

# Engineering high-density endothelial cell monolayers on soft substrates

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## Abstract

This study demonstrates that a confluent monolayer of endothelial cells (ECs) can be tissue engineered on a soft substrate with a cell density and morphology that approximates *in vivo* conditions. We achieved formation of a confluent EC monolayer on polydimethylsiloxane (PDMS) elastomer by microcontact printing of fibronectin (FN) in a square lattice array of 3  $\mu\text{m}$  diameter circular islands at a 6  $\mu\text{m}$  pitch. Uniform coatings of FN or serum proteins on PDMS or on tissue-culture-treated polystyrene failed to support the equivalent EC density and/or confluence. The ECs on the FN micropatterned PDMS achieved a density of  $1,536 \pm 247$  cells  $\text{mm}^{-2}$ , close to the  $3,215 \pm 336$  cells  $\text{mm}^{-2}$  observed *in vivo* from porcine pulmonary artery and significantly higher (2- to 5-fold) than EC density on other materials. The probable mechanism for enhanced EC adhesion, growth and density is increased focal adhesion (FA) formation between the ECs and the substrate. After 14 days culture, the micropatterned FN surface increased the average number of FAs per cell to  $35 \pm 10$ , compared to  $7 \pm 6$  for ECs on PDMS uniformly coated with FN. Thus, microscale patterning of FN into FA-sized, circular islands on PDMS elastomer promotes the formation of EC monolayers with *in vivo*-like cell density and morphology.

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## 1. Introduction

The clinical application of small-diameter artificial vascular grafts (<6 mm) is limited due to high rates of occlusion from neointimal hyperplasia [1–3]. This thickening of the vessel wall by proliferating smooth muscle cells is due at least in part to (i) mechanical mismatch at the anastomosis between the graft and natural blood vessel [4]; and (ii) poor re-endothelialization of the luminal surface [1,5]. Researchers have explored a variety of techniques to address these problems, ranging from surface modification of standard artificial grafts [6–9] to complete tissue-engi-

neered grafts [10–14], all in an attempt to recapitulate the natural vessel. Unfortunately, no approach has achieved a graft with long-term patency that is also practical for clinical use. Further, total tissue engineered approaches require months to fabricate and are labor and material intensive, i.e. expensive. There is need, therefore, for an off-the-shelf option for emergency medicine and low-cost applications. In an attempt to address this problem, we sought to engineer an endothelial cell (EC) monolayer that mimics the cobblestone morphology and high-density of ECs *in vivo* on a Food and Drug Administration (FDA)-approved, low-cost substrate with mechanical properties similar to those of natural artery.

We engineered a smooth, biochemically micropatterned surface designed to provide specific adhesion cues for the support and growth of a high-density EC monolayer. The method used to create these cues was microcontact printing ( $\mu\text{CP}$ ), which has been widely applied over the past decade to selectively pattern extracellular matrix (ECM) proteins

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onto surfaces [15–17]. Using this technique, a number of studies have examined adhesion and growth of single cells, focusing on how the geometric arrangement and type of ECM protein affects cell fate [18], cell spreading [17,19], cytoskeletal architecture [19–21] and formation of focal adhesions (FAs) [18,19,21,22]. Because we were interested in growing high-density, confluent monolayers, we adopted a strategy to control FA density by using  $\mu$ CP to pattern arrays of small ECM islands. Closely spaced arrays ( $<10 \mu\text{m}$  pitch) of fibronectin (FN) islands ( $<25 \mu\text{m}^2$ ) have been shown to support spreading of single ECs and other cell types, with vinculin-positive FAs co-localized on the islands [18,19,21,22]. Specifically, Chen et al. showed that the spreading of single ECs on FN circle arrays increased with decreasing diameter and pitch [21]. Thus, the size and spacing of micropatterned ECM protein islands may be used to control cell adhesion and growth by directing focal contact adhesion formation.

Moving from the single-cell to multi-cell level,  $\mu$ CP methods have been applied to the engineering of two-dimensional (2-D) tissue monolayers [23–27]. It is possible to control cell shape and phenotype [24], direct alignment of Schwann cells [25] and endothelial cells [28,29], and engineer anisotropic myocardial sheets [23,27]. Based on this, we have extended the ability to control FA density at the single-cell level to multi-cell EC monolayers by engineering an array of  $3 \mu\text{m}$  diameter FN circles at a  $6 \mu\text{m}$  pitch on polydimethylsiloxane (PDMS) elastomer surfaces. PDMS elastomer was selected as a model substrate based on a number of key factors. First, compliance mismatch between the artificial vascular graft and host vessel at the anastomosis is known to elicit intimal hyperplasia [1,4,30]. The polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE) graft materials used for large caliber grafts are substantially stiffer ( $E \sim 14 \text{ GPa}$ ) than arteries [3]. In comparison, the elastic modulus ( $E$ ) of the PDMS used is  $\sim 1.5 \text{ MPa}$  by bulk tensile testing [31] and AFM nanoindentation [32,33]. This is within an order of magnitude of the  $E \sim 0.4 \text{ MPa}$  reported for human coronary artery by tensile testing [34] and similar to the  $E \sim 3 \text{ MPa}$  reported for basement membrane—the ECM structure ECs adhere to in vivo—by AFM nanoindentation [35]. Second, PDMS is a medical-grade, FDA-approved polymer that is well characterized as a cell culture substrate for  $\mu$ CP of ECM proteins [15]. As such, engineering the surface of PDMS to support high-density EC growth has potential use in off-the-shelf vascular grafts, ventricular assist devices and other blood-contacting applications.

We cultured porcine vascular ECs on five different substrates in order to demonstrate the enhanced functionality of the micropatterned PDMS surface relative to uniform surface chemistries. These consisted of tissue culture grade polystyrene (PS), tissue culture grade PS uniformly coated with FN ( $\text{PS}_{\text{FN}}$ ), plasma-oxidized PDMS ( $\text{PDMS}_{\text{Plasma}}$ ), plasma-oxidized PDMS uniformly coated with FN ( $\text{PDMS}_{\text{FN}}$ ) and plasma-oxidized PDMS micropatterned with FN circles ( $\text{PDMS}_{\text{FNcircles}}$ ). The EC monolayer confluence, cell density and FA density were evaluated at 4, 7 and

14 day time points. These results were compared to the EC density found in freshly harvested porcine pulmonary artery (PPA) to determine the ability of the  $\mu$ CP PDMS surface to recapitulate in vivo EC density.

## 2. Materials and methods

### 2.1. Substrate fabrication

Tissue culture grade polystyrene (PS) Petri dishes (35 mm diameter, Corning) were used directly as received or were coated with PDMS by dispensing  $\sim 2 \text{ ml}$  of Silastic<sup>®</sup> T2 (Dow Corning<sup>®</sup>) PDMS elastomer prepolymer into the Petri dish and curing at  $\sim 22 \text{ }^\circ\text{C}$  for 24 h. PDMS films cured in the Petri dishes were further modified by exposure to an argon radiofrequency glow discharge (RFGD) plasma for 1 min at 50 W, generating  $\text{PDMS}_{\text{Plasma}}$  substrates. To create uniform FN surfaces the PS- and  $\text{PDMS}_{\text{Plasma}}$ -coated Petri dishes were functionalized by incubating with  $50 \mu\text{g ml}^{-1}$  FN (Sigma<sup>®</sup>) in Hank's balanced salt solution (HBSS) for 1 h at  $\sim 22 \text{ }^\circ\text{C}$ , generating  $\text{PS}_{\text{FN}}$  and  $\text{PDMS}_{\text{FN}}$ , respectively. The array of  $3 \mu\text{m}$  diameter FN circles at  $6 \mu\text{m}$  pitch was patterned onto  $\text{PDMS}_{\text{Plasma}}$  surfaces to generate  $\text{PDMS}_{\text{FNcircles}}$  using a process adapted from standard  $\mu$ CP methods [15,16,28]. Briefly, the micropattern was generated in CAD and transferred to a chrome on quartz photomask by electron beam writing (Compugraphics<sup>®</sup>, Inc.). Silicon wafers were treated with hexamethyldisilazane, spin coated with Shipley 1813 positive photoresist and exposed to UV light through the photomask via contact photolithography. Exposed photoresist was developed and the silicon wafer was etched to a depth of  $3 \mu\text{m}$  by deep reactive ion etching (Bosch process). After processing, the remaining photoresist was removed with a piranha etch, and Silastic T2 PDMS elastomer stamps were created by curing prepolymer against the micromachined silicon wafer masters. Once cured, PDMS stamps were cut into  $1 \text{ cm}^2$  squares and argon RFGD plasma treated at 50 W for a period of 1 min. This plasma treatment step was critical to enable FN adsorption and accurate transfer with the dimensions used in this study. Prior to patterning, all stamps and substrates were sterilized in 70% ethanol and air-dried. Stamps were inked with a  $50 \mu\text{g ml}^{-1}$  solution of FN in HBSS and incubated at room temperature for 1 h, rinsed thoroughly with HBSS and then dried under nitrogen. Substrates were stamped by bringing the PDMS stamp in conformal contact with the PDMS substrate for 1 h and subsequently washed three times with HBSS prior to seeding.

### 2.2. Analysis of substrate topography by atomic force microscopy

Atomic force microscopy (AFM) was used to analyze the topography and surface roughness of the different substrates. Specifically examined were PS, PDMS (before plasma treatment),  $\text{PDMS}_{\text{Plasma}}$  and  $\text{PDMS}_{\text{FN}}$ . All topographic imaging was performed using a Dimension<sup>®</sup> 3100

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