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Micromolded gelatin hydrogels for extended culture of engineered cardiac tissues

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ABSTRACT

Defining the chronic cardiotoxic effects of drugs during preclinical screening is hindered by the relatively short lifetime of functional cardiac tissues *in vitro*, which are traditionally cultured on synthetic materials that do not recapitulate the cardiac microenvironment. Because collagen is the primary extracellular matrix protein in the heart, we hypothesized that micromolded gelatin hydrogel substrates tuned to mimic the elastic modulus of the heart would extend the lifetime of engineered cardiac tissues by better matching the native chemical and mechanical microenvironment. To measure tissue stress, we used tape casting, micromolding, and laser engraving to fabricate gelatin hydrogel muscular thin film cantilevers. Neonatal rat cardiac myocytes adhered to gelatin hydrogels and formed aligned tissues as defined by the microgrooves. Cardiac tissues could be cultured for over three weeks without declines in contractile stress. Myocytes on gelatin had higher spare respiratory capacity compared to those on fibronectin-coated PDMS, suggesting that improved metabolic function could be contributing to extended culture lifetime. Lastly, human induced pluripotent stem cell-derived cardiac myocytes adhered to micromolded gelatin surfaces and formed aligned tissues that remained functional for four weeks, highlighting their potential for human-relevant chronic studies.

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1. Introduction

Cardiotoxicity is a leading cause of market withdrawal for drugs in the United States [1,2]. Many drugs demonstrate cardiotoxicity due to chronic exposure, especially the anthracycline class of cytotoxic cancer drugs, such as doxorubicin [3,4]. Chronic cardiotoxicity is usually identified in animal models, such as mice [5,6] or dogs [7], that are exposed to the drug for at least two months. To reduce costs and improve throughput, cardiotoxicity is screened *in vitro* with neonatal rat ventricular myocyte cultures, but these studies typically do not extend beyond ten days [8,9], limiting their

relevance as models for chronic exposure. Traditional *in vitro* systems also do not recapitulate the native tissue architecture or extracellular microenvironment of the heart, both of which are known to regulate myocyte phenotype [10–13]. Another limitation of many pre-clinical assays is a focus on toxicity [14] or ion channel activity [15], which are not direct indicators of cardiac output. Furthermore, studies with animals and animal cells are not always relevant to humans due to species-dependent differences [16], indicating that it is important to develop *in vitro* systems that are compatible with human-derived cardiac myocytes [17]. Due to the limitations of current *in vitro* systems, there is a need for biomaterials that can support long-term culture of engineered animal and human cardiac tissues and facilitate readouts of contractility to better predict adverse or functional effects of drugs on the heart.

Our group has previously described several *in vitro* platforms for engineering neonatal rat cardiac tissues and quantifying contractile function in response to variables such as tissue architecture [10,18], mechanical stretch [19], or acute drug exposure [20]. These “Heart on a Chip” platforms utilized microcontact printing [18,20] or micromolding [21] to engineer anisotropic cardiac tissues that mimicked the aligned, laminar structure of ventricular

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myocardium. Stress generation was quantified by culturing engineered tissues on polydimethylsiloxane (PDMS) polymer [18,20] or alginate hydrogel [21] cantilevers, forming muscular thin films (MTFs) that curled as tissues contracted. These two substrates each have advantages and disadvantages, from both biological and practical perspectives. PDMS can be spin-coated and laser-cut [20], which simplifies and standardizes the fabrication process, but PDMS is much stiffer than native myocardium, limiting its physiological relevance. Alginate hydrogels better match the elastic modulus of the heart, but alginate is a non-fouling biomaterial that is not native to the heart and requires additional fabrication steps for fibronectin adhesion [21]. Our previous method for fabricating alginate hydrogel MTFs also lacked control over thickness and cantilever shape [21], which compromises the throughput and reproducibility of the assay. To date, these *in vitro* tools have been used to quantify contractile stresses after three to five days in culture.

The extracellular matrix in many organs, including the heart [22], consists mostly of collagen. Gelatin is a derivative of collagen and thus forms hydrogels that are naturally non-toxic and amenable to cell adhesion [23], as shown for endothelial cells [24] and chondrocytes [25]. Gelatin hydrogels also have tunable elastic moduli and can be made thermostable by cross-linking with microbial transglutaminase [26–27], improving their robustness for cell culture. Because of these desirable properties, our objective for this study is to develop gelatin hydrogels as MTF substrates and use them to measure contractile stresses generated by both neonatal rat and human induced pluripotent stem (iPS) cell-derived engineered cardiac tissues. We will also determine if gelatin hydrogels improve the health and culture lifetime of engineered cardiac tissues compared to PDMS micropatterned with fibronectin, a commonly used culture substrate.

2. Materials and methods

2.1. Elastic modulus measurements

Solutions of 5%, 10%, and 20% w/v gelatin from porcine skin (175 Bloom, Type A, Sigma–Aldrich, St. Louis, MO) were prepared with 1%, 2%, and 4% microbial transglutaminase (MTG, Ajinomoto, Fort Lee, NJ), for a total of nine solutions, similar to previously published protocols [24,25]. Gels were cured overnight in 35 mm Petri dishes (6 mL/dish) in triplicate. After curing, a 6 mm diameter biopsy punch was used to remove a cylinder of gel for each dish. The diameter of the gel cylinder was measured and stress–strain curves were derived using an Instron 3342 (Norwood, MA) in uniaxial unconfined compression mode. The compressive Young's modulus was taken as the slope in the linear region where deformation was first detected. For each gel solution, the Young's modulus values were averaged for the three technical replicates. Three independent sets of gel solutions (biological replicates) were fabricated, measured, and averaged together to determine average Young's modulus values.

2.2. Soft lithography and microcontact printing

Elastomeric stamps were fabricated from polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) using previously published protocols [10,28,29]. Briefly, custom photomasks were used to shield wafers spin-coated with SU-8 2002 photoresist (MicroChem, Newton, MA) during UV exposure with a mask aligner. After exposure, wafers were rinsed in propylene glycol monomethyl ether acetate to dissolve un-exposed regions, dried, and silanized. PDMS was poured onto the wafer, cured at 65 °C for at least 4 h, carefully peeled from the wafer, and cut into stamps. For micromolding, stamps with 10 μm lines separated by 10 μm gaps were used. For microcontact printing, stamps with 15 μm lines separated by 2 μm gaps were incubated with fibronectin (50 $\mu\text{g}/\text{mL}$, BD Biosciences, San Jose, CA) for 1 h. Glass coverslips were spin-coated with PDMS and exposed to UV-ozone for 8 min prior to being stamped with fibronectin, as previously described [10,18].

2.3. Gelatin muscular thin film (MTF) substrate fabrication

Glass coverslips (22 mm \times 22 mm) were covered with low-adhesive tape (3M, St. Paul, MN), which was cut using a laser engraving system (Epilog Laser, Golden, CO). The tape was selectively peeled from each coverslip such that the outer border and two inner rectangles remained covered with tape. Coverslips were then immersed in 0.1 M NaOH for 5 min, followed by 0.5% APTES in 95% ethanol for 5 min and 0.5% glutaraldehyde for 30 min, as previously described [30]. Coverslips were

rigorously rinsed and dried. The inner two rectangles were then peeled, leaving a region of selectively-activated glass exposed.

To make gelatin solutions, 20% w/v gelatin (Sigma–Aldrich, St. Louis, MO) was warmed to 65 °C until all powder was dissolved. MTG (Ajinomoto, Fort Lee, NJ) at 8% w/v was warmed to 37 °C. Equal parts of the gelatin and MTG solutions were then mixed to produce a final solution of 10% w/v gelatin and 4% w/v MTG. The solution was quickly pipetted onto the exposed regions of glass coverslips. PDMS stamps with 10 μm \times 10 μm line features were then inverted on top of the gelatin drop and gentle pressure was applied so that the bottom edges of the stamp were touching the taped edges. Gelatin was then left to cure overnight at room temperature with the stamp in place.

After gelatin was cured, the coverslip and stamp were immersed in distilled water to re-hydrate the gelatin. The stamp was then carefully peeled off the gelatin. Gel rehydration minimized damage to the gelatin during peeling. Coverslips with molded gelatin were then dried in a tissue culture hood for 2–3 h.

Cantilevers (1 mm wide \times 2 mm long) were laser engraved into the dehydrated micromolded gelatin using an Epilog laser engraving system (Golden, CO) with the following settings: Power 3%, Speed 6, Frequency 2500. Laser cuts were centered over the un-activated regions of the coverslips. Diagonal cuts through the corners of the tape and a square cut just inside the tape border were also made to ease tape removal. After cutting, the outer border of the tape was carefully removed without disrupting the gelatin cantilevers. Gelatin chips were they re-hydrated in PBS and stored at 4 °C until cell seeding.

To measure gelatin thickness, gelatin solutions were doped with AlexaFluor 488 200 nm fluorescent beads (Invitrogen, Carlsbad, CA) at a concentration of 1:1000 prior to fabricating gelatin MTFs. Z-stacks of hydrated substrates were imaged on a Zeiss LSM 5 LIVE confocal microscope (Oberkochen, Germany) at six locations on each coverslip using a 10 \times objective. Three coverslips were imaged for each of three independent batches of gelatin. Z-stacks were re-sliced in ImageJ (NIH, Bethesda, MD) to acquire side views and the height of each z-stack was manually measured.

2.4. Cell culture

Neonatal rat ventricular myocytes were isolated according to previously published protocols approved by the Harvard University Animal Care and Use Committee [18,31]. Briefly, ventricles were extracted from two day old Sprague–Dawley rats and incubated in Trypsin solution (1 mg/mL) overnight at 4 °C. Ventricles were then subjected to four collagenase (1 mg/mL, Worthington Biochemical Corp., Lakewood, NJ) digestions for 1–2 min at 37 °C followed by manual pipette agitation. Cell solutions were strained, re-suspended in M199 culture media supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 0.1 mM MEM nonessential amino acids, 20 mM glucose, 2 mM L-glutamine, 1.5 μM vitamin B-12, and 50 U/ml penicillin, and pre-plated twice to reduce non-myocyte cell populations. One million cardiac myocytes were seeded for each well of a 6-well plate. Fetal bovine serum concentration was reduced to 2% after two days in culture.

2.5. Immunostaining and image analysis

Engineered cardiac tissues were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and 0.5% Triton-X (Sigma–Aldrich, St. Louis, MO) for 5 min. Tissues were then incubated with primary antibodies against sarcomere α -actinin (Sigma–Aldrich, St. Louis, MO) for 90 min at room temperature followed by secondary antibodies against mouse IgG conjugated to Alexa Fluor 546 (Invitrogen, Carlsbad, CA) and DAPI (Invitrogen, Carlsbad, CA) for 60 min at room temperature. For each coverslip, ten fields of view were imaged using a Leica DMI 6000B inverted fluorescent microscope with a 40 \times objective (Wetzlar, Germany).

For each image, the orientation angles of α -actinin immunosignals were calculated, as previously described [10]. The orientational order parameter (OOP) [18] was then calculated for all orientation angles consolidated from all images taken on each coverslip. The OOPs for each condition were averaged and compared using student's t-test.

2.6. MTF experiments and analysis

Gelatin MTF substrates with engineered cardiac tissues were transferred to a 35 mm Petri dish and soaked in Tyrode's solution (1.8 mM CaCl_2 , 5 mM glucose, 5 mM HEPES, 1 mM MgCl_2 , 5.4 mM KCl, 135 mM NaCl, 0.33 mM of NaH_2PO_4 , pH 7.4). The dish was placed on the stage of a Leica MZ9.5 stereomicroscope (Wetzlar, Germany). Using fine forceps, the excess gelatin and tissue surrounding the MTFs was removed and discarded and each MTF was gently peeled from the glass coverslip. The dish was then placed in a heating block to restore 37 °C within the bath and field stimulation electrodes were inserted into the top of the dish. Rows of contracting MTFs were recorded at 100 frames per second using a Basler A601f-2 camera (Exton, PA) while pacing from 1 to 6 Hz at 5–7 V using a MyoPacer Cell Stimulator (IonOptix, Milton, MA).

To convert movies to stress measurements, movies were thresholded and the radius of curvature for each MTF was calculated using the x-projection and original length [18]. The radius of curvature, thickness, and elastic modulus of each MTF was used to calculate stress using modified Stoney's equation [31]. For each MTF, the

average diastolic, systolic, and twitch (difference between systolic and diastolic) stresses were calculated, averaged, and compared using student's *t*-test.

2.7. Metabolic measurements

Cellular metabolism was measured using a Seahorse Bioscience XFe 24 Extracellular Flux Analyzer (North Billerica, MA). Gelatin solution (5% w/v gelatin, 1% w/v MTG) or PDMS was pipetted into the wells (10 μ L/well) of a XF24 cell culture polystyrene microplate (Seahorse Bioscience, North Billerica, MA). PDMS was cured overnight at room temperature. Wells with cured PDMS were treated in a UVO Cleaner (Jelight, Irvine, CA) for 8 min and incubated with 50 μ g/mL human fibronectin for 10 min. Gelatin and PDMS-fibronectin wells were then rinsed with PBS and stored at 4 $^{\circ}$ C until cell seeding with neonatal rat ventricular myocytes (100 000 cells/100 μ L/well). After seeding, plates were left in the laminar flow hood for 30 min before being placed in the 37 $^{\circ}$ C, 5% CO₂ incubator to prevent cell aggregation at the well edges, as suggested by the manufacturer. After 1–4 h, 500 μ L of media was added to each well. Media was then changed according to the standard cell culture protocol described above.

After four days in culture, culture media was replaced with XF Assay Medium (Seahorse Bioscience, North Billerica, MA) supplemented with 20 mM glucose and the plate was incubated in a 37 $^{\circ}$ C, non-CO₂ incubator for 1 h. Wells of a hydrated sensor cartridge (Seahorse Bioscience, North Billerica, MA) were then loaded with 2 μ M oligomycin (well A), 1 μ M carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (well B), and 1 μ M antimycin A and 1 μ M rotenone (well C), according to manufacturer's instructions (all components from XF Cell Mito Stress Test Kit, Seahorse Bioscience, North Billerica, MA). A cartridge and cell plate were inserted into the Seahorse XFe 24 Extracellular Flux Analyzer (North Billerica, MA), which made three measurements of baseline oxygen consumption rate (OCR) followed by serial injections of the drugs listed above and three OCR measurements after each injection. The difference between baseline OCR (before adding compounds) and OCR after oligomycin injection is a measurement of ATP production. The difference between baseline OCR and OCR after FCCP injection is a measurement

of spare respiratory capacity. The OCR value after antimycin/rotenone injection is a measurement of non-mitochondrial respiration. The difference between baseline OCR and after antimycin/rotenone injection is a measurement of basal respiration. Several wells were collected and averaged across three different neonatal rat ventricular myocyte harvest cycles.

2.8. Human iPS cell-derived cardiac myocyte culture

Cor4U human iPS cell-derived cardiac myocytes were acquired from AxioGenesis (Cologne, Germany) and thawed, seeded, and cultured according to the manufacturer's protocols. Briefly, 500 μ L of thawing media was added to each frozen vial of cells and thawed at 37 $^{\circ}$ C. Thawed cells were cultured for two days on fibronectin-coated 6-well plates in culture media supplemented with puromycin. Cells were then rinsed with PBS, trypsinized, counted, and re-seeded on gelatin MTF substrates. Every two or three days, media was replaced with fresh hiPS cell-derived cardiac myocyte culture media (AxioGenesis, Cologne, Germany).

3. Results

3.1. Gelatin muscular thin film (MTF) fabrication

Ventricular myocardium consists of aligned, elongated cardiac myocytes embedded in a compliant extracellular matrix network [32]. To mimic this *in vitro*, we identified micromolded gelatin hydrogels cross-linked with microbial transglutaminase (MTG) [26,27] as potential muscular thin film (MTF) substrates that would recapitulate the architecture and mechanical properties of ventricular tissue (Fig. 1A). To fabricate hydrogels with different elastic moduli, we used 1%, 2%, and 4% w/v MTG to cross-link 1%, 2%, 5%,

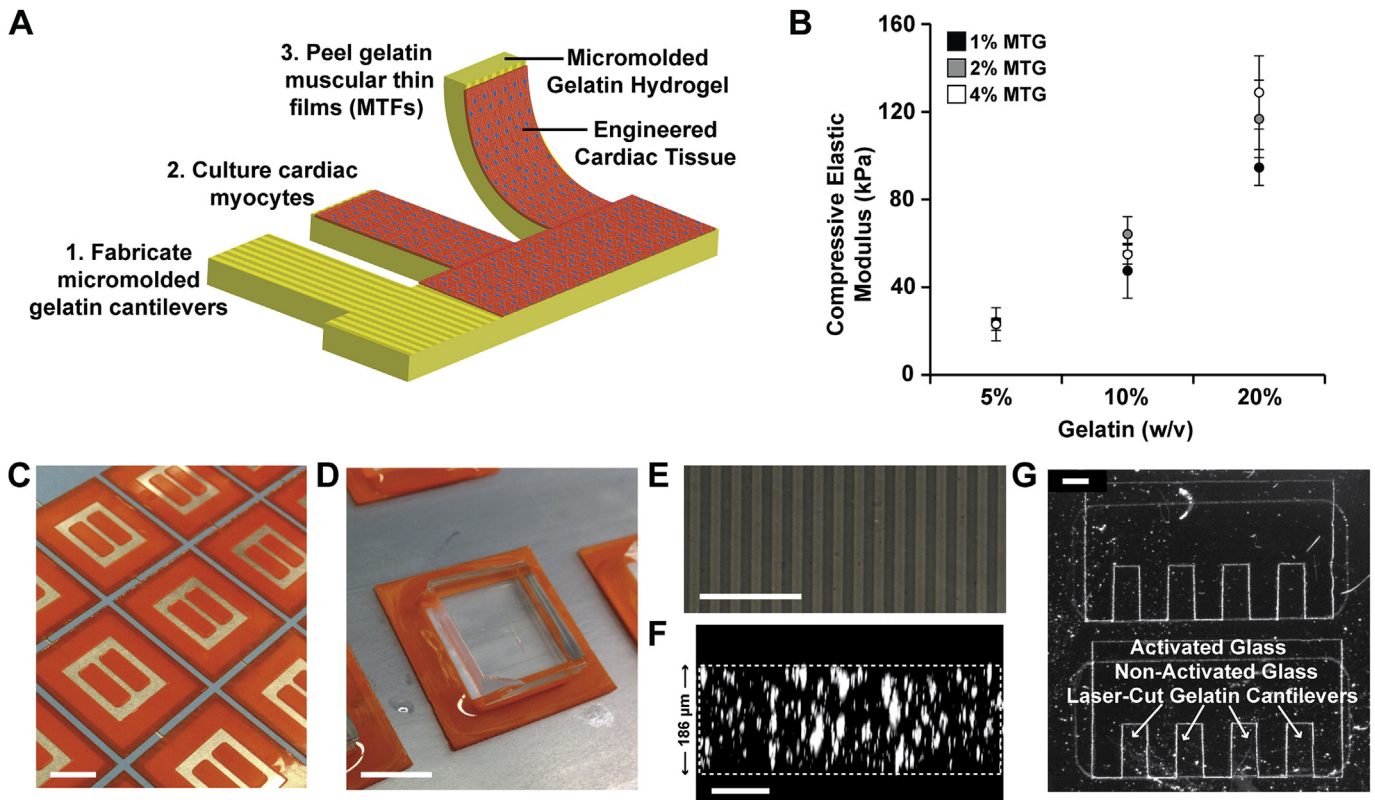


Fig. 1. Fabrication of gelatin muscular thin films (MTFs). (A) Schematic illustrating the fabrication and function of MTFs consisting of engineered cardiac tissues cultured on micromolded gelatin hydrogel cantilevers. (B) Compressive elastic moduli for different combinations of w/v gelatin and w/v microbial transglutaminase (MTG) ($n = 3$, mean \pm standard error). (C) Square glass coverslips (22 mm \times 22 mm) were covered with orange low-adhesive tape, which was laser engraved and selectively peeled to expose designated regions for activation to increase gelatin adhesion. Scale bar is 10 mm. (D) After activation, the middle pieces of tape were removed and warm gelatin solution was dropped onto the glass coverslip. PDMS stamps were placed on top of the gelatin and elevated above the coverslip surface by the tape border, resulting in a gelatin hydrogel with thickness defined by the thickness of the tape. Scale bar is 10 mm. (E) After gelatin was cured, PDMS stamps were removed, resulting in micro-molded gelatin surfaces. Scale bar is 100 μ m. (F) Side-view of confocal z-stack of micro-molded gelatin gel (10% w/v gelatin, 4% w/v microbial transglutaminase) embedded with fluorescent beads for thickness measurements. Scale bar is 100 μ m. (G) Micro-molded gelatin substrates were dehydrated and laser-cut into cantilevers for muscular thin film experiments. Cantilevers were positioned over the non-activated glass regions to facilitate peeling from the coverslip. Scale bar is 1 mm.

10%, and 20% w/v gelatin. Gelatin at 1% and 2% w/v did not form a gel. Hydrogels with 5%, 10%, and 20% w/v had compressive elastic moduli of approximately 24 kPa, 56 kPa, and 114 kPa, which were not dependent on MTG concentration (Fig. 1B). Although 5% w/v gelatin more closely matched the elastic modulus of the heart, which is 10–15 kPa [33], the gels often fractured during later fabrication steps, limiting their utility as a robust experimental platform. Thus, we chose 10% w/v gelatin hydrogels cross-linked with 4% w/v MTG for further study, which have an elastic modulus of approximately 55 kPa and better match the elastic modulus of the native heart compared to traditional culture substrates, such as plastic or glass.

MTFs consist of engineered muscle tissue cultured on polymer or hydrogel cantilevers [29] that are anchored to a glass coverslip and bend away from the coverslip as the tissue contracts [18,20] (Fig. 1A). The radius of curvature, thickness, elastic modulus, and length of each MTF are used as parameters for calculating stresses generated by the tissue [18,34], requiring uniform and defined film thickness for reliable stress measurements. Because hydrogels have a relatively low elastic modulus, hydrogel MTFs must be at least 50 μm thick to prevent over-curling, which is difficult to achieve with spin-coating. Thus, instead of spin-coating, we covered glass coverslips with low adhesive tape and cut the tape in the pattern shown in Fig. 1C using a laser engraver. We first selectively peeled the tape to expose regions not intended for MTF placement and activated those regions with aminosilane and glutaraldehyde to improve gelatin adhesion. After activation, we peeled the inner pieces of tape, but not the pieces of tape covering the edges of the coverslips. Gelatin at 10% w/v with 4% w/v MTG at 65 °C was pipetted onto the glass coverslips within the taped boundary and PDMS stamps with 10 μm -wide line features separated by 10 μm -wide and 2 μm -deep gaps were placed on the gelatin drops (Fig. 1D). The stamps had surface areas large enough such that their edges rested on the taped boundaries, propping the stamps slightly above the surfaces of the coverslips (Fig. 1D). Gelatin constructs were left to cure overnight, after which the hydrogels were rehydrated and stamps were carefully peeled off the gelatin, leaving line features embossed on the gelatin (Fig. 1E). To ensure that the thickness of the hydrogel was consistent, we embedded gelatin with fluorescent beads and used confocal microscopy to image z-stacks of the hydrogel from multiple chips fabricated from three independent gelatin solutions (Fig. 1F). The mean gelatin thickness calculated from all images was 188 μm with a standard deviation of 10 μm , representing $\sim 5\%$ of the mean, which is relatively low variation. Micromolded features were 2 μm deep and therefore contributed a negligible amount of variation to gel thickness. Thus, we fabricated micromolded gelatin hydrogels with control over elastic modulus, adhesion, thickness, and topography.

Another fabrication step important for consistent MTF stress measurements is pre-cutting cantilevers prior to cell culture to minimize user intervention and tissue damage. Previously, we reported using a laser engraver to pre-cut PDMS MTFs [20]. To translate this method to gelatin hydrogels, we dehydrated our gelatin hydrogel chips and used a laser engraver to cut gelatin MTF cantilevers positioned over the non-activated regions of the chip (Fig. 1G). After laser cutting, gelatin constructs were rehydrated and stored at 4 °C until cell seeding. Thus, we fabricated micromolded gelatin hydrogels pre-cut into cantilevers ready for cell culture and MTF measurements of tissue stress.

3.2. Engineered cardiac tissues

Cardiac tissue is highly anisotropic *in vivo* [30], which maximizes sarcomere alignment and stress generation [10]. We previously reported that alginate hydrogels can be micromolded and

functionalized with fibronectin to induce muscle tissue anisotropy [21]. We took a similar approach by micromolding gelatin to align cardiac myocytes. Importantly, gelatin is derived from collagen and thus does not require any additional extracellular matrix linkage steps to facilitate cell adhesion [23–25]. As a result, neonatal rat ventricular myocytes seeded directly onto non-micromolded gelatin and cultured for four days formed isotropic monolayers (Fig. 2A). Myocytes seeded onto micromolded gelatin formed anisotropic monolayers with relatively uniform sarcomere alignment (Fig. 2B), which was quantified by calculating the orientational order parameter (OOP) of α -actinin immunostains (Fig. 2C) [10,18]. Thus, micromolded gelatin hydrogels are favorable substrates for cardiac tissue engineering because they induce tissue anisotropy and sarcomere alignment without requiring additional protein adhesion steps.

3.3. Gelatin MTF contractility assay

To test the utility of micromolded gelatin hydrogels as MTF substrates, we cultured neonatal rat ventricular myocytes for four days on our gelatin MTF constructs, transferred them to a stereomicroscope, and carefully peeled each MTF away from the glass coverslip. Field stimulation electrodes were positioned in the dish and constructs were paced from 2 to 5 Hz. As shown in Fig. 3A, the radius of curvature decreased from diastole to peak systole. The slight sideways curving of the films is likely due to mis-alignment between the longitudinal axis of the tissue and the edge of the cantilevers because micromolding was performed manually. We calculated radii of curvature using the x-projections of the films and translated those values to contractile stresses using a modification of Stoney's equation [31]. We found that, as frequency increased, diastolic stress increased, but systolic stress remained relatively constant, causing a decrease in twitch stress (Fig. 3B). Thus, gelatin hydrogels can be used as MTF substrates to quantify contractile stresses in engineered cardiac tissues.

3.4. Culture lifetime of engineered cardiac tissues

Cardiac myocytes are highly mechanosensitive and factors such as stiffness and extracellular matrix protein are known to regulate their phenotype [11,13]. We hypothesized that engineered cardiac tissues on micromolded gelatin hydrogels would survive longer in culture compared to fibronectin-micropatterned PDMS because gelatin hydrogels are more biomimetic in terms of mechanical and chemical properties. To quantify this, we counted the number of gelatin and fibronectin-PDMS constructs with beating and non-beating engineered cardiac tissues each day for four weeks because spontaneous contraction is correlated to a healthy, functional cardiac phenotype [35,36]. We found that, on PDMS micropatterned with fibronectin, the number of constructs beating spontaneously consistently dropped over two weeks and was approximately 40% after four weeks of culture due to factors such as delamination and loss of striations (Fig. 4A). Conversely, on micromolded gelatin, the number of beating constructs stayed above 60% the entire four weeks of culture and was approximately 80% at the end of four weeks (Fig. 4A). Thus, micromolded gelatin extends the lifetime of engineered cardiac tissues and supports the maintenance of a more physiological phenotype compared to PDMS micropatterned with fibronectin.

To quantify the functional effects of long-term culture on cardiac tissues cultured on micromolded gelatin, we next made MTF measurements of stress in constructs cultured for 25 days and compared them to tissues assayed after four days (Fig. 4B–D). We collected contractile stress measurements from tissues engineered across multiple primary harvest cycles, explaining the variability

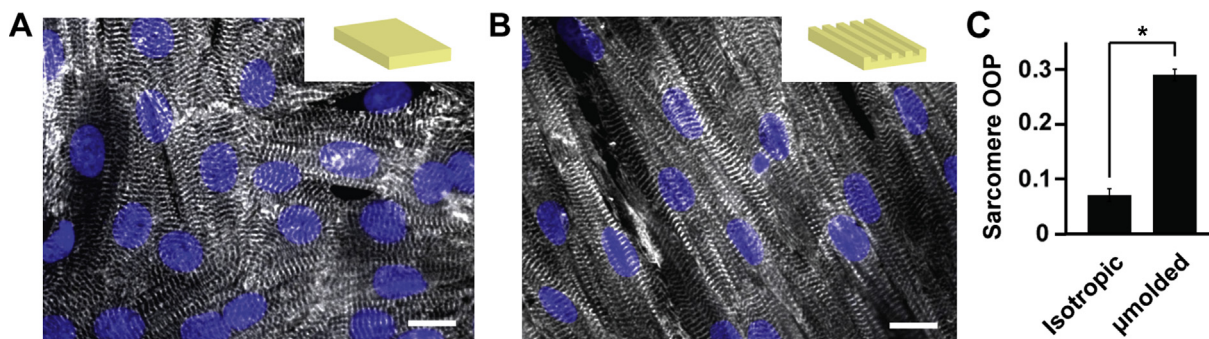


Fig. 2. Engineering anisotropic cardiac tissues with micro-molded gelatin hydrogels. Neonatal rat ventricular myocytes cultured for four days on flat (A) and micro-molded (B) gelatin hydrogels (10% w/v gelatin, 4% w/v microbial transglutaminase) form isotropic and anisotropic monolayers, respectively. White: α -actinin, blue: nuclei. Scale bar is 10 μm . (C) Tissues on micromolded gels have a significantly higher sarcomere orientational order parameter (OOP) compared to flat gels. Gels were 10% w/v gelatin, 4% w/v microbial transglutaminase. Bars represent standard error, $n = 4$ for isotropic, $n = 5$ for micromolded, * indicates $p < 0.05$.

between values in Figs. 3 and 4. On Day 25, MTFs contracted and could be paced from 2 to 5 Hz, similar to tissues cultured for four days. Interestingly, diastolic (Fig. 4B) and systolic (Fig. 4C) stresses were not significantly different between four and 25 days, but twitch stresses were significantly higher at 25 days compared to four days at 2–4 Hz pacing (Fig. 4D). This suggests that cardiac tissues cultured on micromolded gelatin are equally functional after four and 25 days and even show signs of improved function with extended time in culture. Further studies are needed to understand the mechanisms underlying these functional differences, such as rigorous quantification of sarcomere alignment, calcium handling, and gene expression over time in culture.

3.5. Metabolic phenotype of engineered cardiac tissues

Because cardiac tissues cultured on gelatin had longer functional lifetimes compared to those cultured on PDMS, we asked if mitochondrial function was different between myocytes on gelatin and PDMS. To probe differences in metabolism, we used an

extracellular flux analyzer to measure oxygen consumption rates (OCR) at baseline and after addition of compounds that alter mitochondrial function, as shown for two representative tissues in Fig. 5A [37]. The measurement instrument requires measurements to be performed in a 24-well plate, which obstructs microcontact printing and micromolding. Thus, we coated 24-well plates uniformly with 5% w/v gelatin cross-linked with 1% w/v MTG or with PDMS, which was then coated with fibronectin. We could not use 10% w/v gelatin because the plates require a small volume of gelatin that cross-linked too quickly at higher concentrations, preventing formation of a smooth surface for cell culture.

After loading the plate into extracellular flux analyzer, we first measured baseline OCR before adding any compounds. We then added oligomycin, which inhibits ATP synthase and blocks ATP production [37]. The difference between baseline OCR and after oligomycin injection indicates OCR dedicated to ATP production (Fig. 5A). The uncoupling agent carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was then added to deplete the mitochondrial membrane potential, causing OCR to

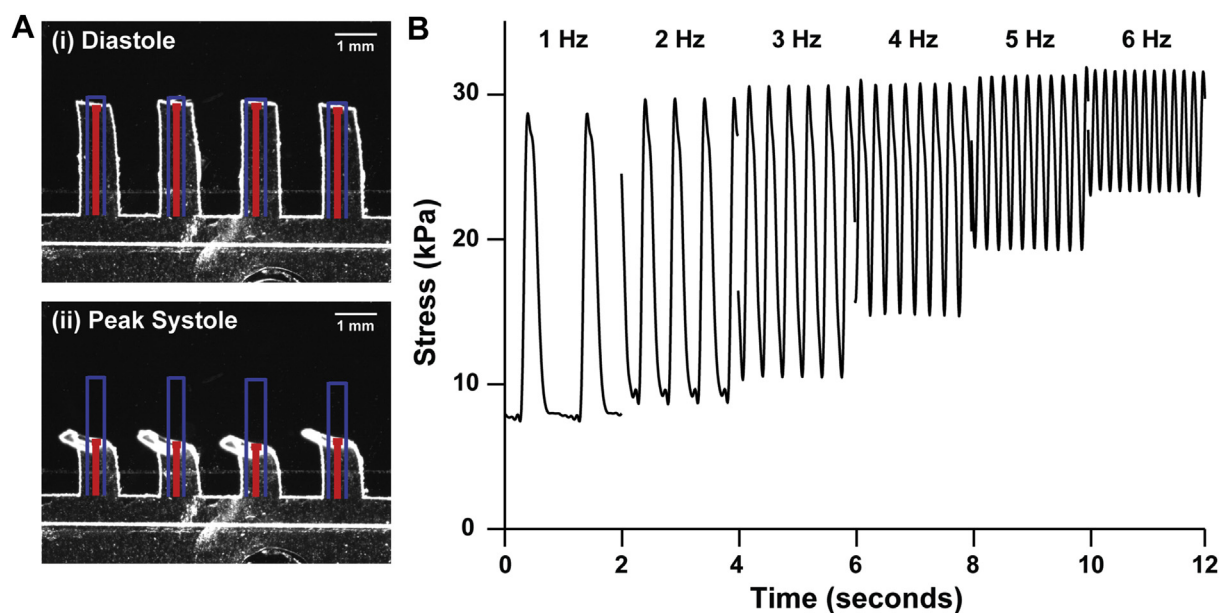


Fig. 3. Measuring contractile stress with gelatin muscular thin films (MTFs). (A) Engineered neonatal rat cardiac tissues cultured for four days on laser-cut, micro-molded gelatin cantilevers (10% w/v gelatin, 4% w/v microbial transglutaminase) that are relaxed at diastole (i) and bend away from the glass coverslip at peak systole (ii). Blue bars indicate initial length, red bars indicate x-projection of the radius of curvature. (B) Stress traces for a representative Day 4 gelatin MTF at increasing pacing frequencies.

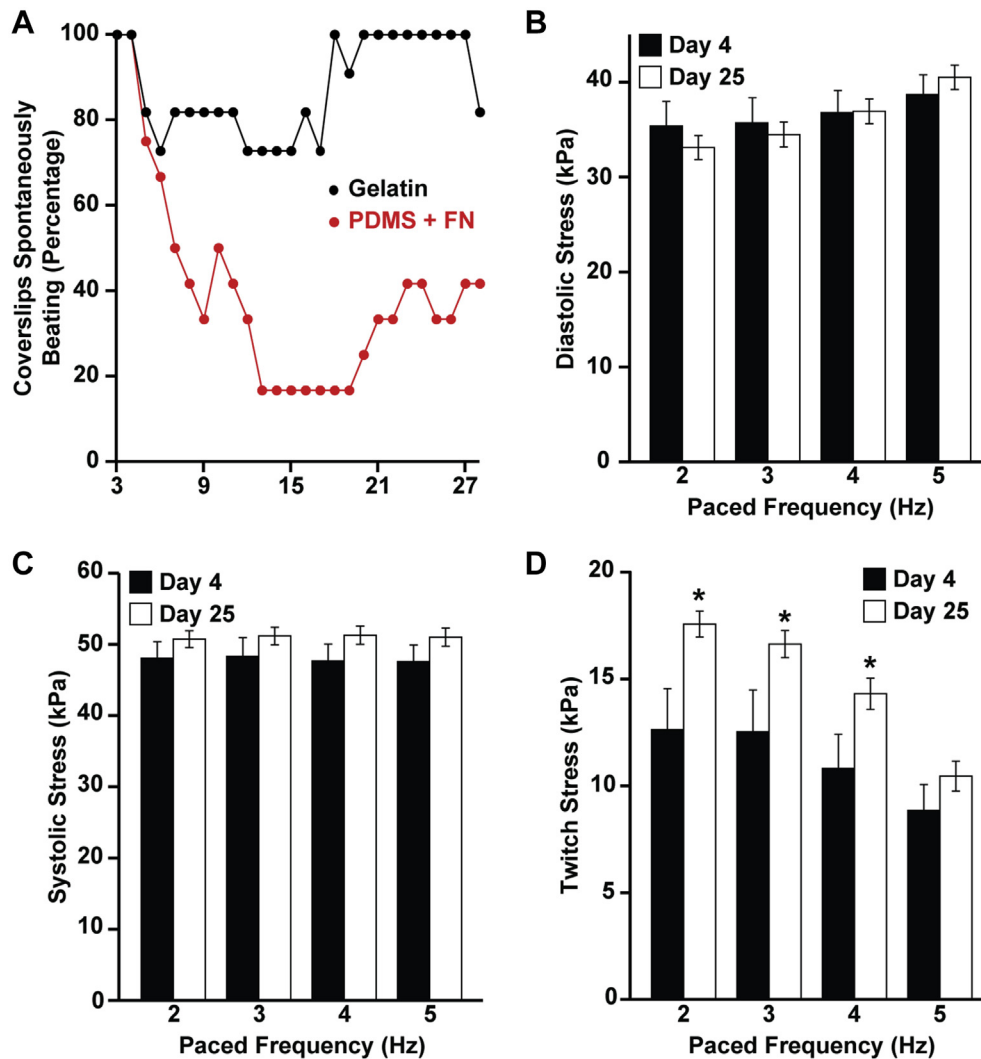


Fig. 4. Gelatin extends the lifetime of engineered cardiac tissues in culture. (A) Percentage of coverslips with spontaneously contracting neonatal rat cardiac myocyte tissues on micromolded gelatin (10% w/v gelatin, 4% w/v microbial transglutaminase, black, $n = 11$) and PDMS micropatterned with fibronectin (red, $n = 12$). Diastolic (B), systolic (C), and twitch (D) stresses for Day 4 (black) and Day 25 (white) gelatin MTFs (10% w/v gelatin, 4% w/v microbial transglutaminase) as a function of pacing frequency. Bars represent standard error, $n = 24$ MTFs for Day 4, $n = 24$ MTFs for Day 25, *indicates $p < 0.05$ relative to Day 4 at the same frequency.

increase to its maximum [37]. The difference between baseline OCR and after FCCP injection indicates the spare respiratory capacity of the tissue (Fig. 5A). Finally, antimycin and rotenone were added to inhibit the electron transport chain and prevent mitochondria from consuming oxygen [37], revealing OCR from non-mitochondrial sources (Fig. 5A). To determine basal respiration, OCR from non-mitochondrial sources was subtracted from baseline OCR (Fig. 5A). Collectively, these measurements provide metrics of mitochondrial function.

As shown in Fig. 5B, average basal respiration and average ATP production were slightly higher on PDMS, but were not statistically different. However, average spare respiratory capacity was significantly higher on gelatin compared to PDMS, suggesting that myocytes cultured on gelatin have a larger bioenergetic reserve to utilize in cases of metabolic stress, which likely improves their health and survival [38,39]. Average non-mitochondrial respiration was equivalent in myocytes on gelatin and PDMS. Thus, our results suggest that myocytes on gelatin potentially survive longer in culture because they have a higher spare respiratory capacity compared to those on PDMS, which equips them to better adapt to increased energy demands and metabolic stress.

3.6. Human iPS-derived cardiac myocyte tissues

Because our data suggests that micromolded gelatin is a favorable substrate for engineering biomimetic cardiac tissues, quantifying contractile stresses, and extending the culture lifetime of neonatal rat ventricular myocytes, we next asked if micromolded gelatin could also be a platform for human iPS cell-derived cardiac myocytes. After two days of pre-plating, we seeded human iPS cell-derived cardiac myocytes on gelatin MTF chips and cultured them for 28 days. Myocytes aligned into anisotropic tissues and expressed sarcomeres (Fig. 6A). We performed MTF experiments with Day 28 human iPS-derived cardiac myocytes on micromolded gelatin MTFs (Fig. 6B) and found that they responded to pacing and generated quantifiable contractile stresses (Fig. 6C). The maximum pacing frequency captured by the human iPS-derived cardiac tissues was 3.5 Hz, which was much lower than neonatal rat tissues and is likely due to species-dependent differences in native beat rate. Above 3.5 Hz, the human tissues only captured every other pacing stimulus. The magnitude of twitch stress in human iPS-derived cardiac tissues was also lower than neonatal rat cardiac tissues. This can be attributed to the immature phenotype of iPS-

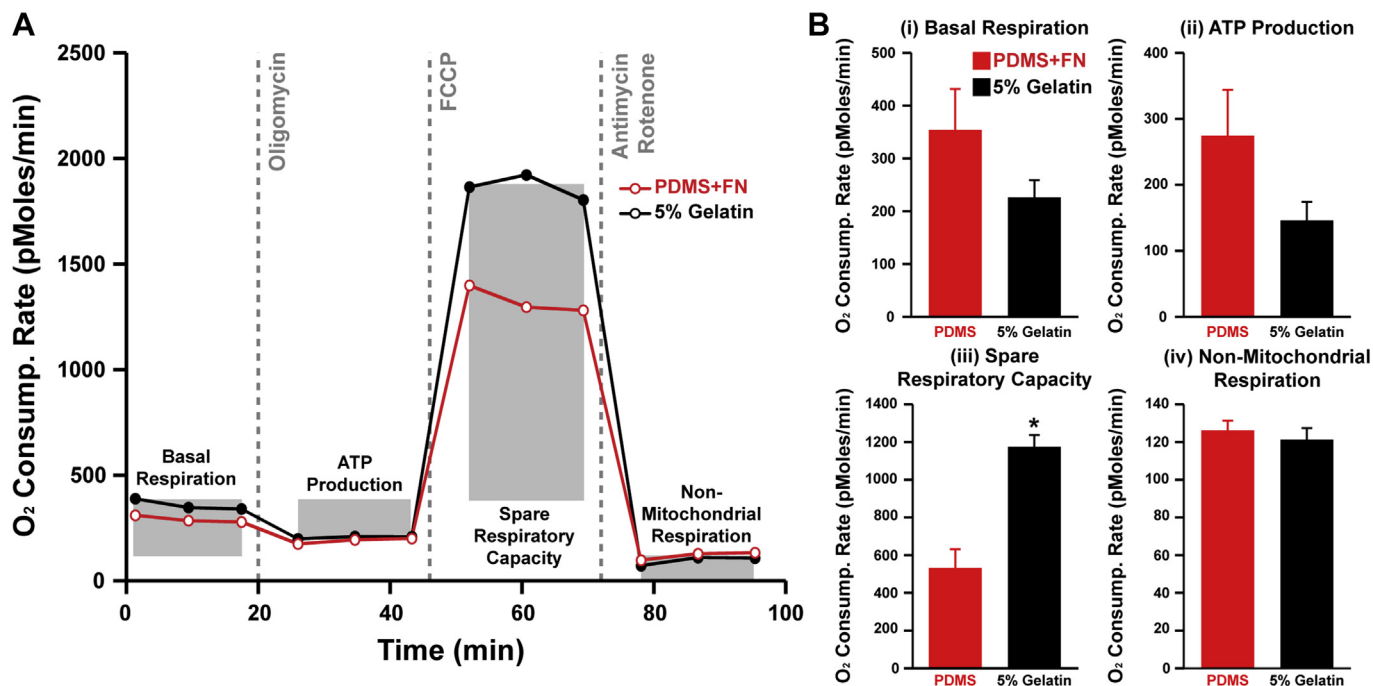


Fig. 5. Metabolic differences in engineered cardiac tissues on gelatin and PDMS. (A) Experimental measurements of oxygen consumption rates for single representative isotropic neonatal rat cardiac tissues cultured on 5% w/v gelatin with 1% w/v microbial transglutaminase (black) and PDMS coated with fibronectin (FN) (red). Compounds added to determine different metabolic parameters (highlighted in gray) are indicated. (B) Average (i) basal respiration, (ii) ATP production, (iii) spare respiratory capacity, and (iv) non-mitochondrial respiration for each substrate. Note that Panel A is from single representative tissues while Panel B is average data for all tissues. Bars represent standard error, $n = 22$ tissues for gelatin, $n = 17$ tissues for PDMS. * indicates $p < 0.05$ compared to PDMS + FN.

derived cardiac myocytes compared to primary cardiac myocytes, similar to previously reported results [40]. As the protocols for differentiation and maturation of human iPS-derived cardiac myocytes improve, the contractile stresses will likely also increase in magnitude. Thus, micromolded gelatin MTFs hold promise as a platform for long-term culture of human-relevant cardiac myocytes.

4. Discussion

With this study, we fabricated MTFs consisting of micromolded gelatin hydrogels and engineered neonatal rat cardiac tissues. Micromolded gelatin had several advantages over alternative substrates, including its composition as a native extracellular matrix protein, its tunable elastic modulus within physiological ranges, and its ability to extend the culture lifetime of engineered cardiac tissues, potentially by reducing metabolic stress. We have also shown that gelatin MTFs are amenable to human iPS-derived cardiac myocyte culture, illustrating their potential as a human-relevant platform for drug screening.

One of the major advantages of micromolded gelatin as a culture substrate is that engineered cardiac tissues maintained spontaneous contractile activity for several weeks, which is much longer than on fibronectin-micropatterned PDMS. There are several important differences between these two culture substrates, each of which could contribute to this phenomenon. First, gelatin hydrogels are much softer than PDMS and are closer to physiological ranges of elastic moduli. Substrates with physiological elastic modulus have been shown to promote the maturation [41,42] and maximize the contractile output [12] of cardiac myocytes. Cardiac myocytes on each substrate are also bound to different extracellular matrix proteins, as gelatin is derived from collagen [23]. Collagen and fibronectin bind to different integrin receptors in the cell membrane [43,44], which regulate processes

such as mechanotransduction and cell signaling [11]. Because collagen is the dominant extracellular matrix protein in the native heart [22], gelatin could improve the health and survival of cardiac myocytes by providing a more biomimetic chemical microenvironment compared to fibronectin. The topography of the substrates is also unique. Fibronectin micropatterned on PDMS has a height of less than 10 nm [45], whereas our micromolded gelatin had features approximately 2 μm high. Substrates with more three-dimensional topography have been shown to improve sarcomerogenesis [46], cell–cell coupling [47], and conduction velocity [48]. Another contributing factor could be that fibroblast attachment and proliferation is higher on stiffer substrates [49,50]. Our primary neonatal rat ventricular myocyte cultures have some contamination from non-myocyte cell populations, which could potentially attach, spread, and divide more easily on PDMS, causing delamination of cardiac myocytes over long-term culture [51]. More studies are needed to tease apart these different cues to identify which parameters are most important for improving the health and lifetime of engineered cardiac tissues.

Cardiac myocytes are highly metabolically active compared to other cell types [52] and have a high density of mitochondria, comprising over 20% of the cellular volume of cardiac myocytes in rats, humans, and other mammals [53]. Under physiological conditions, mitochondria supply myocytes with ATP for normal cardiac function [52]. Mitochondria also possess a reserve capacity, which allows ATP to be generated above the basal level in situations of increased energy demands [37]. It has been suggested that the reserve capacity is important for myocytes to respond to stresses in their environment because they can quickly adapt to elevated energy demands [38,39]. We found that the reserve capacity of myocytes on gelatin was significantly higher than those cultured on fibronectin-coated PDMS, which could contribute to their longer culture lifetime. Interestingly, oxygen consumption increases in hypertrophy and heart failure [54] and the spare capacity is

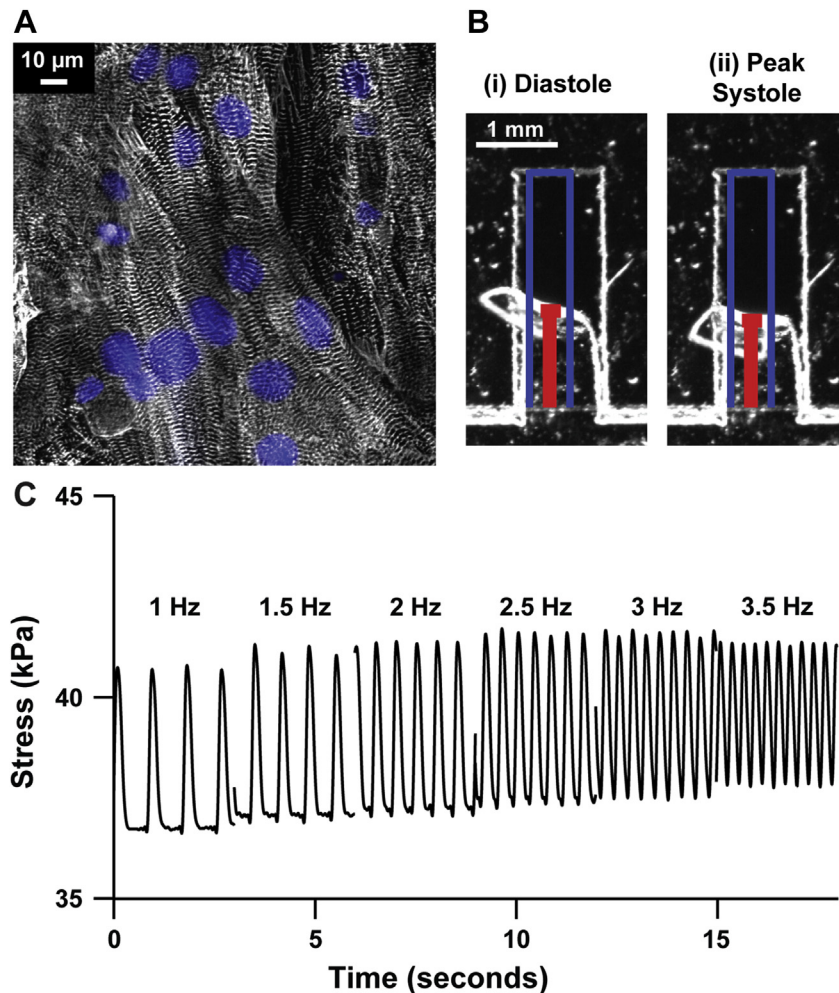


Fig. 6. Extended culture of human iPS-derived cardiac myocytes on micromolded gelatin MTFs. (A) Immunostain of human iPS-derived cardiac tissue cultured on micromolded gelatin (10% w/v gelatin, 4% w/v microbial transglutaminase) for 28 days. White: α -actinin, blue: nuclei. Scale bar is 10 μ m. (B) Images of MTFs with human iPS-derived cardiac myocytes at (i) diastole and (ii) systole. Blue bars indicate initial length, red bars indicate x -projection of the radius of curvature. (C) Representative stress trace from human iPS-derived cardiac tissue on micromolded gelatin MTF (10% w/v gelatin, 4% w/v microbial transglutaminase) cultured for 28 days.

depleted in failing hearts [55]. Our study suggests that parameters such as fibrosis or extracellular matrix remodeling could contribute to the metabolic changes observed in different cardiac pathologies.

Extending the culture lifetime of engineered cardiac tissues is especially beneficial when considering our constructs as a platform for drug screening, particularly for drugs that demonstrate cardiotoxicity on a chronic timescale, such as cytotoxic cancer drugs [3,4]. These drugs warrant extended exposure times to cause an adverse response *in vivo* [5–7], indicating a need for cell culture substrates that can maintain functional engineered cardiac tissues for several weeks for *in vitro* studies to be comparable. Another potential application for gelatin substrates is maturation of human iPS cell-derived cardiac myocytes. Studies have shown that human iPS cell-derived cardiac myocytes need at least 30 days to differentiate and acquire a more mature phenotype in culture [17,56]. Thus, substrates that support longterm culture are useful tools to mature human iPS cell-derived cardiac myocytes in culture and screen drugs for chronic cardiotoxicity.

Currently, the pharmaceutical industry relies on animal models that are not human-relevant and cell culture systems that lack important aspects of native cellular microenvironments. Organs on Chips that combine biomimetic substrates, such as micromolded gelatin, with human-relevant cells, such as human iPS cell-derived cardiac myocytes, have valuable potential to become new *in vitro*

models that can be quickly and cheaply utilized during the pre-clinical stages of drug development and safety screening [57,58]. Continuing to evolve this platform by integrating patient-specific cells, moving towards 3-dimensional systems, and coupling to other Organ on Chip systems via microfluidics, for example, has significant promise for developing new toolsets that can better predict drug toxicity and efficacy.

5. Conclusions

With this study, we engineered anisotropic cardiac tissues on micromolded gelatin hydrogel cantilevers to function as MTFs for quantifying contractile tissue stresses. Gelatin hydrogels had several advantages over previous MTF substrates, such as fibronectin-patterned PDMS and alginate. Unlike PDMS, gelatin could be fabricated with physiological elastic modulus. Because gelatin is derived from collagen, cardiac myocytes adhered directly to the hydrogel without any additional protein adhesion steps, unlike alginate. Neonatal rat cardiac myocyte tissues maintained spontaneous beating activity and consistent levels of contractile stresses for over three weeks in culture, indicating that gelatin hydrogels are better suited for long-term culture compared to fibronectin-patterned PDMS, which typically supports a culture lifetime of about one week. Rat myocytes on gelatin hydrogels had

higher spare respiratory capacity compared to those on fibronectin-coated PDMS, suggesting that myocytes on gelatin can better adapt to elevated energy demands in times of metabolic stress. We also showed that human iPSC-derived cardiac myocytes could be cultured on gelatin hydrogel MTFs for over three weeks, indicating that this platform is compatible with human-relevant cell types. Thus, micromolded gelatin hydrogel MTFs support long-term culture of engineered rat and human cardiac tissues, potentially because they better recapitulate the chemical and mechanical microenvironment of the heart.

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