Contact guidance for cardiac tissue engineering using 3D bioprinted gelatin patterned hydrogel

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Abstract
Here, we have developed a 3D bioprinted microchanneled gelatin hydrogel that promotes human mesenchymal stem cell (hMSC) myocardial commitment and supports native cardiomyocytes (CMs) contractile functionality. Firstly, we studied the effect of bioprinted microchanneled hydrogel on the alignment, elongation, and differentiation of hMSC. Notably, the cells displayed well defined F-actin anisotropy and elongated morphology on the microchanneled hydrogel, hence showing the effects of topographical control over cell behavior. Furthermore, the aligned stem cells showed myocardial lineage commitment, as detected using mature cardiac markers. The fluorescence-activated cell sorting analysis also confirmed a significant increase in the commitment towards myocardial tissue lineage. Moreover, seeded CMs were found to be more aligned and demonstrated synchronized beating on microchanneled hydrogel as compared to the unpatterned hydrogel. Overall, our study proved that microchanneled hydrogel scaffold produced by 3D bioprinting induces myocardial differentiation of stem cells as well as supports CMs growth and contractility. Applications of this approach may be beneficial for generating in vitro cardiac model systems to physiological and cardiotoxicity studies as well as in vivo generating custom designed cell impregnated constructs for tissue engineering and regenerative medicine applications.

1. Introduction
Following myocardial infarction (MI), subsequent cell death and matrix remodeling at the MI foci lead to the degradation of fibrillar collagen network and the accumulation of fibrotic scar tissue. This causes mechanical dysfunction of the ventricular wall that can lead to wall thinning, ventricular dilation and the subsequent sequelae of congestive heart failure and myocardial rupture [1, 2].

It has been proposed that ventricular function can be improved by augmenting or replacing the necrotic tissue with tissue engineered construct referred to as a ‘heart patch’ [1–6]. Decellularized extracellular matrix, synthetic polymers and hydrogels have been studied as possible candidates to form such heart patches [3]. The heart patch may assist in supporting the mechanical contraction of the heart however their functionality can be improved by supporting the delivery of contractile cells [3, 7]. Unfortunately, contractile cardiomyocytes have an extremely low proliferation rate. Hence they are not a feasible source for ex vivo expansion and seeding [3, 8]. Stem cell solutions are currently being sought to generate sufficient functional cardiomyocytes for this application [3, 9, 10].

Additive manufacturing techniques or 3D printing have emerged as useful approaches in tissue engineering to fabricate cell supporting scaffolds [11]. Indeed, 3D printing has been shown to generate features that influence the behavior of the seeded cells such as the printing of cell aligning features [12–14]. Such 3D printed cell aligning grooves and channels are usually fabricated from stiff synthetic polymers by extruding the polymer in a molten state or dissolved in volatile solvents, thus allowing rapid post deposition setting.
through cooling or solvent evaporation, respectively [15]. Natural extracellular matrix protein based polymers, such as gelatin are usually deliverable as aqueous based hydrogels which are more complex in nature to bioprint due to the requirement of a crosslinker for stability [11, 16].

The 3D bioprinting of channels and grooves are of interest to tissue engineering research since behavior, phenotype and morphology of cells are strongly influenced by surface features, an effect referred to as topological or surface guidance [17–19]. In particular, stem cells lineage commitment can be modulated by such surface guidance [20–22]. Aligned fibers, grooves, and ridges have been used to orientate cells and stimulate the differentiation of stem cells towards adipogenic, osteogenic, neurogenic, myogenic and cardiomyogenic phenotype [23–26]. For example, wide channels of shallow nanoscale depth and micron width on a rigid poly(methylcrylate) surface stimulated osteogenesis in human mesenchymal stem cell (hMSCs) [26], whereas hMSCs cultured on 350 nm scale grooves on polydimethylsiloxane expressed neuronogenesis associated markers [27]. Interestingly, aligned orientation has proven to be a strong promoter of myocardial lineage for stem cells. Furthermore, aligning features improve the organization and maturity of cardiomyocytes and ESC derived cardiomyocytes [6, 28–30]. This controlled alignment of the seeded cells within a cardiac patch allows for the matching of the direction of cardiomyocyte beating with the functional anisotropy within the host myocardium [31].

Here we aim to produce a cell aligning hydrogel microchannel scaffold by 3D extrusion bioprinting as demonstrated in (figure 1(A)). The scaffold consists of a gelatin hydrogel film with 3D printed gelatin features forming the microchannel walls. Gelatin has been frequently studied as a potential material to fabricate tissue engineered myocardial scaffolds [3, 32]. Previously, it has also been used to create cardiomyocyte aligning grooves. Tsang et al created hydrogel channels using photodegradable gelatin methacrylamide (GelMa). The inclusion of the photodegradable o-nitrobenzyl ester group allowed for patterning using laser printed photomasks. Channels created in this manner induced alignment in neonatal rat cardiomyocyte and improved beating characteristics [29].

The challenge of 3D printing hydrogels is in producing a printable hydrogel of suitable viscosity that can be delivered in an unbroken trace with minimal spreading whilst retaining its vertical height [33]. Since such a syringe deliverable and biocompatible hydrogels are aqueous, they require post deposition crosslinking for stability. The photoinitiated crosslinking of soluble hydrogels is commonly used to create cell friendly scaffolds. However, in this study, the gelatin hydrogel is stabilized through the enzymatic action of microbial transglutaminase (mTgase). The mTgase is derived from Streptococcus moharaense and widely used in food biotechnology [34, 35]. It has the advantages of calcium independence, broad substrate specificity, a wide range of reaction conditions and low cost [36–39]. It catalyzes the crosslinking transamidation reaction between lysine to glutamine residues forming ε-(γ-glutamyl) lysine bonds [36, 39, 40]. These amino acid ratios have a suitable prevalence in collagen and its derivative, gelatin, to support the production of stable hydrogel [37, 41–43]. The mTgase crosslinks gelatin to form a highly stable and cyto-compatible hydrogel within reasonable time frame [37], without the toxicity of any chemical crosslinker and the reaction occurs in more physiological like environments [38]. We have previously found that mTgase can be used in bioprinting to stabilize the print [33]. The enzymes are viewed as having considerable promise for regenerative bioengineering [37, 39]. However, to date, few applications have been researched. The mTgase crosslinked gelatin has been studied in vivo, for applications such as a potential wound sealant and as a retina adhesive [44].

In this study, we aim to examine the application of 3D bioprinted mTgase crosslinked gelatin in generating a cell guidance scaffold with relevance to use in tissue engineered cardiac regeneration. The gelatin scaffold with 3D bioprinted patterning was assessed for the induction of stem cell myocardial lineage commitment. The effect of varying channel width was also examined as regards to topographic guidance for cell attachment. This was assessed by cell morphology, focal adhesion elongation, and F-actin stress fiber arrangement. The cardiomyogenic lineage commitment of aligned hSMCs was determined with the use of mature cardiomyocyte protein markers and fluorescence-activated cell sorting (FACS) analysis. In addition, the 3D bioprinted microchannel scaffold was seeded with native CMs and the attachment, alignment and rhythmic beating were compared to that of the plain hydrogel surface.

2. Experimental section

2.1. Fabrication and crosslinking of gelatin hydrogel film

A 5% gelatin (Bloom 300, type A; Sigma-Aldrich) solution was prepared by dissolving solid gelatin in phosphate buffer saline (PBS) and warming at 60 °C for 2 h. Then 3 wt% mTgase (TG-BW-MH, 100U/gram, EC 2.3.2.13 with sodium caseinate and maltodextrin additives, Ajinomoto) was mixed with gelatin solution and sterilized with 0.2 μm membrane filter. The gelatin mixture (100 μl) was then immediately added onto a transparency sheet and square shaped glass coverslip was quickly placed on the drop. Gelatin mixture was allowed to crosslink at 37 °C.
overnight followed by gelatin coated coverslip detachment from the sheet.

2.2. 3D bioprinting of gelatin hydrogel

Gelatin line features were 3D printed onto the gelatin hydrogel film to form cell aligning microchannels using a pressure-controlled robotic dispensing system (Janome 2300N [13, 14]). A 5% gelatin/3% mTgase mixture was loaded into a 5 ml dispensing syringe equipped with 30 gauge needle (inner diameter 160 μm) which in turn placed into the robotic dispensing system. The pattern was drawn using manufacturer supplied JR-C points software. The dispensing pressure and robot arm speed were set to 0.1 MPa and 10 mm s⁻¹, respectively. After printing, gelatin patterned samples were placed in the incubator at 37 °C overnight to facilitate gelatin pattern crosslinking. Surface profiler (model: ASIQ, KLA Tencor) was used to measure the thickness of printed gelatin pattern (supplementary figure (S2) is available online at stacks.iop.org/BF/10/025003/mmedia). Samples were kept at 4 °C overnight before the measurement.

2.3. Gelatin hydrogel characterization

The characterization of mTgase crosslinked gelatin hydrogels was performed in detail in previous publication [33]. The shear storage modulus (G’) of noncrosslinked and crosslinked gelatin hydrogel samples (n = 3) was computed using cone and plate rheometer (Physica MCR 501, Anton Paar) according to manufacturer’s protocol. Briefly, CP25 cone-plate geometry with 25 mm diameter was used for in situ time-sweep test. Dynamic time-sweep tests were done by loading 50 μl of gelatin hydrogel solution containing crosslinker on the plate and allowed to crosslink for an hour. All samples were tested at 0.1% strain amplitude at an angular frequency of 1–100 rad s⁻¹ within the linear viscoelastic regime. The elastic modulus was calculated from storage modulus values as an application of the Rheoplus software supplied by the manufacturer.

Hydrogel tensile stress was measured using MTS Criterion Model 42 instrument. Three individual rectangular hydrogel strips were analyzed to measure the tensile stress. The ends of crosslinked hydrogel strips...
(0.5 cm × 2 cm, 1 mm thick) were tightened into two holders in an unstressed state. The strips were then stretched with 250 N load cell at 0.5 mm s⁻¹ speed until sample failure occurred.

The gelatin hydrogel swelling ratios were evaluated to study water uptake capacity [45]. Gelatin patterned samples (n = 3) were immersed in PBS at 37 °C for 7, 14 and 21 days. Samples were weighed before immersing in PBS (W₀). PBS was changed every three days. After a defined time interval, samples were collected, weighed (Wₙ) and vacuum dried completely. Vacuum dried samples were further weighed to determine their weights (W₀). The swelling ratio of each sample was calculated by using the following formula.

Swelling ratio (%): \( W₀ - Wₙ / W₀ \times 100 \).

2.4. Cell culture and seeding
Bone marrow derived hMSCs were purchased from Lonza (Cambrex). Low glucose Dulbecco’s modified eagle’s medium (DMEM) containing L-glutamine (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (PAA) and 1% antibiotic/anti-mycotic solution (PAA) was prepared and used for cell culture. The cells were incubated at 37 °C in humidified atmosphere of 5% CO₂. A solution of 0.25% trypsin-EDTA (Invitrogen) was used for cell detachment purpose. Only early passage stem cells (passage 3–5) were used for cell culture experiments. Before cell seeding, gelatin hydrogel samples were sterilized with 70% ethanol for 1 h and subsequently UV sterilized for 2 h. Samples were then soaked in DMEM for one day for equilibration. Cells were finally seeded on samples in six well plates at a concentration of 5500 cells cm⁻². Neonatal rat cardiomyocytes (CMs) (Lonza) were then seeded (2 × 10⁴ cells cm⁻²) on microchannelled and plain hydrogel scaffolds (22 mm × 22 mm) in six well plate and cell proliferation monitored for 9 days.

2.5. Immunocytochemistry and microscopy
Immunostaining was performed on day 7 and 14 after cell seeding. Cells were fixed with 4% paraformaldehyde (PFA) solution for 10 min at room temperature followed by treatment with 0.1% Triton X-100 for 5 min. Freshly prepared 5% bovine serum albumin (BSA) was used for cell blocking purpose. Samples were incubated with primary antibodies, mouse monoclonal anti cardiac myosin heavy chain IgG (1:400, Abcam), mouse monoclonal anti cardiac troponin T IgG (1:200, Abcam) and mouse monoclonal vinculin IgG (1:400, Millipore) for 1 h at 37 °C, washed thrice in PBS and subsequently labeled with Alexa Fluor 488 goat anti mouse IgG (1:400, Invitrogen). For F-actin staining, cells were stained with rhodamine phalloidin dye (1:400, Invitrogen). For cell nucleus staining, 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used. Fluorescent images were captured with an Eclipse 80i upright microscope (Nikon) using a 20× objective lens. An optical microscope (Nikon eclipse TS100) was used to observe cell morphology and gelatin patterned samples. Bright field images of CMs were captured using Nikon Eclipse Ti-S microscope with a 4× objective lens. To examine the CMs beating frequency, movies were recorded with a microscope with a camera (Nikon Digital Sight DS-Fi1c) and video capture software at 10× and 4× magnification after day 2, 4, 7 and 9. The beating frequency was estimated after analyzing 3 individual samples from each group.

2.6. Cell proliferation assay
The Alamar blue assay (Invitrogen) was employed to determine the cell proliferation rate as well as gelatin hydrogel biocompatibility. The Alamar blue reagent (10% v/v, 0.2 ml) was added to each sample containing 1 ml of culture medium in six well plates and incubated in the dark for 1 h at 37 °C. Then 0.1 ml of culture medium containing reagent and transferred to 96 well plates. Spectrophotometric plate reader was utilized to read excitation and emission fluorescence at 560 nm and 585 nm, respectively.

2.7. FACS analysis
hMSCs were cultured for 7 days on the gelatin microchannelled and plain hydrogel surface. Cells were fixed with 4% PFA solution for 10 min, trypsinized and collected. Cells were then permeabilized with 0.25% Triton X-100 solution for 10 min and blocked with 0.5% BSA for 30 min after washing. Further, cells were incubated with β-MHC primary antibody (1:50, Abcam) for one hour at 37 °C followed by incubation with FITC-labeled secondary antibody (1:250) for 1 h. Finally, after washing, cells were analyzed for β-MHC expression using Millipore GUAVA easyCyte HT flow cytometer. Cell samples incubated with only FITC-labeled secondary antibody were considered as the control.

2.8. Image analysis and statistical study
Cell aspect ratio is the ratio of cell length to cell width. To compute the cell aspect ratio, cell length and width were quantified by ‘Measure’ function of ImageJ software. Cell area in the actin stained cell images was quantified by, firstly, manually marking the cell outline and then using ImageJ ‘Measure’ function. ‘2D fast Fourier transform’ (FFT) function of ImageJ was employed to translate spatial distribution data into mathematically defined optical data [46]. FFT frequency plot of focal adhesion distribution demonstrated the degree of focal adhesion alignment by displaying the grayscale pixels distributed in patterns around the origin. One-way ANOVA method was used to show the statistical significance. A p-value <0.05 was considered statistically significant.
3. Results

3.1. Bioprinting of crosslinked gelatin forms a stable hydrogel and supports cell growth

Previously, we successfully optimized associated parameters (e.g. monomer and crosslinker concentration, working temperature etc) to bioprint a cellularized 3D 5% gelatin construct [33]. Here, we implemented a similar method for gelatin hydrogel formulation. Gelatin film coated on a glass coverslip was fabricated and served as a base for 3D gelatin bioprinting to develop gelatin patterned scaffold. The bioprinted gelatin trace emerges from 30 gauge needle and undergoes limited lateral spreading to form a microchannel of ∼200–250 μm width. Therefore, the microchannels printed with 500 μm spacing (or line pitch) produced an effective spacing of ∼250–300 μm between two adjacent microchannels (figures 1(B) and (C)), (supplementary figures (S1) and (S2)). In addition, microchannels printed with 1000 μm spacing created an effective spacing of ∼800 μm between two adjacent microchannels (supplementary figure (S1)). The plotted print coordinates generated using JR-C points software for the printing could be viewed as graphical representations in excel spreadsheet software [13] (supplementary figure (S3)). For rat neonatal CM seeding, microchannels were printed with 300 μm spacing (since CMs are smaller cells than hMSCs), producing an effective spacing of ∼100 μm between two adjacent microchannels (supplementary figure (S1)).

The elastic modulus of crosslinked gelatin was evaluated and it was found to be ∼80 kPa (figure 1(D)). The stress-strain behavior of the crosslinked hydrogels was also determined to assess their tensile strength (supplementary figure (S4)). The swelling ratio percentage was determined over the period of three weeks to check the water uptake capacity of crosslinked gelatin hydrogel which was in the range of 110% to 120%. This small increase is indicative of the formation of well-crosslinked and stable hydrogel [47]. Transglutaminase facilitates the formation of considerably stable gelatin hydrogel, indeed, we have previously demonstrated the enzyme crosslinked hydrogel underwent little degradation (<20%) over 3 weeks in growth media with stirring [12].

hMSCs were cultured on the gelatin hydrogel scaffolds allowing the monitoring of their growth activities (figure 2). Cells easily adhered to the gelatin hydrogel surface within an hour. Cells on patterned scaffold adopted aligned and elongated morphology along the microchannel direction on the first day and maintained it throughout the culture duration of 14 days. In contrast, cells on the plain scaffold demonstrated random arrangement and spread morphology for entire cell culture period. These findings demonstrate that bioprinted gelatin microchannels successfully acted as the topographic cues to promote cell alignment. In addition, cell proliferation assay demonstrated the biocompatible nature of gelatin hydrogel scaffold with no noticeable difference in the cell viability or proliferation between the unpatterned and microchannel surface (supplementary figure (S5)).

3.2. Patterned gelatin hydrogel steers stem cell myocardial lineage commitment

Cells cultured on gelatin hydrogel scaffolds were subjected to immunocytochemical analysis to analyze the stem cell lineage commitment. Mature markers of cardiac differentiation (β-MHC and cardiac troponin T) were selected to check their expression in the cells. Immunofluorescence images confirmed prominent β-MHC expression in cells seeded on microchannels after 7 and 14 days of culture, whereas for the unpatterned surface, cells failed to demonstrate β-MHC expression (figure 3(A), supplementary figure (S6)). Moreover, after 7 days, patterned cells exhibited upregulation of cardiac troponin T, whereas no traces of troponin expression were observed in cells on unpatterned hydrogel (figure 3(A), supplementary figure (S7)). These results were in agreement with previous literature which reported the myocardial lineage commitment of hMSCs cultured on ECM fibronectin strip pattern [48, 49]. FACS analysis was performed after 7 days to check the cardiomyogenic cell population percentage from both groups (figure 3(B)). FACS data revealed that ∼48% cells from the microchanneled group indicated positive β-MHC expression as compared to ∼21% cells from the unpatterned group. Altogether, the immunostaining and FACS data confirmed that gelatin patterned hydrogel promoted the myocardial lineage commitment of hMSCs.

To examine the effect of spacing between adjacent microchannels on the stem cell lineage commitment, similar cell densities were seeded on the microchanneled scaffolds with varying spacing between two adjacent microchannels (0.5 and 1 mm spacing (figure 4)). F-actin stained images illustrated that hMSCs were aligned and elongated on the microchanneled scaffold with 500 μm spacing. In comparison, cells on the microchanneled scaffold with 1000 μm spacing showed non-aligned and spread morphology. Additionally, elongated cells on microchanneled scaffold with 500 μm spacing displayed significant positive β-MHC expression, whereas spread cells on the microchanneled scaffold with 1000 μm spacing illustrated very weak β-MHC expression [19, 50, 51].

3.3. Influence of 3D patterning on cell cytoskeleton anisotropy and focal adhesion distribution

To investigate the cytoskeletal organization during cell alignment and elongation, F-actin stained cells images were examined after 7 days (figure 5(A)). Cells on microchanneled scaffolds showed well-aligned F-actin stress fibers orientated along the channel direction.
However, cells on the plain scaffold were found with randomly arranged F-actin stress fibers. Overall, obvious differences in actin cytoskeleton arrangement between both groups demonstrate the involvement of actomyosin-mediated contractility in modulating hMSCs morphology \[52, 53\]. To quantify the degree of cell elongation between the two groups, the aspect ratio was determined. The aspect ratio reveals that hMSCs on microchanneled scaffold showed greater elongation and narrow width wise spreading compared to that of well-spread cells on the plain scaffold (figures 5(B) and (C)). The next cell morphological parameter assessed was the nucleus orientation to assess cell alignment (figure 5(D)). The graph in figure 5(D) demonstrates the degree of cell orientation. Cells with nucleus orientation less than 20° from the axis of microchannel direction were considered as aligned cells. Majority of cells on microchannel scaffold demonstrated orientation along the direction of the microchannel. In contrast, cells on the plain scaffold did not display any aligned orientation.

Vinculin, a mechanosensitive focal adhesion protein was chosen to map the spatial distribution of focal adhesions in cells from both groups (figure 6(A)). The microchanneled scaffold supported the formation of long and aligned focal adhesions in cells. In stark contrast, small and randomly distributed focal adhesions were expressed by cells on the plain scaffold. These results reflect that of previous alignment studies which mention the development of ‘long and aligned focal adhesions’ and ‘punctate and randomly expressed focal adhesions’ within cells on microchanneled and unpatterned surface, respectively \[18, 54\]. Additionally, 2D-FFT frequency plot of focal adhesion distribution was generated to check the degree of focal adhesion alignment by displaying the grayscale pixels distributed in the pattern around the origin. FFT analysis data emphasized that channel orientated cells had aligned focal adhesions along the microchannel direction while non-orientated cells on plain scaffold displayed randomly organized focal adhesions (figure 6(B)).

### 3.4. 3D patterned scaffold supports cardiomyocyte growth, alignment and synchronized beating

Neonatal rat CMs were cultured on both microchanneled and plain hydrogel scaffolds to assess the effect of microchannels on CMs attachment and alignment. Since neonatal rat CMs are smaller in size than hMSCs, microchannels were printed with 0.3 mm spacing (supplementary figure (S1)). It was found that microchannels with 0.5 mm spacing did not sufficiently align the neonatal rat CMs (data not shown). A few hours after seeding, CMs readily attached, spread on both hydrogel surfaces and became tightly packed (figure 7(A)). CM alignment was evident on microchanneled scaffold throughout the culture duration. In contrast, CMs on plain hydrogel exhibited random growth and spread morphology.

Subsequently, the beating behavior of the CMs was observed. It may be of note that previous studies involving rat neonatal CMs on aligned gelatin scaffolds have
demonstrated beating rate within a range of \( \sim 20 \text{ bpm} \) at the low end and between 60 and 100 bpm at the high end [55, 56]. The first sign of the beating was observed on day 2. Interestingly, the spontaneous beating was much more visible in CMs grown on microchanneled scaffold compared to those on the plain scaffold. Furthermore, the beating frequency on day 2 was 26 beats min\(^{-1}\) and 16 beats min\(^{-1}\) for CMs on microchanneled and plain hydrogels, respectively (figure 7(B)). From day 4 onwards, a large population of CMs on the microchannels demonstrated stronger and more synchronized beating. In contrast, plain scaffold seeded CMs showed weak, non rhythmic and isolated beating in clusters (supplementary movies S1

Figure 3. hMSC’s expression of the mature cardiac markers, \( \beta \)-MHC and cardiac troponin T as evidence of myocardial lineage commitment. (A) The panel displays immunofluorescence images of scaffold seeded hMSCs investigated for the expression of the mature cardiac markers using either \( \beta \)-MHC or cardiac troponin T primary antibodies with alexa fluor 488 secondary antibody (green fluorescence). Double arrow white line indicates pattern direction. The scale bar = 100 \( \mu \text{m} \). (B) FACS analysis of \( \beta \)-MHC expression. A substantially greater number of cells were found to express \( \beta \)-MHC on the microchanneled surface (\( \sim \)48\%) compared to those on the plain surface (\( \sim \)21\%). The controls ‘microchannel Ab control’ and ‘plain Ab control’ refer to the sample without \( \beta \)-MHC primary antibody.
and S2). On day 4, the beating frequency increased to 34 beats min\(^{-1}\) on microchannel seeded CMs and 26 beats min\(^{-1}\) for unpatterned CMs. Furthermore, a gradual upward trend in CM beating frequency was noticed on the microchanneled scaffold, reaching a maximum 62 beats min\(^{-1}\) on day 9, whereas the frequency on the plain scaffold did not show such increase with 30 beats min\(^{-1}\) on day 7 and 26 beats min\(^{-1}\) on day 9. Overall, CM beating was more noticeable on the microchanneled scaffold, appearing more organized along the orientation of the microchannels and with a higher beating frequency compared to those on the plain hydrogel.

### 4. Discussion

#### 4.1. Bioprinting the hydrogel microchannels to align the cells

We report herein a method of direct free from fabrication of patterned designs using a novel and completely natural bioink that is both crosslinked in ‘physiological’ like conditions and cell supportive while allowing long term construct stability. All of this is achieved while avoiding the need for templating as required using other biofabrication methods. Thus we are able to produce a gelatin hydrogel film and then bioprint aligned features to generate hydrogel microchannels. The viscosity of the printable hydrogel is sufficient to retain the integrity of the deposited trace whilst the action of mTgase crosslinks the protein, as we have found previously [33]. The viscosity of a 5% gelatin solution can be varied considerably from less than 10 cps at 40 °C to 1000 cps at 20 °C. It was found that ideal viscosity for bioprinting occurred at 24 °C as the printable hydrogel produced a continuous 3D trace without collapse or spreading while mTgase activity crosslinked the hydrogel. The mTgase crosslinked hydrogel demonstrated extended stability in aqueous conditions allowing extended cell culture of hMSCs and cardiomyocytes in this study.

The elastic modulus of a surface is known to play a role in the differentiation of stem cells, as it provides a cue to the nature of the target tissue, hence an important consideration when creating a biomimetic scaffold [57, 58]. The crosslinked gelatin had an elastic modulus of 80 kPa, whereas, the modulus of a neonatal rat heart is from 4 to 11.4 kPa and the adult rat heart is from 12 to 46 kPa [59]. However, the measurements of the modulus of the adult rat myocardium have been found to vary with readings as high as 70 kPa, [60, 61] closer to the crosslinked gelatin hydrogel in this case.

hMSCs seeded on the printed microchannels appear visually to become aligned and elongated along the printed microchannels, whereas cells cultured on plain gelatin scaffold displayed spread morphology. Both hydrogel material properties and topography play key role in such stem cell behavior. However, the topography has been found to be considerably influential in determining cytoskeletal organization, cell morphology and aligned orientation [20, 22, 25, 62]. Indeed, Yim et al found that between material properties and surface topography, the latter was found to have the greatest effect on cell cytoskeletal arrangement [62].

To demonstrate that the differences in the cell morphologies are a cellular response to guidance cues...
and not just an artifact of topographical constraints within the printed microchannels, cell cytoskeleton morphology was studied. Prager-Khoutorsky et al demonstrated that the elongation and orientation of cells are preceded by focal adhesion orientation and then the formation of long actomyosin bundles. The visualization of F-actin filaments within microchannel orientated cells revealed that the actomyosin stress fibers were arranged along the direction of microchannels. This alignment of stress fibers with grooves has also been observed with hMSCs aligned on nanogrooves where topographical constraints were not an issue. Focal adhesions staining revealed they were also arranged with a distinct directional bias along the line with the microchannels. Distinct elongated focal adhesion expression was observed in hMSC cultured on microchanneled surface, while randomly distributed focal adhesions were observed in cells seeded on the unpatterned surfaces. The focal adhesions allow the cells to sense and interact with the surface, with the feedback stimulating alterations in cellular behavior to match the current niche. Hence it has been found that the interactions between the focal adhesion and the surface influence F-actin organization and the composition of focal adhesion.

Nuclei elongation was observed in the aligned stem cells, with approximately 55% of cells aligning ≤20° from the direction of the microchannels. This is comparable to the rate of nuclei alignment achieved for both cardiomyocytes aligned on photodegradable gelatin microchannels (≈20 μm width) and closer to that of hMSCs aligned on narrow microchannels (of 25, 50 and 100 μm widths) than the lower level.

Figure 5. The influence of microchanneled hydrogel on F-actin stress fiber arrangement, cell elongation and cell orientation. (A) F-actin immunofluorescence images illustrate the development of aligned F-actin stress fiber (stained red with rhodamine phalloidin) in cells after 7 days of culture on microchanneled and plain hydrogel. Double arrow white line indicates pattern direction. The scale bar = 100 μm. (B) The aspect ratio (ratio of cell length to width) indicates the degree of cell elongation. The higher the aspect ratio, the greater the degree of cell elongation (p < 0.005, n = 100). (C) The cell area observed in cells on microchanneled hydrogel compared to those on plain hydrogel (p < 0.05, n = 50). (D) Nucleus orientation of <20° was considered as an aligned cell. The nucleus orientation profile of both groups is shown as histogram, (n = 200). The scale bar = 100 μm.
achieved by hMSCs seeded on wide and non-aligned microchannels (500 and 1000 μm) [66].

4.2. Effect of channel width on cell alignment

Cells can align themselves on surfaces with channels/grooves of wide range of width and depth from nano to micro in dimensions. It is observed that the cell orientation is triggered by cell filopodia sensing the ridges [67]. The extending filopodia from the cells senses resistance from the grooved ridges and subsequently trigger the elongation. It is generally considered that when the channel width is close to or smaller than the width of the cell, the cell alignment is distinct [17]. In one study, comparing relatively narrow to wide microchannels, it was found that cells on microchannels of 25–50 μm showed distinct alignment, whereas wider ones, 500–1000 μm did not [66]. Similarly, in our study, we found that relatively wide microchannels of approximately 250–300 μm width (500 μm spacing) induced noticeable alignment of hMSCs. However, increased

![Figure 6](image-url). The spatial distribution of focal adhesions in hMSCs cultured with/without bioprinted hydrogel microchannels. (A) Immunofluorescence staining of vinculin demonstrated the arrangement of focal adhesion in response to topographical guidance in the presence/absence of microchannels. Vinculin was stained with alexa fluor 488 secondary antibody (green fluorescence) and the nuclei with DAPI stain. (B) FFT analysis data displays the alignment of focal adhesion from cells on microchanneled hydrogel in contrast to non-aligned focal adhesion from the unpatterned cells. The scale bar = 100 μm. Double arrow white line indicates pattern direction.
spacing between microchannels (∼800 μm spacing) could not support cellular alignment (figure 4).

We assume that wall of microchannels provides contact guidance for immediately contacting cells to align along the channel direction. These aligned cells confer guidance to neighboring cells within the channel to induce further cellular orientation along the channel direction. Hence cells across the channel width become noticeably elongated and aligned. Conversely, on wide microchannelled surfaces (∼800 μm spacing), aligned cells contacting the channel edges tend to have less influence on cells adhered centrally failing to confer any guidance cues [66, 68].

4.3. Assessment of stem cell lineage commitment

The cardiomyocyte markers β-MHC and cardiac troponin T were both strongly expressed within the aligned hMSCs seeded on the microchanneled surface but not for those on the plain surface. Moreover, the loss of cell orientation observed with microchannel expansion from 500 to 1000 μm spacing was also associated with the absence of expression of β-MHC.

hMSC alignment has been well documented to be associated with hMSC commitment towards myocardial lineage even in the absence of any additional growth factors [10, 60]. Of note is that in these studies, and others, hMSC commitment is manifested by phenotypic expression of representative cardiomyocyte markers, but does not result in actual beating. This could be due to lack of yet unknown factors that may directly reprogram hMSC towards beating CM phenotype. Similarly, our experiments did not result in actual hMSC beating following alignment. However, we showed that the printed features and the microchannel spacing strongly influence the lineage commitment of hMSC more than the hydrogel material properties alone.

4.4. Cardiomyocyte activity

The activity and organization of beating CMs have been shown to improve with the seeding on aligning surface [29, 69]. This improvement has considerable potential for the development of custom designed heart patch seeded with beating CMs. Indeed, our
group has recently published on the safety and efficacy of applying similar heart patches [70] and injectable formulations [71] for the treatment MI in a rat model. In those studies, cardiac specific ECM formulations were used which contain predominantly collagen of various types resembling the biophysical properties of the crosslinked gelatin hydrogel tested in this work. In our current work, optical visualization of CMs demonstrated that gelatin scaffolds from both groups facilitated CM adhesion, spreading and confluent cell–cell coverage. When CM beating potential was evaluated, CMs on microchanneled scaffold revealed remarkable consistent rhythmic beating throughout the culture period. Compared to the unpatterned CMs, the beating was more prevalent and better organized with noticeable beating in line and occasionally perpendicular with the patterning and was more persistent over the culture duration. Similarly, in the study by Tsang et al found that CMs on unpatterned hydrogels formed isolated clusters and had eccentric beating rhythms, whereas aligned CMs within gelatin microchannels exhibited more regular beating pattern, proceeding at a greater rate and with better distribution across the construct [23]. Similarly, our time dependent experiment revealed that the beating rate for both patterned and unpatterned CMs increased up to 7 days (168 h). However, after 9 days (216 h), patterned CMs demonstrated an increase in beating rate, whereas the unpatterned CMs showed decrease in beating rate. Such rapid increase in beating rate for the patterned CMs was also shown by other groups [56, 72]. van Spreenhel et al associated this effect with the development of more organized sarcomere structure in aligned CMs. Moreover, the mechanical properties of gelatin have been found to promote CM beating rate and duration as compared to other surfaces such as collagen and polystyrene [55].

Cytoskeletal organization has been shown to have a profound effect on the contractility of neonatal CMs [73–75]. The greater beating rate attained by aligned CM compared to non-aligned CMs has been associated with aligned CMs achieving cytoskeletal organization resembling that of mature CMs, in particular the arrangement of proteins such as sarcomeric α-actinin, connexin 43 and troponin I. On the other hand, in unpatterned CMs these proteins were found to be more randomly organized [56]. Furthermore, Wang et al observed that CMs seeded on unpatterned surfaces gradually lose their contractility, whereas CMs on grooved patterns retained the activity [69]. Interestingly, such channels and topographic guidance were shown to orientate CMs more effectively than electrical stimulation [76].

5. Conclusion

The results presented here show that 3D bioprinting can produce stable cell aligning microchannels from a gelatin hydrogel crosslinked with enzyme mTGase. These microchannels can orientate hMSCs, promote myocardial lineage commitment and improve the organization and rhythmic beating of CMs serving as model cells. Compared to other fabrication methods, our approach has two major and distinct advantages. First, we use completely natural and biocompatible bioink which is both easily printable and enzymatically crosslinked in a physiological manner allowing long term construct stability and cell support. Second, the construct design and pattern is easily modified given the flexibility of a free-form fabrication method compared to other template-dependent approaches. Possible applications for the engineered cardiomiomic construct may include in vitro cardiac tissue modeling for physiological and cardiac cytotoxicity studies as well as in vivo in generating custom designed tissue engineered cardiac patches for future myocardial regeneration—a theme that is currently being evaluated in our lab.

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