Aligned Cell Sheets Grown on Thermo-Responsive Substrates with Microcontact Printed Protein Patterns

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Tissue structure is critical to tissue function. However, despite many recent advances in tissue engineering, recapitulation of native tissue structure remains a key challenge in the field.[1] Traditionally, methods to mimic native tissue architecture require the use of artificial scaffolds,[2–4] but ideally, engineered tissues would be made entirely of biological components. Cell sheet engineering techniques address this challenge as they rely on cells to produce their own extracellular matrix (ECM).[5] However, incorporation of structure into cell sheets, has been limited, and often requires lengthy culture times and mechanical removal that may damage the tissue.[5–7] The thermoresponsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) is an attractive biomaterial substrate for cell sheet engineering and has been used extensively for a variety of tissues, such as skin,[8] cornea,[9] cardiac muscle,[10] and liver.[11] Confluent cell layers and their associated ECM can be non-invasively removed from PIPAAm-grafted surfaces as intact sheets, simply by lowering the temperature.[12,13] Cell sheets can be grown in a matter of days and then stacked in multiple layers[14] or rolled into tubes[15] to form three dimensional, completely biological tissue constructs. However, cell sheets are typically grown on uniform PIPAAm surfaces, which results in randomly oriented cells. Attempts to pattern PIPAAm substrates has thus far been limited to multi-step processes with low resolution (pattern dimensions ≥0.5 mm) that were designed for spatially segregating multiple cells types.[16–18] Therefore, we were interested in developing a simple method to grow intact cell sheets that mimic native tissue organization on PIPAAm-grafted substrates.

Microcontact printing is a straightforward and inexpensive technique to precisely control cell shape, organization, and function on a variety of surfaces.[19] Traditionally, it has been used to pattern substrates with alternating cell-adhesive and non-adhesive regions,[20] thus preventing cells from growing to confluence. However, forming continuous, organized cell layers is desirable for a variety of tissue engineering applications. In this paper, we describe a system for growing and harvesting confluent, aligned cell sheets on PIPAAm-grafted substrates that have been microcontact printed with the cell-adhesive protein fibronectin (FN).

The concept is shown in Scheme 1 and was inspired by the work of Schmalenberg, et al.[21] Briefly, PIPAAm-grafted tissue culture polystyrene (TCPS) dishes were printed with FN using patterned or flat polydimethylsiloxane (PDMS) stamps to create lanes or uniform regions of FN, respectively (Scheme 1, path A and path B, respectively). Cells were seeded onto FN-PIPAAm substrates in serum-free media to restrict cell adhesion to FN regions. After cells attached and spread on FN, serum was added to promote growth into non-FN-printed regions, thus forming confluent sheets.

The presence of printed FN on PIPAAm substrates was confirmed by immunofluorescent microscopy. Patterned stamps created FN lanes while flat stamps yielded uniform FN regions (Fig. 1A and B). For cell sheet studies, vascular smooth muscle cells (VSMCs) were seeded onto FN-PIPAAm substrates in serum-free media and allowed to attach overnight. VSMCs preferentially adhered to and spread on FN-stamped regions, becoming elongated and aligned on patterned substrates but were well-spread and randomly oriented on uniform substrates (Fig. 1C and D). At 24 h, serum was added and cells grew to confluence within a few days, remaining aligned or randomly oriented on patterned and non-patterned substrates, respectively (Fig. 1E and F).

Confluent cell sheets were stained for nuclei, F-actin and FN. VSMCs on patterned FN-PIPAAm had aligned actin fibers in the direction of the pattern while non-patterned cells had randomly oriented actin fibers (Fig. 1G and H). FN staining showed that an organized matrix formed on patterned FN-PIPAAm, as many fibers aligned with the pattern direction; FN on non-patterned substrates was disorganized (Fig. 1I and J). The images demonstrate that patterning influences both cell and ECM organization, both of which are critical to defining tissue structure.
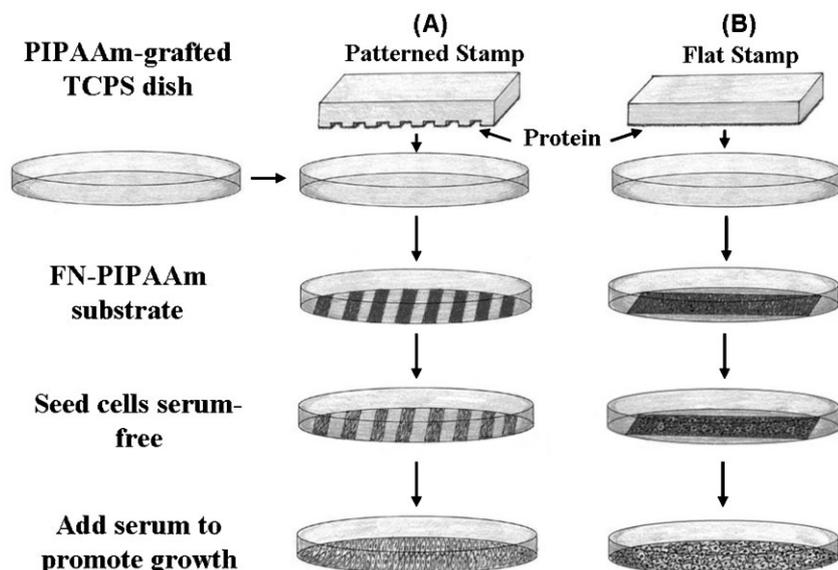
Confluent patterned and non-patterned cell sheets were released uniformly as tissue-like monolayers from FN-PIPAAm substrates by lowering the culture temperature. Cell sheets were incubated at 20 °C and periodically checked for detachment. At 0 min, cell sheets were fully attached to the FN-PIPAAm substrates (Fig. 2A and B). After 90 min, cell sheets were in the process of detaching and by 120 min were fully released from the dish surface (Fig. 2C and D). There was no observed difference in detachment speed between patterned and non-patterned cell sheets. Confluent cells cultured on regular TCPS dishes did not spontaneously detach (data not shown).

We noted that cell sheets harvested by free detachment lost the organization imposed by FN-PIPAAm substrates as the cell sheets contracted during release. Therefore, we further investigated methods to harvest sheets without losing their organization. Previously, gelatin stamps have been used to stack

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(A) Patterned Stamp

(B) Flat Stamp

PIPAAm-grafted TCPS dish

← Protein →

FN-PIPAAm substrate

Seed cells serum-free

Add serum to promote growth

Scheme 1. Development of aligned VSMC sheets on FN-PIPAAm. TCPS dishes grafted with PIPAAm are microcontact printed with fibronectin (FN) protein using A) a patterned stamp or B) a flat stamp. The resulting FN-PIPAAm substrate consists of FN lanes or a uniform FN region, respectively. Cells seeded serum-free preferentially adhere to FN. Serum is then added to promote expansion of cells into regions without printed FN, resulting in a confluent, aligned or randomly oriented cell sheet, respectively.

structural design of a variety of tissues. While we only describe VSMCs in this paper, we have also made aligned cardiomyocyte sheets using the same method (preliminary data, unpublished). Future work will involve layering patterned sheets in a variety of configurations to build 3D tissues with well-defined structure. Additionally, the mechanical, biochemical, and functional properties of aligned cell sheets will be further characterized. In conclusion, our method will be beneficial to advancing the design of engineered tissues that more closely replicate native tissue architecture.

Experimental

Photolithography for PDMS Stamps: Patterned polydimethylsiloxane (PDMS) stamps were made using soft lithography, as previously described [22]. High resolution transparency masks (CAD/Art Services, Bandon, OR, USA) were printed with patterns consisting of 50 μm-wide lines with 50 μm-wide spacing. Silicon wafers were cleaned by acetone, methanol, and isopropyl alcohol. A 5 μm-thick layer of photoresist (SU-8 3005, MicroChem Corp., Newton, MA, USA) was cast onto wafers using a spin-coater (Active Co., Ltd., Saitama City, Japan). The wafers were then exposed to UV light through the patterned mask using a BA100 mask aligner (Nanometric Technology, Inc., Tokyo, Japan). Patterned wafers were developed using ethyl lactate and then treated with a solution of 2% dimethyloctadecylchlorosilane (Shin Etsu Chemical Co., Ltd., Tokyo, Japan) in toluene to facilitate removal of PDMS stamps. PDMS pre-polymer and catalyst (Silpot 184 and Catalyst Silpot 184, Dow Corning, Tokyo, Japan) were mixed at a ratio of 10:1 and cured against the patterned wafers at 80 °C for 2 h. Patterned stamps were then carefully cut from the wafer surface. Flat stamps were cut from non-patterned regions of the wafer. Patterned stamp dimensions were 50 μm-wide grooves and ridges with 5 μm groove depth.

Microcontact Printing of PIPAAm Substrates: PIPAAm-grafted tissue culture polystyrene (TCPS) dishes were made according to previously published methods [12] and sterilized by ethylene oxide gas. Immediately prior to use, PDMS stamps were cleaned with acetone, methanol, and isopropyl alcohol and dried with air. Stamps were then treated with oxygen plasma (PX-1000, SAMCO International, Kyoto, Japan) at 400 W for 180 s to render the surfaces hydrophilic. Stamps were immediately covered with pre-warmed fibronectin (BD Biosciences, Bedford, MA, USA) diluted to 100 μg mL^{-1} in sterile phosphate buffered saline (PBS) and incubated for 10 min at 25 °C. Excess solution was aspirated from the stamp surfaces and the stamps were then thoroughly dried with air. Stamps were brought into conformal contact with the PIPAAm substrate, firmly pressed for 15 s, and carefully peeled away. FN-stamped PIPAAm (FN-PIPAAm) substrates were gently rinsed three times with PBS and either fixed for immuno-fluorescent imaging or stored in serum-free media at 37 °C until cell seeding (<3 h).

Cell Seeding and Culture: Human aortic vascular smooth muscle cells (VSMCs) (AoSMC passage 3, Lonza, Basel, Switzerland) were cultured on regular TCPS dishes (Corning, Tokyo, Japan) in complete media consisting of Smooth Muscle Cell Basal Medium (SmBM) (Clonetics, Walkersville, MD, USA) supplemented with growth factors (SmGM-2 SingleQuots, Clonetics), 5% fetal bovine serum (FBS) (Gibco, Tokyo, Japan) and 1% penicillin-streptomycin (Sigma, Tokyo, Japan). Cells were maintained in a humidified incubator with 5% CO$_2$ at 37 °C. For seeding onto FN-PIPAAm substrates, VSMCs were harvested with trypsin (Gibco, Tokyo, Japan) and collected in serum-free media (complete media without FBS) containing soybean trypsin inhibitor (Sigma). Cells were seeded serum-free at near-confluent density onto FN-PIPAAm substrates and allowed to attach
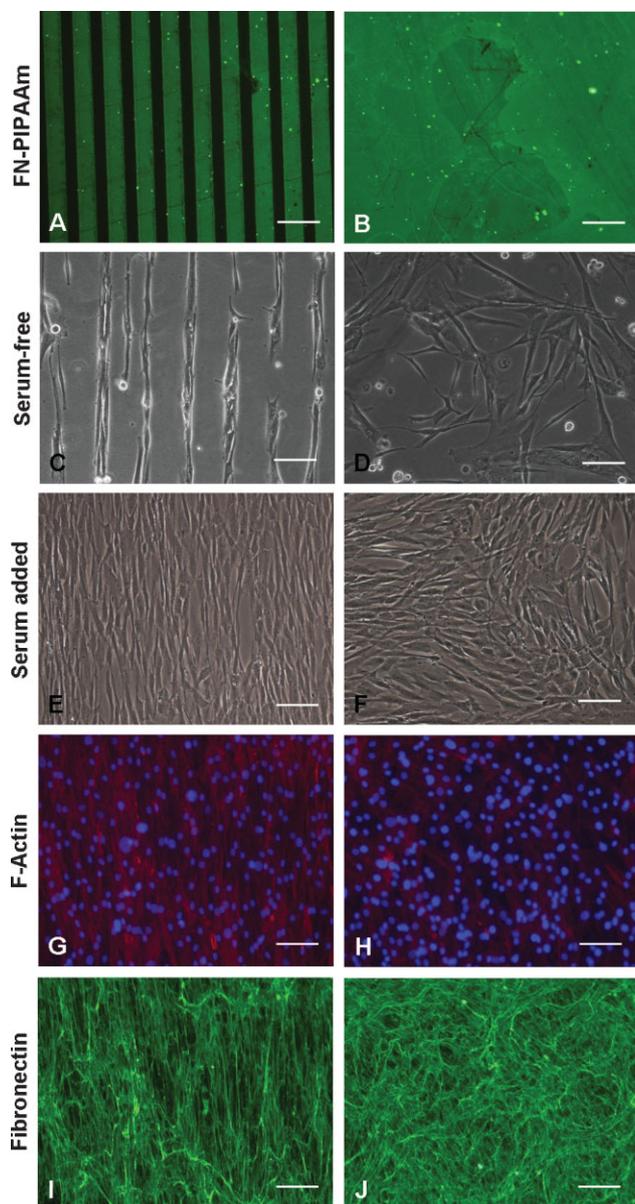
multiple cell sheets;[18] here, we used this technique to maintain cell sheet organization. Cell sheets incubated with gelatin stamps at 20 °C detached from the FN-PIPAAm surfaces and adhered to the gelatin. The stamps were then incubated on regular TCPS dishes at 20 °C to allow transferred cell sheets to adhere to the new surface. The gelatin was dissolved in excess media at 37 °C and removed. Brightfield microscopy showed that cell sheets maintained orientation before and after transfer: patterned cell sheets remained aligned (Fig. 3A and B) and non-patterned remained randomly oriented (Fig. 3C and D). Cell orientation was determined for both patterned and non-patterned VSMCs under serum-free, confluent, and transferred conditions. The majority of patterned cells maintained alignment within 30° of the direction of the FN lanes (serum-free: >90%, n = 419; confluent: > 90%, n = 439; transferred: >70%, n = 541) (Fig. 3E). For non-patterned cells, angles were measured with respect to an arbitrary direction. Non-patterned cells demonstrated no preferred direction for all conditions (serum-free: n = 453; confluent: n = 342; transferred: n = 532) (Fig. 3F).

In summary, we developed a simple method to create confluent patterned cell sheets on thermo-responsive substrates. FN was microcontact printed onto PIPAAm-grafted TCPS dishes to create patterned lanes or non-patterned, uniform regions. Patterned FN-PIPAAm promoted VSMC alignment parallel to the lanes while non-patterned FN-PIPAAm resulted in randomly oriented cells. Cells maintained aligned or random organization on the respective substrates even at confluence. Both patterned and non-patterned cell sheets could be harvested by lowering the temperature. A gelatin stamp was required to prevent cell sheet contraction and thus preserve cell sheet organization. However, the gelatin stamp is advantageous as it allows stacking of multiple cell sheets. We believe that our method will be applicable to other cell types, proteins, and pattern geometries, thus allowing precise

Figure 1. Characterization of FN-PIPAAm and cell sheets. Patterned (left column) and non-patterned (right column) substrates and sheets were created according to Scheme 1. Immunofluorescent staining of FN-PIPAAm shows that patterned stamps yielded FN lanes (A) while flat stamps yielded uniform FN regions (B). When seeded serum free, cells preferentially adhered to FN regions. On patterned FN-PIPAAm, cells became elongated and aligned parallel on lanes (C); on non-patterned FN-PIPAAm, cells were randomly oriented (D). After the addition of serum, cells grew into surrounding regions and became confluent while maintaining alignment (E) or random orientation (F). Staining for actin shows that fibers were highly aligned in patterned cells (G) and randomly oriented for non-patterned cells (H). Cells on patterned substrates formed organized FN matrix (I) while cells on non-patterned substrates had disorganized FN (J). Scale bars = 100 μm.

overnight. The next day, serum-free media was removed and cells were given complete media for the remainder of the culture period. Media was changed every 2–3 days. VSMCs at passage 4–8 were used for the experiments.
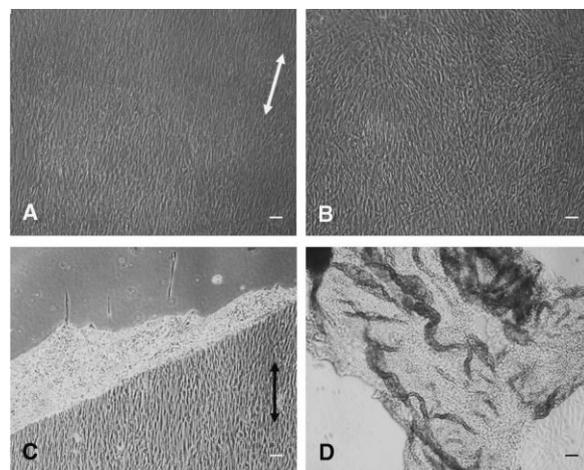

Figure 2. Cell sheet detachment from FN-PIPAAm. Cell sheets were detached from FN-PIPAAm substrates by lowering the culture temperature to 20 °C. At $t = 0$, patterned (A) and non-patterned (B) sheets were fully attached to FN-PIPAAm substrates. At $t = 90$ min, both patterned (C) and non-patterned (D) sheets were in the process of detaching. By $t = 120$ min, both patterned and non-patterned cell sheets were fully detached from the substrates (data not shown). Double-headed arrows indicate direction of alignment. Scale bars = 100 μm.

Substrate Characterization by Immunofluorescent Microscopy: FN-PIPAAm substrates were fixed with pre-warmed 4% paraformaldehyde (PFA) for 15 min and then rinsed three times with PBS. Non-specific binding was blocked with 0.1% bovine serum albumin (BSA) solution for 1hr at 37 °C. Samples were then incubated with fibronectin antibody (rabbit anti-bovine FN polyclonal antibody, Biogenesis, UK) at 1:200 dilution in 0.1% BSA solution for 2 h at 37 °C. Samples were washed three times with BSA solution and then incubated with a secondary antibody (Alexa Fluor 488 conjugate, anti-rabbit, Molecular Probes, Carlsbad, CA, USA) at 1:200 dilution in 0.1% BSA for 1h at 25 °C. Samples were rinsed three times with 0.1% BSA and then covered with PBS for imaging. Images were acquired with a Nikon Eclipse TE2000-U microscope and Axiovision Software release 4.4.

Cell Morphology and Cell Sheet Staining: VSMCs were imaged using brightfield and immunofluorescent microscopy. For staining, samples were fixed with pre-warmed 4% PFA for 15 min and rinsed three times with warmed PBS. Samples were permeabilized with 0.5% TritonX-100 (Sigma) for 10 min at 25 °C and rinsed three times with PBS. Samples were blocked with 0.1% BSA solution for 1 h at 37 °C and then incubated with Hoechst 33258 (Molecular Probes; 1:400 dilution), Alexa Fluor Phalloidin 568 conjugate (Molecular Probes; 1:200) and fibronectin antibody (1:200) for 2 h at 37 °C. Samples were rinsed three times with 0.1% BSA solution and then incubated with Alexa Fluor 488 (1:200) for 1 h at 25 °C. Samples were rinsed 3 times with 0.1% BSA, covered with PBS, and imaged.

Cell Sheet Detachment: Cell sheets were detached from FN-PIPAAm substrates by lowering the culture temperature. Cell sheets were scored around the edges with a micropipette tip and then placed in an incubator set to 20 °C. Samples were monitored every 15 min to assess detachment progress and imaged using brightfield microscopy. Occasionally, samples were gently agitated to facilitate cell sheet release.

Cell Sheet Transfer: Cell sheets were transferred to new TCPS dishes using a gelatin stamping technique[18]. Custom-made cell sheet manipulators were coated with a 1.5 mm-thick layer of gelatin (Sigma) and then placed on top of cell sheets. The gelatin stamps were incubated with the samples for 1hr at 20 °C so that the cell sheets would detach from the FN-PIPAAm substrates and adhere to the gelatin. The gelatin stamps were then removed with the attached cell sheets and transferred to new TCPS dishes. After another incubation period of 1 h at 20 °C, the sheets had
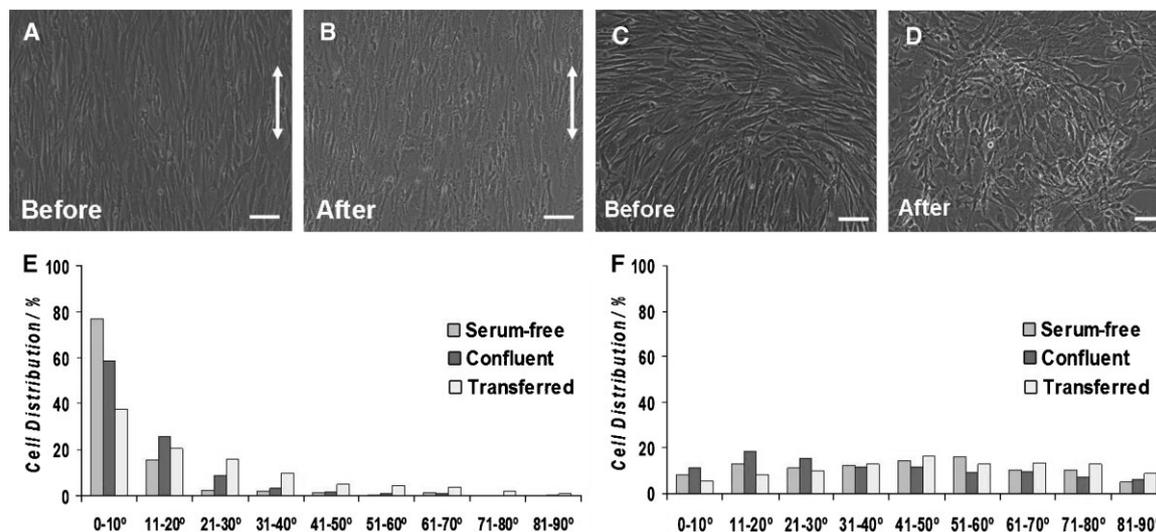
Figure 3. Cell sheet transfer and cell alignment calculations. Patterned cell sheets are aligned before (A) and after (B) transfer using a gelatin stamp. Non-patterned cell sheets remain randomly oriented before (C) and after (D) transfer. Cell angle distribution calculations show that patterned cells maintain a high degree of alignment for all conditions examined (E) while non-patterned show no preferred orientation (F). Scale bars = 100 μm.

attached to the new TCPS surfaces. The gelatin was dissolved in excess culture media at 37 °C and the samples were thoroughly rinsed with fresh media. Brightfield images of the cell sheets before and after transfer were taken.

Cell Orientation Measurements: Cell orientation was measured from fluorescent images of nuclei [23] for serum-free, confluent, and transferred conditions. Nuclear staining with Hoechst 33258 and image acquisition were as described above. Cell orientation was measured using ImageJ software v1.37 (NIH, Bethesda, MD, USA) by determining the angle of the long axis of the nucleus with respect to the parallel direction of the pattern. In the case of non-patterned samples, orientation was measured with respect to an arbitrary direction. A measurement of 0° indicates perfect alignment with the pattern or arbitrary direction, with the maximum angle measurement of 90° indicating perpendicular orientation.

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