Fabrication of Polymeric Replicas of Cell Surfaces with Nanoscale Resolution

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We report an approach for fabricating biomimetic surface replicas of cells with nanoscale resolution. Fixed cells serve as a template for a two-stage replica molding process. Cast from the template, the impression replica contains a reproduction of cellular topographical features indented into its surface, and cast from the impression replica, the relief replica contains a copy of these features protruding from its surface. Various polymers and cells can be utilized, and scanning electron microscopy, atomic force microscopy, and white light interferometry analyses confirm the replication of nanoscale features. These replicas are useful for investigating cellular function and for biomimetic tissue engineering.

Introduction

Advances in tissue engineering, prosthetics, and regenerative medicine require the development of biomaterials that promote specific cellular responses.[1] Current research is directed toward generating substrates and scaffolds that influence cell adhesion, growth, and organization.[2−5] For example, cells may be recruited to an implanted material in order to improve its physical integration into a surgical site,[6] to improve its biocompatibility, as for the patency of a vascular graft,[7] or to attract and direct other cell types, as for nerve regeneration.[8,9]

Cells normally reside and interact in local environments with distinct and organized micro- and nanotopography, provided in part by the cells that surround them. Cells respond to topographical features,[10,11] and these features can influence many important cellular functions.[11−14] Previous studies suggest that material substrates with nanometer- and micrometer-scale surface features can increase cell adhesion, migration, and process extension, as compared to substrates with smooth surfaces.[4,15−17]

Therefore, topography has been incorporated into material surfaces with the goal of tailoring cell function. Earlier studies have utilized substrates with surface features that contain either regular, ordered topography (i.e., repeating grooved features)[18,19] or disordered, random topography.[7,20,21] In some cases, substrates have been developed to replicate the geometry of components of the extracellular matrix.[22−25] Substrates have typically included either features that are on the order of tens of micrometers in size (i.e., the size of a cell) or features that are submicrometer in size (i.e., the size of cellular features such as cellular processes). However, none of these materials that aim to tailor cell function has replicated the complex mixture of micro- and nanotopography that cells encounter in vivo in which the local cellular environment is much richer in its mixture of sizes, shapes, and organization.

In this work, we employed cultured cells as templates to impart the morphology of the cells to a material substrate. A variety of methods have been used in previous studies to incorporate topographical features into materials, including conventional "top-down" techniques such as photo- and scanning beam lithography as well as "bottom-up" techniques such as nanoimprint lithography, step-and-flash lithography, replica molding, and solvent-assisted micromolding.[26] A recent study reported the transfer of features from unfixed cells to poly(dimethylsiloxane) for the purpose of imaging and analysis.[27] Here we report a replica molding approach using fixed cells and elastomeric polymers that is more reproducible and versatile in its applicability to multiple cell types and materials. This approach is novel in its generation of two distinct and corresponding impression and relief replicas that incorporate the external morphology of the cell templates and that can be employed to investigate the growth and differentiation of cells in vitro and in vivo.

Materials and Methods

Microcontact Printing. Poly(dimethyl siloxane) (PDMS, Dow Corning) stamps with repeating grooves and plateaus were fabricated

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as described by Goldner et al.[19] The pattern was designed in AutoCAD LT 2004 (Autodesk), printed at 10 000 dpi onto a Mylar mask, and transferred via photolithography onto a Si wafer spin-coated with a 50 μm layer of negative tone Nano SU-8 50 photoresist (Microtech). The resulting micropatterned wafer contained repeating grooves with a 60 μm groove width, a 60 μm plateau width, and a 50 μm groove depth. Sylgard 184 PDMS elastomer base was mixed with Sylgard 184 PDMS curing agent (10:1 wt/wt) and then degassed, poured onto the micropatterned wafer to a thickness of 1 to 2 mm, cured at 95 °C for 1 h, and removed to produce stamps of 1 cm × 1 cm × 1 to 2 mm for microcontact printing (μCP).

All coating and cell culture reagents were from Invitrogen Life Technologies unless otherwise indicated. Stamps were submerged in 10% sodium dodecyl sulfate (Sigma) in *d*-H_2O, rinsed in *d*-H_2O, and incubated with 50 μg/mL mouse laminin (LN) in Hanks' balanced salt solution without calcium or magnesium (HBSS-CMF) for 1 h. Glass coverslips were plasma activated with a plasma cleaner/sterilizer (PDC-32 G, Med RF level, Harrick), and incubated in contact with stamps overnight.

Cell Culture. Schwann cells (SCs) were obtained from adult rat sciatic nerves using a modification of the method of Morrissey et al.[19,28] Dissociated SCs were cultured on tissue culture plastic dishes coated with 100 μg/mL poly-L-lysine (PLL) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (base media) supplemented with 2 μM forskolin and 10 μg/mL bovine pituitary extract (SC media). In later experiments, SCs were obtained via a generous gift of Dr. Mary Bunge (University of Miami). Cultures of aligned SCs were prepared by a modification of the technique of Thompson and Buettner.[29] SCs were cultured on LN-micropatterned coverslips at 30 000 cells/mL in SC media for 3−7 days to reach the desired level of cell density.

Embryonic rat aortic smooth muscle cells (SMC) were obtained from the American Type Culture Collection (CRL-1444). SMCs were cultured on flasks coated with 100 μg/mL PLL in base medium supplemented with 4 mM L-glutamine at 37 °C and 5% CO_2. SMCs were rinsed with HBSS-CMF, treated with 0.25% trypsin-EDTA for 10 min to detach cells, resuspended in media, and plated on glass slides coated with 100 μg/mL PLL.

DRG were obtained from the spinal columns of postnatal (P0−P4) rat pups according to Goldner et al.[19] Cells were cultured in base medium with 50 ng/mL nerve growth factor.

Cell cultures were fixed with 2% paraformaldehyde (Sigma) and 4% sucrose (CalBiochem) in 0.1 M phosphate-buffered saline at pH 7.4 (PBS) for 15 min at room temperature and rinsed with PBS. Immunocytochemistry was performed according to Goldner et al.[19] using the primary mouse monoclonal antibody RT97 directed against the phosphorylated epitopes of neurofilament (developed by Dr. John Wood and obtained from the Developmental Studies Hybridoma Bank) and a Cy3-conjugated goat antimouse secondary antibody (Jackson Laboratories).

Substrate Preparation. Reagents for cell template preparation were from Electron Microscopy Sciences. For preparation of the cell template, samples were incubated in Karnovsky's fixative overnight, rinsed with 0.1 M cacodylate buffer, incubated in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h, rinsed with *d*-H_2O, incubated in 1% thiocarbohydrazide in *d*-H_2O for 30 min, incubated in 0.5% OsO_4 in *d*-H_2O for 30 min, dehydrated with graded ethanols to 100% ethanol, air dried, and sputter-coated with gold−palladium. For replica molding with PDMS, Sylgard 184 elastomer base was mixed with Sylgard 184 curing agent (10:1 wt/wt) and applied to the template to form a layer of 1−3 mm thickness. The solution was heated to 95 °C for 1 h, and the resulting polymer film was removed. For replica molding with polyurethane (PU), a 7.5% w/v solution of PC-3585A Carbothane (Thermedics Polymer Products) in chloroform (Sigma) was heated to 65 °C until clear, and entrapped air bubbles were removed by bath sonication. The polymer mixture was applied to the template to form a layer of 1−1.5 mm thickness. Following solvent evaporation, the resulting polymer film was removed. The fixed cells were used as a template to generate the impression replica, and the impression replica was used without any additional processing as a template to generate the relief replica.

Analysis. Substrates were examined with a Hitachi S-2700 scanning electron microscope using an acceleration voltage of 8 kV; with a Zygo New View 6000 3D profiler to generate white light interferometry (WIM) profiles of the surface features; and with a DI 3100 atomic force microscope (AFM, Veeco Instruments) with a silicon tip (100 nm diameter; resonance frequency of 378 kHz; Mikro Masch) used in tapping mode with a scan rate of 1 Hz.

Results and Discussion

Here we describe the generation of biomimetic polymer materials with surfaces that contain replicas of cellular topography. Two distinct types of materials were produced: the impression replica contained indented topographical features, and the relief replica contained protruding topographical features. Because of our laboratory's interest in axon guidance and nerve regeneration, prealigned Schwann cells were used as the source of cellular topography for the initial experiments to mimic the organization of Schwann cells that promote regeneration after nerve injury. However, there is no limit to the type of cell that can be employed.

The impression replica was prepared in a four-stage process, and the relief replica was prepared in a five-stage process requiring one additional step beyond the preparation of the impression replica. As depicted in Figure 1, the stages included (1) photolithographic production of a polymeric stamp for microcontact printing (μCP); (2) μCP of a micropatterned laminin-striped glass coverslip; (3) cell culture to generate a cell template containing fixed, aligned SCs; (4) fabrication of the impression replica with indented topographical features; and (5) fabrication of the relief replica with protruding topographical features. The first three stages of the fabrication process were performed to align the SCs; when using other cell types for applications where alignment is not desired, the cell template can be prepared simply by fixing the cultured cells (see below). Use of a strong fixation procedure, similar to that required for SEM, preserved the cell morphology and allowed the cells to act as a template. To generate the impression replica, which contains a replica of cellular topographical features indented into its surface, a solution containing the desired polymer was applied to the fixed cell sample. Once the solution had polymerized or phase separated, the impression replica was removed. A similar procedure was used to generate the relief replica, which contains a replica of cellular topographical features protruding from its surface. A solution containing the desired polymer was applied to the patterned surface of the impression replica, thus using the impression replica as a template. Once the solution had polymerized or phase separated, the relief replica was removed. Note that the cell template, the impression replica, and the relief replica could all be reused as templates for subsequent experiments.

An examination of corresponding regions of the cell template (Figure 2A−C), the impression replica (Figure 2D−F), and the relief replica (Figure 2G−I) under SEM demonstrated that multiple features could be replicated from the cell template, including cell organization and alignment (Figure 2A, D, and G), cell morphology (Figure 2A−I), and subcellular features (Figure 2C, F, and I). At 1800× magnification (Figure 2C, F, and I), the detailed morphological features of somata and cell extensions were visible and reproduced clearly in both replicas. Inherent to the replica process, the impression replica contains

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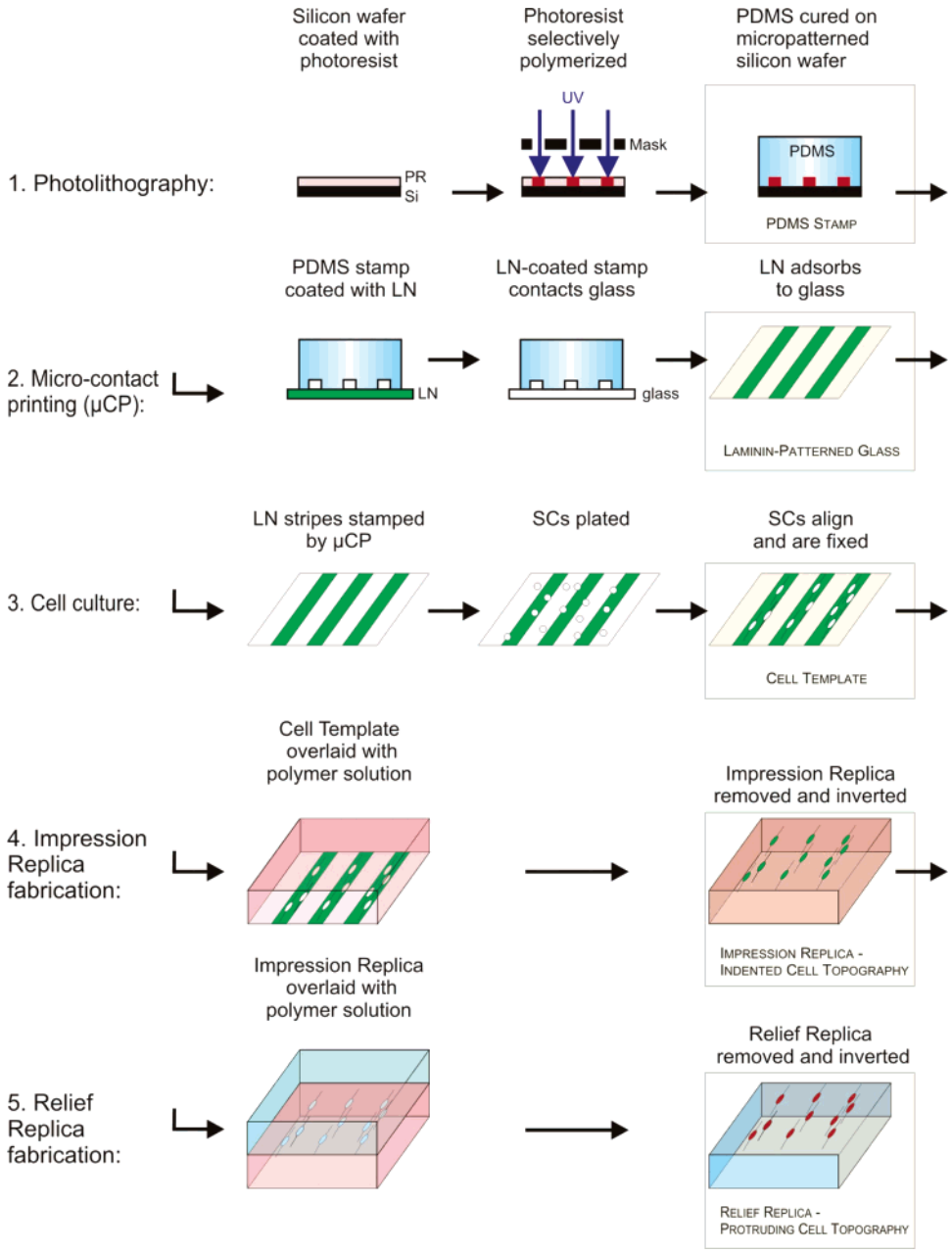

Figure 1. Overview of the technique highlighting the 5 main steps: (1) production of a polymeric stamp for μCP; (2) μCP of laminin stripes onto glass coverslips; (3) cell culture; (4) fabrication of the impression replica with indented topographical features; and (5) fabrication of the relief replica with protruding topographical features. The technique can begin with step 4 using fixed cells if no alignment of cells is desired.

features that are mirrored with respect to both the cell template and the relief replica because the impression replica was cast from the cell template and subsequently functioned as the template for the relief replica.

Polymeric materials with surface replicas of nanoscale features can be achieved with this technique. A comparison of surface features between corresponding regions of the cell template (Figure 3A−C), the impression replica (Figure 3D−F), and the relief replica (Figure 3G−I) with WIM analysis confirmed that feature geometry and dimensions were reproduced by the replica processing. The shapes, lengths, widths, and heights (or depths for the impression replica) of the cell somata were reproduced with this fabrication method, as measured and shown in the WIM oblique images (Figure 3A, D, and G) and overhead images (Figure 3B, E, and H). Shapes and x and z dimensions of cell extensions were replicated accurately to within 5−10 nm, as demonstrated by the corresponding surface profiles (Figure 3C, F, and I) measured along the lines overlaid on the top views of Figures 3B, E, and H. The corresponding surface profiles are presented on a single graph for a closer examination of feature replication in Supporting Information.

Various polymers can be employed using this approach to generate a wide range of material properties. For example, SC cell templates containing cellular processes of approximately 100 nm width were replicated in polyurethane (PU) (Figure 4A and B). Polymers that are useful for this technique are able to flow in the liquid phase, capable of conversion to a rubbery or gelled solid upon curing or phase separation, detachable from a cell template, and capable of maintaining cell-templated dimensions and geometry upon detachment from the cell template.

There is no limit to the type of cell whose topographical features can be replicated with this technique. In addition to SC, smooth muscle cells have served as cell templates (Figure 4C), with their features replicated in the impression replica (Figure 4D) and the

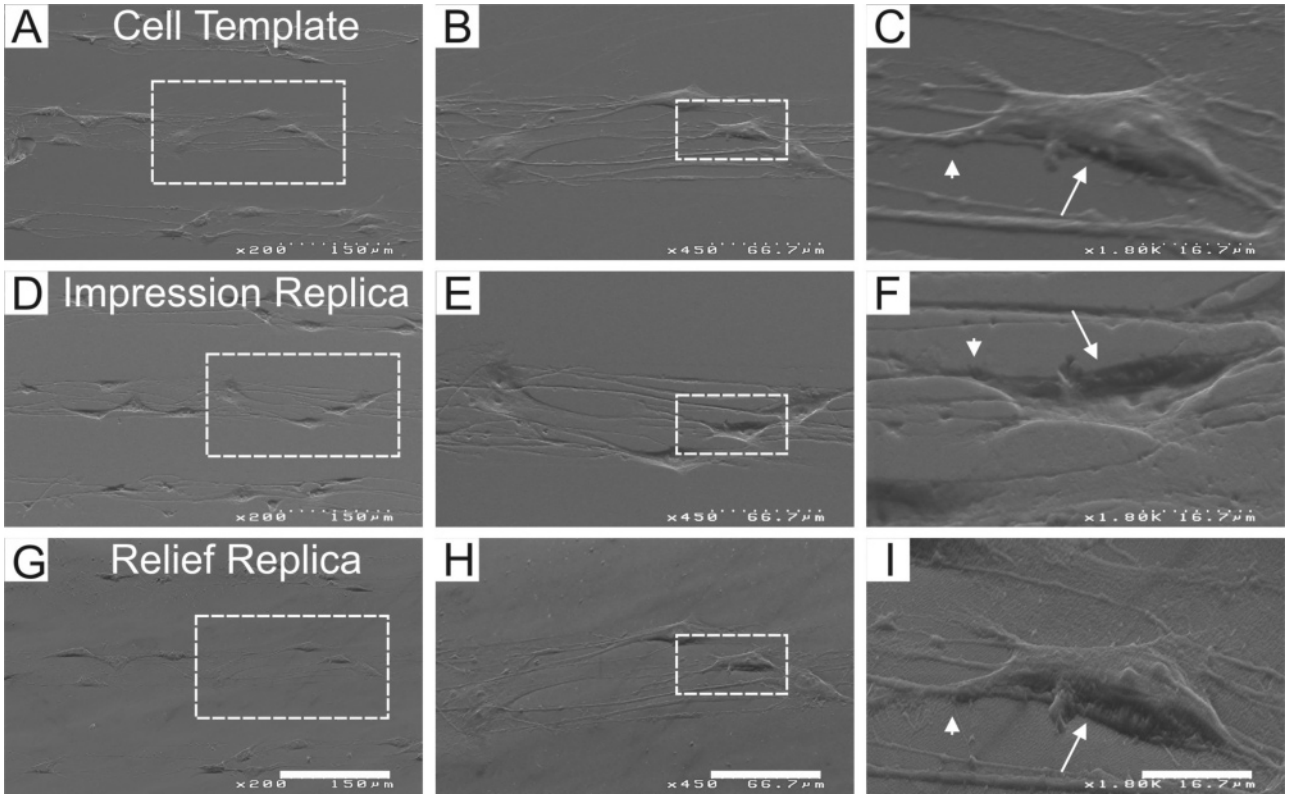

Figure 2. Biomimetic materials replicate cellular features, as visible under SEM. SCs were cultured at 30 000 cells/mL on stripes of LN for 24 h, fixed, and used as cellular templates to prepare the impression replica and the relief replica from PDMS. Corresponding regions of the impression replica and the relief replica show the replication of cellular topographical features in the original template. SEM micrographs of the SC template (A−C), the impression replica (D−F), and the relief replica (G−I). A rectangle outlines the borders of the region that is magnified in the image to the right. Note that inherent to the process, features in the impression replica are inverted relative to those of the template and the relief replica. Arrows, cell somata; arrowheads, cellular extensions. Scale bars, 2G = 150 μm, 2H = 66.7 μm, 2I = 16.7 μm.

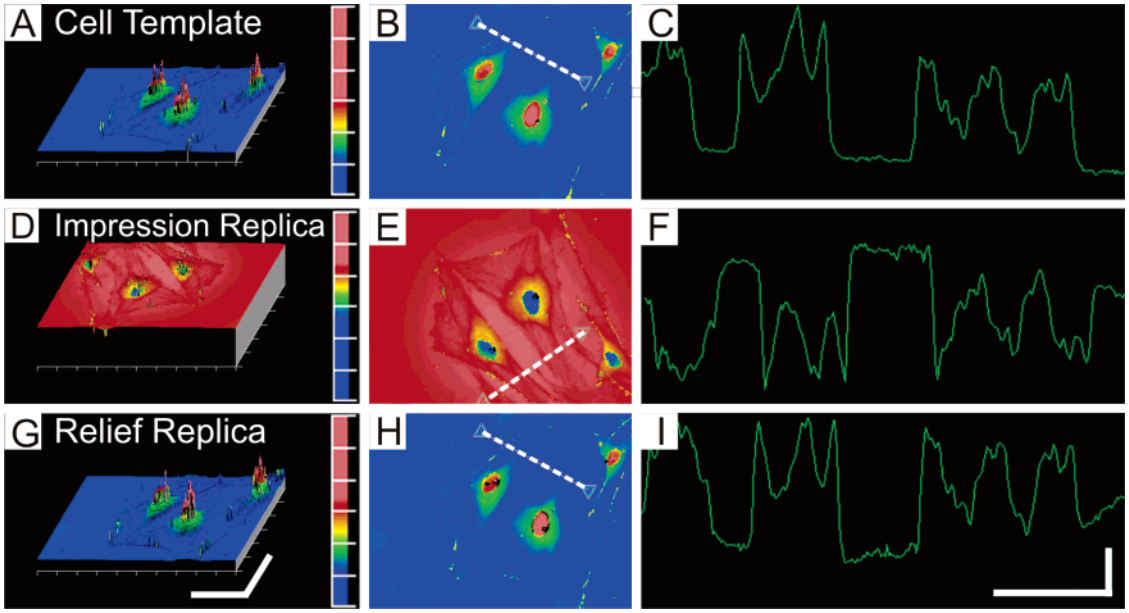

Figure 3. Biomimetic materials replicate cellular features, as quantified by WIM. SCs were cultured at 30 000 cells/mL on stripes of LN for 24 h, fixed, and used as cellular templates to prepare the impression replica and the relief replica from PDMS. Corresponding regions of the impression replica (D−F) and the relief replica (G−I) replicate the sizes and shapes of cellular topographical features in the original template (A−C). Oblique images (A, D, and G) provide a three-dimensional view of cellular topographical features on each substrate; corresponding overhead images (B, E, and H) provide a top view of each surface; and corresponding surface profile plots (C, F, and I) provide measurements of a cross section along the line superimposed on each overhead image. Note that inherent to the process, features in the impression replica are inverted relative to those of the template and the relief replica. Color bars in A, D, and G show pseudocolor scales where the range of each color bar represents a *z*-axis difference of 2.25 μm (A), 2.55 μm (D), or 2.03 μm (G). Scale bars in G: *x* axis = 50 μm, *y* axis = 70 μm; scale bars in I: *x* axis = 20 μm, *z* axis = 20 nm.

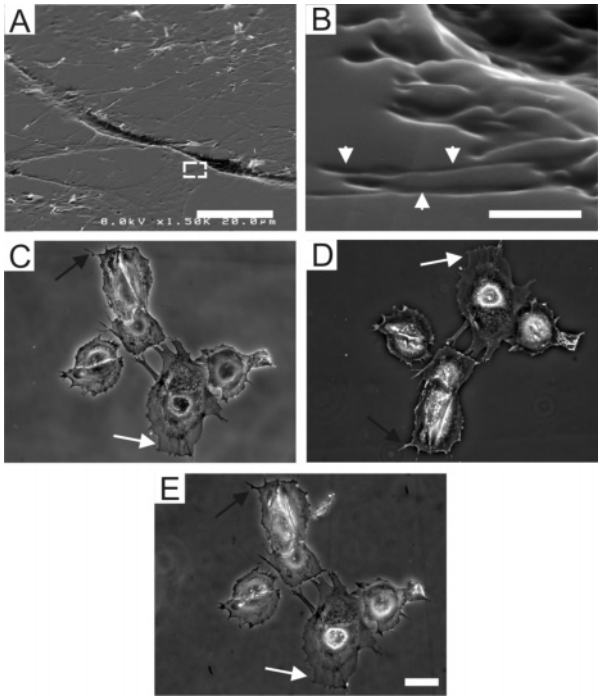

Figure 4. Various polymers and cell types can be utilized. (A and B) SCs were cultured at 30 000 cells/mL on stripes of LN for 24 h, fixed, and used as cellular templates to prepare the impression replica from PU. SEM micrographs of the impression replica film of PU contain indented topographical features of aligned SCs. This region corresponds to cellular extensions (white arrowheads). The rectangle in A outlines the region that is magnified in B, in which nanoscale features are visible. (C−E) SMCs were cultured at 30 000 cells/mL on PLL-coated coverslips for 24 h, fixed, and used as cellular templates to prepare impression and relief replicas from PDMS. Phase-contrast micrographs of corresponding regions of the impression replica (D) and the relief replica (E) show the replication of cellular features in the original SMC template (C). Note that inherent to the process, features in the impression replica are inverted relative to those of the template and the relief replica. Arrows, lamellipodia; black arrowheads, filopodia. Scale bars, 4A = 20 μm, 4B = 1 μm, and 4E = 50 μm.

relief replica (Figure 4E). In general, adherent cells can be utilized with the procedure described here, and cells that are cultured in suspension require just one additional step of immobilization on a solid material in order to function as a template. Future exploration of the use of critical point drying in conjunction with this technique may further enhance the preservation of cellular features in the generation of the initial cell template.

The materials generated with this technique are biocompatible and support the growth of cells. DRG neurons grew on LN-coated PDMS impression replicas and extended neurites that aligned along features that replicated SC topography (Figure 5).

Previous studies have shown that cells react to microscale and nanoscale surface features, where the features are either geometrically patterned or randomly ordered.[4,30,31] The availability of two distinct types of surface replicas with indented cellular features (the impression replica) and protruding cellular features (the relief replica) paves the way for a systematic investigation into the effects of cellular topography on cellular functions such as adhesion, orientation, activation, extension, and migration.

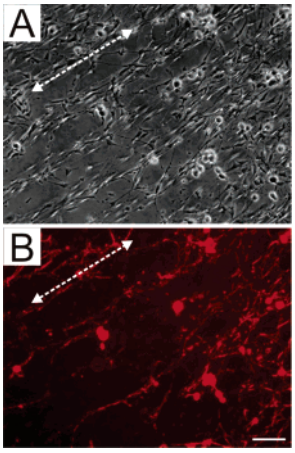

Figure 5. DRG neurons extend neurites on impression replica of SCs. SCs were cultured at 30 000 cells/mL on stripes of LN for 3 days, fixed, and used as cellular templates to prepare the impression replica from PDMS. Dissociated DRG neurons were cultured on a LN-coated impression replica for 3 days and stained for antineurofilament immunocytochemistry. In the phase contrast micrograph (A), both the patterned replica and the neurons are visible, whereas in the corresponding fluorescence micrograph (B), only the neurites are labeled. Dotted arrows indicate the direction of the cellular pattern indented in the replica surface. Many neurites are aligned with the pattern. Scale bar, 100 μm.

Conclusions

We have developed transparent, topographically biomimetic replicas of mammalian cellular surfaces containing combinations of micro- and nanoscale features. With the incorporation of cellular topography onto surfaces, the roles of biomaterials can be expanded from providing simple mechanical support to providing critical cues for the study of cellular function. By combining materials that are biomimetic in cellular topography with technologies to modify bulk material properties, surface chemistry, and the controlled release of diffusible factors, tailored biomaterial systems can be developed toward the ultimate goal of directing cells to form functional tissues.

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Supporting Information Available: Superimposed surface profile plots of a corresponding cell template, impression replica, and relief replica and AFM images of the surfaces of a cell template, an impression replica, and a relief replica. This material is available free of charge via the Internet at http://pubs.acs.org.

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