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Increased cardiomyocyte alignment and intracellular calcium transients using micropatterned and drug-releasing poly(glycerol sebacate) elastomers

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Abstract

Myocardial tissue engineering is a promising therapy for myocardial infarction recovery. The success of myocardial tissue engineering is likely to rely on the combination of cardiomyocytes, pro-survival regulatory signals, and a flexible biomaterial structure that can deliver them. In this study, poly(glycerol sebacate) (PGS), which exhibits stable elasticity under repeated tensile loading, was engineered to provide physical features that aligned cardiomyocytes in a similar manner to that seen in native cardiac tissue. In addition, a small molecule mimetic of brain derived neurotrophic factor (BDNF) was polymerised into the PGS to achieve a continuous and steady release. Micropatterning of PGS elastomers increased cell alignment, calcium transient homogeneity and cell connectivity. The intensity of the calcium transients in cardiomyocytes was enhanced when cultured on PGS which released a small molecule BDNF mimetic. This study demonstrates that robust micropatterned elastomer films are a potential candidate for the delivery of functional cardiomyocytes and factors to the injured or dysfunctional myocardium, as well as providing novel \textit{in vitro} platforms to study cardiomyocyte physiology.

1. Introduction

Myocardial tissue cannot undergo significant regeneration, which is a primary cause of heart failure after myocardial infarction.\textsuperscript{1} To combat this lack of endogenous repair, many studies have attempted to introduce cardiomyocytes into dysfunctional areas to replace damaged cells
and improve cardiac function. However, introducing cells into the scar area by direct injection into the myocardium or via circulating blood results in poor engraftment.\textsuperscript{2}

In an effort to improve cardiac engraftment, cardiac tissue engineering strategies focus on cellular transplantation techniques combined with degradable scaffolds to create viable myocardial replacement tissue.\textsuperscript{3} The degradable scaffolds must, however, meet the mechanical demands of cardiac tissue without cracking or disintegrating. The intrinsic properties of poly(glycerol sebacate) (PGS) make it of interest in myocardium tissue engineering; it is a biodegradable elastomer designed for soft tissue engineering that does not undergo plastic deformation or permanent set under long-term repeated cyclic strain.\textsuperscript{4} PGS also has an adjustable secant modulus which covers the whole range of heart muscle; from 10-20 kPa at the beginning of diastole to 200-300 kPa at the end.\textsuperscript{3}

Altered calcium homeostasis, especially inadequate calcium release, is a cause of pathophysiology of myocardial dysfunction and heart failure.\textsuperscript{5} To limit the progression of myocardial infarction to heart failure, it is of paramount importance to restore normal calcium cycling.\textsuperscript{6} Several applied or potential therapies have been reported to normalize the depressed calcium transients in cardiomyocytes, including intracardiac myoblast transplantation\textsuperscript{7}, introducing pulsed infrared radiation\textsuperscript{8} and increasing sarco-endoplasmic reticulum calcium ATPase mRNA and protein expression in impaired myocardium tissue.\textsuperscript{9} Recent studies have shown an important role of brain derived neurotrophic factor (BDNF) in regulating calcium transients and cardiomyocyte contraction force in cardiomyocytes.\textsuperscript{10-11} BDNF also inhibits apoptosis of ischemic cardiomyocytes by regulating miR-195.\textsuperscript{12} BDNF exerts these actions
through binding to full-size or truncated versions of tropomyosin receptor kinase B (TrkB). The actions of BDNF may be partially or fully-replicated using small molecule agonists to the TrkB receptor. While BDNF mimetics have effects in NIH-3T3 cells, PC12 cells and cementoblasts, little is known about the effects of BDNF mimetics on cardiomyocyte function and survival, and the application of BDNF mimetics to other cardiac cells has not been reported. The compound N,N',N'' tris(2-hydroxyethyl)-1,3,5-benzeneticarboxamide has been reported to activate TrkB receptors. While it was recently shown that the signal transduction processes activated are unclear, animal studies have demonstrated the small molecule mimetic prevents neuronal degradation in rats, provides protective effects on injured spinal cord nerves and promotes recovery after hypoxic-ischemic stroke.

Here we report the development of mechanically robust elastomeric PGS based films with modified (microchannel) topography and the incorporation of a small molecule BDNF mimetic. We then assess how microchannel dimensions and the BDNF mimetic affect cardiomyocyte calcium transients.

2. Experimental Section

2.1 Photolithography and fabrication of polydimethylsiloxane (PDMS) elastomer mould

A PDMS mould was fabricated for patterning of the PGS and BDNF mimetic incorporated PGS (BPGS) films. An epoxy-based negative photoresist SU-8 2002 (Microchem) which contains SU-8 monomers, solvent and the photoinitiator (triarylsulphonium hexafluoroantimonate) was evenly cast on a precut (1 cm × 1 cm) silicon wafer ultrasonically cleaned by ethanol and isopropanol. The silicon wafer with SU-8 2002 was spun at 500 rpm for 10 s with an
acceleration of 100 rpm.s\(^{-1}\) followed by 200 rpm.s\(^{-1}\) for 30 s with an acceleration of 300 rpm.s\(^{-1}\) based on the manufacturer's process to generate a 3 \(\mu\m\) thick film. Photomasks with alternating chrome and clear strips of 10, 20 or 50 \(\mu\m\) (Bandwidth Foundry International Pty Ltd.) were then placed next to the surface of the photoresist film. The photoresist films were selectively polymerized via exposure of UV light at 365 nm for 15 s at 15 mW.cm\(^{-2}\). A visible mask image was created after heating the silicon wafer on a hot plate for 2 min at 95 °C. The non-polymerized photoresist under the chrome strip was immersed in SU-8 developer with gentle shaking for 10 s followed by a second wash with isopropanol for another 10 s at room temperature. The rinse step was repeated if a white film (signifying uncured oligomer) was produced during the isopropanol wash as recommended by the manufacturer of the resin.

A 10:1 (w/w) mixture of Sylgard 184 (Dow Corning) PDMS elastomer base to curing agent was poured onto the patterned photoresist on the silicon wafer described above and heated on a hot plate at 150 °C for 10 min to polymerize the PDMS. After careful removal of the cured PDMS elastomer from the silicon wafer with patterned photoresist, the patterned side of the PDMS mould was then oxidized by air plasma (K1050X plasma asher Quorum Emitech, U.K.) for 1 min at an intensity of 1.5 mW/cm\(^{2}\) to create a hydrophilic surface.\(^{19}\) A 60 %wt sucrose solution in water was coated on the oxidized PDMS surface of the mould within 5 min of plasma treatment. The sucrose coated PDMS elastomer mould was then transferred on a 150 °C hot plate and heated for 10 min to create a 0.2 \(\mu\m\) thin sucrose layer. This acted as a release agent for convenient separation in the follow-up experiments.
2.2 Synthesis of N,N',N'' tris(2-hydroxyethyl)-1,3,5-benzenetricarboxamide (BDNF mimetic)

Solvents (dichloromethane, methanol, isopropanol) were purchased from VWR in HPLC grade. Other chemicals were purchased from Sigma-Aldrich and used as received. NMR spectra were recorded on a Bruker Advance III 400 with a 5 mm broadband auto-tuneable probe with Z-gradients at 293 K. Chemical shifts are reported as δ in parts per million (ppm) and referenced to the chemical shift of the residual solvent resonances (DMSO-d$_6$ $^1$H: δ = 2.50 ppm, coupling are shown as d: doublet, t: triplet, q: quartet and bs: broad singlet). NMR spectra were processed using MestReNova software.

A solution of trimesoyl chloride (10 g, 37.7 mmol) in dichloromethane (100 mL) was added dropwise under stirring to excess ethanolamine (23 mL, 370 mmol) over 2 h. The solution was heated to reflux for 6 h, then allowed to cool to ambient temperature (Fig. 1A). Water (100 mL) was added and the mixture was extracted with dichloromethane/isopropanol solution (4/1 v/v, 100 mL x 3). The combined organic phases were dried (Na$_2$SO$_4$) concentrated in vacuo and recrystallized in hexane/methanol (1:1 v/v) mixture to give product as white crystals (yield: 2.1 g, 16.3%). $^1$H NMR (DMSO-d$_6$) δ (ppm): 8.63 (t, $^3$$J_{HH}$ = 4.96), 8.44 (s), 4.77 (bs), 3.56 (t, $^3$$J_{HH}$ = 5.79), 3.38 (q, $^4$$J_{HH}$ = 5.26, 6.66) (Fig. S1).

Figure. 1 Synthesis of BDNF mimetic (A) and BPGS elastomer which incorporates the BDNF
mimetic via the formation of a prepolymer (130 °C, N2 atmosphere) followed by curing (160 °C vacuum) (B).

2.3 Synthesis of PGS and BPGS

PGS and BPGS prepolymers were synthesized based on previously established protocols, with minor modifications. Glycerol and sebacate with or without BDNF mimetic were mixed at a molar ratio of 45:50:5 or 50:50 respectively, at room temperature. The monomers underwent polycondensation at 130 °C under 130 cm³.min⁻¹ flow of nitrogen for 24 hr. 0.3 g of paste-like prepolymer was then cast on the sucrose coated PDMS moulds at 150 °C on a hot plate to form an approximate 300 μm thick film. The cast films were cured at 160 °C under vacuum for 8 hr (Fig. 1B). After cooling to room temperature, the PGS and BPGS together with the PDMS mould were removed from the oven and submerged overnight in Milli-Q water at ambient temperature. The patterned films were peeled off from the PDMS mould the next day. Unpatterned films with same thickness served as controls and were synthesized on flat PDMS sheets prepared under the same conditions.

2.4 Degradation characterization of PGS and BPGS

Degradation experiments were carried out in Milli-Q (MQ) water-based and Dulbecco's Modified Eagle Medium (DMEM) based solutions. For MQ water-based degradation, about 1 g of the PGS or BPGS films with a surface area of 5 cm² were soaked in 20 mL of MQ water or 1 mol.L⁻¹ hydrochloric acid (HCl) for 1 week. For DMEM-based degradation, BPGS films were cut into round-shape specimens with a surface area of 70 mm² (approximately 50 mg) and these
specimens were soaked in 500 μL DMEM in 48 well culture plates with or without porcine liver esterase (2 μL 1366 units.mL⁻¹, supplied by Sigma) and each group comprised of three samples. 1 unit of esterase is defined by the supplier as the amount of esterase needed to hydrolyze 1 μmol ethyl butyrate per min at pH 8.0 and 25 °C. The culture plate was placed in an environmental shaker (37 °C) with a rotating rate of 70 rpm. 100 μL of media was extracted from the wells every three days. The same amount of fresh media was added in the extracted wells with 0.6 μL of porcine liver esterase. The extracted media were carefully sealed and stored at 4 °C.

2.5 UV-vis spectroscopy characterization

UV–vis absorption spectra of aqueous BDNF standard solutions and BPGS extracts were recorded on a UV-4100 (Shimadzu) spectrophotometer between 180 nm and 500 nm with a scan rate of 100 nm.min⁻¹. The MQ water-based degradation samples were heated on a hot plate at 60 °C overnight and redissolved in 1 L of MQ water to bring the solute to the optimum concentration for its measurement. About 1 mL solution of each sample was added into a 10 mm thick quartz cell. MQ water was used as the background.

2.6 HPLC characterization

High performance liquid chromatography (HPLC) analysis was performed using a Hewlett-Packard 1100 series HPLC system (Agilent Technologies, CA) with a reverse-phase VydaCM analytical (C18, 300 Å, 5 μm, 4.6 mm x 150 mm) column. The eluent gradient was 0 % to 25 % solvent B (solvent A: 0.1 % trifluoroacetic acid (TFA)/H₂O; solvent B: 0.1% TFA/CH₃CN) for the
first 25 min. The flow rate was 1 mL.min\(^{-1}\). A UV detector was used and the wavelength was set to 214 nm to detect the aromatic ring of the BDNF mimetic. The UV-vis response was characterized by known BDNF mimetic concentrations from 0.5 μM to 200 μM. The degradation products in DMEM with or without enzyme were characterized by HPLC. Visual examination of solutions was also undertaken at various periods during the degradation of the BPGS by the DMEM-enzyme media.

2.7 Mechanical characterization

BPGS and PGS specimens with a thickness of approximately 300 μm were cut from a cured unpatterned BPGS or PGS films using a dumbbell shaped mould with a gauge length of 12.5 mm and a width of 3.25 mm. The thickness of each specimen was measured using an electronic Vernier calliper. Tensile and cyclic tests were performed using an Instron 5860 mechanical tester equipped with a 100 N load cell. Extension rates of 10 mm.min\(^{-1}\) and 25 mm.min\(^{-1}\) were applied for the tensile and cyclic tests respectively, to be consistent with earlier studies.\(^{21}\) Specimens for the tensile tests were stretched to failure and those for cyclic tests underwent a strain of 15%, which is typical of the dynamic loading strain of the myocardium under normal physiological conditions.\(^{22}\)

2.8 Morphology characterization

The micropatterned structure of the PDMS moulds and BPGS films were visualized and quantified using optical profilometry (VeecoWyko NT100) without further surface treatment.
2.9 PGS and BPGS film pretreatment before cell culture

PGS and BPGS films were sterilized by soaking in 80% ethanol for 12 hr. The films were soaked in 300 μL of DMEM in a 48 well culture plate placed in an incubator (37 °C in humidified air containing 5% CO$_2$) with a culture medium change every 48 hr to remove unreacted sebacate acid which provides an unfavourable acidic environment for cardiomyocytes. The pH value was measured daily by insertion of a pH meter until the pH change was not significant. Porcine skin gelatin (300 μL of 1% solution in MQ water, supplied by Sigma) was added to cover the surface of the BPGS or PGS film. The plate was incubated for 2 hr and the remaining gelatin solution was washed away with sterile DMEM.

2.10 Cell culture

All animal procedures were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee in accordance with the National Health and Medical Research Council guidelines for the care and use of laboratory animals. Neonatal cardiomyocytes (NCMs) were isolated from 1-2 day old neonatal rat pups by enzymatic digestion as described previously.$^{23}$ NCMs were seeded at $2.2 \times 10^5$ cells per well in 48 well-plates on pretreated BPGS and PGS films with different sized micropatterned features and incubated in minimal essential medium (MEM) containing 10% new born cow serum (NBCS) and 0.1 mM bromodeoxyuridine (BrdU) at 37 °C in humidified air with 5% CO$_2$. After 24 hr, the media was changed to DMEM with 0.1mM BrdU, 10 μg/mL human transferrin (Sigma) and 2.5 U.mL$^{-1}$ human insulin (Sigma). Thereafter, NCMs were maintained in DMEM with the same concentration of apo-transferrin and insulin.
without serum or BrdU and the culture media was changed every 72 hr, as described previously.  

2.11 Immunocytochemistry

After 7 days of culture, cells were fixed using 4 % paraformaldehyde (PFA) and permeabilized by 0.3 % (w/v) Triton-X. Cardiomyocytes were then blocked with 10 % normal goat serum (NGS) in phosphate buffered saline (PBS) and incubated with anti-α-actinin (mouse) (ab9465) (and anti-connexin (rabbit) (ab11370) at 1:200 and 1:1000, respectively, or anti-troponin I (rabbit) (ab47003) at 1:200 in 10 % NGS respectively overnight at 4 °C. The cardiomyocytes were incubated in diluted secondary antibodies (goat-anti-mouse 568 and goat-anti-rabbit 488) in PBS at 1:1000 for 1 hr at 37 °C. The samples were rinsed and incubated with 1:5000 4',6-diamidino-2-phenylindole (DAPI) for 5 min and were then sealed with mounting medium on glass slides. Stained samples were imaged using a scanning confocal microscope (Leica SP5 XMP, Germany) with 10x and 40x water immersion objectives. Due to autofluorescence of PGS, the boundary of cells was not clear. Therefore, a background subtraction was carried out using a 50.0 pixels radius rolling ball followed by brightness/contrast adjustment using ImageJ. An example of a fluorescent image before and after correction is shown in Fig. S2.

2.12 Nuclei alignment analysis

Following a previous reported method, images of DAPI-stained nuclei were taken by fluorescence microscopy (Nikon Eclipse TS 100). The images were converted to binary images by Image J and each individual nucleus was fitted to an ellipse. The angle between the
nuclei elongation direction and the pattern direction was quantified as the angle of deviation and the angles were grouped in 10° increments. Differences between nuclei alignment angles less than 20° were considered as aligned. For each pattern, 6 different sample images from 3 films containing 100-200 cells each were averaged and summarized.

2.13 BDNF treatment

The cardiomyocytes which had been cultured on 20 μm patterned and unpatterned PGS films in DMEM for 72 hr after isolation and seeding were cultured in DMEM with or without 60 nM BDNF mimetic or 20 nM BDNF protein for another 48 hr (three samples for each group). No additional treatment was applied for the cardiomyocytes cultured on BPGS films.

2.14 Intracellular calcium transient imaging and intercellular coupling analysis

Calcium transients (changes in the cytoplasmic Ca^{2+} concentration) were recorded using fluorescence microscopy (Nikon Eclipse TS 100) (excitation at 485 nm, emission at 520 nm) for cardiomyocytes cultured on untreated samples and treated samples. Fluo-4 AM (Thermo Fisher Scientific™), an indicator of intracellular calcium, was dissolved in dimethyl sulfoxide at 10 mM and diluted to a final concentration of 2 μM in DMEM. Cardiomyocytes were loaded with Fluo-4 in DMEM for 30 min at 37 °C in a 5% CO₂ in air humidified incubator. Three replicate experiments for each group were performed to enable comparison with cardiomyocytes cultured on PGS films without any treatment. Whole-frame images obtained at 1360 × 1024 pixels were continuously recorded to produce image sequences. The image sequences were corrected for darkening over time.
(caused by condensation of water vapour on the lens) using ImageJ’s bleach correction tool, and were then analyzed using the ImageJ software plugin LC Pro and previously reported R code.\textsuperscript{27} The region of interest (ROI) was defined as a circle with a diameter of 40 pixels with an area similar to a cardiomyocyte size and only ROIs with significant intensity change (P<0.05) between frames were exported. The results were expressed by fluorescence (F) normalized to baseline fluorescence (F\textsubscript{0}) (F/F\textsubscript{0}) over time.

Intercellular coupling analysis was performed by analyzing eight ROIs randomly chosen from each image sequence following a recently used approach.\textsuperscript{28} The fluorescence intensities in the ROIs over five seconds were combined in a correlation matrix. Three analyses for each type of specimen were carried from three image sequences. The number of significantly correlated ROIs (p<0.05) was counted for each correlation matrix.

2.15 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. The average time between calcium transients were analyzed using Kruskal-Wallis analysis followed by Dunn’s test and the standard deviations were analyzed by F tests between two groups. The fluorescence intensities over time were analyzed by nonparametric Spearman correlation. The other data were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. Results are presented as the mean ± standard deviation (SD) and a p value less than 0.05 was considered as significant.
3. Results and Discussion

BDNF mimetic, \(N,N',N''\)-tris(2-hydroxyethyl)-1,3,5-benzenetricarboxamide, has 3 primary hydroxyl groups like glycerol (Fig. 1A). We therefore hypothesised that the BDNF mimetic could be polymerized into the polymer by polycondensation and subsequently be released via aqueous hydrolysis. As the BDNF mimetic is a stable small molecule, we also hypothesised that it can withstand the elevated temperatures during polymerisation without losing biofunctionality. The polymer synthesis comprised two stages, namely prepolymer formation and crosslinking. BDNF mimetic was mixed with glycerol and sebacate at the prepolymer fabrication stage to achieve copolymerization. The BPGS was subsequently degraded in \(1\text{mol.L}^{-1}\) HCl to determine the amount of BDNF mimetic incorporated into the elastomer using UV-vis spectroscopy (Fig. S3). The percentage of BDNF mimetic recovered from the HCl-degraded BPGS polymer was 32 ± 2% of the initial amount added to the polymerisation mixture and equates to a final 34.2 mg mimetic per g of BPGS elastomer.

Enzymatic degradation of BPGS was explored to determine the ability of the elastomer to release the mimetic under \textit{in vivo} like conditions. For DMEM based degradation, the PGS and BPGS samples degraded faster in the presence of porcine liver esterase. When the films were soaked in DMEM-only solutions with phenol red indicator, the colour remained bright pink after 30 days. When soaked in DMEM solutions with added porcine liver esterase, the solution became orange after 15 days and at day 21, the colour was yellow, indicating an acidic environment introduced by the degradation product of sebacate (Fig. S3). The local acidity increase is considered to be a limitation for the application of PGS for tissue engineering.
however in vivo experiments showed that the inflammation caused by PGS is less compared to another widely used biomaterial poly (lactic-co-glycolic acid). The degradation products were analyzed by HPLC. However, only 0.758 mg BDNF mimetic per gram BPGS elastomer was detected in the accelerated degradation products (Fig. S3). The value is significantly lower than the total amount of mimetic determined using UV-vis spectroscopy of the acid degraded product with 34.2 mg mimetic per gram BPGS. The difference is most likely caused by the detection methods since all partially degraded BPGS soluble fragments will be detected by UV-Vis as they also contain an aromatic group (the aromatic peak at 210 nm in the UV spectrum was used to quantify the amount of mimetic). However, using HPLC, partially hydrolysed fragments containing the mimetic with different molecular weights will be eluted at different times (possibly shown by the peaks at 17.1 min and 27.4 min, Fig. S3) and so the HPLC method provides an indication of the actual amount of “free” BDNF mimetic released into solution. It is assumed that only the free BDNF mimetic is biologically active, although further studies would be required to confirm this. The amount of BDNF mimetic released from the BPGS degraded in DMEM without porcine liver esterase enzyme was below the detection limit of the HPLC until day 12. However, for the experiment with DMEM containing enzyme, the amount released was detectable by day 3. The BDNF mimetic peak area of BPGS degraded in DMEM with enzyme was consistently larger than that in DMEM at different time points (Fig. S3). The amount of BDNF mimetic released from (DMEM-enzyme) solutions accelerated with time due to the reduction in pH which likely further caused acid-catalyzed degradation. At day 30, the concentration of BDNF mimetic in the test using porcine liver esterase enzyme was ~2.6 times higher than for the test using DMEM without enzyme (19.3 μM vs. 7.3 μM).
The secant moduli of the PGS and BPGS films were 625 ± 30 kPa and 587 ± 50 kPa respectively at 10% strain (Fig. 2A), which is greater than the maximum human myocardial stiffness at the end of diastole, which has a secant modulus of 300 kPa.\textsuperscript{31} Although cardiomyocytes cultured on very soft substrates (e.g. those with a Young’s modulus less than 360 Pa) exhibit more intense spontaneous contractions,\textsuperscript{32} stiffer materials implanted on the surface of the infarcted area could provide a greater reduction in myocardium stress and provide a protective effect from ventricular remodelling.\textsuperscript{33} As the myocardium undergoes constant cycles of contraction during its lifetime, repeated extensions need to be performed to characterize the influence of multiple cyclic tensile deformations. For the PGS and BPGS cyclic tests, the resilience, which indicates the elastic recovery ability of the material, was calculated using the ratio between the area under the unloading curve and loading curve (averaged over the test cycles 2 to 50), with 94 ± 4 % and 88 ± 6 % respectively (Fig. 2B, 2C, 2D). Though there was a slight drop in the resilience for BPGS compared with PGS, the difference was not statically significant, indicating that both could endure the working environment of the heart. There were no significant differences in the elongation at break and the ultimate stress for PGS and BPGS, with 134 ± 47% and 213 ± 41%, and 741 ± 70 kPa and 863 ± 50 kPa respectively. These physical properties do not affect beating behavior for cardiomyocytes, since the contraction strain of cardiomyocytes is normally within 15%. However, since they are inversely proportional to crosslinking density, the uptrend in extensibility and ultimate stress indicates a drop in crosslinking density. This can be attributed to loss of BDNF mimetic due to the elevated temperatures during synthesis.
The patterned PDMS moulds (Fig. 3A) and PGS films (Fig. 3B) were visualized and quantified using optical profilometry. From these images, it is facile to measure the widths of the flat channels (bottom sections of the pattern) and the crests (top sections) of the PDMS mould and the cast PGS films. There was no significant variation in channel depth with channel width or between the BPGS and PGS and the average value was determined to be 3.0 ± 0.4 μm. This value corresponds very well with the 3 μm channel depth selected for the thickness of the SU-8.

Figure 2 (A) Stress-strain curves of PGS and BPGS synthesized at 160 °C under vacuum conditions for 8 hr; Plot of the 50 cyclic stress-strain curves of PGS (B) and BPGS (C) synthesized under the same conditions; (D) Comparisons of secant modulus, ultimate strength, elongation at break and resilience between PGS and BPGS.

The patterned PDMS moulds (Fig. 3A) and PGS films (Fig. 3B) were visualized and quantified using optical profilometry. From these images, it is facile to measure the widths of the flat channels (bottom sections of the pattern) and the crests (top sections) of the PDMS mould and the cast PGS films. There was no significant variation in channel depth with channel width or between the BPGS and PGS and the average value was determined to be 3.0 ± 0.4 μm. This value corresponds very well with the 3 μm channel depth selected for the thickness of the SU-8.
photoresist cured onto the silicon wafer. The reason for the selection of a 3 μm photoresist channel was that previous studies have shown that cell alignment increases with an increase in channel depth with a channel depth of 2 μm being the threshold for robust alignment.\textsuperscript{34} However, deeper channels might isolate cells in the channels which could weaken cell-cell communication\textsuperscript{35}. The channel widths of PGS and BPGS were, however, consistently lower and the crest widths were consistently larger than the photomask. That is, the photomask with a nominal width of 10 μm yielded a channel width of 8.6 ± 0.8 μm and a channel crest of 11.3 ± 0.8 μm, while the dimensions for the 20 μm photomask were 17.2 ± 1.0 μm (channel) and 22.2 ± 0.9 μm (crest), and for the 50 μm mask were 44.7 ± 0.9 μm (channel) and 56.4 ± 1.2 μm (crest) as shown in Fig. 3C. As similar dimensions were observed with the PDMS moulds, it is likely the differences between photomask and mouldings were introduced during the crosslinking of the photoresist as a result of UV light diffraction which leads to some crosslinking of the photoresist in shaded areas, which narrowed the channel size, as has also been reported elsewhere.\textsuperscript{36}
Cardiomyocytes grew on both the channels and crests of the BPGS (Fig. 4A) and PGS (Fig. S4), facilitating physical contact of cells between adjacent channels and ensuring intercellular communications. Overall, the alignment of cardiomyocytes was improved on the patterned PGS and BPGS compared with unpatterned films. This was confirmed by fluorescent images of cardiomyocytes stained on day 7 for actin filaments (troponin I) which regulates the contraction behaviour of cardiomyocytes by binding and releasing $\text{Ca}^{2+}$. Compared with the isotropic morphology of cardiomyocytes on unpatterned films, aligned cardiomyocytes showed an elongated cell shape and oval-shaped nuclei along the channel direction of the micropatterned BPGS (Fig. 4B) and PGS (Fig. S4). Cardiomyocytes cultured on 10 μm and 20 μm BPGS films displayed more bridging across the channels (Fig. 4A) (examples shown by arrows) and this...
contrasts with the 50 μm patterned BPGS, where fewer cardiomyocytes bridged across channels. Similar and not statistically significant different results were obtained on the PGS films and are presented in Supplementary Information (Fig. S4). These results may be attributed to the relative size of rat cardiomyocytes which also have a lateral width of approximately 20 μm matching the pattern size. On the other hand, as the longitudinal size of cardiomyocytes is approximately 100 μm, it is more difficult for cardiomyocytes to stretch across the 50 μm channels. Although a previous study on nanodimensional channels (approximately 0.45 μm channel width) showed an improvement in cell alignment with the subcellular nanopatterned structures, those cells are aligned by the confinements of cell–matrix adhesions and actin filaments in channels.

As the nuclei showed an elliptical shape in elongated cardiomyocytes, the alignment of cardiomyocytes could be quantified based on the elliptical nuclei major axis direction. Here, we followed a previous study which defined cells to be aligned when the angle between the major axis of elliptical nuclei and the pattern direction was less than 20. No preference for nuclei orientation was evident on unpatterned PGS and BPGS films as the nuclei orientation was evenly distributed for all directions (inserted figures in Fig. 4B, C and Fig S4). However, cardiomyocytes cultured on patterned PGS and BPGS exhibited preferential nuclei alignment along the channel direction. Although some cardiomyocytes cultured on the 20 μm channel BPGS films bridged across several channels, these films presented the highest proportion of aligned cells with 61 ± 7% of the cells having an alignment angle of less than 20 which was statistically different from the unpatterned films (p < 0.001). Lower alignment was found for
cardiomyocytes cultured on 10 μm patterned films (52 ± 7% cell alignment) and 50 μm patterned films (37 ± 5% cell alignment) and these percentages were each statistically different from the alignment of non-patterned films with p < 0.001 and p < 0.005 respectively (Fig. 4C and 4D). This finding was also supported by the sarcomeric α-actinin in cross-striation sarcomeres (Fig. S5), which is perpendicular to the cell elongation direction. The sarcomers were preferentially perpendicularly aligned in the micropatterned samples (white arrows indicate the pattern direction), while there was no such tendency of cardiomyocytes cultured on unpatterned samples due to the isotropic orientations. Quantification of cardiomyocyte alignment on the PGS, was similar to the BPGS and is provided in the ESI (Fig S4). On all substrates, the immunolabelling for connexin 43, a gap junction protein that enables rapid electrical conduction by spreading Ca$^{2+}$ across cardiomyocytes, was observed between cardiomyocytes (Fig S5).
Figure. 4 (A) Phase contrast images of cardiomyocytes cultured on BPGS films with different patterns showing alignment with channels of nominal width 10 μm, 20 μm, 50 μm and for unpatterned BPGS. The arrows highlight cardiomyocytes which bridged across channels. (B) The alignment of cardiomyocytes was confirmed by fluorescent images stained with troponin I antibody which is a specific marker of myofilaments of cardiomyocytes (green) 10 μm, 20 μm, 50 μm patterns and unpatterned BPGS. The inset images in (B) show elongation of nuclei towards the channel directions in micropatterned BPGS. A similar distribution was seen on PGS that was not statistically different to (C) the percentage of orientation of the nuclei aligned with the channel direction on BPGS and (D) the orientation of the nuclei aligned with the channel direction versus the patterning of the BPGS film. The asterisks indicate the level of statistical difference.
The calcium transients of cardiomyocytes (given as fluorescence intensity relative to baseline, $F/F_0$) cultured on PGS over time were averaged by summation of ROI intensities on a time point divided by the number of ROI (Fig. 5A). As calcium elevation can trigger excitation-contraction coupling which is critical for optimal contractility, the calcium transients over time somewhat reflect the contractile activity of cardiomyocytes. Compared with unpatterned PGS samples that showed beating randomly distributed, most beats in micropatterned PGS samples were coordinated between cells. Synchronized beating behaviour was almost exclusively found on micropatterned PGS samples (Fig. 5B). This effect was particularly strong on 20 μm PGS patterns, while on 10 μm and 50 μm PGS patterns, some beats were not coordinated i.e. minor transients’ peaks overlapped major ones. Though significant differences between time intervals were not found between patterned PGS samples and the unpatterned PGS samples, the beating homogeneity was significantly improved on 10 μm and 20 μm patterns relative to the unpatterned films, as measured by the standard deviation of the average beat time shown in Fig 5C. The non-homogeneous beating behaviour of cardiomyocytes cultured on unpatterned PGS films can be attributed to deficiency of connexin 43 (Fig. S5), which is an essential regulator of cardiomyocyte connectivity and coordinate depolarization and repolarization. We tested the idea that myocytes cultured upon unpatterned surfaces have weaker connectivity by assessing the synchronicity of spontaneous calcium oscillations in different regions of the substrate. Using a correlation matrix, we showed an increase in the number of synchronously active myocytes when cells were cultured upon micropatterened PGS. Thus, using eight regions (for a maximum possible 28 correlations) we identified $11.7 \pm 1.5$ significant correlations between regions on unpatterned PGS; $18.6 \pm 2.1$ correlations at 10 μm patterning; $26.7 \pm 1.1$
correlations at 20 μm; and 19.7 ± 3.5 at 50 μm (Fig. 5D). These data indicate that patterning promotes increased cellular connectivity. Compared with unpatterned PGS samples that showed oscillations in calcium concentration randomly distributed spatially and temporarily (Video 1 ESI), the 20 μm PGS patterned films showed synchronized calcium transients across the field of view (Video 2, ESI). The 50 μm PGS patterned films only showed synchronized calcium transients within small cell clusters (Video 3, ESI).
Figure 5 Averaged calcium transients (A) and peak amplitude (B) of cardiomyocytes cultured 10 μm, 20 μm, 50 μm patterns and for unpatterned PGS. (C) Comparison of the average time between beats and beat homogeneity between different patterned PGS and unpatterned PGS films. (D) Comparison of correlated cells between different patterned PGS and unpatterned PGS films.
The calcium transients of the cardiomyocytes were enhanced on the unpatterned and patterned PGS films in the presence of added BDNF protein (Fig. 6A, 6B) and results are summarized in Fig 6C, 6D. The concentrations of added BDNF protein and BDNF mimetic chosen here were based on previous studies 10-11, 13 to allow comparison of the results. The concentration of the BDNF mimetic was three times higher than the BDNF protein (60 nM vs. 20 nM), but the difference in amplitude and number of cardiomyocytes involved indicated by the number of events in the scattergrams was not significant compared with negative control groups especially on micropatterned PGS samples. For the BPGS film, the release of BDNF mimetic is most likely caused by continuous degradation of the elastomer network. The calcium transient amplitude of the BPGS samples was greater compared with BDNF mimetic treated PGS samples (both unpatterned and patterned). One possibility is the formation of high local concentrations of BDNF mimetic at the interface of the BPGS compared to the PGS with added BDNF mimetic added to the media (60 nM). However, the concentration of BDNF mimetic released from BPGS in media is still below the detection limit of the HPLC (the minimum detectable amount was approximately 5 μM). The other possibility may be the continuous stimulation by the BDNF mimetic for a longer time during culture. Compared with the other two treated groups, where the treatment started at day 4 for 48 hr, the cardiomyocytes cultured on BPGS films would be exposed to the BDNF mimetic at the beginning of cell culture. The longer treatment time might lead to a greater amplitude. Previous studies showed a 10-25% increase in the average calcium transients in cardiomyocytes after 20 nM BDNF protein treatment for 5-20 mins.10-11 In this study, the prolonged treatment time can further enhance the calcium transients up to approximately 80% and such enhancement does not fade with time.
We also tested the intercellular coupling of cardiomyocytes in the presence of BDNF full protein and BDNF mimetic, but no significant increase was found in the number of paired correlated cells (Fig. S6), indicating that the BDNF protein or mimetic influences the intensity of calcium transients and not the intercellular coupling between cardiomyocytes.
Figure 6. Example scattergrams of calcium transients of cardiomyocytes cultured on (A) unpatterned samples and (B) 20 μm patterned PGS without any treatment, with 20 nM BDNF protein or with 60 nM BPGS. Unpatterned and 20 μm patterned with no added BDNF mimetic or protein are also shown. Calcium transients of cardiomyocytes cultured on unpatterned samples (C) and 20 μm patterned samples (D).
Conclusion

Highly elastic BPGS substrates were fabricated with defined micropatterned channels. Compared with unpatterned substrates, cardiomyocytes cultured on the patterned substrates showed increased nuclear alignment and sarcomere alignment and increased regularity of Ca\(^{2+}\) transients, especially for samples patterned with 20 μm channels. The incorporated BDNF mimetic enhances the calcium transients in a similar way to soluble BDNF protein and we speculate that the mimetic is released from the PGS network as it degrades. Future studies should establish the detailed pharmacological and genetic effects of BDNF mimetic and investigate the \textit{in vivo} release of mimetic from BPGS or similar biomaterials to combat myocardial electrical dysfunction.

Supporting Information Available

The following files are available free of charge: Figures showing the NMR data of the BDNF mimetic; example of brightness/contrast adjustment of image; analysis of the degradation of BPGS; fluorescent images of cardiomyocytes cultured on PGS and fluorescent images of cardiomyocytes stained with connexin and sarcomeric α-actinin; videos comparing cardiomyocytes Ca\(^{2+}\) transients cultured on micropatterned and unpatterned samples.

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References


Increased cardiomyocyte alignment and intracellular calcium transients using micropatterned and drug-releasing poly(glycerol sebacate) elastomers

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