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Using living radical polymerization to enable facile incorporation of materials in microfluidic cell culture devices

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Abstract

High throughput screening tools are expediting cell culture studies with applications in drug discovery and tissue engineering. This contribution demonstrates a method to incorporate 3D cell culture sites into microfluidic devices and enables the fabrication of high throughput screening tools with uniquely addressable culture environments. Contact lithographic photopolymerization (CLiPP) was used to fabricate microfluidic devices with two types of 3D culture sites: macroporous rigid polymer cell scaffolds and poly(ethylene glycol) (PEG) encapsulated cell matrices. Cells were cultured on-device with both types of culture sites, demonstrating material cytocompatibility. Multilayer microfluidic devices were fabricated with channels passing the top and bottom sides of a series of rigid porous polymer scaffolds. Cells were seeded and cultured on device, demonstrating the ability to deliver cells and culture cells on multiple scaffolds along the length of a single channel. Flow control through these rigid porous polymer scaffolds was demonstrated. Finally, devices were modified by grafting of PEG methacrylate from surfaces to prevent non-specific protein adsorption and ultimately cell adhesion to channel surfaces. The living radical component of this CLiPP device fabrication platform enables facile incorporation of 3D culture sites into microfluidic cell culture devices, which can be utilized for high throughput screening of cell-material interactions.
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1. Introduction

As biomaterials evolve for use in cell culture and tissue engineering, there is a growing need to provide rapid, efficient screening techniques for evaluating cell-material interactions. Microfluidic devices provide a powerful, yet versatile platform for such screening tools, since they necessitate reduced reagent amounts relative to conventional cell culture techniques, while allowing for a high density of test sites to be evaluated on a single device. Recent efforts have yielded a variety of microfluidic device designs to study specific aspects of cell culture. For example, laminar flow guided by microfluidic channels has been used to generate controlled gradients of soluble chemotactic factors for the study of single cell movement [1] and for adsorption of proteins to channel surfaces for the study of axon extension of neurons [2]. Gradients of soluble factors have also been delivered to large arrays of two-dimensional cell culture sites to screen simultaneously for effects on an entire population of cells [3,4]. Perfusion culture devices, in which one media composition flows over cells, have been designed to provide microenvironments suitable for the culture of several mammalian cell types including human carcinoma cells (HeLa) [5], hepatocytes [6,7], osteoblasts [8], fibroblasts [9], pancreatic islets [10], umbilical vein endothelial cells [11], and umbilical vein smooth muscle cells [12].

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Media flow is necessary for cell culture as a means to deliver a fresh nutrient supply, to remove waste, and more specifically, to deliver material gradients to affect a range of cell responses.

A subset of research related to cell culture with microfluidics focuses on modifying surface chemistry to direct specific ligand interactions or to pattern spatially cell adhesion. Several of the more commonly used patterning techniques include micromolding in capillaries [13], microcontact printing [14], etching with oxygen plasma [15], and photolithographic techniques [16]. Three-dimensional cell constructs have been patterned by photoencapsulating cells in single layers [17] and in multiple layers, each containing a different cell type [17,18], on flat surfaces. Photopatterning interpenetrating networks of cell-adhesive materials [19] is among the more recent approaches to cell patterning of biomaterials for microfluidic applications. Most of these cell-patterning techniques are applied to glass or silicon surfaces and involve multiple steps to create patterns, limiting the applicability of these techniques for use within microfluidic channels and with polymeric devices.

Many of these previous microfluidic devices were fabricated using poly(dimethylsiloxane) (PDMS) patterned with soft-lithography and molding [20]. This fabrication scheme has many benefits including inexpensive polymeric materials relative to silicon devices and the use of photolithography to make molds with complex channel shapes. Limitations of PDMS devices for cell culture stem from the inability to incorporate additional materials easily via covalent attachment of biomolecules to surfaces or attachment of biomolecules to three-dimensional cell culture sites in PDMS. For example, one method has recently been reported for modifying surfaces within PDMS microfluidic channels [21], involving a multistep process that first swells PDMS to load it with a photoinitiator, then photopolymerizes a dilute monomer solution to the channel walls while rotating the device. This method generates an interpenetrating network of polymer on the device surface with a thickness of about 10 μm, partially interpenetrating the PDMS network and partially rising above the device surface. Limitations of this method include non-covalent attachment of the polymer network to the device material, and a time consuming, multi-step procedure. The ability to easily modify device surfaces covalently, in a spatially controllable manner, would be advantageous, as it would allow for incorporation of multiple materials into a single device to facilitate more complex cell culture studies.

Contact lithographic photopolymerization (CLiPP) is a fabrication approach used to produce polymeric microfluidic devices, which perform a variety of unit operations ranging from pumping and mixing for detection of specific biological factors [22–26]. CLiPP provides not only a rapid device fabrication platform via lithographic photopatterning of polymers, but also an adaptable fabrication platform with the ability to readily incorporate a wide range of polymers and functional materials through the living radical feature of the CLiPP polymer system [27,28]. It is the goal of this contribution to demonstrate how CLiPP fabricated microfluidic devices are useful for cell culture and reveal the unique advantages of these devices in incorporating polymers of relevance to the biomaterials community. Demonstrations include static culture of cells encapsulated in hydrogels, static and dynamic culture of cells situated on porous polymer scaffolds, controlled treatment of three-dimensional cell culture sites with multiple fluid streams, cell patterning on channel surfaces, and on-device cell analysis in CLiPP fabricated devices.

2. Materials and methods

2.1. Microfluidic device materials

Microfluidic devices were fabricated from 49.25 wt% hexanedioldiacrylate (HDDA, UCB Chemicals), 49.25 wt% Polyfox 3320 (fluorinated diacrylate, donated by Polyscience), 1.0 wt% Irgacure 184 (photoinitiator, Ciba) and 0.5 wt% tetraethylthiuram disulfide (TED, photoiniferter precursor, Aldrich). This formulation will be referred to as the device monomer or, once polymerized, device polymer.

Glass coverslips (1″ by 1″) were treated to attach methacrylate groups, and used as transparent caps for several devices. First, glass coverslips were cleaned with piranha solution (30%(v/v) H_2O_2, 70% (v/v) sulfuric acid) for 45 min, rinsed with deionized water, then acetone, and stored in a container purged with argon. This cleaning step exposes hydroxyl groups on the coverslip surfaces, providing reactive sites for silanes. Next, a methacrylated silane, methacryloxypropyltrimethoxysilane (MAPTMS, Gelest, Inc.), was reacted with the coverslip surfaces by vapor deposition [29]. For vapor deposition, 30 μl MAPTMS and piranha cleaned coverslips were placed in a Teflon jar, purged with argon and sealed with a screw-on top. The Teflon container was placed in an oven at 60 °C, about 8 °C below the boiling point of MAPTMS, for 2 h, then removed and left to cool to room temperature. The coverslips were washed with acetone and stored in a container filled with argon. During this process, silane ends of the MAPTMS react with hydroxyl groups on the coverslip surface, leaving pendant methacrylate groups on the glass surface. The methacrylate groups provide sources for covalent attachment of photopolymers to the glass coverslip. Finally, coverslips were coated with a thin layer (<20 μm) of device monomer, purged with argon, covered with a non-patterned photomask, and exposed to UV light for 200 s to polymerize the monomer to the coverslip. The glass coverslip with polymer was then incorporated into devices by the transfer method as described in Section 2.2 below.

2.2. Device fabrication

Cell culture devices were fabricated with contact lithographic photopolymerization (CLiPP) as previously described [22,26]. Briefly, the device monomer was covered with a patterned photomask and exposed to collimated UV light for 500 s (45 mW/cm, 365 nm). In the exposed areas of the photomask, the photoinitiator generates active radicals that propagate through carbon–carbon double bonds of the diacrylate materials to form a crosslinked network in the shape of the negative image of the photomask. As this reaction occurs, TED groups are also cleaved into two dithiocarbamate (DTC) groups. The DTC groups are sufficiently stable to prevent initiation of significant amounts of polymerization and instead react primarily in two distinct manners. One path results in the recombination of DTC groups to reform TED. The second path results in DTC groups that form reversible ‘end-caps’ on kinetic polymer chains, and thus impart a living radical nature to the polymer network. This mechanism was first explored and reported by Otsu et al. [30]. When a device layer is photopolymerized, some of the DTC end-caps remain exposed on the device polymer surface. Upon subsequent exposure to UV light, the end-caps provide sites for covalent attachment of various materials as illustrated in Fig. 1.

Here, multilayer microfluidic devices were fabricated using the transfer method as illustrated in Fig. 2. After photopolymerization, unreacted monomer was removed from channels with low-pressure air and flushed with ethanol. The transfer method was used because it allows for facile alignment of multiple layers and for simple cleaning of channels and features. Teflon tubing (150 μm i.d., 360 μm o.d., Scivex) was inserted into the microfluidic channel

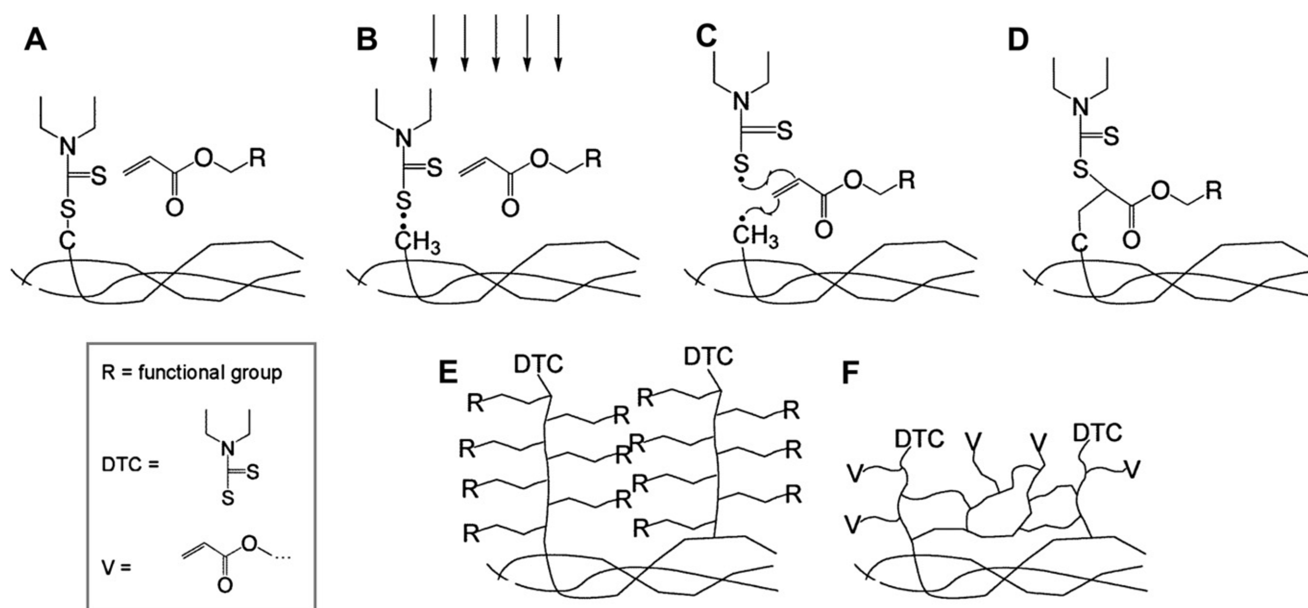

Fig. 1. Illustration of the living radical feature of CLiPP system. (A) The CLiPP photopolymer system forms a crosslinked polymer network upon exposure to UV light, incorporating DTC groups as polymer chain end-caps. DTC groups remain on the polymer surface. (B) Upon subsequent exposure to UV light, DTC and carbon radicals are reformed. (C) Carbon radicals can propagate through other vinyl groups. (D) The DTC radicals then re-cap the new carbon radical. Steps (A) through (D) continue until UV light is terminated or all of the materials are consumed. This process enables facile modification of polymer surfaces with brush grafted functional materials (E), or with crosslinkable materials (F).

inlets. The space between the square microfluidic channel and the round tubing was sealed by filling with device monomer and exposing to UV light. Tubing was connected to syringes with Scivex MicroTight fittings. Cell culture devices were cleaned and sterilized for at least 24 h by pumping 70% (v) ethanol through channels at 10 μl/min.

2.3. Scaffold materials

Cell scaffolds were fabricated from 48.3 wt% Ebecryl 4827, an aromatic urethane diacrylate (UDA, donated by UCB Chemicals), 48.3 wt% triethylene glycol diacrylate (TEGDA, Polysciences), 1.4 wt% Irgacure 184 (photoinitiator, Ciba), and 1.0 wt% tetraethylthiuram disulfide (photoiniferter precursor, Aldrich). This formulation will be referred to as the scaffold monomer or scaffold polymer, before or after exposure to UV light, respectively. Scaffold pores were created by mixing 20 wt% monomer with 80 wt% salt, polymerizing with exposure to 365 nm light at 45 mW/cm^2 for 500 s, and finally leaching the salt out with water. Salt particles were ground and sieved to specific size ranges, which correspond to the final pore size in the polymerized scaffolds. This scaffold fabrication process has been explained in detail previously [24].

2.4. Grafting materials

Channel surfaces were modified with poly(ethylene glycol) methacrylate (PEGMA, average molecular weight 360, Sigma Aldrich Chemical Co.). As purchased, PEGMA contains 650 ppm inhibitor, methoxyhydroquinone (MEHQ), which was removed from PEGMA by passing it through a column of basic alumina (Sigma Aldrich Chemical Co.). PEGMA was then purged with argon and protected from light exposure. Surfaces were covered with PEGMA, purged with argon, covered with a photomask and exposed to 365 nm light at 45 mW/cm^2 for 900 s, resulting in a grafted layer of PEGMA on the device surface. Grafted surfaces were cleaned and sterilized by submerging in 70% ethanol, while on a shaker plate, for 24 h prior to use for cell culture.

2.5. Cell culture

NIH 3T3 fibroblasts were used for cell culture experiments. Cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco), 0.2% (v/v) gentamicin (Gibco), 0.2% (v/v) Fungizone (Gibco) and 1.0% (v/v) Pen-Strep (Gibco). All samples and microfluidic devices were placed in an incubator to maintain high humidity, 37 °C, and 5% CO_2 for cell culture experiments. Cells were passaged at confluency from plate 1–6 until used in seeding experiments. Cells were detached from culture plates with trypsin, and cell suspension concentrations were measured by hemacytometer.

2.6. Static cell seeding

NIH 3T3 fibroblast cell suspensions were adjusted to 10^6 cells/ml and manually dispensed onto cell culture sites with pipettes. Generally, the culture sites were covered with 155 μl of cell suspension per cm^2 of culture surface. Polymer samples were placed in 6 well tissue culture plates, covered with cell suspension and placed in an incubator set to 37 °C, 5% CO_2 for 30 min. Samples were then covered with another 2 ml of culture media and stored in the incubator until ready for cell analysis.

2.7. Dynamic cell seeding

For seeding cells via microfluidic channels, the channels were preconditioned with DMEM with 10% serum for 60 min at 10 μl/min, or approximately four changes of media per minute. This allows for removal of residual ethanol from the channel walls and for media proteins to adsorb to device surfaces, which promotes cell attachment. NIH 3T3 fibroblast cell suspensions were then loaded into 1 ml syringes and connected to inlet tubing to the culture devices. Dynamic cell seeding devices integrated multiple device layers, including a layer with cylindrical porous scaffolds embedded in device polymer with top and bottom sides of the scaffolds open to a channel layer that directed media flow across the top sides of the scaffolds and a separate channel layer that directed flow across the bottom sides of the scaffolds. The cell suspensions were pumped through the channels above the scaffolds at 10 μl/min, and DMEM with 10% serum was pumped through the channels below the scaffolds at 2 μl/min. These flow rates were maintained for 10 min, then both flow rates were set to 0 for 10 min to allow cells to settle into features of the porous scaffolds. Flow was reintroduced for another 10 min, followed by another 10 min with no flow to allow for cells to settle. Next, 10 ml syringes

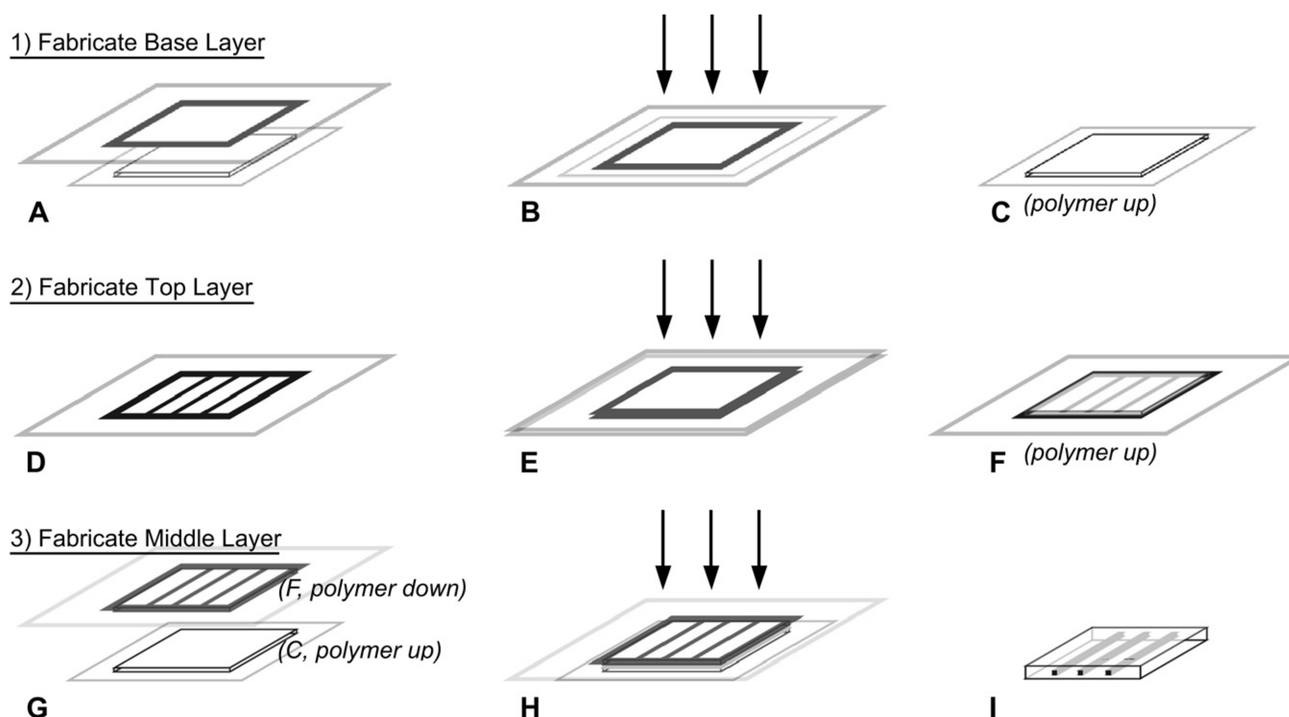

Fig. 2. Illustration of the transfer method for fabrication of multilayer microfluidic devices. (A) Glass and photomask are aligned over device monomer. (B) The photomask is brought into contact with device monomer, height is adjusted and setup is exposed to UV light. (C) Glass and photomask are removed leaving a polymerized base layer. (D) Photomask for middle layer is attached to glass. (E) A glass with attached photomask is aligned over the middle layer photomask, monomer is added between the photomasks, height is adjusted and the setup is exposed to UV light for 500 s. (F) The top glass with attached photomask is removed leaving a polymer lid layer attached to the middle layer of photomask and glass. (G) The glass-top layer assembly and the bottom layer are aligned so that the polymer layers are facing each other. (H) Device monomer is placed between the top and base polymer layers, height is adjusted and the setup is exposed to UV light. (I) Both sets of glass with attached photomasks are removed and device channels are cleaned out with low-pressure air.

were filled with DMEM with 10% serum and connected to all inlets. DMEM was pumped through all channels at 2 μl/min for the duration of the culture experiment. Care was taken to avoid introducing air bubbles into the devices when switching syringes.

2.8. Cell labeling

To demonstrate viability, cells were treated in the device with a membrane integrity assay (calcein AM and ethidium homodimer-1, Invitrogen). Briefly, the assay solution was prepared in culture media and pumped through all channels at 1 μl/min for 30 min, or approximately 12 full changes of media in each of the channels. Live cells are labeled green as calcein AM passes through the cell membrane and is hydrolyzed into a fluorescent form of the molecule. Dead cells are stained red as ethidium homodimer-1 passes through compromised cell membranes and preferentially binds with nucleic acids. Cells were imaged with laser scanning confocal microscopy (Zeiss, LSM5 Pascal, Axioplan 2 imaging).

3. Results and discussion

The microenvironment of mammalian cells is extremely complex, involving combinations of soluble and surface-bound signaling factors in a three-dimensional space. One of the primary limitations in the field of microfluidic cell culture is that cell patterning has been conducted primarily on modified glass surfaces, restricting treatment with soluble factors to at most, a one-dimensional gradient across the population of cell culture sites. This limits the ability to combine large numbers of soluble factors and material properties simultaneously for high throughput studies of cell-material interactions. The goals of this work were to demonstrate the ability to incorporate various materials into a polymeric microfluidic device for cell culture in a manner that enables for three-dimensional culture sites to be addressed on two discrete surfaces simultaneously, providing a platform that can be used for high throughput screening of cell-material interactions.

3.1. Cell culture in three-dimensional sites of single layer devices

To demonstrate that cytocompatible, three-dimensional culture sites can be integrated into CLiPP microfluidic devices, two types of materials were photopolymerized into holes or wells in a layer of device polymer. Macroporous polymer scaffolds and PEG hydrogels were attached to the device polymer through living radical photopolymerization as illustrated in Fig. 1.

Macroporous scaffolds are often used as cell culture sites for bone tissue engineering studies requiring rigid, cell-adhesive surfaces that are meant to more closely mimic the native cellular environment [31]. It would be advantageous to incorporate this type of scaffold into microfluidic cell culture devices to allow for high throughput cell culture studies to be conducted with various scaffold materials in combination with soluble cell stimulants. In addition to conducting high throughput

cell culture studies, the benefits of microfluidic devices would be realized by reducing the amounts of materials required, compared to conventional culture techniques. Macroporous polymer scaffolds were incorporated into a layer of a CLiPP cell culture device by photopolymerization of UDA and TEGDA with salt particles, followed by leaching to remove the salt and creation of a porous polymer scaffold as described in Section 2. Fibroblasts 3T3 were manually seeded onto the porous scaffolds and cultured for up to 3 weeks, demonstrating material cytocompatibility as shown in Fig. 3A and long-term cell viability in these devices. Cell staining and confocal microscope images were used to verify that cells infiltrated and attached to surfaces throughout the pores of the scaffolds, and that the tortuous paths through the scaffold allowed for nutrient transport through the scaffolds to maintain cell viability for up to 3 weeks. To date, CLiPP microfluidic cell culture devices are the only devices to incorporate three-dimensional culture sites into polymeric devices. The macroporous three-dimensional scaffolds can be readily incorporated in microfluidic cell culture devices, to transform the dimension of high throughput cell culture studies in microfluidic devices.

Another type of three-dimensional cell culture structure that has been widely explored is cell encapsulation in poly(ethylene glycol) (PEG) hydrogels [32]. Prior work with PEG cell encapsulations on microfluidic devices primarily uses microfluidic channels as molds to pattern gel formation on treated glass surfaces [17]. It would be advantageous to incorporate PEG hydrogels into polymeric devices to facilitate fabrication of microfluidic cell culture devices with integrated channels which address specific hydrogel sites. To address this limitation, 3T3 fibroblasts were encapsulated in PEG hydrogels within wells of a CLiPP cell culture device by filling the wells with a cell–monomer suspension, followed by exposure to 8 mW/cm^2 UV light for 10 min. The encapsulated cells were then cultured in media supplemented with serum. Cells remained viable for over 24 h as shown by the large number of green labeled cells in Fig. 3B indicating cytocompatibility with the on-device culture procedure. When hydrogel scaffolds were allowed to dry, they did not detach from device walls, indicating that hydrogels were attached to the device polymer. This structure may be incorporated into a device with microfluidic channels by first making the device with channels, sterilizing the device, then photopatterning the hydrogels into the wells of the device, or by clamping presterilized device layers around the device hydrogel layer. The latter method is simpler and also allows for easy retrieval of hydrogel samples for histological analysis. In addition to consuming smaller amounts of materials, microfluidic channels allow for different media compositions to be directed to individual gel sites, facilitating the study of combinations of gel and media compositions on one device.

3.2. Cell culture in three-dimensional sites of multilayer devices

While CLiPP materials allow for incorporation of cytocompatible, three-dimensional cell culture sites into a single layer of polymer, integrating these culture sites into multilayer

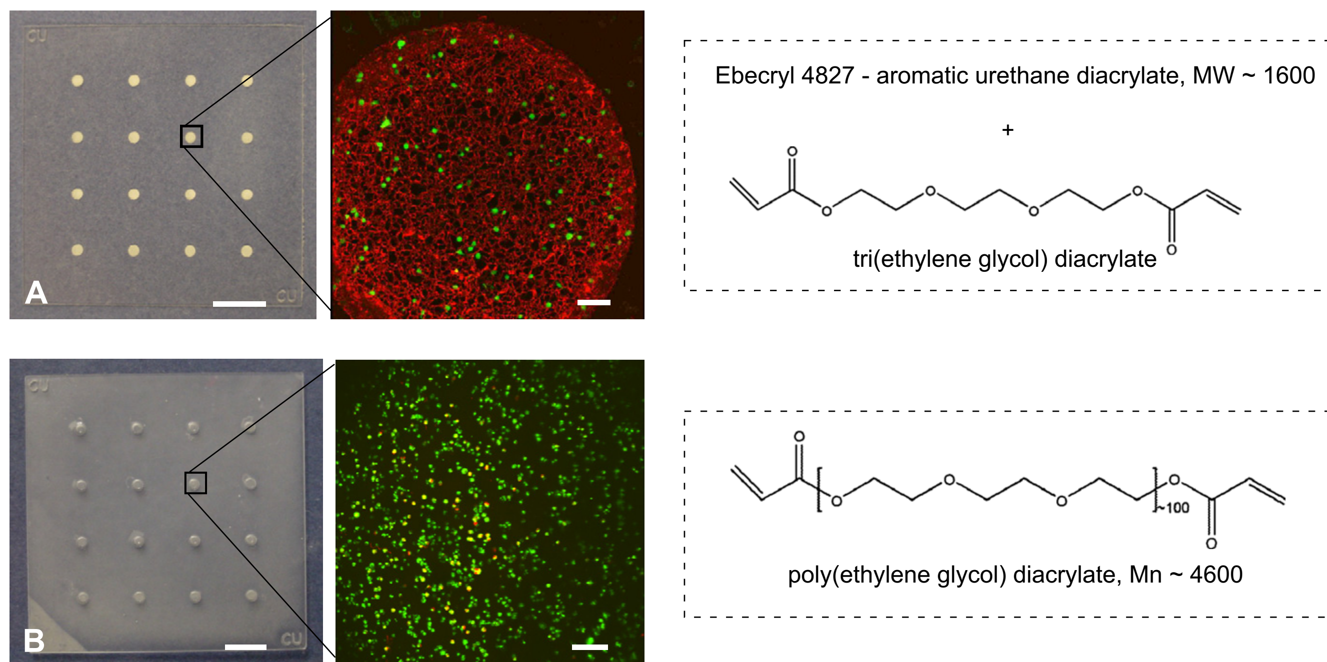

Fig. 3. Multiple biomaterials are easily covalently incorporated into CLiPP fabricated microfluidic devices for culture of adhesion dependent mammalian cells. (A) (left) Photograph of one layer of a microfluidic device with a 4 × 4 array of porous rigid cell scaffolds made of a mixture of diacrylated materials, scale bar = 5 mm, (middle) confocal laser scanning microscope (CLSM) image with live cells stained green and dead cells and the scaffold stained red, scale bar = 100 μm. (B) (left) photograph of one layer of a microfluidic device with a 4 × 4 array of hydrogel scaffolds, scale bar = 5 mm, (middle) CLSM image of cells encapsulated in hydrogel with live cells stained green and dead cells stained red, scale bar = 100 μm.

devices is necessary to introduce more complex gradients of soluble factors to the culture sites than is possible with culture sites patterned on flat surfaces. For example, if an array of culture sites is patterned onto a flat surface, the sites can be treated with a one-dimensional gradient of soluble factor compositions, dependent on flow rate and diffusion coefficients of the selected materials. More complex gradients are possible when compositions of soluble factors are controlled on the top and bottom sides of the culture sites simultaneously. For example, if an array of three-dimensional culture sites is attached within a layer of a microfluidic device with channels on the top and bottom sides of the array of scaffolds, then a gradient of compositions may be introduced to the top side, as well as to the bottom side of the array of scaffolds. This approach not only allows for more combinations of soluble factors to be studied at once, but it also enables each individual cell culture site to be exposed to two media compositions on the top and bottom sides concurrently. To fabricate a device as described above, the scaffolds must be securely attached in a device layer, while leaving the top and bottom sides of the scaffold exposed, and the cell scaffold layer must be able to be integrated into a multilayer device with microfluidic channels on each side of the scaffold layer. Prior methods of fabricating cell culture microfluidic devices are limited by their inability to covalently attach three-dimensional culture sites within polymeric devices. The living radical feature of the CLiPP fabrication system, however, does allow for three-dimensional culture sites to be incorporated into polymer layers as shown in the previous section of this contribution. To demonstrate that the scaffolds can also be integrated into devices with channels to treat cell culture sites, multilayer devices were fabricated as illustrated in Fig. 4. After the devices were fabricated, they were cleaned by pumping 70% (v) ethanol through the channels for a minimum of 24 h. After conditioning the channels with cell culture media to ensure that all of the ethanol was removed, a suspension of 3T3 fibroblasts was pumped through the microfluidic channels to seed the cells onto the UDA–TEGDA porous scaffolds in the device. The cells were cultured with growth media via microfluidic channels for 4 h, labeled and imaged with confocal microscopy as shown in Fig. 4. Similar results were obtained with culture times of 24–48 h as well. These images correlate with viability seen in the one-layer device studies and demonstrate that a large number of cells can be seeded onto a series of scaffolds along the length of a channel. The apparent lack of cells on part of the fourth scaffold was due to a defect in the scaffold that occurred during the fabrication of the device. All of the scaffolds maintained mechanical integrity throughout the culture experiments. These results verify that three-dimensional cell culture sites are integrated into multilayer devices with channels enclosing the top and bottom sides of the scaffolds and suggest that the devices can be designed and made to conduct high throughput screening of large combinations of cell-material interactions.

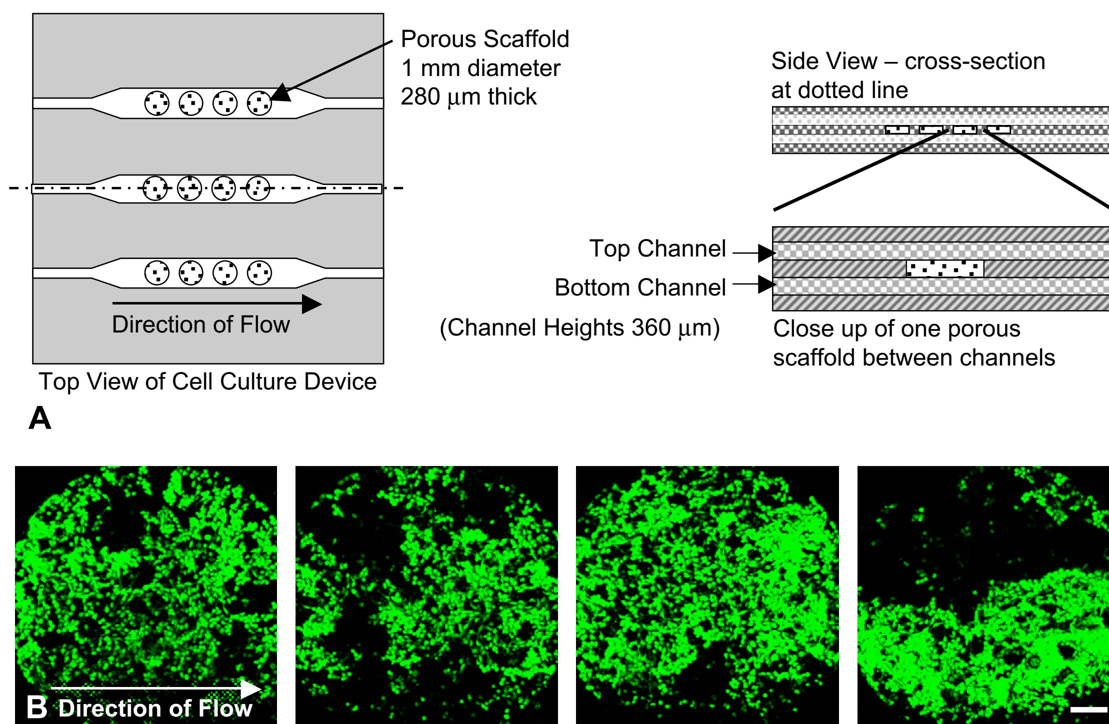

Fig. 4. A) Illustration of a multilayer microfluidic cell culture device. Fibroblasts 3T3 were seeded and cultured on porous scaffolds within microfluidic channels with flow. A cell suspension was passed through the top channel for two cycles of 10 min at 10 µl/min, followed by 15 min with no flow. Cell culture media was pumped through the bottom channel at 1 µl/min/channel to ensure porous scaffolds did not dry out on the bottom sides. After cell seeding, cell culture media was delivered to the cells via channels on the top and bottom sides of the scaffold at 2.5 µl/min/channel for 4 h. (B) CLSM images of live cells. Scale bar represents 100 µm.

3.3. Flow control through three-dimensional sites of multilayer devices

In the previous section, it was suggested that a gradient of soluble factors could be established between the top and bottom of the scaffolds by controlling flow rates on each side of the three-dimensional culture sites. To demonstrate the ability to control flow through the porous scaffolds and verify that the mechanical integrity of the scaffolds is maintained, another multilayer microfluidic device was fabricated with a channel above a scaffold and an orthogonally oriented channel below the scaffold, as illustrated in Fig. 5. To visualize fluid paths, fluids were chosen that were both optically clear until mixed. Thymolphthalein remains clear below pH 9.3 and turns to an intense blue when at or above pH 10.5. Thymolphthalein was pumped through the bottom channel and phosphate buffered saline (PBS) at pH 10.5 was pumped through the top channel. To quantify the amount of fluid mixing indirectly in porous scaffolds, images of the color change due to fluid mixing were captured and analyzed with NIH image. Higher intensity correlates to more fluid mixing in the scaffold, or more of the thickness of the scaffold being exposed to a mixture of streams. Scaffold intensity for each image was normalized with the intensity of a set region of the device polymer in the image. The flow rate through the top channel was held constant at 1 μl/min, and the flow rate through the bottom channel was varied from 1 μl/min to 10 μl/min. Fig. 5D shows that equal flow rates of 1 μl/min resulted in the highest color intensity, and the largest differential in flow rates of 1–10 μl/min resulted in the lowest color intensity. The pressure required to direct flow through the scaffold was calculated with Darcy's Law of flow through porous media, ($k = 9 \times 10^3\ \mathrm{cm^2\ cp\ s^{-1}\ atm^{-1}}$) to be 1.13 times greater than the pressure drop, calculated with Hagen–Pouiseuille law, over the length of the channel. The difference in pressure drops through each path, indicates that flow is not directed through the scaffold and that color change is due solely to diffusive mixing through the scaffold. For diffusion of a small molecule like thymolphthalein the characteristic time for diffusion through a 300 μm thick scaffold is approximately 10 s, while for the flow rate of 1 ml/min, a point in the fluid stream is in contact with the porous scaffold for 22 s and for a flow rate of 10 ml/min, contact time is 2 s. The greater contact time for lower flow rates allows for greater mixing time and a more intense color in the scaffold. Porous scaffolds remained attached to device layers with flow rates used in this demonstration and up to 100 μl/min flow difference on either side of the scaffold, without detaching from the device. These results demonstrate that the amount of fluid mixing that occurs through the thickness of the scaffold is varied by controlling flow rates on either side of the scaffold and that the scaffolds are securely attached to the polymeric device.

Perfusion of fluid through the scaffolds is also possible with the same multilayer device design. Perfusion through the porous scaffolds would be beneficial for cell culture experiments targeted at studying the effects of shear forces on three-dimensional culture sites, as is done with some macro-scale bone tissue engineering strategies. Fig. 5A–C demonstrate that color change is localized to the porous scaffold, or to either channel by varying flow rates and hence time for diffusive mixing to occur. The flow patterns are what would be expected for flow through porous scaffolds with open, interconnected pores through the thickness of the scaffold. It has been shown that cells can be seeded and cultured on porous scaffolds within multilayer devices and that the scaffolds maintain

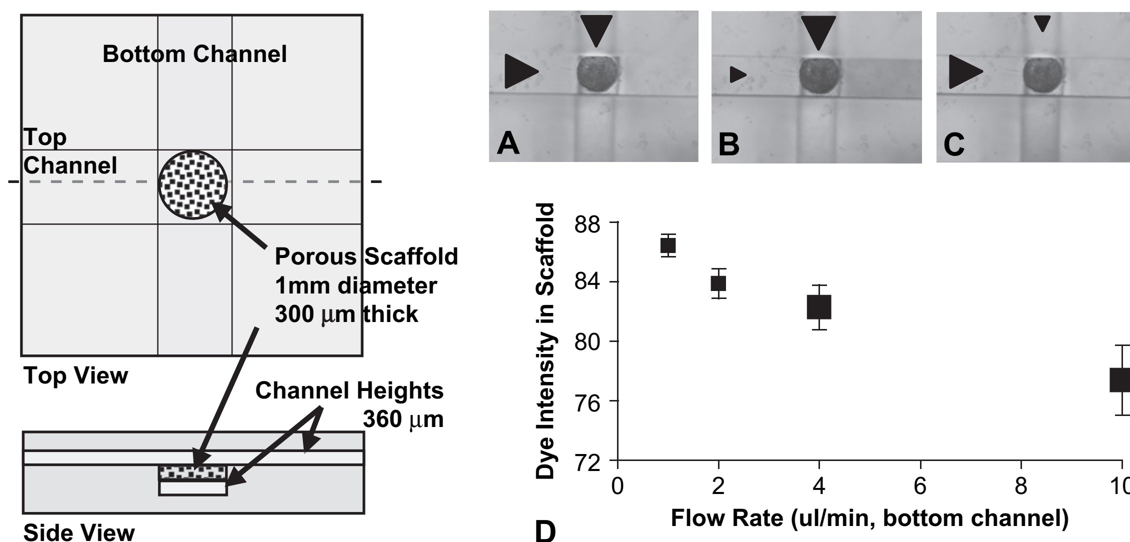

Fig. 5. Temporally controllable gradients were created through the thickness of a biomaterial scaffold in a five-layer device. When fluid from the top and bottom channels mixes, via the scaffold, the solution will change from clear to blue. (A) When flow rates are balanced above and below the scaffold, diffusive mixing is primarily restricted to the scaffold area and there is no increase in color change in the top channel relative to the bottom channel downstream of the scaffold. (B) The flow rate of the top channel is smaller, and allows for increased contact time with the scaffold and greater diffusive mixing in the top channel. (C) The flow rate of the bottom channel is smaller and allows for greater contact time with the scaffold and greater diffusive mixing in the bottom channel. (D) Color intensity of mixing fluids in the scaffold is measured by color analysis with NIH image. Flow rate through the top channel is held constant at 1 μl/min. Higher grey value indicates greater dye intensity in the scaffold, and hence a greater volume of mixed fluids in the scaffold.

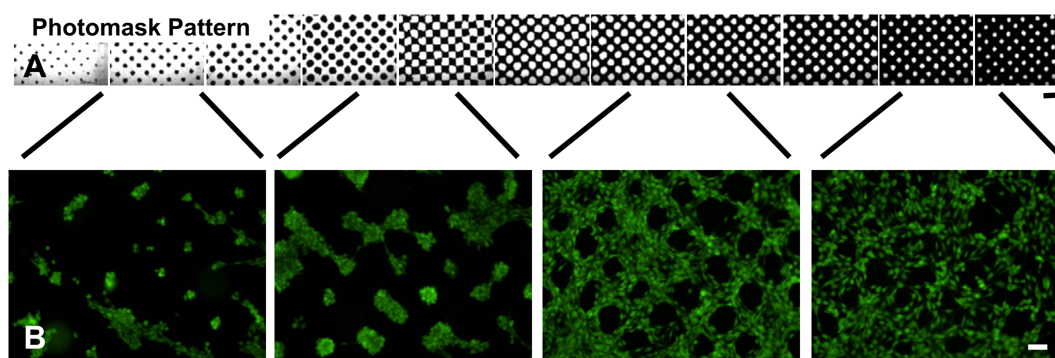

Fig. 6. Poly (ethylene glycol) methacrylate was photografted onto channel surfaces. Photomasks were used to create graft patterns with varying sizes of dots and spacing between dots (A). This pattern allows for systematic study of cell adhesion as it depends on adhesive region size and spacing. Preliminary grafting studies were done on flat surfaces, and NIH 3T3 fibroblasts were subsequently seeded on the modified substrate. (B) Live cells are highlighted, indicating adhesion in non-grafted regions. Scale bars = 100 μm.

mechanical integrity with flow through the scaffolds, indicating that multilayer devices with the porous scaffolds can be designed for high throughput studies with perfusion through the scaffolds.

3.4. Modifying device surfaces by photografting

While materials have been incorporated as three-dimensional cell culture sites on-device, the living radical feature of the CLiPP system can also be useful for modifying channel surface properties to control protein adsorption or elicit specific cell responses. For multilayer cell culture microfluidic devices, it would be beneficial to prevent protein adsorption on channel surfaces to ensure proteins intended to treat cells are not lost to the channels and to prevent non-specific cell adhesion in the channels. Previous work has shown that poly(ethylene glycol) methacrylate (PEGMA) grafted from a CLiPP polymer surface, similar to the polymer used for CLiPP cell culture devices, via DTC groups prevented non-specific cell adhesion and directed fluid flow in microfluidic channels [33]. To verify that cell culture devices could similarly be modified to prevent protein adsorption and non-specific cell adhesion, PEGMA was photografted from a device polymer surface through a grayscale photomask. Due to the printer limitations, the grayscale printed as a range of dot sizes with a range of spacing between the dots (Fig. 6A). The negative pattern of the photomask was transferred to the device surface with grafted PEGMA, and as seen in Fig. 6B, cells only adhered to the non-grafted regions. The culture device surfaces are easily modified by photografting of PEGMA to control protein adsorption and cell adhesion and could be further modified with a host of methacrylated materials to present other desirable surface chemistries.

4. Conclusions

Multilayer microfluidic cell culture devices were fabricated incorporating covalently attached three-dimensional cell culture sites and channels to address the top and bottom sides of the scaffolds separately. The living radical feature of the device polymer system allows for facile incorporation of the three-dimensional culture sites and surface modifications, which enables fabrication of a multilayer device that will be useful as a high throughput screening tool to study combinations of cell-material interactions not easily achieved otherwise.

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