large molecules such as TGF- β 1 (MW 25 kDa) into the collagen matrix, finite element simulations were

used to compare passive diffusion of large molecules in the conventional assay (100 μ L of collagen in a 96-well plate cylindrical form) against the ATPS-mediated production of microgels (5 μ L hemispherical droplets; Fig. 6A, B). Concentration of the diffusing molecule reaches at least 90% of equilibrium throughout the construct within 30 min in the ATPS droplet, while the conventional assay reaches 90% equilibrium after 28 h; demonstrating that while the conventional collagen contraction assay responds to applied soluble cues, this response is not necessarily homogenous throughout the system, nor does the system allow for burst or timed stimulation by soluble cues. Equilibration of smaller droplets occurs much more rapidly.

To explore the possibilities in using this system to apply dynamic stimulation profiles of large molecules to cells within the collagen matrix, we applied timed TGF- β 1 stimulation profiles to ATPS-produced microgels and to the conventional 100 μ L assay (Fig. 6C). After polymerization and rinsing of the microgels, they were exposed to 1 ng/mL of TGF- β 1 for short bursts of time, before washing the gels thoroughly with PBS and allowing them to contract for 1 day. At equilibrium conditions, 1 ng/mL of TGF- β 1 was sufficient to induce increased contraction of the collagen matrix within this time frame (Fig. 5C); and even extremely rapid 1 min exposures to this concentration of TGF- β 1 was sufficient to induce increased levels of contraction in ATPS-mediated gels. In contrast, 5 min of exposure was not sufficient to prompt a similar effect in the conventional assay (Fig. 6C). This data indicates that penetration of collagen into the large gels is minimal, while penetration into the microgels is completed quite rapidly. Hence, utilizing collagen microgels in the contraction assay enables rapid stimulation of cell culture constructs for timed stimulation studies.

Though this effect will be less noticeable with smaller molecules, the ability to remove molecules from the assay will also be of importance in timed stimulation assays. To demonstrate such an application, contracting microgels were exposed to Latrunculin-A, known to disrupt the actin filaments of the cytoskeleton, and hence abrogate contraction of the tissue construct. 24 h of exposure to Latrunculin A (or Nocodazole, a microtubule disruptor; data not shown) prevented collagen contraction, but limiting exposure to 60 min enabled the microgels to recover their contractile ability, showing no significant differences from the control ($p = 0.513$; Fig. 6D).

3.4. ATPS-collagen patterning within cell culture environments

In addition to exploiting transport properties resulting from the ability to produce low-volume gels, aqueous two-phase mediated patterning of contractile collagen gels also enables the unique ability to fabricate collagen microgels within existing cell-culture models (Fig. 7A), to provide a cell-based functional readout for signaling factors produced in that system. While possible to manually transfer pre-made gels into an existing cell culture environment [54], the low mechanical rigidity of collagen makes this challenging. As the in situ ATPS-based polymerization method presented here utilizes fully aqueous environments, this technique should be broadly and easily applicable to a wide variety of cell culture systems. To demonstrate proof-of-principle of this technique, we patterned collagen microgels containing MC 3T3 pre-osteoblasts over a confluent monolayer of MCF10A breast epithelial cells (Fig. 7B, C). The epithelial cell sheet is a simple cell culture system, characterized by tight junctions between cells. After 4 h in culture the collagen microgel contracted, and damage was observed to the underlying epithelium (Fig. 7C, D). Damage occurs close to the contractile droplet, while areas further away remain intact, suggesting that the mechanical insult provided by the contractile droplet may cause this effect. This simple co-culture system

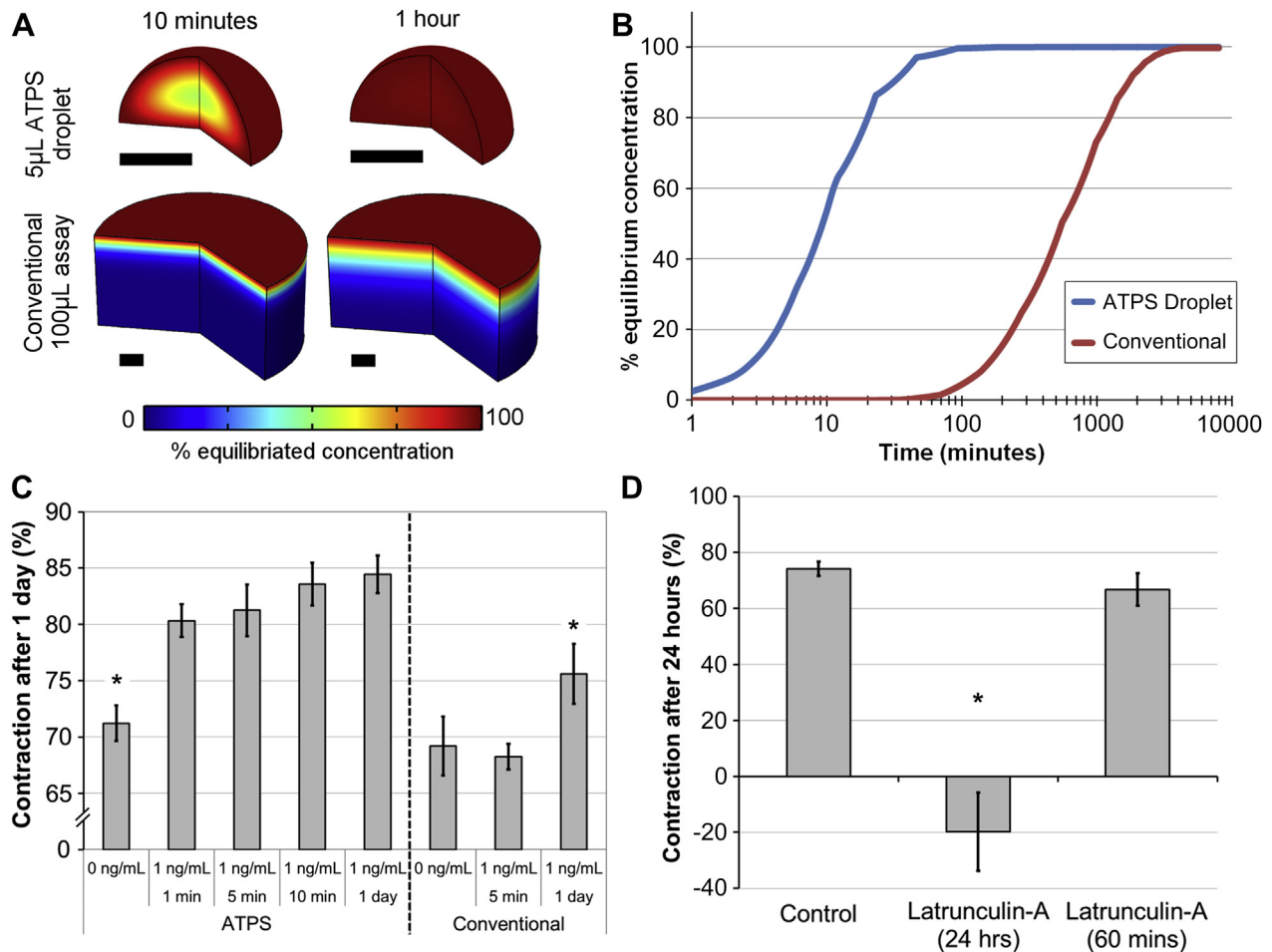


Fig. 6. Miniaturization of droplets enables rapid diffusive transport through the collagen matrix. (A) Simulations indicate that diffusive transport enables equilibration of the TGF- β 1 molecule (MW 25 kDa) within 1 h, as compared to the conventional collagen contraction assay. Scale bar = 1 mm. (B) Percent equilibrium concentration at the base of the collagen droplets over time. Conventional assays require \sim 24 h to completely equilibrate to added concentrations of TGF- β 1. (C) Timed stimulation of ATPS-collagen and conventional collagen droplets with TGF- β 1. Small volume (5 μ L droplets) of ATPS-collagen respond to 1 min of stimulation with TGF- β 1, as compared to conventional assays ($n = 7-8$ for ATPS assays; $n = 3$ for conventional assays; * indicates $p < 0.001$ compared to all other conditions in the same assay). (D) Small droplet size also enables simple removal of soluble factors for pulsed stimulation experiments. After 24 h, collagen contraction is strongly inhibited by Latrunculin-A, but contraction is recovered if the surrounding media is changed ($n = 7-8$; * indicates $p < 0.001$).

demonstrates the ability to study interactions between different tissue types fabricated in situ, and may be used to simulate certain physiological processes, such as in development or wound healing in respiratory airways, in which fibroblasts interact with a wounded epithelial layer. Furthermore, this approach may also be used to measure the influence of soluble cues generated by relevant cell culture model systems on contraction of an in situ collagen microgel.

4. Discussion

The utility, versatility and applicability of the collagen contraction assay in studying biological systems of development, healing and disease has prompted the use of this assay for over three decades. Miniaturization of this assay presents critical advantages over conventional macroscale analogs, including enabling the study of small populations of cells, reducing the cost per assay, and allowing rapid diffusion of large molecules through the collagen construct, thereby enabling timed studies in applying chemical stimuli. However, miniaturization techniques typically require expensive equipment and extensive expertise in microfabrication or additional processing steps [44–46], which can hinder translation to conventional biological wet-labs.

To enable rapid and versatile formation of collagen microgels, we explore collagen formation within an aqueous two-phase system, in which collagen is localized to one phase, where it is allowed to polymerize. Maintaining collagen in an aqueous environment prevents evaporation of the droplet phase, which can be particularly important in maintaining cell viability while polymerizing microgels in an air environment (Fig. 3D). While it may be possible to fabricate microgels in an air environment, using an ATPS eliminates the need to optimize for variables such as polymerization time and humidity. The use of aqueous phases also reduces the need for thorough washing and enables the delivery of large protein molecules to the construct, which can be challenging to implement when polymerizing collagen droplets in an oil-water system. As demonstrated in Fig. 7, the use of the ATPS technology also enables the in situ polymerization of collagen microgels within existing cell culture systems, which may greatly improve the versatility and applicability of this technique.

ATPS-mediated collagen microgels were formed using either a manual pipette, or an automated liquid handler system, suggesting that this approach is likely compatible with high-throughput screening techniques and infrastructure. Collagen microgels formed via ATPS were found to have similar microstructures (Fig. 2A, B), and contractile function (Figs. 4 and 5A) as their

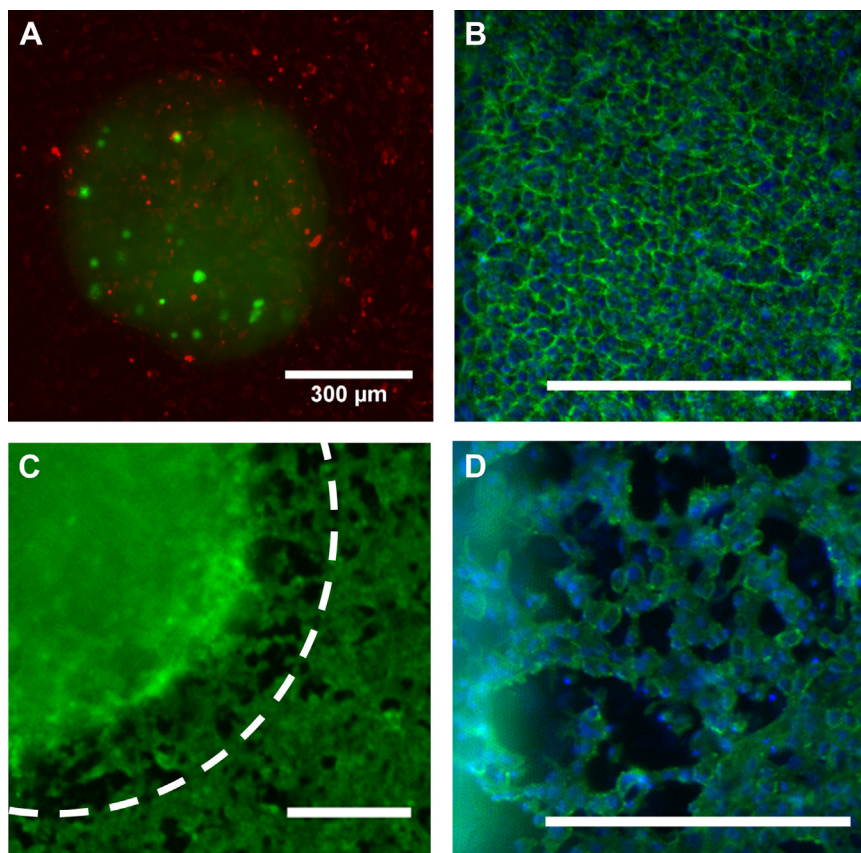


Fig. 7. Patterning contractile collagen microdroplets within existing live-cell culture environments. (A) NIH 3T3 cells (green) are encapsulated within a collagen droplet formed over a layer of HEK 293 cells (red). (B) A monolayer of MCF10A cells prior to collagen patterning, stained for actin (green) and nuclei (blue). Actin structures indicate a well-connected epithelial monolayer. (C,D) MC 3T3 cells encapsulated in collagen (formed via ATPS) on the existing epithelial monolayer. The original boundary of the collagen droplet is marked with a white dashed line. Within 4 h, the MC 3T3 cells induce contraction of the gel, and we observed damage to the underlying epithelial layer. Scale bars = 300 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

macroscale counterparts. We demonstrate that initial collagen gel volumes ranging from one to 100 μL do not influence contraction rates, supporting the use of miniaturized gels in contraction assays. Consistent with conventional macroscale contraction assays, contraction rates are most strongly influenced by the type of cells embedded in the matrix (Fig. 4C), the density of cells (Fig. 5B), and the presence of soluble factors in the media (Fig. 5C). Taken together, these findings strongly indicate that the macroscale collagen contraction assay may be used as a substitute experimental platform that reduces reagent consumption, enables the study of small cell populations, and provides increased throughput over conventional macroscale systems.

An additional advantage in miniaturizing the contraction assay arises from the ability to rapidly equilibrate large molecules in the bulk of the collagen material. Rapid diffusion of signaling molecules into microgels (Fig. 6A, B) enables experimentalists to precisely define soluble stimulation profiles, which should enable well-defined studies in fields such as systems and synthetic biology. We demonstrate that short ‘pulses’ of TGF β are sufficient to initiate contraction of the whole microgel (Fig. 6C) while similar pulses do not prompt a macroscale gel to completely contract. Similarly, the ability to remove inhibitory molecules (demonstrated in Fig. 6D) should enable studies on the effects of timing in stimulating biological systems. Timing is emerging as an important feature in mimicking *in vivo* physiological systems [32–36], and extending the collagen contraction assay to include a time-dependent component may yield interesting insights into the biology of such systems.

5. Conclusions

We demonstrate the use of aqueous two-phase systems to produce sub-microliter hemispherical constructs of collagen on a variety of substrates including live cell monolayers. The aqueous environment prevents evaporation within the small droplet volume, allowing encapsulated cells to remain viable. Contractile cells remodel the matrix at a rate dependent on cell type, cell density, and the presence of soluble factors, mimicking results in conventional macroscale collagen contraction assays. The ability to use collagen microgels for contraction assays reduces the volume of reagents and number of cells needed, and significantly improves transport of molecules into the bulk of the hydrogel droplet. We demonstrate the use of microgels for time-dependent and burst stimulation profiles, which would not be possible with conventional approaches. Finally, the aqueous two-phase processing system enables production of collagen microgels within existing culture environments to potentially conduct collagen contraction assays *in situ*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.biomaterials.2013.08.046>.

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