Published in final edited form as:

Biomaterials. 2010 March ; 31(7): 1683. doi:10.1016/j.biomaterials.2009.11.056.

Directed Growth of Fibroblasts into Three Dimensional Micropatterned Geometries via Self-Assembling Scaffolds

Mustapha Jamal^a, Noy Bassik^{a,b}, Jeong-Hyun Cho^a, Christina L. Randall^c, and David H. Gracias^{a,d,*}

Mustapha Jamal: mjamal1@jhu.edu; Noy Bassik: noy@jhu.edu; Jeong-Hyun Cho: jcho33@jhu.edu; Christina L. Randall: rchris10@jhu.edu; David H. Gracias:

^a Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, Maryland 21218, USA

^b School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205, USA

^c Department of Biomedical Engineering, Johns Hopkins University, Maryland 21205, USA

^d Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, USA

Abstract

We describe the use of conventional photolithography to construct three dimensional (3D) thin film scaffolds and direct the growth of fibroblasts into three distinct and anatomically relevant geometries: cylinders, spirals and bi-directionally folded sheets. The scaffolds were micropatterned as two-dimensional sheets which then spontaneously assembled into specific geometries upon release from the underlying substrate. The viability of fibroblasts cultured on these self-assembling scaffolds was verified using fluorescence microscopy; cell morphology and spreading were studied using scanning electron microscopy. We demonstrate control over scaffold size, radius of curvature and folding pitch, thereby enabling an attractive approach for investigating the effects of these 3D geometric factors on cell behaviour.

Keywords

tissue engineering; photolithography; curvature; cell culture

1. Introduction

Cells cultured in three-dimensional (3D) microenvironments often exhibit physiological differences from those cultured on flat substrates [1,2]. Tumor models, for example, have shown markedly improved efficacy when cultured in 3D systems that promote the organization of cells into microstructures observed *in vivo* [3–5]. In order to explore the effect that 3D environments have on cell behaviour, a variety of 3D scaffolds have been utilized including porous hydrogels [6], electrospun fiber meshes and rigid polymeric substrates [7]. One limitation shared by these scaffolds is their inherently random spatial orientation. Solid free-form fabrication methods have been used to overcome this limitation by enabling precise

*Corresponding author: +410 5165284, dgracias@jhu.edu (D.H. Gracias), Johns Hopkins University, 3400 N Charles Street, 221 Maryland Hall, Baltimore, MD 21218. Fax: +4105165510.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

control over 3D scaffold features such as pore size and connectivity [8]. Although these methods have provided new capabilities for the study of cells in 3D, they are serial processes which often require specialized equipment that is not easily accessible. Additionally, these processes have limited utility in cost-effective fabrication of curved and simultaneously patterned structures.

Lithographic methodologies including electron beam lithography, soft-lithography, and photolithography have increasingly been utilized to precisely pattern materials for cell culture [9–11]. For example, soft-lithography has been used to functionalize two-dimensional (2D) surfaces and subsequently study cell attachment [12], cell-matrix [13] interactions, as well as the influence of cell geometry on their fate [14]. Cell morphology and adhesion have also been modulated by lithographically patterning materials of varying stiffness [15] and micro- and nano-topographical features [16,17]. Recently, lithographic approaches have also been developed to create 3D patterned cell substrates such as micropatterned hydrogels [18]. By encapsulating cells in photopatternable hydrogels, multi-layered cell culture geometries with well-defined micron scale features such as channels for improved nutrient diffusion are feasible [19,20]. Still, curved geometries such as cylinders, spirals and folded sheets are difficult to construct and pattern using these layer-by-layer lithographic methods. Multilayered cylindrical scaffolds have been demonstrated [21,22], but these scaffolds were rolled-up manually.

In this paper, we adapted a self-assembly strategy [23,24] to construct 3D thin film cell scaffolds and directed the growth of fibroblasts into three geometries that are reminiscent of microstructures abundant in tissues within the body: cylinders (e.g. vasculature, ducts), spirals (glandular coils, cochlea), and bi-directionally folded sheets (gyri/sulci, intestinal villi). An overview of our 3D cell culturing strategy is illustrated in Fig. 1. Briefly, micro-patterned scaffolds were fabricated on 2D silicon (Si) substrates using conventional photolithography (Fig. 1a). Hundreds of 4 mm square 2D templates with well defined sizes, shapes, and materials could be simultaneously fabricated on a single 3 inch diameter Si wafer (Fig. 1b). Upon dissolving the underlying sacrificial layer (Fig. 1c), the scaffolds spontaneously folded into their final curved geometries due to the differential in stresses across multilayer flexible hinges. The scaffolds were then rinsed, coated with the extracellular matrix protein fibronectin to promote cell attachment, and used to guide the 3D growth of fibroblasts (Fig. 1d). The highlight of our technique is that it harnesses the strengths of 2D lithographic patterning while enabling the fabrication of curved, complex 3D cell scaffolds.

2. Material and methods

2.1 Chemicals and materials

Cell culture—L929 mouse fibroblast cells were purchased from ATCC (cat # CCL-1). The following chemicals were purchased from Sigma-Aldrich (Sigma): Minimum Essential Medium Eagle, horse serum, sodium pyruvate, nonessential amino acids and fibronectin from bovine plasma. Phosphate-buffered saline (PBS) was purchased from Gibco.

Fluorescent microscopy—Calcein AM was purchased from both Sigma and Invitrogen. Ethidium homodimer-1 was purchased from Invitrogen.

Fabrication—Si wafers were purchased from Montco Silicon Technologies. Gold (Au) and copper (Cu) slugs were purchased from Alfa Aesar, chromium (Cr) rods from Fil-Tech Inc, Au plating solution from Technic Inc, and the following chemicals from MicroChem Corp.: SC 1800 series photoresist (PR), 351 developer, SU-8 2015 photoresist (SU-8), and SU-8 developer. Iron (III) chloride was purchased from Sigma. Acetone, 2-propanol, and ethanol solvents were purchased from J.T. Baker and Fisher Chemicals.

Scanning electron microscopy—Sodium cacodylate, formaldehyde, glutaraldehyde, and uranyl acetate were purchased from Electron Microscopy Sciences. The following chemicals were purchased from J.T. Baker: sucrose, sodium hydroxide, sodium acetate anhydrous, hydrochloric acid, and osmium tetroxide. Calcium chloride was purchased from Fischer Chemicals and sodium barbiturate from Sigma.

2.2 Fabrication of the scaffolds

The fabrication process is an adaptation of that used in prior work [23,24] specifically designed to include the bio-inert materials Au and SU-8 for cell culture. The details of the fabrication process are as follows: Si wafer substrates were first rinsed with acetone, 2-propanol, and distilled water and were then dried under nitrogen gas. A 15 nm Cr adhesion layer followed by a 150 nm Cu sacrificial layer were thermally evaporated on the wafer (Fig. 2a) at a pressure of approximately 10^{-5} Torr. Next, a lift-off metallization step was used to precisely pattern the stress driving layer that was composed of Cr and Au. Briefly, a 2.7 μm layer of PR was spin-coated at 3000 rpm and baked for 1 minute at 115°C on a hot plate. The micrometer features were patterned by selectively exposing the Si wafer to ultraviolet light through a photomask that was designed using AutoCAD and laser printed on plastic film at 40,640 dots per inch by Fineline Imaging, Inc. The wafer was developed in Microposit 351 Developer resulting in regions of bare Cu and regions of PR. For structures with a single direction of curvature, a stressed bilayer of 140 nm Cr and 30 nm Au was thermally evaporated as before, and upon removing the PR in acetone, only the patterned bilayer remained (Fig. 2b). The lithography step was repeated in order to electrodeposit 270 nm of Au. A similar process was used to deposit 11.5/245/59 nm of Cr/Au/Cr which is necessary for the bi-directionally folded structures. Once the Cr/Au bilayer or Cr/Au/Cr trilayer was complete, we patterned the areas that would become rigid panels in the folded scaffolds. This was achieved by either electrodepositing a 3 μm thick layer of Au or photopatterning a 10 μm thick layer of SU-8 (Fig. 2c). Finally, PR was photopatterned atop the flexible hinge regions (Fig. 2d) and the 2D templates were released by dissolution of the Cu sacrificial layer in a 40 wt % aqueous solution of iron (III) chloride with 5 wt % hydrochloric acid (Fig. 2e). On dissolution at 40°C, the templates lifted-off and spontaneously assembled into 3D scaffold geometries in less than one minute (Fig. 2f). They were rinsed multiple times in deionized water and stored in 1x PBS, pH 7.4, for cell culture.

2.3 Preparation of substrates for cell culture

Scaffolds were transferred from PBS at room temperature to chilled (4°C) PBS containing 25 $\mu\text{g/mL}$ bovine fibronectin. They were incubated for at least 1.5 hours in a bio-safety hood at room temperature before being rinsed with warm 1x PBS, pH 7.4, followed by one rinse with warm cell culture medium as specified in the following section.

2.4 Cell culture

L929 mouse fibroblast cells were cultured in 75 cm^2 culture flasks containing 85% Minimum Essential Medium Eagles with l-glutamine, sodium bicarbonate, 10% horse serum, and less than 1% of both sodium pyruvate and nonessential amino acids. Cells were maintained at 37 °C in a humidity controlled incubator with 5% CO_2 . Cells were suspended using trypsin just prior to culturing on the scaffolds. After rinsing the fibronectin coated scaffolds with media, they were moved to a freshly suspended fibroblast solution ($\sim 10^4$ cells/mL) and incubated for 2 hours to allow cells to adhere. The scaffolds were then rinsed with fresh media to remove non-adhered cells. Finally, they were pipetted into multi-well plates and placed in the incubator as above, with media exchanged daily.

2.5 Cell viability assay

The materials used in the construction of the scaffolds (namely Au, Cr, Cu, SU-8, and PR) were deposited on Si wafers and screened for viability using a two-color fluorescence assay. Cells were first grown to approximately 80% confluency in a tissue culture treated BD Falcon clear 96-well plate. Wells were then exposed to approximately 25 mm square pieces of each test material as well as bare Si. The positive and negative controls were media and latex, respectively. Cells were stained for viability by incubating with calcein AM (2.5 μ M) and ethidium homodimer-1 (4 μ M) in warm PBS after 24 and 48 hours of incubation with test substrates. Fluorescence measurements were made using a Spectra Max Gemini XPS fluorescent spectrophotometer and Softmax Pro analyzer (Molecular Devices). Wells were read at nine discrete sections and samples were done in duplicates. An analysis of variance was performed on the cell viability data obtained along with a post-hoc Scheffe test to investigate the biocompatibility of the individual components of the scaffolds.

2.6 Fluorescence microscopy of cell culture on self-assembling sheets

Cells were stained for viability by incubating with calcein AM (2.5 μ M in warm PBS) for 45 minutes prior to imaging. Fluorescence microscopy was primarily conducted using a Nikon AZ100 multi-zoom microscope fitted with a Nikon DS-Fi1 camera. Focused 3D images were acquired by taking multiple images in the z plane using a motorized stage. The focused images were created using the extended depth of field (EDF) algorithm within Nikon's NIS-Elements software. The multiday cell study (Fig. 3) was performed by taking a z-stack manually on a Nikon Labophot fitted with a QImaging camera and the composite image was again created via EDF using NIS-Elements.

2.7 Scanning electron microscopy (SEM)

Samples were prepared for SEM following a fixation and post-fixation protocol by Perkins *et al* [25]. Briefly, cells were fixed for one hour in a solution containing 0.1M sodium cacodylate, 3.0% formaldehyde, 1.5% glutaraldehyde, 2.5% sucrose, and 5 mM calcium chloride at a pH of 4. Samples were then washed three times in 0.1M sodium cacodylate with 2.5% sucrose at a pH of 7.4 for 15 minutes each, post-fixed with Palade's osmium tetroxide [25] for one hour on ice, then rinsed and incubated at room temperature with Kellenberger's uranyl acetate for two hours in the dark before being dehydrated in chilled ethanol. Afterwards, the samples were dried using a Tousimis SAMDRI-795 critical point dryer, mounted with carbon tape, and sputter-coated with 3 nm of platinum before being imaged using a JEOL JSM-6700F cold cathode field emission SEM.

3. Results and discussion

The self-assembly of the scaffolds is driven primarily by spatial differences of stresses, elastic moduli and Poisson ratios of rigid panels, flexible hinges and open segments within 2D thin film sheets. These differences are engineered by multiple steps of photolithography, thin film deposition and etching. Guidelines and geometric design rules required in the patterning of 2D sheets to enable assembly of specific 3D curved structures are described elsewhere [23,24]. However, in previous work the criterion for material selection was based on ease of patterning and accessibility of different materials. In order to utilize these structures as *in vitro* scaffolds it was necessary to construct them with bio-inert materials, so that cells could be cultured for extended periods of time (e.g. up to a month). Hence, metals like Cu and Ni were replaced by Au and SU-8 [26,27]; thicknesses were retargeted with these materials to achieve self-assembly. The process flow required two major modifications. First, lift-off metallization and electrodeposition were used to pattern the Cr/Au bilayer due to concerns regarding poor wet and dry etching selectivity of Au. Second, the use of a Cu film as compared to a previously used polymeric sacrificial layer provided several desirable features. It served as a conductive

seed layer for electrodepositions and reduced cracking of the 2D sheets. Cu could also be rapidly and selectively etched which enabled the lift-off metallization step and the release of the 2D templates leading to self-assembly.

To demonstrate the feasibility of growing cells on the 3D micro-patterned scaffolds, we investigated the biocompatibility of individual materials for fibroblast culture. In both the 24 and 48 hour cell viability assays, all components with the exception of Cu and the negative, latex exposure control were found to be non-toxic when compared to the media-only positive control ($p < .05$). The viability assay confirmed the suitability of the materials for cell culture. We then evaluated the viability of fibroblasts grown on the self-assembled scaffolds.

Fibroblasts were cultured on a patterned cylindrical volute and the viability of the population was monitored over 9 days (Fig. 3). It should be noted that by day 9, the 3D scaffold was completely covered with a film of cells. We have independently verified that cells are viable on these structures for at least 30 days. Calcein AM was used to visualize the live fibroblasts since it could be administered on multiple days without eliciting a cytotoxic effect. Initially, cells were interspersed along the scaffold (Fig. 3a) and exhibited lamellipodia and an elongated morphology characteristic of fibroblasts grown on planar tissue culture substrates [28,29]. By day 5 (Fig. 3b), the rigid panels and flexible hinges were covered by a confluent layer of cells. The open regions of the scaffold were clearly distinguishable by the absence of fluorescent cells and are an important feature of the design of the scaffolds since they can improve the diffusion of oxygen and growth factors to the cells, which becomes severely limited in cell layers 100–200 μm thick [30]. By day 7 (Fig. 3c) the cells began to grow into multiple layers and the open regions began to fill-in until, by day 9 (Fig. 3d–f), these regions were completely covered by a film of cells.

We fabricated scaffolds with varying geometries, materials, feature sizes, and curvatures. Shown in Fig. 4a and b are zoomed-in optical images of cylinders formed from 2D arrays of 15×15 , 160 μm square panels of Au or SU-8 polymer, respectively. The rigid panels were connected to their neighbours by 80 μm long hinges, resulting in triangular hollow regions. Fibroblasts could be seen on the reflective surfaces of both scaffolds. The SU-8 panels were transparent, which may be useful for cell-migration experiments. We believe that these scaffolds can be constructed with most photopatternable materials; however, retargeting of the width, length and thickness of individual layers may be required based on the thin film stresses, moduli and Poisson ratios of the materials.

Another highlight of the construction methodology is that many 2D patterns can be included while retaining a similar overall 3D curvature and geometry. The cylinder shown in Fig. 4c was processed simultaneously with that of Fig. 4d and although they have different sized patterns, they have approximately the same radius of curvature. This similar curvature was accomplished by halving the dimensions of all the rigid, flexible and open areas while doubling their number. As a consequence of the change in feature size, the cylinders also had similar porosities ($21 \pm 1\%$), but different pore densities (approximately 1 pore per 254 μm^2 for the cylinder in Fig. 4c versus 1 pore per 120 μm^2 for Fig. 4d). Additionally, since the rigid panels and hinges can be coated with specific cell adhesion proteins, one could in principle manipulate the initial cell-cell spacing on the scaffolds. The self-assembly strategy thus enables the precise control over the porosity and patterning on 3D scaffolds which is of importance in studying cell behaviour [7,8,19,22].

Recent work by Rumpler *et al* suggests that cells are able to sense radii of curvature many times greater than their size [31]. Our 3D scaffolds can enable studies of the influence of curvature on cell behaviour since the curvature can be readily manipulated. Scaffolds constructed with (Fig. 4e) and without (Fig. 4f) the rigid PR layer within the hinges showed a

variation in curvature of approximately 30%. The local curvature within the scaffold can also be easily manipulated by changing the thickness of the Cr/Au/PR layers within the hinge. For example, while the folding angle of hinges was approximately 20° in the cylindrical scaffolds (Fig. 4a–d) the folding angle was approximately $\pm 90^\circ$ in the bi-directionally curved 3D scaffold (Fig. 4g and h). This bi-directional scaffold was formed from a single continuous 2D array of 450 μm square Au panels connected by 45 μm long hinges. The bi-directional curvature was generated by micropatterning both Cr/Au and Cr/Au/Cr hinges. The undulations of this cell culture geometry are reminiscent of intestinal villi and the gyri/sulci in the brain, where the increased surface area for a given volume is critical.

Finally, there is considerable versatility in the overall geometry of the curved structure. Shown in Fig. 4i and j is a curved, spiral-like geometry. Unlike the highly interconnected cylinders and bi-directional sheet, the spiral-like geometry was formed by culturing cells on a scaffold ribbon formed by joining two strips of 40 μm square rigid panels connected to their neighbours by 40 μm long hinges. As the ribbons spiralled outward from the core of the scaffold, the flexible hinges were less constrained in their position and could twist into highly convoluted yet less predictable geometries.

Shown in Fig. 5 are SEM images of fibroblasts cultured on a cylindrical double volute scaffold after 5 days. The difference in this volute scaffold design as compared to a normal cylinder was that the square rigid panels of the double volute were only connected to their neighbours at the corners of each panel. Volute tended to roll up much faster at the edges as compared to the centres of the 2D sheets. These double volutes are interesting since they allow easy visualization of cellular interactions across different layers of the scaffold which come in to close contact along a central plane.

The fibroblasts populated the entire length of the double volute (Fig. 5a) and were observed on both the inner (Cr) and outer (Au/PR) scaffold surfaces (Fig. 5b). Interestingly, the cells that were adhered to the inner surface exhibited fewer filipodia and had elongated cell bodies compared to cells adhered to the outer surface. The filipodia were approximately 200 nm in diameter, which closely matches observed values for adherent fibroblasts [32]. We hypothesize that the difference in cell morphology can be attributed to the dissimilar material properties, which we are currently exploring. In addition to these morphological differences, the two cylindrical halves of the double volute scaffold were in close proximity to one another along a central plane. In this region, fibroblasts were observed to adhere to adjacent panels (Fig. 5c). At higher magnification (Fig. 5d), we observed that fibroblasts were not only in contact with cells on adjacent faces via filipodia but many cells bridged the physical gap between the two faces. Although it is not surprising that fibroblasts, a migratory cell line, would be able to populate the entire scaffold, the potential for monitoring cell-cell interactions and migration between two discontinuous regions has important implications for multilayered cultures and tissue engineering [21,22].

From the multi-day cell culture and the SEM images, it is evident that fibroblasts cultured on thin film scaffolds not only adhere to the inner and outer surfaces but over time they become overgrown and form multilayer thick films. We therefore envision multilayered 3D cultures with different cell types populating well-defined regions of the scaffold. To this end, we are exploring methods to culture cells on the 2D templates prior to their self-assembly, which would provide a hands-free alternative to manually stacked and rolled multicellular scaffolds. Our fabrication method allows dissimilar materials to be patterned on the surfaces of the 3D scaffold. Hence, scaffolds can be patterned with regions where the adhesion of proteins and cells is either promoted or eliminated [14,33,34]. For example, the Au and SU-8 surfaces utilized in the scaffolds can be functionalized using specific thiols and silanes respectively [12,35,36] to manipulate cell adhesion in different regions on the surfaces of the 3D scaffold.

Since the methodology is compatible with conventional 2D lithography, other micro- and nanoscale factors (such as surface topography) [16,17,32] that are observed to influence cell behaviour can be readily incorporated onto the surfaces as well. Furthermore, adapting the self-assembly process to generate biodegradable scaffolds using polymers such as polycaprolactone will be of value for *in vivo* tissue engineering applications [7,37,38].

4. Conclusions

We utilized a self-assembly strategy to fabricate multi-material 3D cell scaffolds which enable the directed growth of cells into 3D micropatterned geometries that were previously difficult to achieve. By simply varying the 2D pattern, feature sizes, and thicknesses of the hinge layers, a variety of complex 3D scaffolds reminiscent of fundamental architectural anatomical subunits could be made. These self-assembled scaffolds can enable the systematic study of the effects that geometric cues such as curvature and pitch have on cell cultures.

Acknowledgments

We acknowledge the Integrated Imaging Center, Michael McCaffery and Ed Perkins for their assistance with SEM preparation. We also thank George M. Stern, Chelsey A. Wood, and Martin Rietveld. This work was funded in part by the NIH Director's New Innovator Award Program, part of the NIH Roadmap for Medical Research, through grants DP2-OD004346-01 and DP2-OD004346-01S1, and the National Science Foundation IGERT Program (DGE-0549350).

References

1. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking Cell-Matrix Adhesions to the Third Dimension. *Science* 2001;294:1708–12. [PubMed: 11721053]
2. Abbott A. Biology's new dimension. *Nature* 2003;424:870–2. [PubMed: 12931155]
3. Debnath J, Brugge JS. Modelling Glandular Epithelial Cancers in Three-Dimensional Cultures. *Nat Rev Cancer* 2005;5:675–88. [PubMed: 16148884]
4. Fischbach C, Chen R, Matsumoto T, Schmelzle T, Brugge JS, Polverini PJ, et al. Engineering tumors with 3D scaffolds. *Nat Methods* 2007;4:855–60. [PubMed: 17767164]
5. Feder-Mengus C, Ghosh S, Reschner A, Martin I, Spagnoli GC. New dimensions in tumor immunology: what does 3D culture reveal? *Trends Mol Med* 2008;14:333–40. [PubMed: 18614399]
6. Cushing MC, Anseth KS. Hydrogel Cell Cultures. *Science* 2007;316:1133–4. [PubMed: 17525324]
7. Yang S, Leong K-F, Du Z, Chua C-K. The Design of Scaffolds for Use in Tissue Engineering. Part I. Traditional Factors. *Tissue Eng* 2001;7:679–89. [PubMed: 11749726]
8. Hollister SJ. Porous scaffold design for tissue engineering. *Nat Mater* 2005;4:518–24. [PubMed: 16003400]
9. Li N, Tourovskaia A, Folch A. Biology on a Chip: Microfabrication for Studying the Behavior of Cultured Cells. *Crit Rev Biomed Eng* 2003;31:423–88. [PubMed: 15139302]
10. Chen CS, Jiang X, Whitesides GM. Microengineering the Environment of Mammalian Cells in Culture. *MRS Bull* 2005;30:194–201.
11. Khademhosseini A, Langer R, Borenstein J, Vacanti JP. Microscale technologies for tissue engineering and biology. *PNAS* 2006;103:2480–7. [PubMed: 16477028]
12. Mrksich M, Dike LE, Tien J, Ingber DE, Whitesides GM. Using Microcontact Printing to Pattern the Attachment of Mammalian Cells to Self-Assembled Monolayers of Alkanethiolates on Transparent Films of Gold and Silver. *Exp Cell Res* 1997;235:305–13. [PubMed: 9299154]
13. Balaban NQ, Schwarz US, Riveline D, Goichberg P, Tzur G, Sabanay I, et al. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat Cell Biol* 2001;3:466–72. [PubMed: 11331874]
14. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric Control of Cell Life and Death. *Science* 1997;276:1425–8. [PubMed: 9162012]

15. Gray DS, Tien J, Chen CS. Repositioning of cells by mechanotaxis on surfaces with micropatterned Young's modulus. *J Biomed Mater Res A* 2003;66:605–14. [PubMed: 12918044]
16. Curtis A, Wilkinson C. Topographical control of cells. *Biomaterials* 1997;18:1573–83. [PubMed: 9613804]
17. Flemming RG, Murphy CJ, Abrams GA, Goodman SL, Nealey PF. Effects of synthetic micro- and nano-structured surfaces on cell behavior. *Biomaterials* 1999;20:573–88. [PubMed: 10213360]
18. Nguyen KT, West JL. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* 2002;23:4307–14. [PubMed: 12219820]
19. Tsang VL, Chen AA, Cho LM, Jadin KD, Sah RL, DeLong S, et al. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB J* 2007;21:790–801. [PubMed: 17197384]
20. Du Y, Lo E, Ali S, Khademhosseini A. Directed assembly of cell-laden microgels for fabrication of 3D tissue constructs. *PNAS* 2008;105:9522–7. [PubMed: 18599452]
21. Seunarine K, Meredith DO, Riehle MO, Wilkinson CDW, Gadegaard N. Biodegradable polymer tubes with lithographically controlled 3D micro- and nanotopography. *Microelectron Eng* 2008;85:1350–4.
22. Papenburg BJ, Liu J, Higuera GA, Barradas AMC, de Boer J, van Blitterswijk CA, et al. Development and analysis of multi-layer scaffolds for tissue engineering. *Biomaterials* 2009;30:6228–39. [PubMed: 19674783]
23. Bassik N, Stern GM, Jamal M, Gracias DH. Patterning Thin Film Mechanical Properties to Drive Assembly of Complex 3D Structures. *Adv Mater* 2008;20:4760–4.
24. Bassik N, Stern GM, Gracias DH. Microassembly based on hands free origami with bidirectional curvature. *Appl Phys Lett* 2009;95:1–3.
25. Perkins EM, McCaffery JM. Conventional and Immunoelectron Microscopy of Mitochondria. *Methods Mol Biol* 2007;372:467–83. [PubMed: 18314746]
26. Merchant B. Gold, the Noble metal and the paradoxes of its toxicology. *Biologicals* 1998;26:49–59. [PubMed: 9637749]
27. Voskerician G, Shive MS, Shawgo RS, von Recum H, Anderson JM, Cima MJ, et al. Biocompatibility and biofouling of MEMS drug delivery devices. *Biomaterials* 2003;24:1959–67. [PubMed: 12615486]
28. Hodges GM, Muir MD. A Scanning Electron-Microscope Study of the Surface Features of Mammalian Cells *In Vitro*. *J Cell Sci* 1972;11:233–47. [PubMed: 4672341]
29. Grinnell F. Fibroblast biology in three-dimensional collagen matrices. *Trends Cell Biol* 2003;13:264–9. [PubMed: 12742170]
30. Lightfoot, EN. *Transport Phenomena and Living Systems*. New York: John Wiley; 1974.
31. Rumpler M, Woesz A, Dunlop JWC, van Dongen JT, Fratzl P. The effect of geometry on three-dimensional tissue growth. *J R Soc Interface* 2008;5:1173–80. [PubMed: 18348957]
32. Dalby MJ, Riehle MO, Sutherland DS, Agheli H, Curtis ASG. Changes in fibroblast morphology in response to nano-columns produced by colloidal lithography. *Biomaterials* 2004;25:5415–22. [PubMed: 15130726]
33. Kleinfeld D, Kahler KH, Hockberger PE. Controlled Outgrowth of Dissociated Neurons on Patterned Substrates. *J Neurosci* 1988;8:4098–120. [PubMed: 3054009]
34. Chien H-W, Chang T-Y, Tsai W-B. Spatial control of cellular adhesion using photo-crosslinked micropatterned polyelectrolyte multilayer films. *Biomaterials* 2009;30:2209–18. [PubMed: 19150738]
35. Ostuni E, Chapman RG, Holmlin RE, Takayama S, Whitesides GM. A Survey of Structure-Property Relationships of Surfaces that Resist the Adsorption of Protein. *Langmuir* 2001;17:5605–20.
36. Tao SL, Popat KC, Norman JJ, Desai TA. Surface Modification of SU-8 for Enhanced Biofunctionality and Nonfouling Properties. *Langmuir* 2008;24:2631–6. [PubMed: 18275232]
37. Agrawal CM, Ray RB. Biodegradable polymeric scaffolds for musculoskeletal tissue engineering. *J Biomed Mater Res A* 2001;55:141–50.
38. Martina M, Huttmacher DW. Biodegradable polymers applied in tissue engineering research: a review. *Polym Int* 2007;56:145–57.

Figure 1.

Schematic diagram illustrating the 3D cell culture strategy. (a) Conventional photolithography was used to fabricate scaffolds *en masse*. (b) Scaffolds were initially micropatterned as 2D templates. (c) Scaffolds self-assembled from 2D to 3D geometries on release from the Si substrate. (d) Cells were cultured on the scaffolds and later analyzed using standard microscopy techniques.

Figure 2.

Side-view schematic of the microfabrication process. Illustrated is the cross-section of two rigid panels connected by a flexible hinge. (a) A Cu sacrificial layer was deposited onto the wafer to enable the final scaffolds to release and self-assemble. (b) Lift-off metallization and electrodeposition were used to pattern the Cr/Au stress bilayer that drove the assembly process. (c) Rigid Au panels were patterned via electrodeposition which prevented bending. (d) Similarly, PR was photopatterned onto the flexible hinges. (e) Once the 2D templates were complete, they were released from the flat substrate enabling their self-assembly into 3D scaffolds (f).

Figure 3.

Fluorescently stained fibroblasts cultured on a cylindrical volute scaffold and imaged over 9 days. Cells were stained with calcein AM and viable cells fluoresce green. (a) Cells were interspersed along the scaffold at day 2. They exhibited spread morphologies characteristic of fibroblasts cultured on flat surfaces. (b) At day 5, the cell population had increased in size and covered both the rigid panels and flexible hinges. The open regions were clearly visible by the absence of fluorescently stained cells. At day 7, fibroblasts began growing in the open regions (c) until the entire 3D scaffold was covered in a multilayered film of cells by day 9 (d–f). With the open regions filled-in (d), the location of cells relative to the underlying scaffold features could be distinguished by using an appropriate filter to observe the underlying autofluorescent PR hinges (e). The multilayer film of cells could also be observed using brightfield microscopy (f). Scale bar is 200 μm (same scale bar is 70 μm for insets).

Figure 4.

Versatility of geometry in 3D cell culture. (a–f) Cylindrical cultures grown on scaffolds with varying materials, feature sizes, and diameters. (a,c,d) Cylinder consisting of a 15×15 array of $160 \mu\text{m}$ square rigid panels connected by $80 \mu\text{m}$ long polymer flexible hinges. (a) Optical image of fibroblast culture grown on a cylinder with rigid Au panels connected by PR coated hinges. (b) Optical image of fibroblast culture grown on a scaffold with transparent SU-8 panels and polymer coated hinges of equal dimensions and pattern as that in 'a'. (c) Fluorescent image of the underlying PR polymer layer of the cell-covered scaffold shown in 'a'. (d) Fluorescent image of the underlying PR layer of a cell-covered scaffold exhibiting the same diameter and total segment areas as scaffold 'a', but 4 times as many segments. (e) Fluorescent image of

calcein AM stained cells of culture shown in 'a'. (f) Fluorescent image of cells cultured on an identical scaffold to 'a' but without PR on the hinges. The scaffold diameter was reduced by 30%. (g,h) Bi-directionally folded scaffold with 450 μm wide rigid panels and 45 μm long hinges that folded to $\pm 90^\circ$. (g) Optical image of a bi-directionally folded scaffold with cells. Schematic overlay illustrates the undulatory cross-section of the scaffold. (h) Fluorescently labeled fibroblasts on a bi-directional scaffold. (i, j) Fluorescent images of PR hinges and calcein AM stained fibroblasts cultured on spiral-like ribbons. Ribbons are composed of two columns consisting of rigid panels and PR hinges with 40 μm lengths. Scale bars are 160 μm .

Figure 5.

SEM of fibroblasts cultured on a cylindrical double volute scaffold fixed after 5 days of culture. (a) Confluent layer of cells covered the entire scaffold with clearly visible open regions. (b) Zoom-in of the scaffold showed that fibroblasts were adhered to both the inside and outside of the scaffold. Cells on the inner Cr surface were more elongated and had fewer filipodia than cells grown on the outer Au/PR surfaces. (c) Close-up of the central region of the scaffold where the two cylindrical regions were in close proximity. Fibroblasts were observed bridging the gap between the two adjacent rigid panels (arrow heads). (d) Zoom-in of fibroblasts bridging the gap between the two discontinuous panels. Scale bars are (a) 150 μm , (b,c) 30 μm , and (d) 5 μm .