Simplified prototyping of perfusable polystyrene microfluidics

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Cell culture in microfluidic systems has primarily been conducted in devices comprised of polydimethylsiloxane (PDMS) or other elastomers. As polystyrene (PS) is the most characterized and commonly used substrate material for cell culture, microfluidic cell culture would ideally be conducted in PS-based microsystems that also enable tight control of perfusion and hydrodynamic conditions, which are especially important for culture of vascular cell types. Here, we report a simple method to prototype perfusable PS microfluidics for endothelial cell culture under flow that can be fabricated using standard lithography and wet laboratory equipment to enable stable perfusion at shear stresses up to 300 dyn/cm² and pumping pressures up to 26 kPa for at least 100 h. This technique can also be extended to fabricate perfusable hybrid PS-PDMS microfluidics of which one application is for increased efficiency of viral transduction in non-adherent suspension cells by leveraging the high surface area to volume ratio of microfluidics and adhesion molecules that are optimized for PS substrates. These biologically compatible microfluidic devices can be made more accessible to biological-based laboratories through the outsourcing of lithography to various available microfluidic foundries. © 2014 AIP Publishing LLC.
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INTRODUCTION

Polydimethylsiloxane (PDMS) has been highly favored by bioengineers due to its prototyping cost-effectiveness, ease of use, and high level of reproducibility from master molds, enabling a large range of microfluidic applications for biological research. However, polystyrene (PS)-based materials have been the mainstay substrate for cell and tissue culture for the past century and the material effects PS has on cell viability, growth, and physiology have been well-characterized.¹ This is certainly not the case for cell culture in PDMS-based systems and as such, how cultured cells are affected by the PDMS substrate is not fully known. In addition, potential drawbacks of PDMS-based devices such as leaching of uncrosslinked oligomers into the culture media, uptake of small hydrophobic molecules from the media, and hydrophobic recovery of the channel surfaces create conditions that may not necessarily be ideal for cell culture.²

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A number of recent advances in the fabrication of PS microfluidics have produced high quality fluidic patterns capable of supporting static cell culture in microchannels.^{3–10} These methods have been instrumental in enabling PS microfluidics, but are difficult to implement in more biologically focused labs as these techniques require complex fabrication techniques,^{3,4} expensive and complicated tooling,⁵ custom fixtures for solvent assisted bonding,^{6,7} and long solvent evaporation times.⁸ Although cells were successfully cultured in these devices, all relied on passive pumping using pipets to seed cells and exchange media periodically. For *in vitro* experiments involving cell culture, various studies have shown that the incorporation of continuous flow for recapitulation of physiologic hydrodynamic forces exerted on cells has a profound effect on preserving *in vivo* cellular structure and function, especially when vascular cells, such as endothelial cells, are involved.^{11,12}

Furthermore, recent computational studies have shown that the oxygen levels in thermoplastic microfluidics may deplete within several hours due to the gas impermeability of the material, adversely affecting cell viability. This oxygen consumption, however, can be counteracted by perfusing media into the microfluidic system.¹³ To these ends, we present a simplified method, using standard lithography and wet laboratory equipment, for fabricating PS microfluidics capable of connecting to syringe pumps for controlled perfusion of cell media and other biological fluids. These PS microsystems have the advantage of being fabricated from the same material as tissue culture flasks and also enable tight control of the hydrodynamic conditions during cell culture. Here, we demonstrate that by incorporating perfusion: (1) endothelial cells can be successfully cultured under controlled hydrodynamic and physiologically relevant conditions in PS-based microfluidics and (2) the PS-PDMS microfluidics can be easily fabricated and leveraged to facilitate viral transduction of non-adherent hematopoietic cells using adhesion proteins that are optimized for PS substrates.

MATERIALS AND METHODS

Endothelialized PS device fabrication

PDMS channels were cut from a microfabricated SU-8 master to be used as a PDMS mold, exposed to oxygen plasma for 1 min, and treated with hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, MO) as shown in Figure 1, step 1. Casting additional PDMS (Sylgard 184, Dow Corning, Midland, MI) mixed at a 5:1 w/w ratio of elastomer base: curing agent on the HMDS-treated PDMS mold resulted in a PDMS die with raised features as shown in Figure 1, step 2. The PDMS die was then used to emboss channels into a 25 × 75 mm PS microscope slide (Ted Pella, Inc., Redding, CA) using binder clips to apply pressure in a convection oven at 160 °C for 15 min, similar to the embossing method described by Goral *et al.* (Figure 1, step 3). Edge beads formed during the embossing process (Figure 1, step 4) were removed and de-burred to ensure flatness of the PS surface, which is critical for optimal thermal bonding conditions. 1/32" holes were then drilled into the inlet and outlet regions of the channel and de-burred, as shown in Figure 1, step 5. The non-embossed side of the PS was plasma treated for 1 min using either a plasma cleaner (Harrick Plasma, Ithaca, NY) or corona gun (Electro-Technic Products, Inc., Chicago, IL) and silanized with 1% v/v aqueous (3-Aminopropyl)triethoxysilane (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature as described by Sunkara *et al.*¹⁴ (Figure 1, step 6).

The final device was created using thermal bonding and by attaching PDMS ports to the top silanized surface. A thin sheet of PS (Papertec, Inc., Garfield, NJ) was cut to the size of the channel to seal the embossed PS. Approximately 10 kPa of pressure was applied to the PS and PS sheet for 5 min on a hot plate (Wenescio, Chicago, IL) set at 98 °C to achieve thermal bonding as shown in Figure 1, step 7. PDMS blocks were aligned with the channel, and tapered inlet and outlet ports were punched out using a 1.2 mm Harris Uni-Core punch (Ted Pella, Inc., Redding, CA). The silanized top surface of the PS device and the tapered side of the PDMS port were both exposed to oxygen plasma for 1 min and brought into contact for bonding (Figure 1, step 8). The completed device was then stored in an oven overnight at 60 °C to

FIG. 1. Schematic of perfusable PS device fabrication for cell culture. (1) Cut out a PDMS mold from patterned photoresist of the desired channel geometries and treat with HMDS. (2) Pour PDMS (5:1 ratio PDMS:crosslinker) onto the HMDS-treated mold and cure to produce a PDMS die with raised features of the channel. (3) Emboss the channel into a PS slide using the PDMS die in a convection oven. (4) Allow the PS to cool before removing pressure. Edge beads will form around the perimeter. (5) Trim the edge beads off and drill inlet and outlet ports. (6) Silanize the top surface. (7) Thermally bond a thin sheet of PS to the embossed PS using approximately 10 kPa pressure for 5 min. (8) Create a PDMS port with tapered holes and plasma treat the device and PDMS to covalently bond.

finalize the bond. The completed device can then be injected with cells and perfused with growth media to culture cells under flow for several days.

Hybrid PS-PDMS device fabrication

The hybrid PS-PDMS devices consisted of an embossed PS base bonded to a PDMS channel using the silanization method described above. The silicon mold consisted of a $1.5 \text{ mm} \times 22 \text{ mm}$ channel with a second layer consisting of a checkerboard pattern of $15 \mu\text{m}$ squares and circles $2 \mu\text{m}$ high within the channels. A PDMS die was produced and embossed into a PS slide, producing a channel with patterned divots on the bottom surface (Figure 2, steps 1–4). The embossed PS was then silanized (Figure 2, step 4), and a flat slab of PDMS with pre-punched inlet and outlet holes was bonded to the PS following 1 min of O_2 plasma exposure (Figure 2, step 5). Non-adherent cells can then be immobilized in the device to

FIG. 2. Schematic of hybrid PS-PDMS device, which can be used for lentiviral cell transduction. Steps 1–4 are similar to those shown in Figure 1. Briefly: (1) Cut PDMS from silicon mold and HMDS treat. (2) Pour PDMS (5:1 PDMS:crosslinker) in HMDS-treated PDMS mold to produce a PDMS die. (3) Emboss. (4) Allow to cool and remove die. Additional trimming is not necessary. Silanize the top surface. (5) Punch inlet and outlet ports in a flat slab of PDMS and align with the embossed channels. (6) The completed device will enable non-adherent cells to be immobilized in the embossed divots within the embossed channels, allowing for an immobilized cell layer to come into homogeneous contact with a lentiviral media layer.

create an immobilized cell layer and a lentiviral media layer when lentiviral media is perfused over the cells (Figure 2, step 6), which makes this device useful for transduction of non-adherent cells.

PS device characterization

Channel dimensions were measured via an Olympus LEXT 3D material confocal before thermal bonding, and fluorescent visualization of the channels was performed by taking Z-stacks of the channels filled with FITC-conjugated albumin from bovine serum (Life Technologies, Carlsbad, CA) on a Zeiss LSM 700 laser scanning confocal microscope. Failure tests were performed by flowing colored water through devices at various shear rates ($n=3$) for at least 100 h or until failure with a peristaltic pump. Computational fluid dynamic simulations were conducted using the COMSOL Multiphysics software suite (COMSOL Inc., Palo Alto, CA) to calculate the maximum pressure in the device for each shear stress.

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cell (HUVEC) culture under perfusion was performed as previously described.¹⁵ For this device, an extra section of silicone tubing was included to allow for gas exchange to the media, and cells were seeded in static with a 1-h incubation time to allow for adherence to the fibronectin. Perfusion occurred at shear stress of 10 dyn/cm² based

on the channel dimensions and flow rate for 24–48 h until confluency and cell alignment to flow was achieved. Static controls were cultured in PS 24-well cell culture plates, and media was periodically changed until the cells reached confluency. All cells were stained with SYTO[®] 13 Green Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, CA) and CellMask[™] Orange Plasma Membrane Stain (Life Technologies, Carlsbad, CA) and imaged via confocal and epifluorescence microscopy.

Lentiviral vector transduction

The PS-PDMS hybrid devices were coated with 50 $\mu\text{g/ml}$ Retronectin[®] (Takara Bio Inc., Otsu, Shiga, Japan) overnight at 4 °C. K562 myelogenous leukemia cells were then seeded into the channels at a concentration of 23 000 cells/ml; enough to form a monolayer on the Retronectin-coated PS base of the device. The cells were allowed to settle and adhere in an incubator for 4 h. After incubation, the device was connected to growth media (IMDM, L-glutamine, pen/strep, 20% FBS) containing HIV-GFP lentivirus (MOI = 0.75, based on static culture transduction of HEK293 cells) and Polybrene (8 $\mu\text{g/ml}$). The viral vector containing media was perfused through the system for 24 h before switching to growth media to culture the cells for an additional 96 h. After the 96 h incubation period, cells were visualized with epifluorescence microscopy to assess transduction efficiency.

RESULTS AND DISCUSSIONS

Consistent channel dimensions and device reliability are critical parameters for long term cell culture in a microfluidic device with constant perfusion. To this end, we performed extensive characterization of the channel cross-sections, dimensions, and durability under various flow rates. The devices also performed as expected in our cell-based assays including endothelialization and lentiviral transduction of suspension cells.

Device characterization

As with any thermal bonding process, channel deformation is always a concern due to the need to heat the PS near its glass transition temperature of $\sim 100^\circ\text{C}$. Optimal bonding was achieved at 98°C . Figure 3(a) shows a topographical image of the channel obtained from material laser confocal microscopy, demonstrating the smooth and uniform sidewalls of the embossed channel. Post-thermal bonded devices were found to maintain the cross-sectional geometry and smooth sidewalls as shown in the reconstructed fluorescent confocal Z-stack images (Figure 3(b)), though a 10%–15% decrease in width and height was observed. Performing thermal bonding under low pressure produces a stable bond that is lower in bond strength compared to more robust methods involving expensive heated presses. However, our device was strong enough to remain leak-free well over the range of shear stresses typically used for culturing cells under flow as seen in Figure 3(c). Endothelial cells are cultured at shear stresses ranging from 1 to 40 dyn/cm^2 for physiological conditions,¹⁶ while our devices were able to perfuse fluid continuously for over 100 h at a shear stress as high as 300 dyn/cm^2 ($n=3$). Past that range, devices would occasionally leak, and at shear stress $>700\text{ dyn/cm}^2$, all devices leaked within 100 h of operation, typically at the PS-PS bond. In addition, the pumping pressures in the device were within the physiological range of blood pressure in the microvasculature,¹⁷ which our microfluidic devices model, and withstood pressures well above an order of magnitude beyond that range surpassing arterial blood pressure (Figure 3(d)). Although our device may not be as robust as traditional PDMS-based devices that can withstand pressures up to the MPa range, it is suitable for the microvascular physiological ranges of shear stress and pressures necessary for cell culture in microfluidics. We selected PDMS as the material of choice for the external ports because of its stable and elastomeric properties which makes it ideal for creating an interference fit with tubing for perfusion that can be easily removed and inserted with

FIG. 3. PS device characterization. (a) 3D laser material confocal scan of embossed channel profile. (b) Reconstructed z-stack of bonded channel filled with fluorescent BSA. (c) Plot of the percentage of devices that were able to be perfused continuously for at least 100 h without leakage for various shear stresses. $n = 3$. (d) Plot of maximum pressures within the devices for each flow rate tested in (c). White scale bars = 200 μm .

minimal risk of introducing air bubbles. Fabrication of these ports is also simple since inlet and outlet holes can simply be punched out.

Endothelial cell culture under flow

HUVECs formed a monolayer within 24 h and grew to confluency over all surfaces of the device after 48–72 h as shown in Figures 4(a) and 4(b). White arrows indicate the direction of flow. The cells restructure and spread in line with the flow instead of taking on the typical cobblestone-like phenotype of HUVECs grown in static conditions (Figures 5(a) and 5(b)). This distinct phenotype is directly linked to perfusion^{11,18} and is demonstrated for the first time in a PS microfluidic. The cells also spread to the sidewalls and top surface of the device to form a tube as shown by the orthographic views in Figure 4(b). To our knowledge, this was also the smallest channel size in which HUVECS were cultured to confluency in a PS microfluidic ($<100 \mu\text{m}$). As channel size decreases, cells will require fresh media more frequently to obtain nutrients and flush out metabolic waste, which is easily achieved with constant perfusion.

FIG. 4. Endothelialization of PS device. (a) Phase contrast images of zoomed out branch region. (b) Confocal fluorescence image of endothelial cells in the smallest branch (width $< 100 \mu\text{m}$) with orthographic views showing flow alignment and confluency along all channel walls. Red = cell membrane and green = cell nuclei. Black scale bars = $200 \mu\text{m}$ and white scale bars = $50 \mu\text{m}$.

Lentiviral transduction of suspension cells

Chuck and Palsson showed that transduction efficiency of adherent mammalian cells can be increased using microfluidic systems.¹⁹ By the same principal, we sought to determine if non-adherent cells, such as hematopoietic cells, which are the target cell type of many clinical gene therapy applications, could be immobilized and efficiently transduced via perfusion of

FIG. 5. Comparison of morphology in cells grown under perfusion in the PS microfluidic with cells grown in static culture in a PS well plate. (a) Cells restructure to take on an elongated morphology in the direction of the flow in the PS microfluidic with constant perfusion. (b) Cells retain a cobblestone-like morphology when cultured in static conditions. Red = cell membrane and green = cell nuclei. Scale bars = $100 \mu\text{m}$.

viral vector-containing media. The current state of the art for clinical lentiviral vector transduction involves bathing the target cells (e.g., CD34+ cells or T cells) in viral vector-containing media within Retronectin-coated gas permeable bags.²⁰ Retronectin is a recombinant human fibronectin fragment that increases the efficiency of lentiviral and retroviral transduction by binding both target cells and recombinant lentiviral or retroviral vector particles to facilitate gene transfer. Despite advances in vector production,²¹ 10–1000 ml of vector are still needed to treat each patient with a high enough efficiency. The scale and yield limitations of vector manufacture and the low efficiency of gene transfer are currently major barriers to the commercialization and widespread implementation of lentiviral vector-based gene therapy, which otherwise has the potential to cure many human diseases. Other widely used gene transfer techniques, such as nucleofection, electroporation, and lipofection, have been shown to have lower transfection efficiencies and cell viability when compared to lentiviral transduction.²² For this proof of concept study, K562 cells were used as a surrogate of CD34+ cells to test our hybrid PS-PDMS cell transduction.

Cells were seeded onto a Retronectin-coated PS slide that was embossed with 15 μm divots and bonded to a PDMS channel. Retronectin works best when coated onto PS, cyclo-olefin, polyethylene, or Teflon, which is the reason a hybrid PS-PDMS device was essential for this application.²³ Silanization had no observable effect on the adsorption of Retronectin on the PS surface. The ability to emboss patterns into the PS also assists with the adhesion of cells by providing more contact surface area. Following a brief recovery period for the cells post-seeding, HIV-GFP vector containing media was perfused through the device for 24 h. Several days post-transduction, the cells were analyzed for GFP expression as a surrogate for viral vector-mediated gene transfer. This novel device design enables an immobilized non-adherent cell layer to be homogeneously exposed to lentiviral media. Using phase contrast and epifluorescence images, several observations were made (Figures 6(a) and 6(b)). First, many of the K562 cells appear to have nestled into the embossed divots as predicted. Second, from image analysis, we estimated that approximately 30.3% of the immobilized K562 cells successfully integrated the transgene from the viral vector, as indicated by GFP expression. All fluorescent cells were counted in a binary manner and compared to the total amount of cells counted in the phase contrast image. Via image analyses with ImageJ (National Institutes of Health, Bethesda, MD), cells with a gray value between 1350 and 4095 (at 12 bpp) fluorescence units were considered to be positive for GFP, which served as our marker for successful lentiviral vector gene transfer. The variability in transgene expression, demonstrated by the heterogeneity of fluorescence intensity among the cells, is due to position-effect variegation, which is caused by integration of the transgene at semi-random genomic sites that display differential permissivity to the transcriptional machinery and subsequently lead to varying levels of mRNA production.²¹ It

FIG. 6. K562 cells transduced in the hybrid PS-PDMS device. (a) Phase contrast image of K562 cells immobilized in the divots. (b) Fluorescence image of cells expressing GFP after 24 h of perfusion of lentiviral media followed by a 96 h incubation period. Cells can be seen expressing GFP at various levels. Scale bar = 100 μm .

was expected from our previous lentiviral transduction studies using this cell line under static tissue culture conditions that, at the MOI utilized, approximately 5%–10% of the K562 cells would be genetically modified.^{24,25} Luni *et al.* have shown that transduction efficiency can be improved by automating a syringe pump to perfuse, come to rest, and reperfuse in cycles to allow viral particles to come into contact with cells through Brownian motion before exchanging viral media through perfusion.²⁶ A similar protocol should be amenable to the hybrid PS-PDMS device and should allow for even more efficient transduction and reduced viral vector requirements, which currently is a major barrier to clinical use as vector manufacturing is inefficient and costly.

CONCLUSIONS

Here, we described key advantages of using PS-based microfluidics for biological experiments and demonstrated that the inclusion of controlled perfusion rather than passive pumping or other methods of fluid exchange could improve this class of devices to create more physiological cell culture environments, as well as extend the applications to other areas such as research and clinical gene therapy. This technique also has the advantage of being accessible to nearly any type of laboratory as it uses mostly low cost or standard laboratory equipment. Extensive characterization was also performed to show that the device was robust enough for the applications demonstrated. We anticipate that the simplified PS microfluidic fabrication technique presented here will allow PS devices to find greater utility in the field of biomedical sciences and microfluidics research as it has all of the major capabilities of a PDMS device while being more biologically compatible.

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