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A Cell Culture Substrate with Biologically Relevant Size-Scale Topography and Compliance of the Basement Membrane

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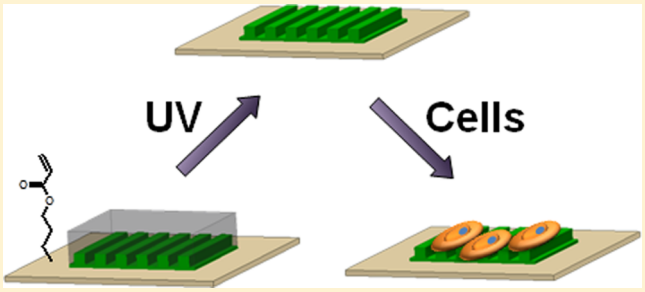
Supporting Information

ABSTRACT: A growing body of literature broadly documents that a wide array of fundamental cell behaviors are modulated by the physical attributes of the cellular microenvironment, yet *in vitro* assays are typically carried out using tissue culture plastic or glass substrates that lack the 3-dimensional topography present *in vivo* and have stiffness values that far exceed that of cellular and stromal microenvironments. This work presents a method for the fabrication of thin hydrogel films that can replicate arbitrary topographies with a resolution of 400 nm that possess an elastic modulus of approximately 250 kPa. Material characterization including swelling behavior and mechanics were performed and reported. Cells cultured on these surfaces patterned with anisotropic ridges and grooves react to the biophysical cues present and show an alignment response.



INTRODUCTION

A growing body of literature broadly documents that a wide array of fundamental cell behaviors are modulated by the physical attributes of the cellular microenvironment.[1−3] Analogous to chemical signaling, physical signaling can alter a cell's gene and protein expression,[4] nuclear compaction,[5] proliferation,[6] state of differentiation[7−10] and migration[11,12] as well as modulate how cells respond to soluble signaling molecules and therapeutic agents.[3] In aggregate, it is clear that physical cues represent ubiquitous and potent signals in determining cell behaviors. *In vitro* assays are typically carried out using tissue culture plastic or glass substrates that lack the 3-dimensional topography present *in vivo* and have stiffness values that far exceed that of cellular and stromal microenvironments. Although elastic modulus is the canonical descriptor of mechanical property of a material, we would like to point out that use of the term *stiffness* is favored and better understood by our colleagues in biology to describe the apparent rigidity of a material and is used as such in this work. Indeed, these *biophysical* attributes vary from one cell location to another, and thus the concept of a one-substrate-fits-all approach is largely inappropriate for *in vitro* investigations. Therefore, to make *in vitro* results more predictive of events *in vivo*, researchers have sought ways to modify cell culture substrates to better mimic *in vivo* physical properties. These biomimetic surfaces have been fabricated from a limited number of natural materials[13−15] or synthetic polymers[16] which support cell viability and functionality. Because of the complexity of fabrication, they have also typically been created possessing *either* tissue-like topography *or* tissue-like stiffness. We demonstrate here the incorporation of *both* topography and stiffness in a single polymeric substrate (poly(ethylene glycol) diacrylate, PEG-DA) that can be fabricated using materials suitable for cell culture and which mimics aspects of the topography and stiffness of *in vivo* tissue.

The basement membrane (BM) is a specialization of the extracellular matrix through which many cell types (e.g., corneal epithelial and vascular endothelial cells) attach to, and interact with, the underlying stroma. The topographic architecture of the BM is described by stochastic arrays of bumps and pores as well as fibers with nanoscale to submicrometer dimensions.[17,18] The majority of reports in the literature using patterned substrates have employed soft lithographic and photolithographic patterns composed of a monotypic feature (bumps or alternating ridges and grooves) with controlled spatial dimensions. These substrates are often created as highly ordered arrays possessing anisotropic order (e.g., alternating ridges and grooves) and having an elastic modulus that

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approximate tissue culture polystyrene (~GPa). Important insights into cell behaviors have been determined from these studies;[4,15] however, they fail to replicate the complex topographic architecture of the native BM. Recently, we have demonstrated[11] that the submicrometer stochastic features of *in vivo* BM topography can be mimicked by producing highly porous polyelectrolyte membranes[19] (PEMs). Through the use of soft lithography, we were able to replicate stochastically ordered topographic features of the porous PEMs into any cell culture substrate, thereby ensuring that the topographic cue was identical for all surfaces and experiments. Although these surfaces are an important step in continuing to understand how biomimetic topographic cues influence cell behavior, they do not replicate the stiffness of the extracellular matrix.

The elastic modulus of extracellular matrix has been measured by a number of techniques, and often the elastic component of a viscoelastic tissue is quantified and then subsequently used to create compliant cell cultureware. This can create difficulties, as the range of published elastic modulus (EM) values for a given tissue typically spans several orders of magnitude. A recent review on the published values of EM for soft tissues has demonstrated that a major contributor to the variation due to the method by which the EM was measured.[20] Values for EM of biological tissues as measured by atomic force microscopy (AFM) are generally less than values obtained by tensile measurement for the same tissue.[20] Typical AFM values range from 0.1 to 200 kPa.[20] Because the strains imparted on the tissue samples in nanoindentation methods are of similar length scales to those of cells, the measured mechanical properties likely better mimic what cells "sense" *in situ*.

Hydrogels are an attractive way to recreate the stiffness of soft tissues in cell culture due to the ease in which the elastic modulus of the gel can be modified as well as being compatible with many cell types. The drawback of very soft hydrogels (<200 kPa), with respect to topographic cueing, is that their large swelling ratios result in distortion of the molded features when swollen, especially those features in the bioactive range (150−1000 nm).[21] To overcome this problem, we have UV cross-linked very thin films (<3 μm) of PEG-DA containing a small amount of Irgacure 2959 (a cytocompatible photoinitiator[22]) to chemically modified (acrylated) glass slides. We find that sufficiently thin films will hold the submicrometer topographic features of a PDMS mold when swollen in solution because lateral expansions were mechanically constrained in the plane of the glass but were free to expand vertically. By selecting an appropriate molecular weight PEG-DA, the elastic modulus of the topographic surfaces can be within the range of many soft biological tissues (elastic moduli ~100 kPa). These surfaces are ideal for the study of cell behavior, as PEG hydrogels are nontoxic, fouling resistant, can be used as a structural material,[23] and can be modified by a large number of small molecules, including saccharides, peptides, DNA, inorganics, and proteins to target specific cell−substrate interactions.[24,25]

RESULTS AND DISCUSSION

Fabrication Process. The process used to fabricate the topographically patterned soft hydrogel substrate is schematically outlined in Figure 1 and is further detailed in the Methods. A small volume (3 μL) of a prepolymer solution consisting of PEG-DA, type I collagen (to promote cell adhesion), and Irgacure 2959 was placed on an acrylated glass substrate and sandwiched with a topographic PDMS mold and cross-linked

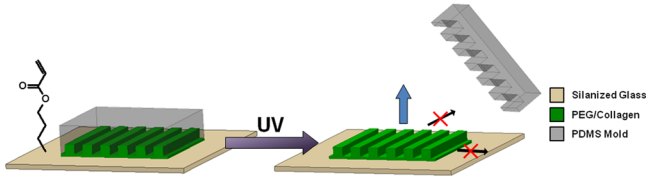

Figure 1. Fabrication process for producing topographically patterned thin PEG hydrogel films. Covalent anchoring of the hydrogel to the substrate prevents lateral expansion (crossed out arrows) to maintain the topography but allows vertical swelling of the molded features to result in a soft topography.

with UV. Equilibration of these very thin films and their photoexposure in an inert atmosphere was found to be critical as oxygen acts as a radical scavenger and prevents the compete polymerization of the PEG membrane, leading to poor feature fidelity and inconsistent mechanical properties. After cross-linking, the PDMS was gently peeled from the substrate and the samples were either immediately imaged with an atomic force microscope or placed in ultrapure ethanol for later imaging. No crack propagation or peeling was observed for these very thin films, indicating a strong bond formed between the acrylate groups on the PEG backbone and the acrylate groups on the glass surface.

We hypothesize that the thickness and anchoring of the hydrogel to the substrate is critical in maintaining the molded topography. Covalent attachment of the polymer network to the glass substrate mediated through the acrylate surface modification is necessary to resist PEG's considerable swelling capability in the lateral directions and restrict volumetric expansion to the vertical direction. If lateral expansion were allowed, the imprinted topography would become distorted and ultimately result in nearly complete loss of any desired features. In addition, the initial thickness of the hydrogel film is important, as the effect of the lateral constraints is lost as the aspect ratio of the features increases resulting in their collapse. In this case, the thickness was minimized through the utilization of a very thin prepolymer capillary film that formed between the stamp and substrate. Vertical swelling is highly desirable as it reduces the effective density of the polymer matrix, reducing its apparent stiffness.

Replication of Complex Topographies. This fabrication method is easily extended to complex, stochastic topographies such as those of biological basement membranes. We recently demonstrated[11] that the formation of highly porous features in polyelectrolyte membranes of poly(acrylic acid) and poly(allylamine hydrochloride) closely approximated *in vivo* basement membrane topography which display ridges, grooves, bumps, and pores with submicrometer dimensions and stochastic surface order (see Methods). Figure 2a shows a height image in air acquired by AFM of a hard epoxy replica (NOA81, Norland Products; suitable for cell culture) of our synthetic basement membrane. The artificial topography encapsulates the sum of the topographic components of *in vivo* topography, e.g. fibers, bumps, and pores, with stochastic surface order and submicrometer dimensionalities. Because of its nonhygroscopic nature, NOA81 molds easily maintain complex topographies but are ultimately too stiff to accurately mimic the mechanical properties of biological BM. However, using the thin hydrogel film process described here, PEG replicas of the stochastically ordered surfaces have the potential to approximate the topographic surface as well as the elastic

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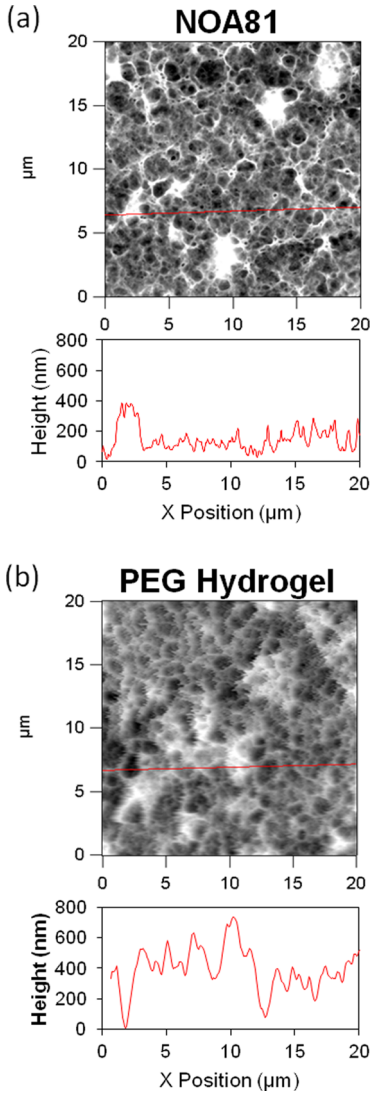

Figure 2. Fabrication of a basement membrane-like stochastic soft topography. An AFM height map and cross of (a) a hard epoxy and (b) soft PEG hydrogel replica of a basement membrane-like topography molded from PEMs. Data for the cross sections are traced from the red lines in the height maps.

modulus of the native BM. Figure 2b is an AFM height map molded PEG replica swollen in ethanol demonstrating that, consistent with our description in Figure 1, swelling principally occurred perpendicular to the surface and retained the complex topographic features of the NOA81 master. Ethanol was used as the swelling solvent to reduce imaging artifacts caused by adhesions between the AFM tip and the soft sample. Control experiments with PEG surfaces possessing a greater elastic modulus using lower molecular weight PEG-DA that could be imaged in physiological saline showed a very similar equilibrium cross-sectional area to those swollen in ethanol (Supporting Information Figure S2).

Swelling Behavior. The motivation for creating topographical surfaces with cross-linked hydrogels is that once swollen in solvent, the elastic modulus of the material decreases, yielding a soft substrate. To investigate this effect, AFM height images were acquired of swelling thin PEG hydrogel films. For the purpose of characterization, anisotropic ridge and groove patterns were used for the rest of the study and were not considered to be simulants of the native membrane. In other words, we exploited the attributes of the anisotropic topographic patterns in the context of characterization and are fully aware that they do not possess a biomimetic surface order as we achieved with our stochastically ordered patterned surfaces as depicted in Figure 2. PDMS molds with anisotropic ridge and groove patterns were employed as mold masters as previously reported.[15,26] The pitch of the molded ridges and grooves ranged in size from 800 to 4000 nm pitch (pitch = ridge width + groove width) with a depth of 600 nm. Figure 3a,b shows a height map and 3-D rendering of the dried 4000 nm pitch film after fabrication. The ridges were approximately 2 μm wide and were slightly ruffled with occasional punctate features that were regions of very high sample−tip interactions. The grooves were larger, closer to 3 μm. Overall the molded pattern was observable, but the drying process resulted in collapse of the relatively high aspect ratio ridges that measured nearly 150 μm in height. The sample was then placed in ethanol and reimaged at 2, 20, and 46 h. Figure 3c,d shows the height map and 3-D rendering of a fully swollen 4000 nm pitch pattern in ethanol after 20 h. The features became very smooth with the ridges swelling to nearly 4 μm in width, and the horizontal dimensions were relatively unchanged but the height of the ridges strikingly increased from 150 to 600 nm. Consistent with the proposed model, the covalent anchoring of the thin PEG film restricted much of the lateral swelling of the features but allowed for vertical expansion.

To capture the swelling kinetics, cross-section data normal to the ridge and groove pattern was extracted from the height maps taken at 0, 2, 20, and 46 h after immersion in ethanol and are shown in Figure 3e. A rapid height increase from 150 to 450 nm was observed upon initial swelling at 2 h and appears to reach a steady state height of 600 nm by 20 h that persists for at least 46 h. Additionally, we found that so long as only one dry to wet transition occurred (i.e., the first one), the sample would maintain its morphology. A similar procedure was repeated for a 4000 nm pitch anisotropic surface that remained in ethanol for 120 h, and it demonstrated excellent stability with the most prominent difference being more rounded corners (data not shown). This stability is maintained so long as the sample remains wet and does not transition between wet and dry states; if the thin film hydrogels went through multiple wet/dry transitions or were left out in a dry environment, the topography would increasingly deteriorate. Although 5 days is sufficient for most cell culture experiments, future work will be necessary to characterize the shelf life of the samples.

The swelling ratio of the thin film was determined by normalizing the cross-sectional area of a ridge in the wet state to that of the ridges in the dry state (A_{wet}/A_{dry}) and is reported in Figure 3f. The temporal trend of the swelling ratio follows that of the cross sections where there is rapid swelling initially around 2 h and then becomes stable beyond 20 h at a ratio of 5.1. As other hydrophilic polymer networks have been found to swell with first-order kinetics,[27] swelling ratios between data points were estimated by applying a first-order fit (solid line in Figure 3f; see Methods for details). The swelling time constant was found to be 2.39 ± 0.27 min and suggests that swelling overnight ($5\tau \approx 12$ h) is necessary for the thin PEG hydrogel to reach an equilibrium volume and, by extension, stabilize its topographical and mechanical properties.

Topographical and Mechanical Characterization. Several thin PEG films were molded into anisotropic ridges and grooves with 800−2000 nm pitches and were topo-

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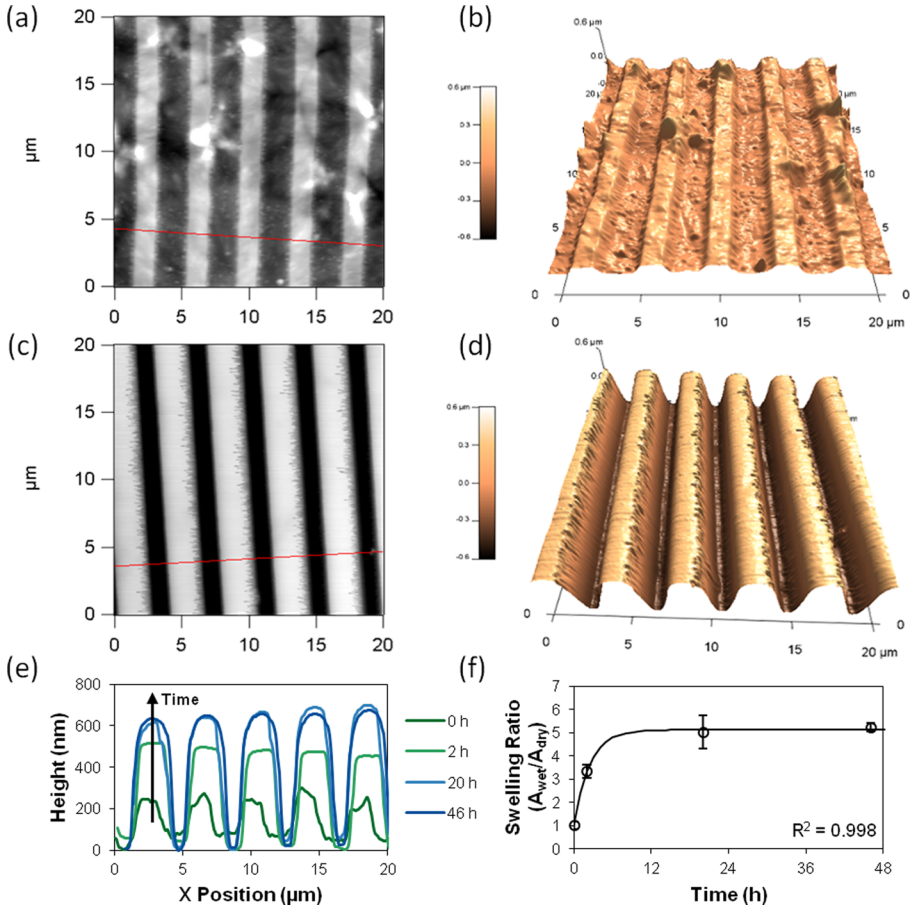

Figure 3. Swelling properties of micropatterned thin PEG films. (a) AFM height map and (b) 3-D rendering of a dried 4000 nm pitch ridge and groove anisotropic pattern. (c) AFM height map and (d) 3-D rendering of the same surface (different position) after swelling in ethanol for 20 h. (e) Cross sections of height map images of the same surface between 0 h (dry, red line in (a)) and 46 h (fully swollen, red line in (c)). (f) Swelling ratio (A_{wet}/A_{dry}) of the ridges as a function of time taken from the cross-sectional data in (e).

graphically and mechanically characterized by AFM as shown in Figure 4a–d. Height maps are displayed with accompanying cross-sectional traces of hydrogels swollen overnight in ethanol. Imaging artifacts from sample–tip interactions were observed on the smaller features sizes and should not be interpreted as bridges between ridges. While the topography is easily observed, the apparent height of the ridges seems to be pitch dependent which we believe is due to obstruction of the AFM probe entering very tall, narrow grooves. The ridge heights stabilize at 600 nm when grooves were at least 1 μm wide which provide sufficient clearance for the tip to fully interrogate the groove.

The ability of these substrates to maintain the topographic features of the mold was also dependent on the thickness of the thin film between the PDMS stamp and glass surface. A 400 nm pitch ridge and groove substrate was also attempted, but we found that the ridges occurred as doublets, indicating that the high aspect ratio soft ridges were not capable of resisting a strong attractive force between the 200 nm spaces of the groove and represent the maximum resolution/aspect ratio for anisotropic features using this method. We attempted to find the threshold thickness necessary for the hydrogel to maintain 400 nm topographic features when swollen by spin-coating thin films of PDMS to create spacers between the mold and glass. We tested ~18, 30, and 50 μm spacers and found that the hydrogel was unable to maintain, with fidelity, the topographic features of the PDMS mold when swollen. Also, for these thicker films, crack propagation and peeling occurred after the mold was removed. Therefore, a very thin film is required to ensure that the expansion of the hydrogel occurs predominantly normal to the rigid support as well as to ensure proper adhesion to the base substrate. If assumed that the entire 3 μL of prepolymer solution was contained under the 1 cm^2 area of the PDMS mold, then the thin film would be expected to be 3 μm thick. However, some liquid buildup was always observed around the edges of the mold, indicating the films were likely less than 3 μm.

The mechanical rigidity of the patterned substrates was subsequently characterized. Force vs indentation data were generated by AFM using calibrated cantilevers over raised surface features of anisotropic topography swollen in ethanol, and representative data for each topography are shown next to the height maps in Figure 4a–d. Using established techniques, the elastic moduli of the surfaces were extracted from the data by assuming a rigid cone (AFM tip) indenting a plane elastic solid[28] and fitting the data to the Hertz model. Figure 4e shows a summary of the average elastic moduli determined for each anisotropic pitch. No significant trends in pitch dependence on elastic moduli were observed (p = 0.68, see Methods), and the mean elastic modulus of flat and topographic surfaces was found to be 256 ± 66 kPa (mean ± SD). The incorporation of collagen also did not significantly impact the EM of the surfaces (Supporting Information Figure S3). To our knowledge, these 800 nm pitch surfaces (400 nm features) are the smallest

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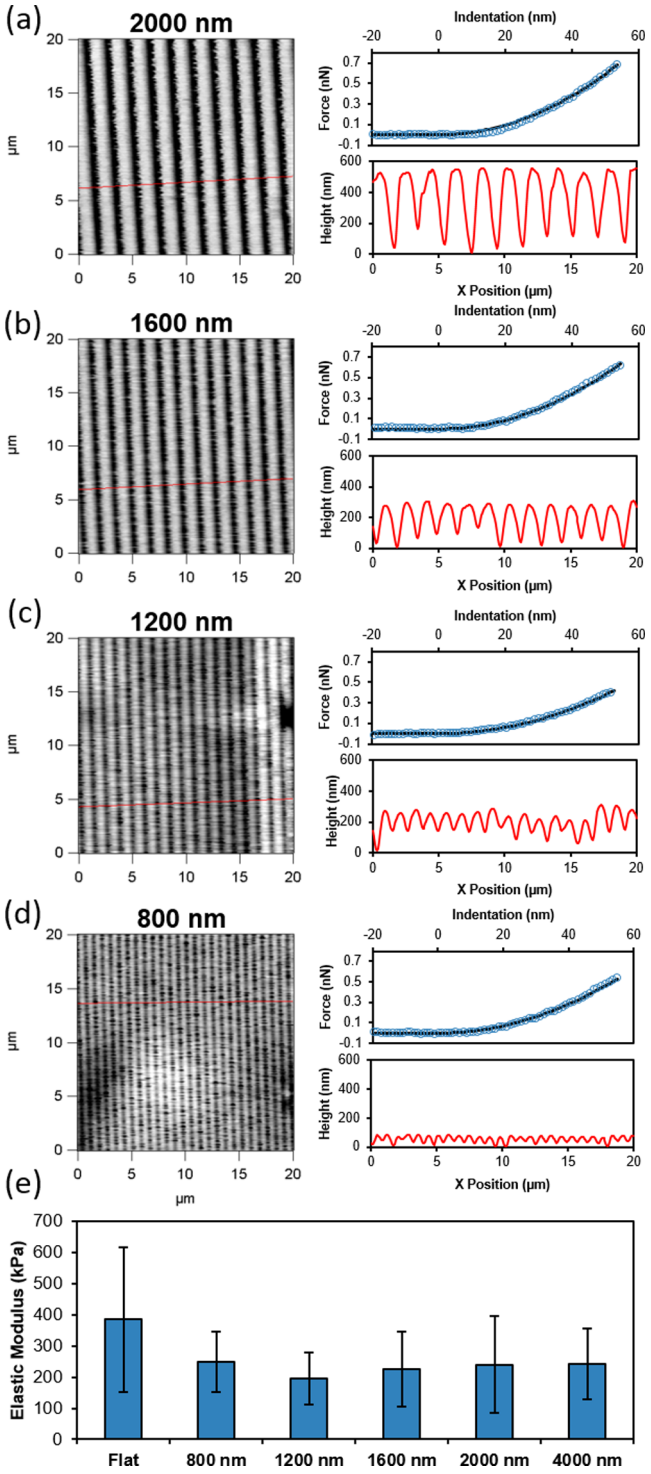

Figure 4. Topographical and mechanical characterization. AFM height images, cross sections, and representative mechanical data for (a) 2000, (b) 1600, (c) 1200, and (d) 800 nm pitch ridges and grooves. (e) Average elastic moduli measured for each pitch. Grand average = 256 kPa.

topographic features that have been molded with such a low elastic modulus. The thickness of thin films is an important parameter to control as contributions from an underlying rigid surface are known to increase the apparent elastic modulus of the material. Because the indentations used in the characterization were 50−100 nm, which is less than 5% of the estimated film thickness, we concluded that substratum effects were negligible. The constancy of the of the values obtained suggests that our use of capillary-controlled thickness of the thin PEG films is a stable and repeatable process. Although possessing slightly higher elastic moduli in comparison to some basement membranes (e.g., 256 kPa vs 2−80 kPa in different BM of the cornea[29]), these surfaces were still within the range of many other biological tissues.[20] The elastic modulus of the material can be further tuned by adjusting a number of factors in the fabrication, such as modifying the PEG concentration, molecular weight of the PEG-DA, film thickness, adding additional cross-linkers in the prepolymer solution, or monovinyl-terminated compounds. When both the elastic modulus of the material and the proper topographies are employed, the apparent stiffness should present a mechanical surface reminiscent of *in vivo* tissues to cells.

Cellular Response to Soft Anisotropic Topographies. To demonstrate the *in vitro* utility and the response of cells to these soft topographic surfaces, immortalized human corneal fibroblasts (htHCF) were cultured on flat, 800 nm, and 4000 nm pitch anisotropically patterned hydrogels. Initial experiments with PEG surfaces formulated without collagen showed that cells were unable to attach to the surface due to PEG's fouling-resistant properties. Therefore, we incorporated type I collagen into the prepolymer solutions provided the cellular adhesion sites on a biologically inert PEG surface. Figure 5a−c shows cells cultured for 24 h on the different surfaces and fluorescently stained for F-actin (red, phalloidin), cell membrane (green, wheat germ agglutinin), and nuclei (blue, DAPI). Cells seeded on the 800 and 4000 nm pitch surfaces responded to the soft ridge and groove topography by aligning with the long axis of the topography while cells on the flat hydrogel surfaces were randomly oriented. The morphological change of the cells on the molded substrate suggests that the soft topography will contact guide cells in a similar fashion to previous reports of cells on noncompliant ridge and groove substrates.[5] Figure 5d shows an AFM deflection image of an htHCF cell cultured on a 4000 nm surface. Close inspection of the top of the cell shows that membrane protrusions were either traveling along the edges or within the grooves of topographic surfaces and provide further evidence of biophysical cues through contact guidance. Previous studies have shown that cell adhesion onto anisotropic substrates is largely mediated by the surface of the ridge rather than the grooves.[26] However, the apparent attachment to the grooves in this study may be due to the unoptimized concentration of cellular adhesion moieties (from collagen) and that larger surface areas within the grooves are necessary to provide a sufficient density of attachment sites.

■ CONCLUSIONS

When developing cell cultureware for use in the laboratory, it is important to consider not only the design of the surfaces but also the difficulty and cost associated with replicating the surfaces in bulk. The method presented here is both cost-effective and easy to implement, given the small volume of PEG-DA that is necessary for the thin film. For example, from a single gram of PEG-DA, one could potentially fabricate ∼1600 individual square centimeter surfaces, and while this demonstration was limited to ridge and grooves and a simulated BM, the soft lithographic method could be extended to any number of topographic features contained in a PDMS mold.

Most importantly, however, we have demonstrated here for the first time that stable *submicroscopic* topographic features,

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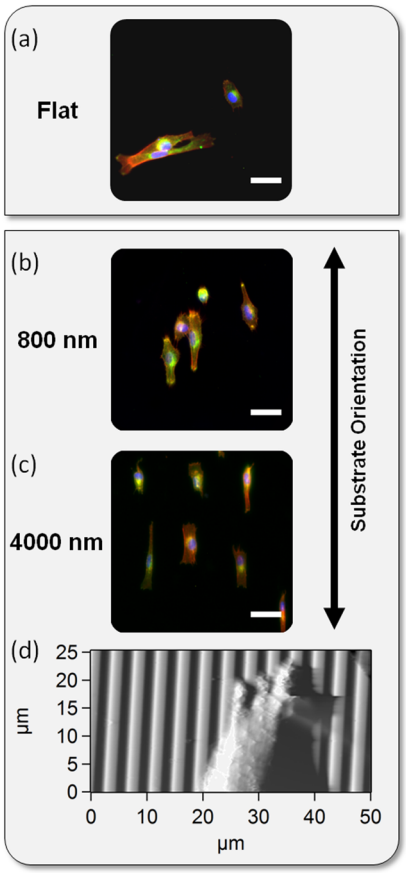

Figure 5. Cellular response to soft topographic surfaces. Fluorescent images of cells cultured on soft (a) flat, (b) 800 nm, or (c) 4000 nm pitch ridges and grooves. Alignment parallel with topography was observed on patterned substrates but not flat surfaces. Cells were stained against actin (red), cell membrane (green), and nuclei (blue). Arrow follows the orientation of the ridges. Scale bar = 50 μm. (d) AFM deflection image of a cell cultured on a 4000 nm pitch ridge and groove surface. Protrusions along the top edge of the cell were following along the grooves.

which mimic *in vivo* topography, can be created with an elastic modulus that is comparable to that sensed by cells in the body.

METHODS

PDMS Molds of Topographies. Molds of master topographical surfaces were made using soft lithography techniques.[1,30] Stochastic molds were made from PEMs using an established technique.[11] Briefly, master surfaces were PEMs formed on glass slides by alternatingly dipping slides in 0.01 M poly(allylamine hydrochloride) (PAH, Alfa Aesar) at pH 7.5 and 0.01 M poly(acrylic acid) (PAA, Polysciences) at pH 3.5. PEMs were dipped in an HCl solution at pH 2.3 for 1 min to form highly porous structures. Anisotropic ridge and groove molds in PDMS were also fabricated according to an established protocol.[15,26] Silicon masters were fluorocarbon-silanized wafers with etched anisotropic topographical ridges and grooves of varying pitch. To maintain high fidelity of nanotopographical features of stochastic and anisotropic master surfaces, a coating layer of hard PDMS (hPDMS) was principally composed of 7.0−8.0% vinymethylsiloxane−dimethylsiloxane copolymer and 25−30% methylhydrosiloxane−dimethylsiloxane copolymer (see ref 11 for details). A thin layer of hPDMS was formed on the masters by spin-coating the hPDMS mixture at 4000 rpm for 40 s. After curing the hPDMS layer in an oven at 60 °C for 30 min (stochastic molds) or 65 °C for 60 min (anisotropic molds), a second, thicker layer of PDMS (Sylgard 184, Dow Corning; 10:1 elastomer to curing agent ratio) was mixed, degassed, and poured onto the hPDMS coated master surfaces and cured for 3 h at 60 °C (stochastic molds) or for 1.5 h at 65 °C (anisotropic molds). The composite PDMS mold was removed and cut into approximately 1 cm^2 pieces and later used in PEG film fabrication. PDMS stamps used for the 4000 nm pitch cell culture substrate did not require an initial hPDMS layer.

PEG Film Fabrication. Glass microscope slides (Fisher Scientific, 12-544-1) served as the underlying substrate for the PEG films. Briefly, glass slides were cut into approximately 1 cm^2 pieces, rinsed with ultrapure ethanol, dried, and plasma treated (Harrick Plasma Cleaner) for 1 min. Glass slides were then immediately acrylated with 3-acryloxypropyltrichlorosilane (3-APT, Gelest, SIA0199.0) via vapor deposition for 2 h. After silanation, glass slides were stored under vacuum for a minimum of 24 h in order to remove unreacted 3-APT or kept under vacuum to minimize exposure to moisture until used for experiments. Prepolymer solutions consisted of 20% w/v PEG-DA (Sigma-Aldrich, 701963, 6000 g/mol), 5% v/v type I collagen (Advanced BioMatrix, PureCol, 5005-B), and 0.15% w/v Irgacure 2959 (Ciba, from a 15% stock solution in ultrapure ethanol) in ultrapure deionized water (Millipore, Milli-Q). The prepolymer solution was well mixed and allowed to equilibrate overnight prior to fabrication.

A 3 μL volume of the prepolymer solution was placed on top of an acrylated glass slide, and then a 1 cm^2 PMDS mold was gently placed on top, resulting in the a thin capillary film. The samples were then placed in an airtight container purged with ultrahigh purity nitrogen gas and allowed to stand for 2 h. The sample and container were placed under high-intensity UV (365 nm, ~35 mW/cm^2) for 20 min. After cross-linking, samples were removed from the nitrogen container the PDMS molds were gently peeled off from the cross-linked thin film.

Characterization. An Asylum Research MFP-3D-BIO atomic force microscope (Santa Barbara, CA) was used for investigating the surface topography and mechanical properties. All AFM experiments were done using PNP-TR-50 silicon nitride cantilevers with a spring constant of 0.32 N/m and 35° half-angle opening (NanoWorld, Switzerland). Imaging was done in contact mode in either air (dry state) or ultrapure ethanol (swollen state). Profiles of 4000 nm ridges were taken from height map images after 0, 2, 20, and 46 h of swelling in ethanol at 27 °C. Swelling ratios were determined by dividing the average cross-sectional area of each ridge at each point by that of the dry (0 h) time point. Swelling ratios were plotted against time and fitted to a first-order equation

$$Q = \alpha + \beta(1 - e^{-t/\tau}) \quad (1)$$

where Q is the swelling ratio, α and β are constants, and τ is the time constant. The value of $\alpha + \beta$ yields the ultimate swelling ratio at infinite time.

For force measurements, substrates were soaked in ultrapure ethanol overnight and briefly imaged to identify ridges and grooves. Five force curves were taken at five different locations on top of ridges for each pitch. Data were fitted to the Hertz model for a conical tip (as an approximation to the pyramidal tip used) to determine the local elastic modulus.[28,31] We assumed a Poisson ratio of 0.5 due to the high content of incompressible water in the swollen material.[32−35] Averages of elastic moduli from all groups were analyzed for statistical significance by a one-way ANOVA.

Cell Culture. For cell culture experiments, PEG films were fabricated on acrylated 15 mm diameter glass coverslips (Ted Pella, 26024) following the same procedure described above. After fabrication, substrates were soaked overnight in ultrapure ethanol. Prior to cell culture experiments, substrates were placed in a sterile environment and soaked in 70% ethanol for at least 30 min then replaced with fresh 70% ethanol and repeated a total of three times. Ethanol was then removed and washed with sterile phosphate buffered saline (PBS, ThermoScientific, SH30256.01) every 30 min for a total of three times. Immortalized human corneal fibroblasts (a kind donation from Dr. James V. Jester, University of California, Irvine) were plated on the topographies and incubated for 24 h at 37 °C and

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5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum. Cells were fixed and stained for the F-actin cytoskeleton (phalloidin, Invitrogen), cell membrane (wheat germ agglutinin), and nucleus (DAPI, Invitrogen) and then imaged with a Zeiss inverted light microscope (Carl Zeiss, Axiovert 200M) using a 20× objective.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1–S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E. Micropatterned Surfaces for Control of Cell Shape, Position, and Function. *Biotechnol. Prog.* **1998**, *14* (3), 356–63.

(2) Discher, D. E.; Janmey, P.; Wang, Y. L. Tissue Cells Feel and Respond to the Stiffness of Their Substrate. *Science* **2005**, *310* (5751), 1139–43.

(3) McKee, C. T.; Wood, J. A.; Shah, N. M.; Fischer, M. E.; Reilly, C. M.; Murphy, C. J.; Russell, P. The Effect of Biophysical Attributes of the Ocular Trabecular Meshwork Associated with Glaucoma on the Cell Response to Therapeutic Agents. *Biomaterials* **2011**, *32* (9), 2417–2423.

(4) Klymov, A.; Prodanov, L.; Lamers, E.; Jansen, J. A.; Walboomers, X. F. Understanding the Role of Nano-topography on the Surface of a Bone-implant. *Biomater. Sci.* **2013**, *1* (2), 135–151.

(5) McKee, C. T.; Raghunathan, V. K.; Nealey, P. F.; Russell, P.; Murphy, C. J. Topographic Modulation of the Orientation and Shape of Cell Nuclei and Their Influence on the Measured Elastic Modulus of Epithelial Cells. *Biophys. J.* **2011**, *101* (9), 2139–2146.

(6) Cousins, B. G.; Zekonyte, J.; Doherty, P. J.; Garvey, M. J. Manufacturing a Nanometre Scale Surface Topography with Varying Surface Chemistry to Assess the Combined Effect on Cell Behaviour. *Int. J. Nano Biomater.* **2008**, *1* (3), 320–338.

(7) Engler, A. J.; Sen, S.; Sweeney, H. L.; Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **2006**, *126* (4), 677–89.

(8) Dupont, S.; Morsut, L.; Aragona, M.; Enzo, E.; Giulitti, S.; Cordenonsi, M.; Zanconato, F.; Le Digabel, J.; Forcato, M.; Bicciato, S.; Elvassore, N.; Piccolo, S. Role of YAP/TAZ in Mechanotransduction. *Nature* **2011**, *474* (7350), 179–83.

(9) Dalby, M. J.; Gadegaard, N.; Tare, R.; Andar, A.; Riehle, M. O.; Herzyk, P.; Wilkinson, C. D.; Oreffo, R. O. The Control of Human Mesenchymal Cell Differentiation Using Nanoscale Symmetry and Disorder. *Nat. Mater.* **2007**, *6* (12), 997–1003.

(10) Curran, J.; Chen, R.; Stokes, R.; Irvine, E.; Graham, D.; Gubbins, E.; Delaney, D.; Amro, N.; Sanedrin, R.; Jamil, H.; Hunt, J. Nanoscale Definition of Substrate Materials to Direct Human Adult Stem Cells Towards Tissue Specific Populations. *J. Mater. Sci.: Mater. Med.* **2010**, *21* (3), 1021–1029.

(11) McKee, C. T.; Wood, J. A.; Ly, I.; Russell, P.; Murphy, C. J. The Influence of a Biologically Relevant Substratum Topography on Human Aortic and Umbilical Vein Endothelial Cells. *Biophys. J.* **2012**, *102* (4), 1–10.

(12) Pelham, R. J., Jr.; Wang, Y. Cell Locomotion and Focal Adhesions Are Regulated By Substrate Flexibility. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (25), 13661–5.

(13) Dickinson, R. B.; Guido, S.; Tranquillo, R. T. Biased Cell Migration of Fibroblasts Exhibiting Contact Guidance in Oriented Collagen Gels. *Ann. Biomed. Eng.* **1994**, *22* (4), 342–56.

(14) Kleinman, H. K.; Martin, G. R. Matrigel: Basement Membrane Matrix with Biological Activity. *Semin. Cancer Biol.* **2005**, *15* (5), 378–86.

(15) Teixeira, A. I.; Abrams, G. A.; Bertics, P. J.; Murphy, C. J.; Nealey, P. F. Epithelial Contact Guidance on Well-defined Micro- and Nanostructured Substrates. *J. Cell Sci.* **2003**, *116* (Pt 10), 1881–92.

(16) Odom, T. W.; Love, C.; Wolfe, D. B.; Paul, K. E.; Whitesides, G. M. Improved Pattern Transfer in Soft Lithography Using Composite Stamps. *Langmuir* **2002**, *18* (13), 5314–5320.

(17) Hironaka, K.; Makino, H.; Yamasaki, Y.; Ota, Z. Renal Basement Membranes by Ultrahigh Resolution Scanning Electron Microscopy. *Kidney Int.* **1993**, *43* (2), 334–45.

(18) Inoue, S. Basic Structure of Basement Membranes is a Fine Network of "Cords", Irregular Anastomosing Strands. *Microsc. Res. Technol.* **1994**, *28* (1), 29–47.

(19) Mendelsohn, J. D.; Barret, C. J.; Chan, V. V.; Pal, A. J.; Mayes, A. M.; Rubner, M. F. Fabrication of Microporous Thin Films from Polyelectrolyte Multilayers. *Langmuir* **2000**, *16* (11), 5017–5023.

(20) McKee, C. T.; Last, J. A.; Russell, P.; Murphy, C. J. Indentation Versus Tensile Measurements of Young's Modulus for Soft Biological Tissues. *Tissue Eng., Part B* **2011**, *17* (3), 155–64.

(21) Fraser, S. A.; Ting, Y.-H.; Mallon, K. S.; Wendt, A. E.; Murphy, C. J.; Nealey, P. F. Sub-micron and Nanoscale Feature Depth Modulates Alignment of Stromal Fibroblasts and Corneal Epithelial Cells in Serum-rich and Serum-free Media. *J. Biomed. Mater. Res.* **2008**, *86A* (3), 725–735.

(22) Williams, C. G.; Malik, A. N.; Kim, T. K.; Manson, P. N.; Elisseeff, J. H. Variable Cytocompatibility of Six Cell Lines with Photoinitiators Used for Polymerizing Hydrogels and Cell Encapsulation. *Biomaterials* **2005**, *26* (11), 1211–1218.

(23) Kobel, S.; Limacher, M.; Gobaa, S.; Laroche, T.; Lutolf, M. P. Micropatterning of Hydrogels by Soft Embossing. *Langmuir* **2009**, *25* (15), 8774–8779.

(24) Elbert, D. L.; Hubbell, J. A. Conjugate Addition Reactions Combined with Free-radical Cross-linking for the Design of Materials for Tissue Engineering. *Biomacromolecules* **2001**, *2* (2), 430–441.

(25) Guarnieri, D.; De Capua, A.; Ventre, M.; Borzacchiello, A.; Pedone, C.; Marasco, D.; Ruvo, M.; Netti, P. Covalently Immobilized RGD Gradient on PEG Hydrogel Scaffold Influences Cell Migration Parameters. *Acta Biomater.* **2010**, *6* (7), 2532–2539.

(26) Karuri, N. W.; Liliensiek, S.; Teixeira, A. I.; Abrams, G.; Campbell, S.; Nealey, P. F.; Murphy, C. J. Biological Length Scale Topography Enhances Cell-Substratum Adhesion of Human Corneal Epithelial Cells. *J. Cell Sci.* **2004**, *117* (Pt 15), 3153–64.

(27) Ogawa, I.; Yamano, H.; Miyagawa, K. Rate of Swelling of Sodium Polyacrylate. *J. Appl. Polym. Sci.* **1993**, *47* (2), 217–222.

(28) Love, A. E. H. Boussinesq's Problem for a Rigid Cone. *Q. J. Math.* **1939**, *10*, 161–175.

(29) Last, J. A.; Liliensiek, S. J.; Nealey, P. F.; Murphy, C. J. Determining the Mechanical Properties of Human Corneal Basement Membranes with Atomic Force Microscopy. *J. Struct. Biol.* **2009**, *167* (1), 19–24.

(30) Duffy, D. C.; McDonald, J. C.; Schueller, O. J. A.; Whitesides, G. M. Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Anal. Chem.* **1998**, *70* (23), 4974–4984.

(31) Harding, J. W.; Sneddon, I. N. The Elastic Stresses Produced by the Indentation of the Plane Surface of a Semi-infinite Elastic Solid by a Rigid Punch. *Math. Proc. Cambridge Philos. Soc.* **1945**, *41* (01), 16–26.

(32) Anseth, K. S.; Bowman, C. N.; Brannon-Peppas, L. Mechanical Properties of Hydrogels and Their Experimental Determination. *Biomaterials* **1996**, *17* (17), 1647−1657.
(33) Dimitriadis, E. K.; Horkay, F.; Maresca, J.; Kachar, B.; Chadwick, R. S. Determination of Elastic Moduli of Thin Layers of Soft Material Using the Atomic Force Microscope. *Biophys. J.* **2002**, *82* (5), 2798−2810.
(34) Ahearne, M.; Yang, Y.; El Haj, A. J.; Then, K. Y.; Liu, K. K. Characterizing the Viscoelastic Properties of Thin Hydrogel-based Constructs for Tissue Engineering Applications. *J. R. Soc. Interface* **2005**, *2* (5), 455−463.
(35) Vinckier, A.; Semenza, G. Measuring Elasticity of Biological Materials by Atomic Force Microscopy. *FEBS Lett.* **1998**, *430* (1−2), 12−16.