Perforation Does Not Compromise Patterned Two-Dimensional Substrates for Cell Attachment and Aligned Spreading

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Supporting Information

ABSTRACT: Polymeric sheets were perforated by laser ablation and were uncompromised by a debris field when first treated with a thin layer of photoresist. Polymer sheets perforated with holes comprising 5, 10, and 20% of the nominal surface area were then patterned in stripes by photolithography, which was followed by synthesis in exposed regions of a cell-attractive zirconium oxide-1,4-butanediphosphonic acid interface. Microscopic and scanning electron microscopy analyses following removal of unexposed photoresist show well-aligned stripes for all levels of these perforations. NIH 3T3 fibroblasts plated on each of these perforated surfaces attached to the interface and spread in alignment with pattern fidelity in every case that is as high as that measured on a nonperforated, patterned substrate.

KEYWORDS: polymer laser ablation, perforated substrate, two-dimensional patterned cell alignment

A challenge for tissue engineering is to direct cell organization in a three-dimensional (3D) device; an ideal device should induce cells to adopt the specific, native-like organization characteristic of a particular tissue type that is required for proper function. Among such scaffold models are 3D printed structures, hydrogels, porous foams, and exogenous extracellular matrix (ECM). These scaffold classes have been constructed artfully using synthetic or natural materials to incorporate, for example, growth factors or cell-attachment peptides to improve bioactivity; others have been designed to incorporate microfluidic channels to mimic vascularization. Several of these have shown clinical promise for tissue repair. Templating spatially aligned cell growth in a 3D construct that leads to similarly aligned ECM might provide the basis for a scaffold model to recapitulate native tissue properties. 3D printing occurs, effectively, through layer-by-layer two-dimensional (2D) printing. 3D constructs can thus be envisaged as a stack of 2D patterned sheets in which spatially controlling cell spreading on their surfaces is key.

Our method to accomplish aligned cell spreading and ECM assembly was demonstrated on a range of biomaterial polymers. It involves patterning with a cell-attractive, two-component interface consisting of micrometer-dimension stripes of a zirconium oxide (ZrO₂) layer that is terminated with a self-assembled monolayer of an α,ω-diphosphonate (SAMP). This nanometer-thin construct templates attachment and proliferation of plated cells in register with the chemical pattern; cell proliferation is unconstrained and remains aligned over the entire surface, and cell-assembled ECM is also in register with the ZrO₂-phosphonate pattern. Stacking such sheets in a 3D construct could, however, be unfavorable for cell viability in the depths of the device: lateral transport of oxygen and nutrients from the device periphery to cells growing toward its center, and removal of waste products from it, could be limiting. It is well-established that perforation facilitates transport in biological and engineered systems. Therefore, were perforations to be present in each 2D-patterned layer of a 3D construct, transport of nutrients and waste products throughout the device would be facilitated, yet these very holes could compromise our cell templating approach. Here, we show that films of polyetherether ketone (PEEK) can be perforated systematically to 20% of their nominal surface areas by laser ablation and that such perforations do not compromise controlled patterning with a cell-adhesive ZrO₂-phosphonate interface. Furthermore, we show that these surfaces template cell spreading with spatial alignment control as strong as that achieved using nonperforated surfaces.

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In a typical experiment, a 355 nm diode pumped, solid state pulsed Nd:YVO₄ laser was used to perforate 50 μm-thick PEEK films. The laser beam was focused to a diameter ca. 8 μm at a energy ca. 40 μJ, yielding a fluence of ca. 80 J/cm², which is in accord with published values for the ablation threshold and hole depth for PEEK films ablated by a 308 nm XeCl eximer pulsed laser; it was adequate to cut through the PEEK with one pass. The laser pulsed every 1 μm while tracing out the perforations, and the stages moved at a speed of 10 mm/s, giving a laser repetition rate of 10 kHz. Initial attempts at ablating clean 1 × 1 cm PEEK substrates showed that the perforation process yielded a debris field that could be visualized by optical microscopy as large, dark areas surrounding the ablated holes (Figure 1A); sonication in ethanol did not remove the debris. This problem was obviated through the simple expedient of first coating the polymer coupons with the same photoresist that was used subsequently for surface photolithographic patterning (Figure 1B).

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When photoresist AZ-5214-E was spin-cast on both sides of the PEEK coupons and then heated (95 °C) to cure, debris from ablation procedure landed on top of the photoresist; removal by sonication in ethanol showed the formation of well-defined perforations that were surrounded by clean polymer (Figure 1B).

Perforations 130 × 30 μm were chosen to be large enough to prevent blockage by a single cell; perforations on PEEK surfaces were introduced in several patterns such that the distance from an attached cell to any hole would be no more than 200 μm, the estimated maximum distance that oxygen diffuses from a capillary to support metabolically active tissue. We prepared substrates in which 5, 10, and 20% of their nominal surface areas consisted of holes to test the limits of our patterning procedures. Doubling the number of holes to convert 5% nominal surface coverage to 10% was done either “head to tail” (Figure 2B) or “side to side” (Figure 2C); surfaces with 20% nominal hole coverage were created by...
ethanol (0.25 mg/mL); this ethanol treatment also removed any residual photoresist. Optical microscopy showed the patterned stripes of the photomask were oriented parallel to the perforated holes. Treating with AZ MIF 300 developer for 35 s gave oriented, striated patterns on the perforated PEEK (Figure 2). The ensembles were then exposed to vapor of zirconium tetra(tert-butoxide) (1) for 5 min at 10⁻³ Torr; oxygen-containing functionalities of the PEEK enable its coordinative bonding to 1.²⁹ Heating to 65 °C gave a surface-bound, cross-linked, mixed zirconium oxide/tert-butoxide pattern, which was converted to patterned ZrO₂−phosphonate by immersion in a solution of 1,4-butanediophosphonic acid in ethanol (0.25 mg/mL);²⁷ this ethanol treatment also removed residual photoresist. Optical microscopy showed the patterned regions to be parallel with the ablated holes; both holes and patterns were uniform over the entire PEEK surface, with hole coverage of 5, 10, and 20% of the substrates nominal areas (Figures 2A–E). Apparently, even when 20% of the surface has been ablated away, the substrate could still be spin-coated with an adequate layer of photoresist in both uniformity and thickness for photolithographic patterning. EDX analysis (Figure 2F–G) confirmed the composition of these patterns to be the desired ZrO₂−phosphonate. Because the ZrO₂ interface is only about 1 nm-thick and its phosphonate termination is a monolayer,³⁹ EDX signals for Zr and P are weak and difficult to distinguish from background; line scans of EDX data are supportive of the assignments made here (Supporting Information, Figure 1).

NIH 3T3 fibroblasts were plated on ZrO₂−phosphonate-patterned PEEK substrates with 5, 10, and 20% nominal surface hole coverages to determine if perforation adversely affected cell attachment and spreading in alignment with the pattern. Cells were plated at 30 000 cells/1 cm² of nominal substrate surface in DMEM with 10% bovine calf serum¹⁹ and were grown for 3 days before being fixed with 3.7% formaldehyde in phosphate-buffered saline, permeabilized with NP-40, and stained with rhodamine–phalloidin to visualize actin filaments. Analysis showed that the cells had spread around and between, but not over, the holes and were in alignment with the ZrO₂−phosphonate pattern (Figure 3). Actin was found to be aligned on the surfaces in register with the patterns after cells had reached confluence (Supporting Figure 1).

Quantitatively comparing cell alignment on patterned, nonperforated versus patterned, perforated surfaces was done by measuring aspect ratios of Fast Fourier Transform (FFT) outputs from stained actin images of adhered cells (Figure 3).³² FFT analysis was done on four 10x images each a perfect square of 1024 × 1024 pixels. Image contrast was normalized with pixel saturation set at 0.4%; FFT was determined using ImageJ software in which grayscale output was colorized using the “spectrum” table. We define the aspect ratio by dividing the width of the FFT output oval by its length; thus, the smaller the aspect ratio, the better the alignment. This ratio was 0.69 ± 0.09 for cells on patterned, nonperforated PEEK (Figure 3A); patterned, perforated substrates had ratios of 0.53 ± 0.05, 0.59 ± 0.09, 0.62 ± 0.09, and 0.59 ± 0.03 for cells on surfaces with 5% hole coverage (Figure 3B), 10% hole coverage via head-to-tail ablation (Figure 3C), 10% hole coverage via side-to-side ablation (Figure 3D), and 20% hole coverage (Figure 3E), respectively. These data show that the cells are at least as well-aligned on patterned, perforated PEEK as they are on patterned, nonperforated PEEK; this conclusion is further confirmed by the FFT analysis of stained actin images of adhered cells.
supported by ANOVA (α = 0.05), where no statistical difference was found for the aspect ratio among all of the PEEK surfaces (p = 0.114). It is not known to what extent, if any, the presence of holes affects the FFT output, although there is no apparent correlation between hole number or alignment with measured aspect ratios.

We showed that clean perforation of a surface-protected polymer film can be accomplished by laser ablation and that this material can be patterned to template spatially controlled cell spreading in a way that is as effective as on unperforated polymer substrates. Protecting PEEK with a thin layer of photoresist prior to laser treatment causes all debris from ablation to be deposited on top of this layer, which is then removed by sonication in ethanol. Ablation-perforated PEEK is easily patterned by photolithography to prepare cell-adhesive ZrO2−phosphate striations; patterned PEEK films template cell spreading in alignment with these striations, even when perforations account for 20% of the nominal surface area. Given the ease of implementing our methods, our procedures for material transformation might provide an effective approach to envisaged 3D constructs based on stacked 2D-patterned, perforated polymer sheets that would be designed also to envisage 3D constructs based on stacked 2D-patterned, perforated polymer sheets that would be designed also to obviate layer-to-layer compression. Coupled with demonstrated excellence in spatially determined surface chemical modification of polymer films and of ECM assembly on them,35 these methods may help inform new routes for fabricating tissue scaffolds comprising cell-assembled matrix38 or organ-on-a-chip technologies39 that effectively recapitulate native tissue architectures in which biodegradable polymeric substrates can be accommodated.

**REFERENCES**


