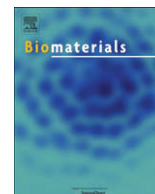




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Integrating polyurethane culture substrates into poly(dimethylsiloxane) microdevices

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ABSTRACT

Poly(dimethylsiloxane) (PDMS)-based microdevices have enabled rapid, high-throughput assessment of cellular response to precisely controlled microenvironmental stimuli, including chemical, matrix and mechanical factors. However, the use of PDMS as a culture substrate precludes long-term culture and may significantly impact cell response. Here we describe a method to integrate polyurethane (PU), a well-studied and clinically relevant biomaterial, into the PDMS multilayer microfabrication process, enabling the exploration of long-term cellular response on alternative substrates in microdevices. To demonstrate the utility of these hybrid microdevices for cell culture, we compared initial cell adhesion, cell spreading, and maintenance of protein patterns on PU and PDMS substrates. Initial cell adhesion and cell spreading after three days were comparable between collagen-coated PDMS and PU substrates (with or without collagen coating), but significantly lower on native PDMS substrates. However, for longer culture durations (≥ 6 days), cell spreading and protein adhesion on PU substrates was significantly better than that on PDMS substrates, and comparable to that on tissue culture-treated polystyrene. Thus, the use of a generic polyurethane substrate in microdevices enables longer-term cell culture than is possible with PDMS substrates. More generally, this technique can improve the impact and applicability of microdevice-based research by facilitating the use of alternate, relevant biomaterials while maintaining the advantages of using PDMS for microdevice fabrication.

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1. Introduction

Cellular function is regulated by several features of the external microenvironment, including substrate, chemical, and mechanical factors. Rapid progress is being made in developing high-throughput experimental approaches to study cellular response to various stimuli: growth factors, chemical cues and culture parameters [1], mechanical forces [2–5], extracellular matrix proteins [6], gradients in chemical stimuli [7], and co-culture conditions [8]. These approaches are building towards techniques to determine the integrated cellular response to combinations of stimuli, thereby improving understanding of fundamental cell-microenvironment interactions and guiding developments in biomaterial design and tissue engineering.

Advances in many high-throughput methods can be attributed to the development of microfabricated technologies. Specifically,

soft lithography fabrication of polydimethylsiloxane (PDMS) microdevices shows great promise in developing miniaturized systems to study cell biology. PDMS is an excellent material for microfabrication: it is elastomeric, transparent, non-toxic, gas permeable, inexpensive, chemically stable, can be cured at low temperatures, and can retain micropatterned features during processing [9]. The availability of this material has been an important factor in driving microdevice development.

Multilayer soft lithography [10] has been used to form more complex PDMS structures, and has led to fully integrated devices capable of systematically, combinatorially and rapidly probing biological systems. However, many multilayer soft lithography systems are limited to culturing cells directly on a PDMS substrate [1–4,11–17]. PDMS is poorly suited for and rarely used in standard cell culture experiments, particularly those requiring extended culture periods of several days. The substrates on which cells are grown can have a substantial impact on cellular function [18,19], and using PDMS for this purpose is one factor that hampers the adoption of microdevices as a mainstream technology for biomedical research. Chemical modification of the PDMS surface to

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improve cell culture is possible [20,21], but challenging and not easily accessible to many research groups with expertise in microfabrication.

The inability to use more common biomaterial substrates in novel microdevices limits their utility for applications in cell biology, biomaterials, and tissue engineering research. For example, PDMS is a poor material for tissue engineering scaffolds because it is highly resistant to degradation, has non-physiological mechanical properties, and is difficult to fabricate into porous structures. Biological results obtained using PDMS microdevice research platforms may not be applicable to the systems for which the studies are intended, because the substrate materials will, by necessity, be different. Hence, there is a need to conduct these microdevice studies on relevant culture substrates and biomaterials. The use of alternative materials to create hybrid devices [22], or to completely replace PDMS with conventional biomaterials as the primary structural material in microdevices [23–25] are potential approaches; however, material properties and processing requirements of these alternative materials can make complex multilayer microfabrication difficult.

To improve the relevance of biological results obtained using such PDMS microfabricated platforms, we have developed a simple, robust and easily accessible method to integrate polyurethane (PU) substrates into the multilayer PDMS soft lithography process. The PU class of polymers was selected based on the ease with which it can be chemically tailored for a specific application, as well as its widespread use in implantable medical devices [26,27] and tissue engineering scaffolds [28–31]. Thus, our approach retains the advantages of using PDMS in microfabrication while enabling studies with a well-established, clinically-relevant biomaterial. In this paper, we describe and characterize the process to integrate Tecoflex[®], a generic formulation of PU, into multilayer PDMS microdevices. In addition to the general advantages of being able to conduct cellular experiments on substrates relevant to clinical applications, we have quantitatively demonstrated specific improvements in long-term cell culture conditions when using PU over PDMS as a culture substrate in microdevices.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma–Aldrich (Oakville, ON, Canada); fluorescent dyes from Invitrogen (Burlington, ON, Canada); and all other materials from Fisher Scientific Canada (Ottawa, ON, Canada).

2.1. Polyurethane film preparation

Polyurethane films were formed by dip-coating handling slabs into a solubilized polymer. Tecoflex[®] aliphatic PU pellets (SG-80A; Lubrizol Corporation; Wickliffe, OH, USA) were dissolved in tetrahydrofuran (THF) at concentrations ranging from 2 to 10 wt%, in preparation for dip-coating. PDMS was used to make the handling slabs, as it is flexible, and the PU films were easily released from this silicone material. Sylgard 184 PDMS (Dow Corning, purchased through A.E. Blake Sales Ltd., Toronto, ON, Canada) was mixed in a standard 10:1 curing ratio and poured into an Omnivell tray (Nunc; Rochester, NY, USA). The PDMS was then degassed in a vacuum chamber to remove air bubbles, before curing overnight at 80 °C. The PDMS was then peeled from the tray and cut to size. This handling slab was then dip-coated in the PU solution. THF causes the PDMS to swell, so the immersion time was minimized to less than 2 s. After dip-coating, samples were quickly transferred to a beaker, stored vertically, covered and left to dry for at least 2 h at room temperature. Drying the samples in a container was critical, as excessive airflow was shown to cause rough, opaque surfaces. A Wyko optical profilometer (Veeco Instruments Inc., Woodbury, NY, USA) was used to characterize the dip-coated PU films. A section of the film was cut and peeled away, and the step-height and surface roughness were measured for each of the solution concentrations used in the dip coating procedure ($n = 3$). A concentration of 5% PU in THF was used in all subsequent experiments.

2.2. Integrating PU films into PDMS microfabrication

Integration of a PU film into microfabricated PDMS devices was achieved using a 'mortar layer' of partially cured PDMS as an intermediary layer [32] between a fully

cured PDMS sheet and the PU coated on the handling slab. Briefly, the process (schematic outlined in Fig. 1A) involved preparation of PDMS in the standard 10:1 ratio, and spin-coating a 15 µm film of PDMS on a polyethylene surface (Ink-jet transparency, Grand & Toy; Toronto, ON, Canada). This thin PDMS film was partially cured for 10 min at 80 °C. The PDMS device layer and the partially cured PDMS film were treated with oxygen plasma (corona discharge treatment, Electro-Technic Products; Chicago, IL, USA), and placed in contact with each other. The sandwich was cured for an additional 10 min at 80 °C, and the transparency was peeled away, leaving a partially cured 'mortar layer' of PDMS adhering to the fully cured PDMS device layer. This mortar PDMS layer and the PU film (mounted on the handling slab) were then treated with oxygen plasma and placed in contact with each other, for a further 10 min at 80 °C. The handling slab was then peeled from the PU–PDMS structure, which was then fully cured for 4 h at 80 °C.

2.3. Multilayer microfabrication of PU–PDMS structures

A simple microchannel device, which mimics the fabrication process for a complex multilayer PDMS system while maintaining experimental simplicity, was formed from two layers of PDMS (Fig. 1B–D). In this study, the channels were fabricated with widths of 1500 µm and lengths of 35 mm. An upper layer with a rectangular channel relief pattern was formed by replica molding on microfabricated SU-8 masters. Briefly, the channel patterns were drawn in AutoCAD, and printed on a transparent film using a high-resolution laser plotter (City Graphics; Toronto, ON, Canada). SU-8 25 photoresist (Microchem; Newton, MA, USA) was spin-coated at 1000 RPM on pre-cleaned 3" × 2" glass slides. The slides were pre-baked, exposed to UV light through the printed film, post-baked and developed, using parameters outlined by the resist manufacturers. The resulting SU-8 structures were optically profiled and determined to be 50 µm high. PDMS was then mixed, poured, degassed, cured, and peeled from the SU-8 master.

Fully cured sheets of unpatterned PDMS were also prepared for the base layer of the microchannel, and both layers were then cut to size. For devices without PU films (Fig. 1B), the PDMS channel and base layers were bonded to each other and then to a clean glass slide, by treating the surfaces with oxygen plasma. For microchannels with PU culture substrates (Fig. 1C), a PU film was bonded to the PDMS base layer using a mortar layer as described in Section 2.2. The process was then repeated with the upper channel layer: a partially cured PDMS film was transferred to the upper channel layer, plasma-treated, and placed in contact with the PU–PDMS structure before completing the curing process.

To test the adhesion strength between PU and PDMS using this bonding method, a test structure was fabricated, consisting of a PU membrane suspended over a hole cored into a PDMS slab (Fig. 1E and F). The base was formed from a fully cured PDMS slab with holes cored in it using a 5 mm disposable biopsy punch (Sklar Instruments; West Chester, PA, USA). The PU–PDMS structure was formed by first applying a partially cured PDMS mortar film (on a polyethylene sheet) to the PDMS base. Because the PDMS mortar film had not fully solidified, the mortar did not form a suspended membrane over the cored PDMS slab and remained on the polyethylene surface in these regions. The PU film was then bonded to the PDMS base. The PDMS base was then plasma-treated and sealed to a glass slide. To visualize the resulting structures, the suspended membranes were coated with 15 nm of platinum and imaged in a scanning electron microscope (Hitachi). To test the adhesion strength between the PU and PDMS, increasing pressure was applied across the PU membrane by connecting the PDMS cavity to a tank of compressed nitrogen via a pressure regulator, and visually observing membrane distention at various pressures.

2.4. Evaluation of cell response to PU versus PDMS substrates

In addition to the general advantages of being able to integrate a clinically-relevant biomaterial into the microfabrication process, the demonstrated incorporation of the Tecoflex[®] material into PDMS devices may yield specific technical advantages in improving device utility and biological relevance, particularly for long-term culture. To test this hypothesis, we compared cell adhesion, spreading, and initial attachment strength to native PDMS and PU substrates. Because cell adhesion to PDMS substrates is often promoted by adsorption of extracellular matrix proteins prior to cell seeding, we also compared cell adhesion to plasma-treated PDMS coated with type I collagen (PDMS + ECM) and plasma-treated PU coated with type I collagen (PU + ECM). Tissue culture-treated polystyrene (TCP) was used as a positive control substrate in some experiments.

2.4.1. PAVIC isolation and cell culture

Primary porcine aortic valve interstitial cells (PAVICs; a fibroblast-like cell) were used as model primary cells. Cells were isolated by enzymatic digestion as described [33] and used between passage 2 to 6 for all experiments.

2.4.2. Adhesion and spreading measurements

Cell adhesion and spreading were measured on coupons of PDMS and PU–PDMS cut to size and placed in 12-well plates. The substrates were soaked in 70% ethanol, air-dried, and exposed to UV light for 30 min to sterilize them. Samples to be coated with matrix proteins were exposed to oxygen plasma and incubated with 50 µg/mL

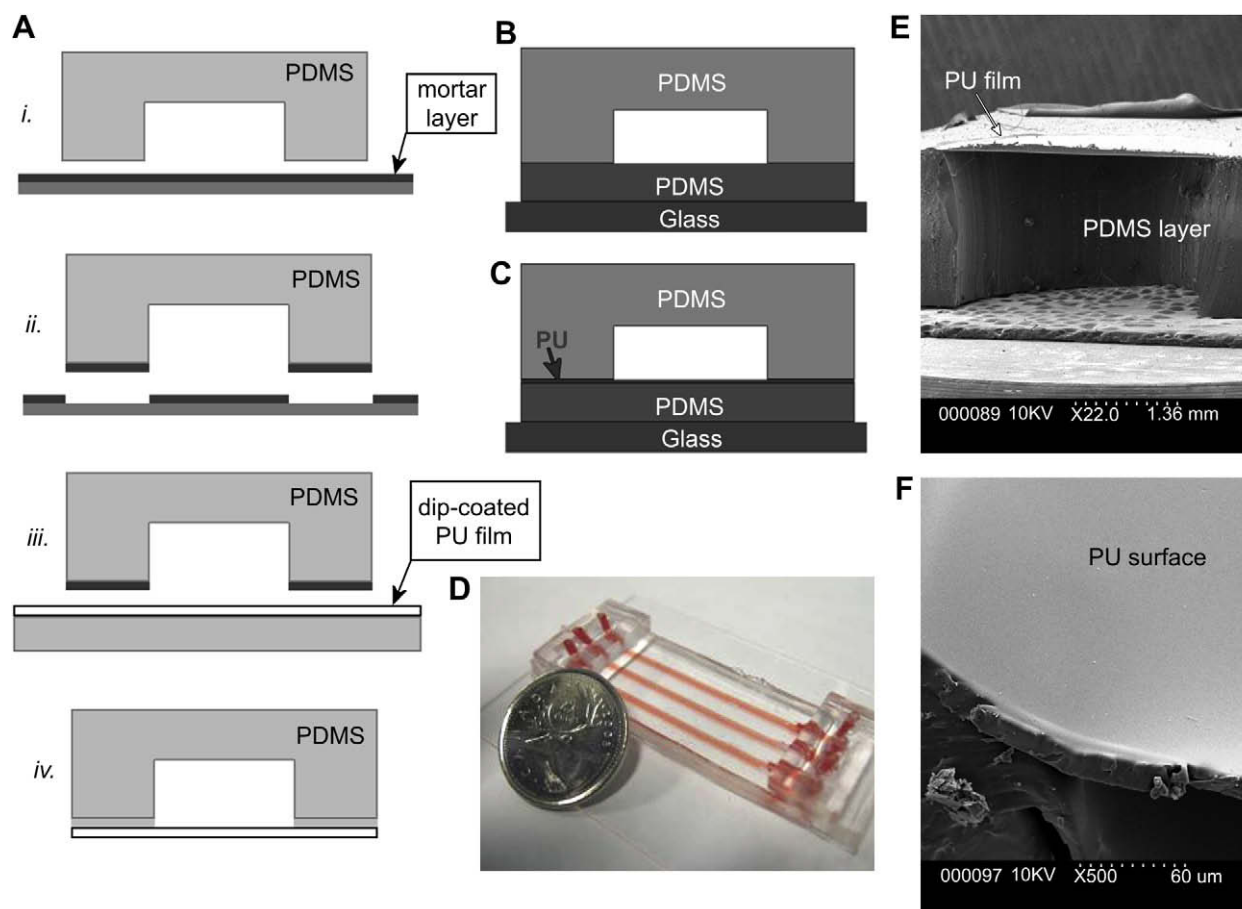


Fig. 1. (A): Fabrication process flow to bond a PU film to a PDMS slab. (i) The PDMS layer is placed in contact with a partially cured 'mortar layer' of PDMS. (ii) The PDMS is peeled away, retaining the partially cured PDMS in the regions of contact. (iii) The PDMS is then brought in contact with a dip-coated PU film, (iv) which is subsequently peeled away from the handling slab. (B and C): Schematic cross-sections of multilayer PDMS microchannels with (B) a PDMS culture surface and (C) a PU culture surface. (D): Fabricated microchannel with integrated PU culture surface. The channel was filled with a red dye to allow easy visualization. (E and F): Scanning electron micrographs of a PU membrane suspended over a cored PDMS slab.

type I rat-tail collagen (Becton Dickinson, Mississauga, ON, Canada) overnight at 4 °C. The substrates were then washed three times in phosphate-buffered saline (PBS) before seeding PAVICs at 3000 and 10,000 cells/cm² for spreading and adhesion experiments respectively, and incubating at 37 °C, 5% CO₂. To assess initial adhesion, the samples were washed with PBS 12 h after seeding and fixed in 10% neutral-buffered formalin. They were then stained with Hoechst 33342 nuclear dye and imaged with a 10× objective under a fluorescent microscope (Olympus IX71; Olympus Microscopes; Markham, ON, Canada) with a CCD camera (Retiga 2000R; QImaging; Surrey, BC, Canada). The number of adhering cells was counted manually in five fields of view for each condition and repeated at least three times.

To determine spread area, samples were cultured for three and six days, washed in PBS, fixed and stained for 10 min in 1% (w/v) crystal violet stain solubilized in 100% ethanol. They were then washed thoroughly in water and imaged using bright-field microscopy. Cell spread area was measured using ImageJ (NIH) and determined in at least three fields of view, for four samples per condition.

2.4.3. Microfluidic adhesion strength assay

The strength of cell adhesion to the various substrates was measured in microchannels using a shear assay, as described by Young et al. [5]. Microchannels with and without PU layers were fabricated as described, mounted to glass slides, and assembled with polyethylene tubing as inlet ports for fluid injection and withdrawal. Channels were sterilized by flushing them with 70% ethanol for 10 min and with sterile PBS for an additional 10 min. Sterile air was flushed through the device to evacuate the channels. Channels were dried by placing them on a hotplate at 50 °C for 20 min. For those channels to be treated with oxygen plasma before collagen coating, the tip of a corona discharge unit was inserted into the inlet port of the microchannel. The plasma was observed to traverse the channel, and this treatment was maintained for 30 s. To coat the channels with an ECM protein, type I collagen was flushed into the channels at a concentration of 50 µg/mL and incubated at room temperature for 30 min. Sterile PBS was used to displace the collagen, and the channels were rinsed three times.

PAVICs were fluorescently labeled with a vital nuclear dye prior to loading them into the microchannels by incubating with Hoechst 33342 (2 µg/mL in fully supplemented DMEM) for 30 min at 37 °C, 5% CO₂. They were then washed with PBS and maintained in fully supplemented media for at least 30 min. Cells were trypsinized from the culture flasks and re-suspended in supplemented media at a concentration of 10 × 10⁶ cells/mL. Using a syringe, 200 µL of the cell suspension was injected into each of the microchannels, and incubated for 2 h at 37 °C, 5% CO₂ to allow initial cell adhesion and spreading on the microchannel substrate.

A multi-channel syringe pump (Cole-Parmer, Montreal, QC, Canada) was used to clear the microfluidic channels of non-adhered cells by flushing them with supplemented DMEM at a rate of 3 mL/h for 2 min. To investigate initial adhesion strength, cells were subjected to shear treatments for 2 min each at rates of 30 mL/h and 60 mL/h. These flow rates translate into shear levels of 98 and 195 dynes/cm², respectively [5].

The number of cells in each channel was determined before and after shear treatments by imaging the nuclei at five marked locations along the channel length, using a fluorescent microscope (Leica; Wetzlar, Germany) and camera (Hamamatsu Photonics; Hamamatsu, Japan). These experiments were repeated for two to four channels per condition.

2.5. Assessment of protein pattern maintenance in long-term culture

Maintaining the ECM protein microenvironment in cell culture conditions is an important requirement for long-term cell culture. In order to assess the maintenance of ECM protein patterns on PU and PDMS substrates, protein pattern fidelity was monitored over time. PDMS samples were prepared by bonding a thin PDMS sheet to a glass substrate. PU samples were prepared by dip-coating a silane-coated glass slide (Sigma) in 5% PU solution. An elastomeric stencil [34,35] was used to pattern proteins into an array of 300 µm diameter circles. The stencil was constructed by exclusion molding [36] to create a thin film of PDMS with through-holes which was then bonded to a thick PDMS gasket. The sample and stencil were then

sterilized by exposure to UV light for 2 h. The stencil was carefully placed on the substrate where it formed a conformal contact. The sample was then plasma-treated through the stencil, and 50 $\mu\text{g}/\text{mL}$ of FITC-labelled bovine type I collagen (Exalpa Biologicals Inc.; Watertown, MA) was incubated in the gasket for 2 h at room temperature. The protein was aspirated, and the sample washed with sterile PBS, before peeling away the stencil. The sample was then washed with 3% bovine serum albumin (BSA) in PBS to block the non-patterned regions on the substrate.

The patterned substrates were incubated with DMEM supplemented with 10% FBS, and fluorescently imaged at several time points over the course of four weeks. At each time point, the media was aspirated and samples were washed with sterile deionized water. A consistent exposure time of 500 ms with a gain of 10 was used to ensure an appropriate intensity comparison between samples. To quantify protein pattern fidelity, the total fluorescent intensity was measured inside and outside the patterned circles ($n = 20$), and the results scaled to the ratio of the measured areas. The ratio between the fluorescing pattern and the background was recorded and normalized to the ratio for that pattern at day zero.

2.6. Statistical analyses

Data was analyzed using one and two-way ANOVA, as appropriate. Post-hoc pairwise comparisons were conducted using the Student–Newman–Keuls method. All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA). Graphical results are plotted as means \pm one standard deviation.

3. Experimental results

3.1. Polyurethane film characterization

PU film thickness was found to increase for increasing concentrations of solubilized polymer (Fig. 2A). For concentrations ranging from 2 to 10 wt%, film thickness increased from $4.6 \pm 1.2 \mu\text{m}$ to $40.4 \pm 3.6 \mu\text{m}$. The increase was not linear over the range of concentrations tested, but increased sharply at concentrations greater than 6 wt%. Surface roughness was found to increase from $56 \pm 18 \text{ nm}$ to $202 \pm 91 \text{ nm}$ with higher concentrations of PU in the

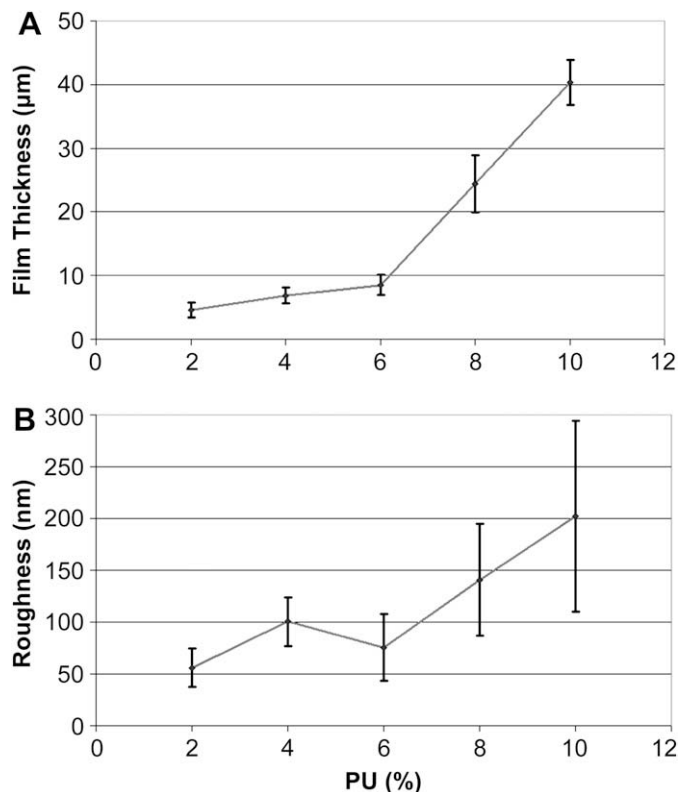


Fig. 2. Characterization results for dip-coated polyurethane on a PDMS handling substrate. (A) Polyurethane film thickness and (B) surface roughness, measured as a function of the concentration of solubilized polyurethane (PU) used in the dip-coating process.

Table 1

Contact angle measurements for native and oxygen plasma treated polyurethane (PU) and polydimethylsiloxane (PDMS) surfaces. Measurements are based on the contact angles of drops of deionized water (5 μL) pipetted onto the material surface ($n = 5$).

	Native substrate	O ₂ plasma treated
PU	$61.6 \pm 1.8^\circ$	$38.0 \pm 3.6^\circ$
PDMS	$104.4 \pm 6.7^\circ$	$3.1 \pm 2.8^\circ$

solvent (Fig. 2B). The variation in roughness across each sample increased dramatically at concentrations higher than 6 wt%. The surface of the PU film was smooth and free of defects. Visual inspection confirmed the thickness of the films determined via surface profilometry (Fig. 1E and F). Qualitatively, we found that films less than 6–7 μm thick were more prone to pin-hole defects, due to dust particles in the immediate environment. The surface wettability of native and plasma-activated PU were determined by water contact angle measurements (Table 1) and compared to the wettabilities of PDMS. The results indicated that plasma-activated PU and PDMS are more hydrophilic than native PU and PDMS substrates.

3.2. Integrated PU films in microfabrication

PU culture surfaces were successfully integrated into multilayer microchannels (Fig. 1A–D) using the mortar layer method, and were tested at flow rates of up to 7 mL/min, at which they maintained structural integrity and did not leak. On testing the adhesion strength between the PU and PDMS films in the suspended membrane structure (Fig. 1E and F), the PU membranes underwent elastic deformation up to pressures of $\sim 35 \text{ kPa}$ without peeling away from the PDMS. At $\sim 70 \text{ kPa}$, the membrane integrity was compromised before exceeding the adhesion strength at the interface of the two materials. The PU–PDMS structures were stored under PBS in a humidified incubator for three days, and this did not affect the bond strength.

3.3. Initial cell adhesion

Significant differences were found in the number of cells initially adhered to each of the substrates ($p < 0.001$; Fig. 3). The number of cells that adhered initially to the PU, PU + ECM and PDMS substrates was significantly lower than that of the TCP control ($p < 0.01$). No statistically significant difference was found between initial adhesion to TCP and PDMS + ECM. Plasma treatment and collagen coating of PDMS improved initial cell adhesion significantly over native PDMS ($p < 0.01$). No differences were found between the PU, PU + ECM and PDMS + ECM substrates; or between the PU, PU + ECM and PDMS substrates.

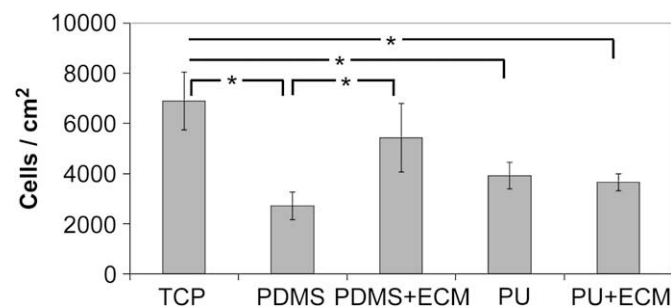


Fig. 3. Initial adherent cell density to tissue culture polystyrene (TCP), polydimethylsiloxane (PDMS), collagen-coated PDMS (PDMS + ECM), polyurethane (PU) and collagen-coated PU (PU + ECM) 12 h after seeding (* $p < 0.01$).

3.4. Initial cell adhesion strength

To determine the strength of initial cellular adhesion to various substrates and treatments, cells were introduced into the microfluidic channels (Fig. 1D) and sheared by fluid flow. The proportion of cells remaining after two levels of shear (Fig. 4A) provided a quantitative measure of adhesion strength for the various substrates and treatments.

The number of cells remaining after shear depended significantly on the substrate material ($p < 0.001$) and the shear level ($p = 0.022$) but there was no interaction between these factors ($p = 0.901$), by two-way ANOVA (Fig. 4B). The PDMS substrate retained fewer cells than any other substrate ($p < 0.001$), and there were no significant differences in the number of cells remaining after shear on the PU, PU + ECM or PDMS + ECM substrates.

3.5. Cell morphology and spreading area

The morphology of PAVICs seeded on TCP is typically well-spread with long processes. For the first 12 h after seeding, there was no significant difference between cell areas on each of these substrates. However, after three days in culture, cells were significantly less spread on PDMS than any of the other substrates ($p < 0.01$). Furthermore, cells seeded on the PDMS + ECM substrate exhibited a significantly smaller spread area than those on the TCP control ($p = 0.023$). Cells on the PU, PU + ECM and TCP control samples spread similarly (data not shown). By day six (Fig. 5), cell spreading areas remained similar on TCP, PU and PU + ECM, and each case exhibited significantly better spreading than on the PDMS and PDMS + ECM substrates ($p = 0.036$).

3.6. Protein pattern maintenance

The maintenance of extracellular matrix protein (type I collagen) on plasma-treated PDMS and PU substrates was compared in culture conditions (Fig. 6). The protein patterns degraded quickly on PDMS substrates, such that by day six in culture, the fluorescently labeled patterns on PDMS were undetectable. In contrast, protein patterns were maintained for at least 26 days on PU substrates (after an initial drop to 30% of the original level over the first 12 days). The normalized contrasts were statistically different based on both substrate material ($p < 0.001$) and culture time ($p < 0.001$), and showed a strong interaction between these two factors ($p < 0.001$). The pattern fidelity was significantly reduced on the PDMS substrate as compared to the PU substrate for every time point after the first 24 h ($p < 0.001$). After day 8, there were no significant reductions in pattern fidelity on the PU substrate.

4. Discussion

The ability to answer many complex questions in cellular biology is significantly facilitated by the utility and availability of microfabricated devices for cell culture applications. These microdevice-based approaches can have a substantial impact on fields such as tissue engineering, drug discovery, and fundamental cell biology. However, in many multilayer microfabricated systems, cells must be cultured on PDMS substrates, which can significantly impact their function. To address this issue, we have developed a technique to integrate PU, a well-established, clinically relevant class of biomaterials, into the PDMS microfabrication process, and have demonstrated this using a generic formulation of PU as a model biomaterial. Thus, we maintained the advantages of using PDMS as a structural material in microfabrication while (1) improving long-term cell culture potential

of soft microdevices and (2) enabling the use of alternative biomaterials in microfabricated systems, thereby improving the applicability of microdevices in cell biology and biomaterials science.

Polyurethane elastomers are easily customizable and can be designed for specific biomedical applications [26,37]. Tecoflex® solution-grade PU has excellent physical properties as a cell adhesion substrate [38], and can be processed by a variety of techniques. For this application, dip-coating was the most suitable for several reasons. Without expensive equipment or materials, dip coating rapidly and reproducibly forms PU films with thicknesses and optical properties consistent with those typically used in microfabrication. Surface roughness can be easily minimized by isolating the sample from airflow during drying. Dip-coating also allows flexibility in the type of handling slabs used, which can be particularly important in the microfabrication process. In this case, a flexible PDMS handling slab was used to facilitate peeling away from the rigid glass–PDMS–PU device layer. If the device layer is flexible, a glass slide can be used as the handling slab, and produces more uniform PU films. However, the adhesion between bare glass and PU is stronger than that of dip-coated PDMS to PU. Hence, this requires greater experimental skill to complete the film transfer, and was not used in this study. When transferred to the PDMS substrate, the PU film is complete and free of wrinkling, and the bond strength between the two materials is sufficient for most microfabricated devices, as demonstrated by bulge testing and the operation of hybrid PU–PDMS microfluidic channels.

We found that native PDMS is a poor substrate for culture of adherent cells, as it was significantly worse than the control TCP, matrix-coated PDMS, bare PU and matrix-coated PU in terms of initial cell adhesion, adhesion strength, and cell-spreading area. This is likely due to the strong surface hydrophobicity, which has a demonstrated negative impact on cellular adhesion and function [39]. In microdevices in which cells are cultured on PDMS, the substrate is often coated with an ECM protein to improve cellular adhesion. Several methods to coat PDMS with matrix protein exist [40–42]. Wipff et al. [42] compared several techniques of coating PDMS with ECM and found significantly improved cellular response for their multi-step PDMS preparation process over other more common techniques. However, they did not compare their method with protein adsorption to plasma-activated PDMS surfaces, which can potentially impact the activity of the adsorbed matrix protein and is commonly used when conducting biological experiments on microfabricated PDMS platforms [43]. Though it has been demonstrated that greater quantities of collagen adhere to hydrophobic surfaces [44] such as native PDMS, there are concerns that matrix proteins adsorb to these surfaces in different conformations, depending on surface wettability [45–47]. These conformational differences can affect cellular adhesion and function. In the case of fibronectin and vitronectin, adsorption to a hydrophobic surface has been shown to significantly reduce the bioactivity of the molecule [45–47]. Whether this applies to collagen specifically has not been addressed. For the purposes of this study, we elected to deposit proteins on plasma-activated substrates, based on lower cell spreading areas and adhesion strengths observed on matrix-coated hydrophobic PDMS, as compared to matrix-coated hydrophilic PDMS (data not shown). Furthermore, others have demonstrated that protein adhesion to plasma-activated PU is covalent [48], which was supported by our observation that protein patterns survived for close to four weeks in culture conditions on plasma-activated PU substrates. Characterization of surface wettability by contact angle measurements indicated that plasma-activated PU is more hydrophilic than native PU (Table 1). Since covalent ECM binding on PU is associated with plasma-activated, hydrophilic

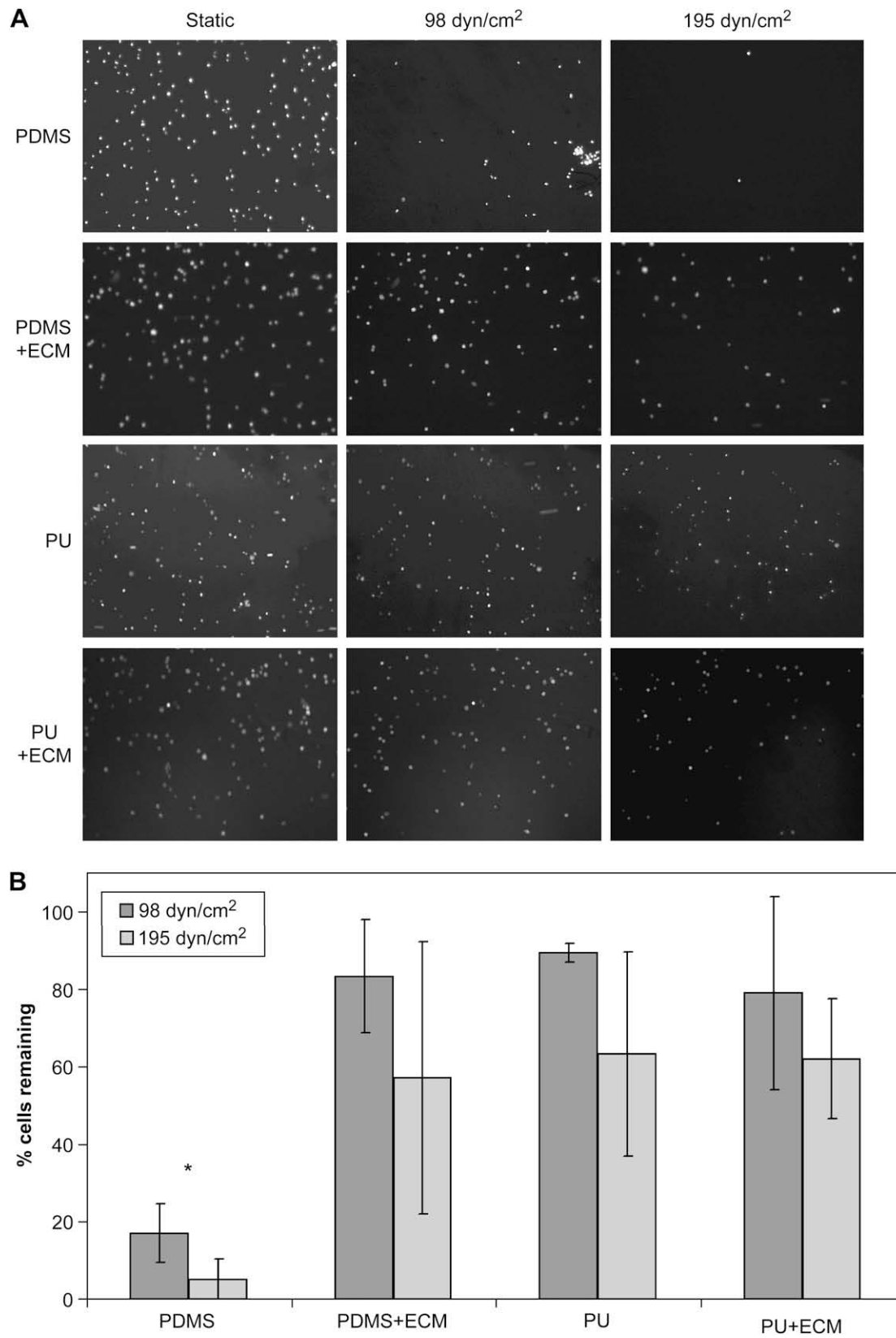


Fig. 4. Results of the microfluidic shear assay to determine initial cellular adhesion strength to various substrates. (A) Representative images of Hoechst nuclear-stained cells remaining in microchannels after increasing shear treatments. (B) Comparison of cells remaining on each substrate following two shear treatments, normalized to the number of cells adhered in the static condition (* $p < 0.001$ compared to other substrates).

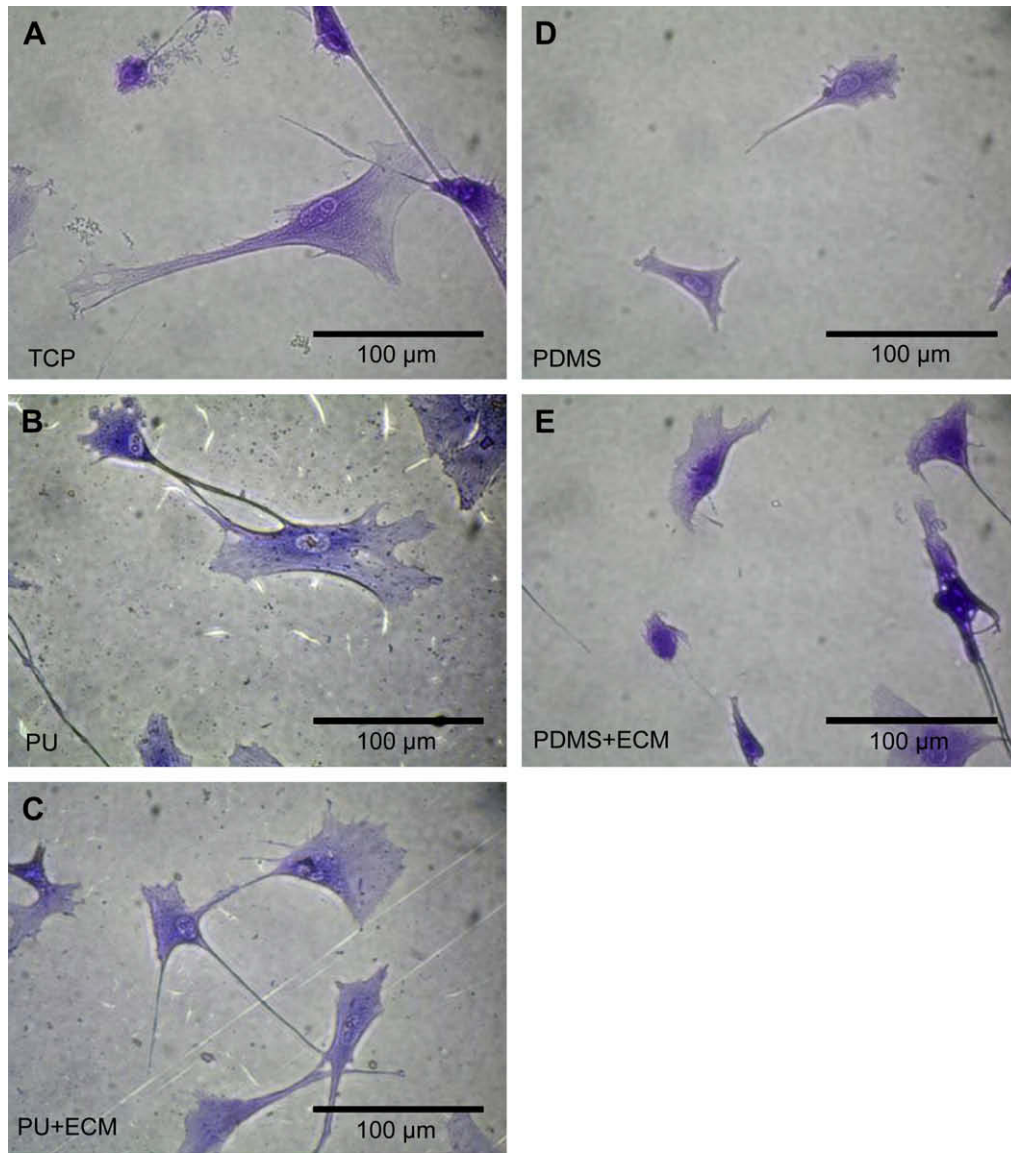


Fig. 5. A comparison of cell spreading areas on different substrates, six days after seeding. (A–E): Representative pictures of cells under these conditions. (F): comparison of cell areas across the samples ($*p < 0.05$; between all conditions in each group).

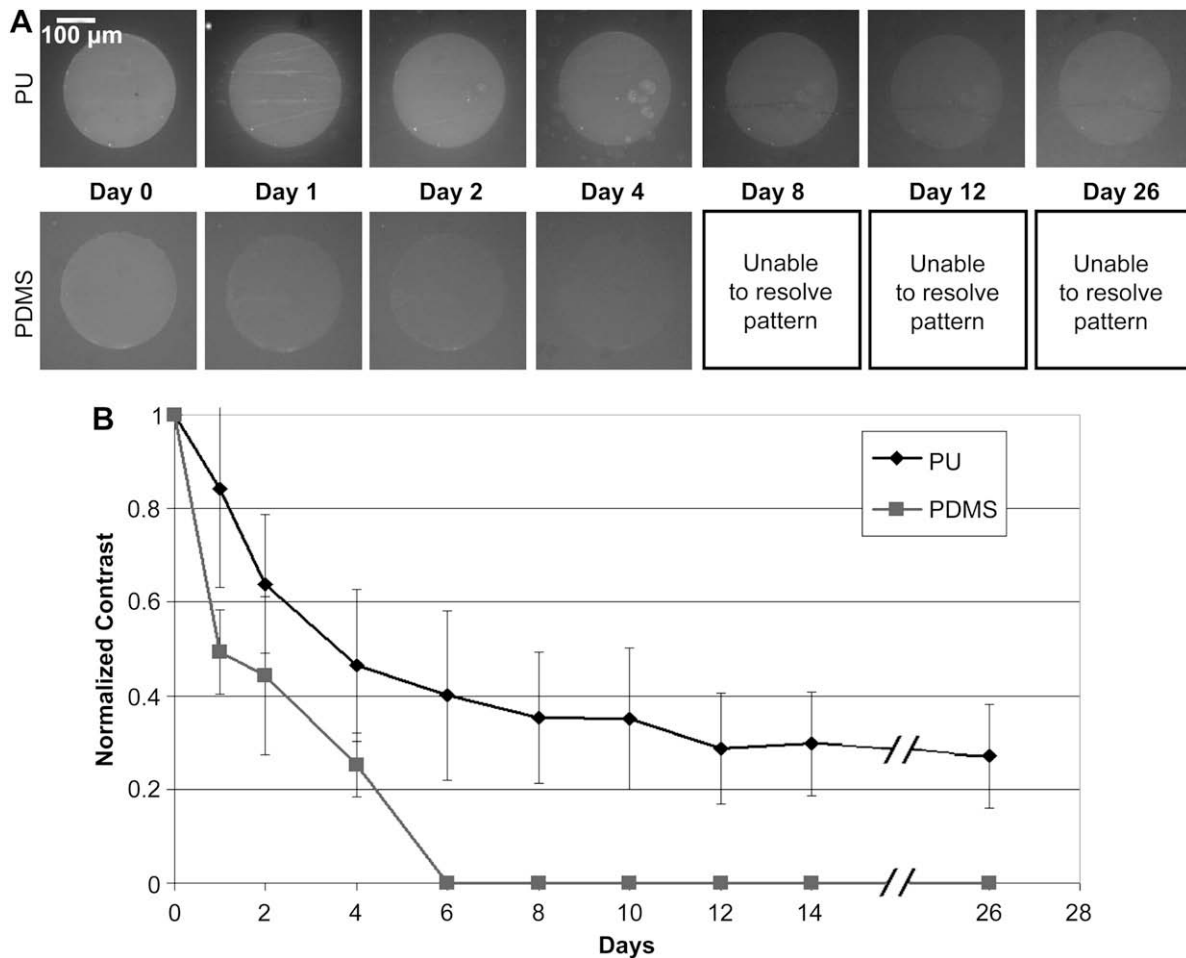


Fig. 6. Assessment of protein pattern fidelity over time in culture conditions. (A): Evolution of a single protein spot patterned on PDMS and PU substrates. (B): Normalized contrast of the patterns ($n = 20$ for each time point) over 26 days.

surfaces, we made the PDMS surfaces hydrophilic as well, in order to minimize experimental differences caused by conformational changes in collagen, and to accurately compare cellular response based on substrate material properties alone.

In terms of initial cell adhesion and adhesion strength, there were no significant differences between collagen-coated PDMS, PU and collagen-coated PU, suggesting that these substrates are interchangeable in terms of suitability for short-term cell culture. It should be noted however, that some polyurethanes have been designed or modified specifically for cell adhesion. For example, Dennes and Schwartz reported a method to modify the Tecoflex[®] PU with RGD peptide, achieving a several-fold increase in cellular adhesion [49]. Techniques have been reported to improve and optimize initial cell adhesion to generic polyurethanes by modifying the surface with oxygen plasma prior to seeding [50]. For this study, PU was neither designed nor optimized for cell adhesion, and these modifications can only further improve this polymer's desirability as a culture substrate over PDMS.

For longer-term cell culture, there are notable differences in cell response for the PDMS and PU materials. Cell spreading area is a measure of cellular response to a substrate, and has a demonstrated impact on cellular function [51]. After three days in culture, cells grown on the PDMS and PDMS + ECM substrates were significantly less spread, while cell spreading area on the PU substrates was comparable to those on TCP. After six days in culture, cells on the PU substrates remain similar in spreading

area to those on TCP, and cell spreading area in this group was significantly larger than on the PDMS substrates. This could be due to the dimethoxysilane chains in PDMS migrating to reduce the surface energy, thereby renewing the surface hydrophobicity [52], or to a loss of adsorbed ECM protein from the surface. The loss of protein pattern fidelity on PDMS substrates (Fig. 6) supports this hypothesis, although it is unclear as to whether those proteins are being removed, or are being replaced in competition with proteins in the supplemented media. The poor cell spreading area observed on PDMS as early as three days after seeding suggests that the proteins are not being replaced by adhesion proteins in the serum-supplemented media (e.g., vitronectin), and the resulting functional differences could have a significant impact on cell function. The inability of PDMS to maintain a controlled matrix environment under culture conditions, even in the absence of cells, demonstrates the unsuitability of PDMS for well-defined, long-term cell culture studies. The Tecoflex[®] PU substrates performed just as well as PDMS in short-term culture conditions and showed significant improvement over PDMS for longer experiments. More generally, polyurethanes have a well-established chemistry and are already in widespread use for implanted devices, tissue engineering scaffolds, and cell biology experiments. Hence, the integration of PU into PDMS microdevices not only extends culture time, but also enables the incorporation and control of clinically relevant culture substrate conditions in multilayer microfabricated research platforms.

5. Conclusion

Polyurethane was successfully integrated into the PDMS multilayer soft lithography fabrication process, so as to improve the long-term cell culture capabilities of microdevices. While initial cell adhesion, spreading and adhesion strength on PU and matrix-coated PDMS were similar, the PDMS surface was not able to maintain matrix proteins and cell spreading in long-term culture. Hence, integrating PU into PDMS devices maintains the advantages of using PDMS in soft lithography, while improving the long-term cell culture capabilities of these devices, thereby increasing the overall impact, accessibility and applicability of microfabricated devices in biomedical research.

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Appendix

Figures with essential colour discrimination. Parts of Figure 5 in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.05.066.

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