Patterned cell arrays and patterned co-cultures on polydopamine-modified poly(vinyl alcohol) hydrogels.

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Abstract. Live cell arrays are an emerging tool that expand traditional 2D *in vitro* cell culture, increasing experimental precision and throughput. A patterned cell system was developed by combining the cell repellent properties of polyvinyl alcohol hydrogels with the cell adhesive properties of self-assembled films of dopamine (polydopamine). It was shown that polydopamine could be patterned onto polyvinyl alcohol hydrogels by microcontact printing, which in turn effectively patterned the growth of several cell types (HeLa, HEK293, HUVEC and PC3). The cells could be patterned at levels down to single-cell confinement, and it was demonstrated that cell patterns could be maintained for at least 3 weeks. Further, polydopamine could be used to modify poly(vinyl alcohol) *in situ* using a cell compatible deposition buffer ($1\,\mathrm{mg\,mL^{-1}}$ dopamine in 25 mM tris with a physiological salt balance). The treatment switched the PVA hydrogel from cell repellent to cell adhesive. By pre-patterning one cell population and depositing polydopamine *in situ*, a second cell population could be seeded, and adhered to the newly switched surface. Patterned co-cultures of HeLa/HeLa and HeLa/HUVEC were thus realized through simple chemistry and could be studied over time. The combination of polyvinyl alcohol and polydopamine was shown to be an attractive route to versatile live cell arrays with minimal infrastructure requirements and low experimental complexity.

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1. Introduction

Patterning live cells in arrays is emerging as powerful technique to enable the study of single cells or clusters of cells in a high throughput manner, with increased control over cell-cell interactions and cell morphology [1, 2, 3, 4, 5]. Patterned cell arrays have been used to assess the relation between cell shape and function [6, 7] assay biological functions at a single cell level [8, 9, **?**], study stem cell biology and growth [10, 11, 12, 13, 14] and analyze cell migration [15, 16]. A range of materials and techniques have emerged to allow micropatterning of cells in a controlled manner, typically combining anti-fouling and cell repellent materials such as poly(ethylene glycol) (PEG) [17] with chemistries to which cells can bind [18]. Micropatterning of cell-directing materials can be achieved in numerous ways[19], such as by photo lithography [20], stencil patterning [21] or soft lithography [22]. An extension of patterning a single cell type is micro-patterned co-cultures, where spatial positions of two or more different cell types is controlled through the patterning process. Co-cultures can enable the design of more tissue-reminiscent *in vitro* cell culture systems [23], allowing the interactions between different cell types to be studied [24, 25].

Hydrogels are becoming widely used as cell culturing materials due to their large potential within tissue engineering [26] and the ability to tune properties such as elasticity, cell ligand density, porosity and other physical and chemical factors in a controlled manner [27, 28]. Poly(vinyl alcohol) (PVA) is a widely available synthetic biocompatible polymer with interesting applications within tissue engineering [29]. PVA can form stable hydrogels through chemical or radiation-induced cross-linking or freeze thaw-methods [30]. However, the simplest method to form PVA hydrogels is thermal treatment above the glass transition temperature of PVA (85 °C [31]), resulting in gels that are stable for long periods in solution [32]. PVA has good film-forming and surface adhesion properties, allowing it to form thin, surface-bound hydrogel films through e.g. dip-coating or spin coating on substrates [33, 34]. Pristine PVA has low protein adsorption [35] and inhibits cell attachment [36], and cell adhesive areas can be made by patterning PVA films. Micropatterning methods of PVA include micro photoablation[37], micromolding on surfaces [38] or in capillaries[39], UV light-enabled degradation[36], photo-crosslinking [40, 41] or inkjet printing of oxidizing solutions[36]. However, so far the available patterning methods are still somewhat limited in either resolution, chemical functionality or by the complex chemistry and instrumentation involved.

Recently, self-assembled films of catecholamines such as dopamine have emerged as versatile and multifunctional coatings for a variety of materials [42]. By exposing a surface to a mildly alkaline solution containing dopamine, the dopamine oxidizes and self-assembles into a thin surface layer of "polydopamine" that is highly stable, but still reactive towards several nucleophiles [43]. Polydopamine has been shown to be cell compatible [44], and has been applied to surfaces by microcontact printing to generate cell patterns [45, 46], also on the typical non-fouling surface of PEG-containing

monolayers [47, 48]. Further, polydopamine has been blended with PVA to make a partially cell-repellent polymer [49], and has been used to make PVA hydrogels more cell adhesive [50].

In this paper we present the development of a cell micropatterning system based on PVA and polydopamine. Through the use of polydopamine as an adhesive layer, stable, cell repellent PVA hydrogel films were formed on multiple substrates such as glass, polystyrene, PDMS and aclar. Microcontact printed polydopamine on PVA is shown to pattern cells for up at least 3 weeks without pattern degradation. Polydopamine on PVA is demonstrated as a versatile patterning method, through the patterning of several cell types, modification of polydopamine with PEI for altered functionality, and integration with microfludic channels. Finally, we present a method to deposit polydopamine during cell culturing, allowing *in situ* switching of non-patterned PVA areas. By combining microcontact printing and *in situ* polydopamine deposition, both same-cell (HeLa/HeLa) and dissimilar cell (HeLa/HUVEC) co-cultures are demonstrated.

Thus, the current study demonstrates that through the use of off-the-shelf chemicals such as PVA and polydopamine and using only simple, mild chemistry and methods, several advanced cell culture applications are achievable. Thereby this system can expand the possibilities available to many cell biology labs without the need for custom materials or extensive equipment.

2. Materials and Methods

2.1. Production of PDMS stamps

A poly(dimethyl siloxane) (PDMS) stamp was made from an SU-8 master in a standard soft-lithography process [51]. Briefly plastic foil masks were designed using Clewin 4 (Wieweb software) and printed by JD Photo-Tools (Lancaster, UK). SU-8 masters were made by performing photolithography with the foil masks on SU-8 5 (MicroChem corp.) on silicon wafers according to the manufacturer's instructions. An anti-adhesion coating was applied to the master by placing it in a vacuum desiccator with a drop of trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma) for 20 minutes. PDMS (Sylgard 184, Dow Corning), mixed at a 10:1 (base:crosslinker) ratio was cast over the master, and cured at 80 °C for 2 hours, then carefully peeled off the master.

2.2. Substrate preparation and PVA coating

Glass coverslips (VWR) were cleaned by immersion in 1M HCl (Sigma) at 60 °C for 2 hours, then sonicated for 5 minutes in deionized water (DIW). They were stored in DIW until use. Aclar film (Ted Pella) was cleaned by sonication in acetone, ethanol and DIW before use. An adhesion layer of polydopamine was applied by floating the substrate on a $1\,\mathrm{mg\,mL^{-1}}$ solution of dopamine hydrochloride (Sigma) in 10 mM tris (Tris(hydroxymethyl)aminomethane, Sigma) buffer, pH=8.5 for 30 minutes [42]. Dopamine solutions change color from clear to dark brown over time, indicating the

oxidation of dopamine [42]. This is a rapid way of determining if dopamine oxidation can occur in a given solution. However, part of the color change is also due to the appearance of larger (micron-sized) polydopamine particles in the bulk solution that appear after 30-60 minutes. To avoid the precipitation of the particles on the surface, the substrates were floated upside down on a drop of the dopamine solution.

A 1% poly(vinylalcohol) (PVA, Mw=22000, degree of hydrolysis >98%, BDH, VWR) solution was prepared by dissolving the PVA in DIW at 90 °C. After stirring for 15 minutes all the PVA had dissolved and the solution was cooled to room temperature. To produce a thin PVA film a small drop was placed onto the substrate and spin-coated for 30 s at 3000 rpm on a custom-built spin coater giving a PVA film of about 15 nm in thickness. Unless otherwise stated, the PVA film was heat annealed for 30 minutes at 130 °C, which was necessary to immobilize the film [36]. For live-cell imaging experiments, PVA was spin-coated in the bottom of a Willco confocal dish (Willco Wells, The Netherlands).

2.3. Microcontact printing

To produce cell patterns microcontact printing of polydopamine was performed [46]. The PDMS stamp was immersed in a $1\,\mathrm{mg\,mL^{-1}}$ solution of dopamine hydrochloride in 10 mM tris buffer, pH=8.5 for 30 minutes, then blown dry with compressed air. To transfer the polydopamine, the stamp was placed face-down on the PVA-coated substrate under a 100 g weight for 2 minutes. Before reusing the stamps residual polydopamine was removed by sticky tape. The stamps could be reused multiple (at least 50) times without observable changes in the patterns.

In some cases $0.1\,\mathrm{mg\,mL^{-1}}$ poly(ethyleneimine) (PEI, 25kDa, branched, Sigma Aldrich) was added to the printing solution. To visualize if PEI had been incorporated into the patterns, PEI was labeled with FITC as described in [52] to produce FITC-PEI. FITC-PEI was purified by dialysis against DIW for 3 days (changed after 4 hours, then every day) with a 10kDa MWCO dialysis membrane (VWR). To compare the stability of micropatterns of PLL and PEI with polydopamine micropatterns, 1 mg/mL of FITC-PLL (Sigma) or FITC-PEI was incubated with a PDMS stamp for 10 minutes, and stamped onto the substrate. Substrates were rinsed with DIW after stamping.

2.4. Solution modification of PVA with polydopamine

Prior to co-culturing experiments, it was observed if polydopamine could modify initially cell repellent films to allow cell adhesion. Immobilized PVA films were post-modified with polydopamine by first shortly equilibrating them in DIW for 30 minutes, then immersing or floating them in a 10 mM tris buffer, pH=8.5 with $1\,\mathrm{mg\,mL^{-1}}$ dopamine. Cells were then cultured as described below.

For *in situ* post-modification of PVA with polydopamine during cell culturing and for dopamine toxicity tests, the cells were washed in PBS, then incubated with $1\,\mathrm{mg\,mL^{-1}}$ dopamine in a modified tris-buffered saline (modified TBS, consisting of

25 mM tris, 140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, pH=8.5) to maximize cell health while still allowing polydopamine deposition. The deposition buffer pH was adjusted at 37 °C as the pH of tris buffers has a high temperature dependence [53]. The cells were then washed in PBS before changing back to growth medium for further culturing.

2.5. Cell culturing

All cell lines were purchased from ATCC. Human cervical cancer cells (HeLa), human embryonic kidney cells (HEK293) and human prostate cancer cells (PC3) were grown in DMEM (Gibco, Invitrogen) supplemented with 1 mM nonessential amino acids (Gibco, Invitrogen), 10% FBS (Gibco, Invitrogen), and 1 mM L-glutamine (Sigma Aldrich). Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium (Lonza). The cells were cultivated at 37 °C in a humidified atmosphere with 5% CO2 and passaged regularly. HUVEC cells were used in passages 4-6. For cell culture experiments, the samples were sterilized in 70% ethanol and rinsed in PBS before use. The samples were then placed in 24-well plates. Typically, 20 000 to 100 000 cells were seeded into each well in 0.5 mL of cell medium.

2.6. Cytotoxicity assay

For dopamine toxicity testing an MTT assay (In Vitro Toxicology Assay Kit, MTT based, Sigma) was performed. 10 000 HeLa cells were cultured in each well in a 96-well plate for 24 hours, then exposed to a dopamine deposition solution (1 mg mL^{-1} in modified TBS) for 0-60 minutes or buffer controls. In some samples the solution was refreshed, this was performed without a rinsing step in between. 24 hours after dopamine exposure, 10 µL of 10 mg/mL MTT was added to each well in a 96-well plate with cultured cells. The cells were incubated for 4 hours, after which the medium was aspirated and the formazan crystals were dissolved in 50 µL DMSO. The absorbance was the measured at 570 nm, subtracting a 690 nm background reference using a Tecan Infinite M200 Pro microplate reader. Each sample was done in 4 duplicates, and p-values were found using a two-tailed paired student's t-distribution. To assess the effect of different radical oxygen scavangers on dopamine cytotoxicity, 1 mM of ascorbic acid (Sigma Aldrich) or 0.25 mg/mL (500U/mL) of catalase (Bovine Serum Catalase, Sigma Aldrich) were added to the dopamine deposition solution.

2.7. PDMS microfluidic device

PDMS microchannels 300 µm wide 100 µm high and were made in the same manner as the PDMS stamps above. To bond them to the samples, they were exposed to oxygen plasma at 0.5 millibar with a power of 50 W for 18 s in a Diener Femto Plasma System (Diener Electronics), placed with the channel down on the samples and cured for 10 minutes at 130 °C.

For cell culturing in microfluidic channels the cells were injected at a density of 10×10^6 cells/ml using a micropipette into channels bonded to pre-patterned PVA/polydopamine surfaces. Once the channel was filled, cell medium was pipetted on top of the entire PDMS device (maintained there by surface tension) to equilibrate pressure between the entrances and maintain a suitable channel environment without the need to include a pumping and tubing system in the cell incubator.

2.8. Co-culturing

To assess the effect of various polydopamine deposition treatments on co-culture efficacy, 50 000 cells/cm^2 (HeLa) were cultured overnight on microcontact printed patterns. Then, 4x5, 4x10 or 4x15 minute polydopamine depositions were performed in modified TBS, before 50 000 cells/cm^2 (HeLa) were seeded. Cell counts were were performed at 16 and 40 hours using the CellCounter plugin for FIJI [54]. For each sample, 3 areas were imaged and counted, discriminating between cells on and off the microcontact printed patterns. A total of 4868 cell were counted. P-values were calculated as above.

For patterned co-culturing experiments, HUVEC or HeLa cells were first grown in patterns as above (typically over night, but from 4 hours to several days gave similar results). These cells were then labeled with 1 µM calcein-AM (Invitrogen) in cell growth medium for 1 hour, and rinsed several times in PBS. The surface was then modified *in situ* as described above. A second batch of HeLa or HUVEC cells was labeled with 5 µM CellTracker Red (Invitrogen) in DMEM or 1 µM calcein red-orange-AM (Invitrogen) for 1 hour, rinsed several times in PBS, then removed by trypsin/EDTA treatment and seeded onto the activated surfaces with patterned calcein-labeled cells.

2.9. Optical and Atomic Force Microscopy

Cells were imaged live using a Leica SP5 confocal microscope, a Leica SP8 confocal microscope or a Nicon Diaphot widefield fluorescence microscope. For actin filament and nucleus imaging, the cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized in 0.1% Triton X-100, and labeled with 165 nM Alexa488-phalloidin (Invitrogen) for actin filaments, while the nuclei were labeled with 1 µg mL^{-1} PI (Sigma Aldrich) and 10µgmL RNase A (Sigma) in PBS, or Hoechst 33248 in PBS. For live cell imaging, the medium was exchanged to HEPES-buffered Live Cell Imaging medium (Invitrogen) for short-term (up to 1 hour) imaging, while for long-term time lapse imaging the medium was exchanged to Leibovitz L-15 (Invitrogen) for HeLa cells or 1:1 L-15 and EBM-2 for HUVEC or HUVEC/HeLa co-cultures. Willco 35 mm glass bottom confocal dishes were used for long-term time lapse imaging. 1.5 mL of medium was used, then covered with white mineral oil (VWR) and maintained on the microscope stage which was maintained at 37 °C with a closed box system.

For AFM microscopy, microcontact printed polydopamine patterns, buffer-soaked patterns and post-modified samples were prepared as described above. The samples were then rinsed in DIW and blown dry using compressed air, before imaging in a Veeco

Nanosight V AFM in ScanAsyst mode. The data was analyzed using Gwyddion [55]. The data was leveled by matching the height median and fitting to a plane, and the root mean square roughness was analyzed at 4 distinct positions on each sample. Sample p-values were compared using a paired student's t-test.

3. Results

To produce patterned cell cultures we present two alternate methods of modifying the cell repellent hydrogel poly(vinyl alcohol) (PVA) using polydopamine: microcontact printing and solution deposition. We show that the first method can be used to pattern cells on the polyvinyl alcohol surface, and explore further aspects of this system, such as patterning on alternate substrates (Aclar films) and PDMS-microchannel integration. We further show that PVA can also be made cell adhesive by solution deposition of polydopamine, and find parameters that allow this to be done *in situ* during cell culturing. Finally we show how the combination of cell patterning by microcontact printing of polydopamine with *in situ* polydopamine deposition can be used to make patterned cell co-cultures.

3.1. Cell-repellent PVA films

Cell repellent surfaces were made by spin-casting and heat annealing poly(vinyl alcohol) thin films (Figure 1) [36]. To minimize potential swelling effects in the film during cell culturing the thickness of PVA was adjusted to 15-20 nm (Figure 2) by using a PVA concentration of 1% in the spin-casting solution. This still gave homogeneous, cell-repellent coatings after heat treatment at 130 °C for 30 minutes (Figure 2). If no heat annealing step was performed the surfaces were not cell-repellent, presumably due to rapid film detachment and dissolution (Figure S1). However, even with heat annealing film detachment could occur if the experiments lasted longer than around 2-3 days or if fully hydrated samples were extensively handled. To increase the PVA adhesion aldehyde-containing silanes or silane-linked glutaraldehyde have been used in the past to chemically immobilize the PVA hydrogel [36, 37]. However, to avoid introducing chemicals which can potentially be cytotoxic if not completely removed prior to cell culture experiments, we instead used an adhesive film of polydopamine, applied to the substrate prior to PVA film spin-casting under mild aqueous conditions [42]. With this treatment no film detachment was observed at 37 °C in cell medium for extended periods (several weeks), displaying the usefulness of polydopamine as a simple adhesive layer.

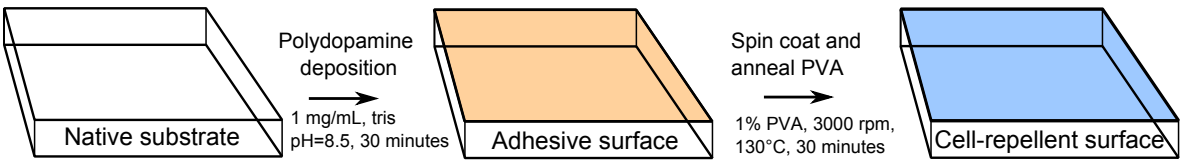

Figure 1: Production of the initial cell-repellent poly(vinyl alcohol) surface.

3.2. Microcontact printing of polydopamine on PVA

Microcontact printing is a simple method to transfer patterns of an "ink" (e.g. polymers, proteins or reactive chemicals) onto a substrate, and can be used to generate patterns of cell adhesive and cell repelling areas for cell patterning [22]. However, when common cell-adhesive polymers such as poly(L-lysine) (PLL) or poly(ethyleneimine) (PEI) were microcontact printed onto PVA, they were quickly washed off due to the anti-fouling nature of PVA (Figure S2). Thus, we investigated microcontact printing of polydopamine onto PVA, due to its reported highly adhesive properties [42, 47]. Polydopamine was first deposited as a thin film on a PDMS stamp [46], and then transferred to the PVA-coated substrate by microcontact printing (Figure 2A). The patterns could be visualized by phase contrast microscopy (Figure 2B) and showed a high degree of fidelity to the relief patterns of the stamp, indicating complete film transfer. At the same time polydopamine patterns adhered well to the PVA surface and were not removed in the washing steps or during long term immersion in cell culture media. Due to it's amine-reactivity, polydopamine has been reported to be co-deposited with amine-containing polymers such as PEI [56]. We were able to produce polydopamine patterns that contained fluorescent PEI (PEI was added to the solution used to deposit the polydopamine film on the PDMS stamp, see Materials and Methods) that remained stable after rinsing when deposited onto PVA films (Figure 2B and Figure S2). The height and microscale morphology of the PVA and polydopamine films was investigated by AFM. In the initial dry state, the PVA film was around 15 nm, while the polydopamine film was around 5 nm (Figure 2C and D). The films appeared homogeneous with few defects, indicating complete coverage by both spin casted PVA and microcontact printed polydopamine (Figure 2E).

3.3. Cell micropatterning on polydopamine/PVA

Polydopamine is reported to be bio-compatible and cell adhesive [44, 46], so microcontact printed polydopamine on PVA hydrogel films should be a simple method to pattern cells in culture. Initial tests were done using HeLa cells, a common model cell line. HeLa cells were successfully cultured on the patterned substrates, and adhered well and spread on the polydopamine areas (Figure 3). No spreading onto pristine PVA areas was observed, even after 3 weeks in culture (Figure 3C). Initial experiments showed similar patterning ability with Human Embryonic Kidney (HEK293), Prostrate Cancer (PC3) and Human Umbilical Vein Endothelial (HUVEC) cells after 3 days on the patterns (Figure 3D-F). The attachment rate depended on the cell type, but typically the cells were adhered to the patterns within a few hours, and un-attached cells could be rinsed away. A time lapse of the attachment of HUVEC cells to polydopamine patterns can be seen in Figure S3. Other cell types, such as HEK293, had lower adhesion, and often detached during routine handling (washing, media change, etc). To increase the adhesion, polyethyleneimine (PEI) (0.1 mg/mL) was co-deposited with dopamine (1 mg/ml) onto the PDMS microcontact printing stamp. Patterns containing PEI

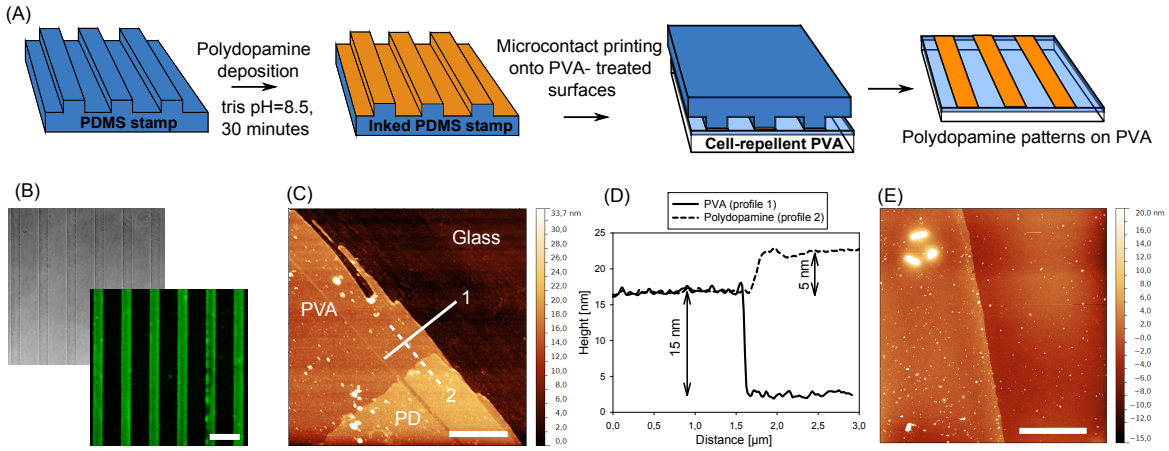

Figure 2: (A) Schematic of the production of polydopamine patterns on PVA surfaces. (B) Upper: Phase contrast image of polydopamine micropattern on polyvinylalcohol surface. Lower: Polydopamine incorporating fluorescently labeled PEI. Scale bar 100 µm. (C) AFM micrograph of microcontact printed polydopamine (PD) onto a PVA film. A large scratch was made into the PVA, exposing the glass underneath and allowing a thickness measurement of the PVA. Scale bar 2 µm. (D) Height profiles of the PVA (profile 1) and polydopamine on PVA (profile 2), respectively, along the lines marked by (1) and (2) in (B). (E) Large-scale AFM scan demonstrating the homogeneous surface coverage of the spin-casted PVA and the microcontact printed polydopamine films. Scale bar 10 µm.

increased the HEK293 cell adhesion sufficiently to avoid detachment during handling.

Microcontact printed patterns could be used to control areas for cell growth in areas of multiple cells, but could also be used to control the geometry of single cells (Figure 3G-J). Although true single-cell arrays, with only one cell at each array site, should be achievable with this method, the exact feature size and seeding procedures were not optimized for this purpose in this work. Additionally, the optimal pattern size varied by cell type. Typically 2-3 HeLa cells were attached at each array spot after 24 hours when using 37 µm circular features, while PC3 cells typically were single cells on the same features (Figure S4). Our minimum feature size was limited to 8 µm due to the photomasks that were used, although much smaller features can be achieved with the soft lithography approach [51].

To investigate cell patterning on substrates other than PVA-coated glass, we performed identical experiments on PVA-coated Aclar films. Due to its low adhesion with epoxy, Aclar is a polymer film typically used for 2D cell cultures, if the cells are to be epoxy embedded for studies with transmission electron microscopy. Identical results as those on glass were obtained for cell patterns on Aclar (Figure S4).

Increased environmental control of cultured cells through microfluidic systems is an important route to to further extend the possibilities of *in vitro* experiments [57]. To this end, we investigated if the PVA-polydopamine cell patterning system could be integrated with microfluidic channels. We found that PDMS bonds irreversibly with the patterned PVA/polydopamine surfaces after only the surface of the PDMS

microchannels device was activated with oxygen plasma, therefore preserving cell patterning ability. This allowed the placement of a PDMS microchannel onto a pre-patterned PVA/polydopamine surface. The bond was maintained with liquid in the microfluidic channel for more than one week, although in this case the PVA had to be limited to the channel area, as stress around the plugs for the associated tubing could rupture the PDMS-PVA bond (Figure S5A). However, underneath the channel area where no stress occurred no leaks were detected over at least one week (Figure S5B). By bonding PDMS microchannels on top of prepatterned polydopamine-PVA surfaces, cells could be grown on pre-defined patterns inside the microfluidic channel (Figure 3K). Interestingly, if the entire device was immersed in an aqueous solution the PVA film was slowly hydrated from the edges, reducing the PVA-PDMS bond strength. This allowed for simple release of the microchannel device if desired for e.g. further microscopy or extraction of the cultured cells.

3.4. Solution polydopamine modification of hydrated PVA films

Next, we explored the possibility of using the versatile surface deposition properties of polydopamine to modify PVA films by solution deposition (the same methods as used to produce polydopamine coating on PDMS stamps). This would enable switching of an initially cell-repellent hydrogel surface to cell adhesive using only mild aqueous chemistry. PVA films were pre-hydrated in DIW to mimic the state during cell culturing, and modified by floating the samples on top of a polydopamine deposition solution (1 mg/mL in 10 mM tris, pH=8.5) to avoid precipitation of polydopamine particles. Surfaces modified for 15-60 minutes all supported cell adhesion and spreading (Figure 4), while unmodified surfaces did not support cell adhesion. Thus polydopamine was able to modify a typical anti-fouling hydrogel while hydrated, allowing it to support cell adhesion and growth, highlighting the strong surface modification properties of polydopamine. The morphology of the cells on the polydopamine-activated PVA surfaces was visualized by actin filament staining (Figure 4). The Hela cells showed a spindly morphology for shorter activation times, but resembled glass controls more with somewhat longer (30-60 min) surface activation times. This change in cell morphology is presumably a result of increasing number of cell attachment sites created by longer polydopamine treatment of the PVA surface.

To observe the changes that occurred on PVA films and microcontact printed polydopamine patterns on PVA when subjected to the polydopamine deposition solution, samples were modified as above, rinsed, dried and then imaged by AFM, and the surface roughness was analyzed (Figure 4B, AFM images shown in Figure S6). Buffer (10 mM tris, pH=8.5) for 60 minutes slightly increased the roughness of both the polydopamine layer and the PVA layer, although the reason for this is not known. When subjected to a dopamine deposition solution for 60 minutes, there was an significant increase ($p<0.05$ compared to buffer treatment) in surface roughness both on the polydopamine and PVA areas, likely indicating deposition of polydopamine on

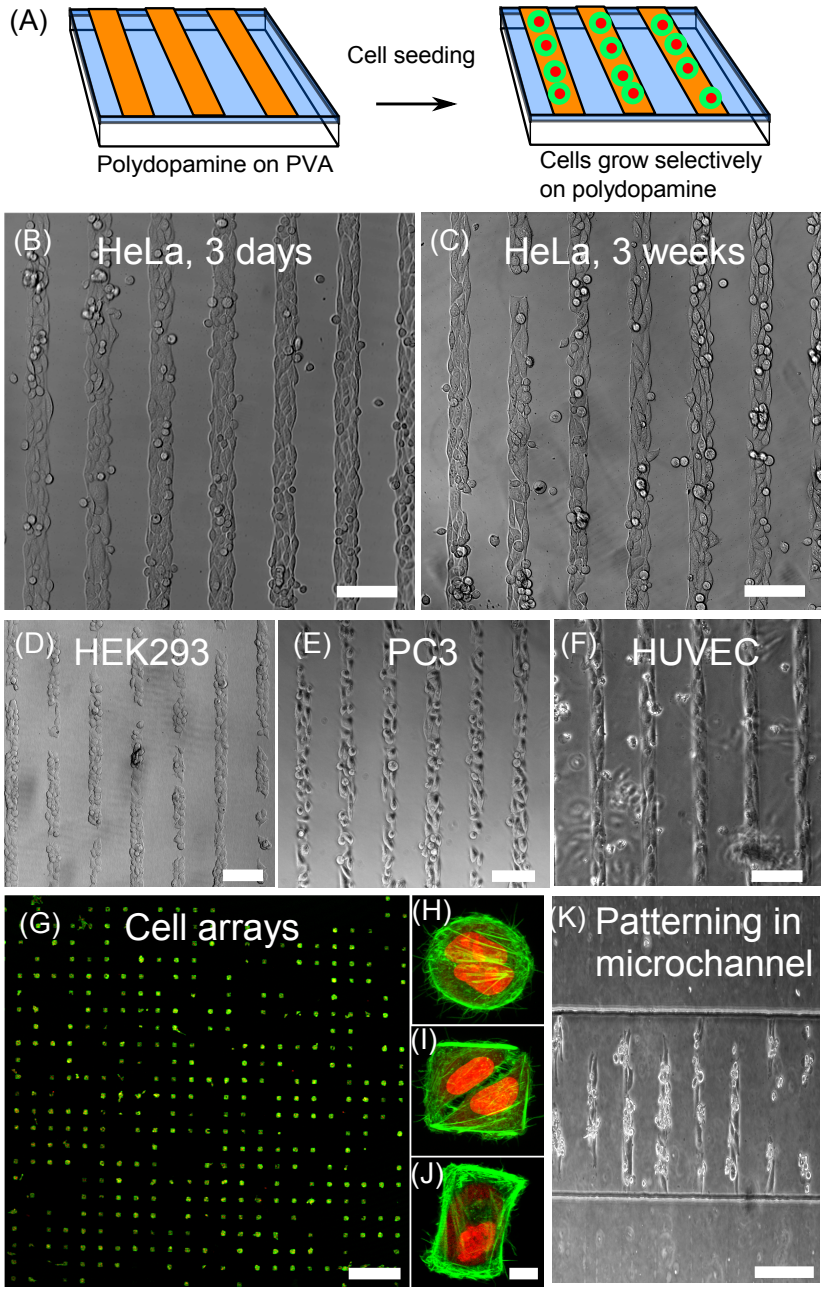

Figure 3: (A) Cells only adhere to the polydopamine areas, and are unable to attach to the PVA background. (B,C) Hela cells tightly follow the polydopamine patterns, both short term (B, 3 days) and long term (C, 3 weeks). The patterns are 40 µm lines with 80 µm gaps. (D-E) Other typical model adherent cell lines also can be patterned, as shown here for (D) HEK293, (E) PC3 and (F) HUVEC cells after 3 days on the patterns. (G) Actin filaments and nuclei in HeLa cells visualized on an array of 37 µm squares, showing single-cell patterning over large areas. (H-J) Close-up of HeLa cells (actin and nucleus labeled green and red respectively) on different pattern geometries, such as (H) circles, (I) squares and (J) H-shaped, demonstrating cell shape control with the patterns. (K) Microchannels could be bonded on top of the patterned surfaces, and HeLa cells were cultured inside the channels for 3 days, growing only on vertical lines (channel is in the middle part of the figure). Scale bars (A-F, J) 100 µm, (G) 200 µm, (H-I) 10 µm.

these samples. To investigate if gravity played a role in polydopamine deposition, e.g. through the precipitation of larger particles first formed in solution, samples were also floated on top of the deposition solution. The roughness was similar to the immersed sample in this case, indicating a formation of a polydopamine layer on the PVA.

3.5. Cytotoxicity of in situ *dopamine deposition*

Switching of initially cell-repellent PVA to cell adhesive could allow for co-culturing of two cell populations in a patterned geometry, as shown schematically in Figure 5A. A requirement is that the switching must occur after the first cell population is cultured on the microcontact-printed patterns, so the switching chemistry must be cell compatible. To comply with this, the polydopamine deposition solution was modified, where the standard 10 mM tris buffer was substituted with a modified tris-buffered saline (TBS) to maintain a typical cell culture salt balance, although at an increased pH of 8.5, which is optimal for polydopamine deposition [58]. A typical deposition solution with 1 mg/mL dopamine in the modified tris-saline buffer changed color to brown, indicating that the altered buffer allowed dopamine oxidation and likely also polydopamine formation (Figure S7)[58].

As a measure of cytotoxicity, the metabolic activity of HeLa cells 24 hours after exposure to different polydopamine deposition conditions was assessed using the MTT-assay (Figure 4C). The modified tris-saline (TBS) buffer at pH=8.5 was not significantly cytotoxic for up to 60 minutes. It was seen that shorter dopamine incubations were not appreciably cytotoxic either. However, longer deposition times did induce somewhat of a cytotoxic effect, especially if the deposition solution was replenished at regular intervals. Dopamine is reported to be toxic to cell culture due to the generation of radical oxygen species (ROS) during dopamine oxidation [59, 60]. Thus an ROS scavenger could reduce the cytotoxic effect of dopamine deposition. Ascorbic acid, a common antioxidant, was investigated first, but this inhibited any oxidation of dopamine, and thus prevented the formation of polydopamine (Figure S7). Therefore, catalase, an enzyme that catalyzes the degradation of hydrogen peroxide, was introduced as a more specific ROS scavenger. Indeed, catalase did not inhibit dopamine oxidation, as indicated by color change in solution and the formation of precipitate particles if left for a long time, but it did significantly reduce dopamine cytotoxicity for longer incubation times (p¡0.05).

3.6. Patterned co-culture by in situ *polydopamine modification of PVA*

To produce patterned co-cultures the first cell population was seeded as before on microcontact printed patterns (Figure 5A). Then, the initially cell repellent areas of the PVA were rendered cell adhesive by a cell compatible polydopamine deposition in modified TBS, before the second cell population was introduced. To maintain an effective surface activation while avoiding the formation of visible polydopamine particles in the deposition solution, the solutions were replenished at intervals of 5-15 minutes (typically the particles became visible after 30 minutes without a solution change). The

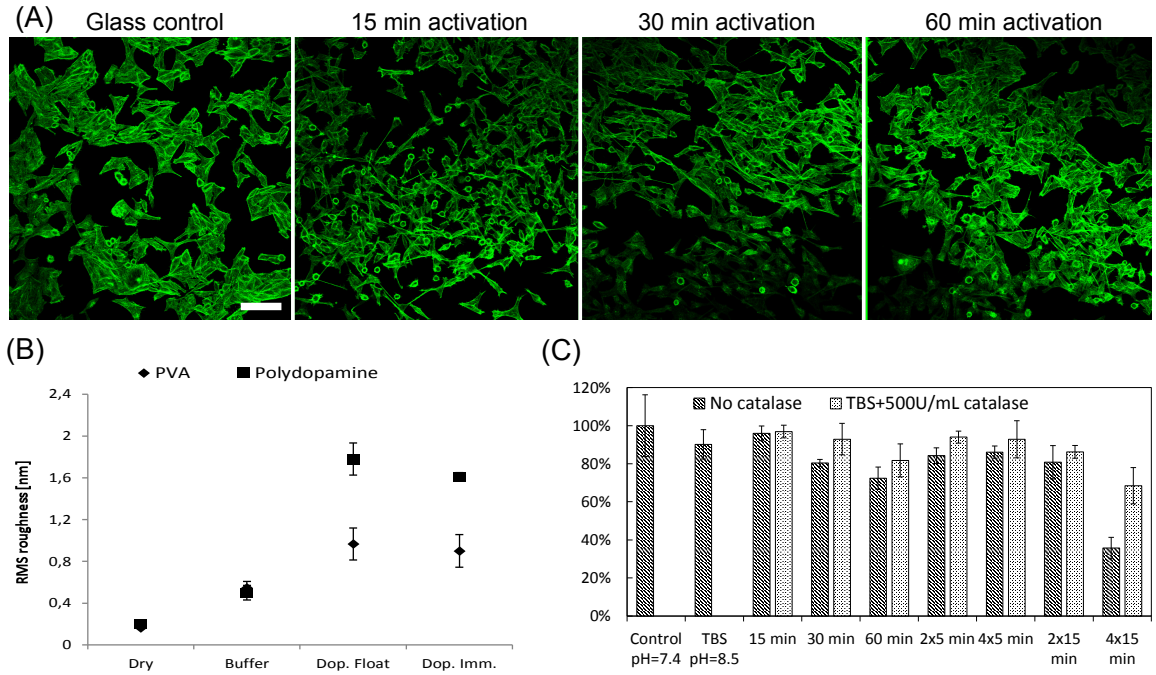

Figure 4: (A) HeLa cells grown for 1 day on PVA hydrogels modified with polydopamine before cell growth. Actin filaments are labeled in green. Increasing the dopamine deposition time gives a cell morphology more similar to glass. Scale bar 100 µm. (B) Root mean square roughness of micro-contact printed polydopamine and PVA areas as measured by AFM on untreated samples (Dry), samples immersed for 60 minutes in 10 mM tris buffer at pH=8.5 (Buffer) and floating (Dop. Float) or immersed (Dop. Imm.) in 1 mg mL^{-1} dopamine in 10 mM tris buffer at pH=8.5. Dopamine immersion significantly ($p<0.05$) increases the roughness of both PVA and polydopamine compared to dry samples and buffer treatment alone. Error bars show ± SEM. (C) Cell viability measured by MTT assay, 24 hours after the cells have been treated with cell medium (control, 60 minutes), modified TBS (TBS, 60 minutes) and 1 mg mL^{-1} dopamine in modified TBS for 0-60 minutes (Dop.), in some cases refreshing the dopamine solution at given intervals. The data is normalized compared to the control. Error bars show ± SEM.

number of cells per area was measured on the microcontact printed (μCP) polydopamine and on the *in situ* deposited polydopamine (Figure 5B, both a 16 hour and 40 time-point is included in Figure S8). Increasing treatment length reduced the cell density difference between μCP and *in situ* deposited polydopamine. A larger cell density was observed on the μCP areas of the less activated samples, presumably due to cell crowding in these areas since spreading into the *in situ* modified areas beyond was limited. Although catalase was shown by the MTT assay to reduce cytotoxicity of the *in situ* polydopamine treatment, it also reduced the strength of the *in situ* activation for shorter activation times ($p<0.05$). For the 4x5 minute and 4x10 minute activations the cell density on the μCP areas also increased in the presence of catalase, presumably due to a stronger cell crowding effect. With a 4x15 minute activation time the *in situ* polydopamine deposition was sufficient to generate cell densities on the previously cell repellent PVA

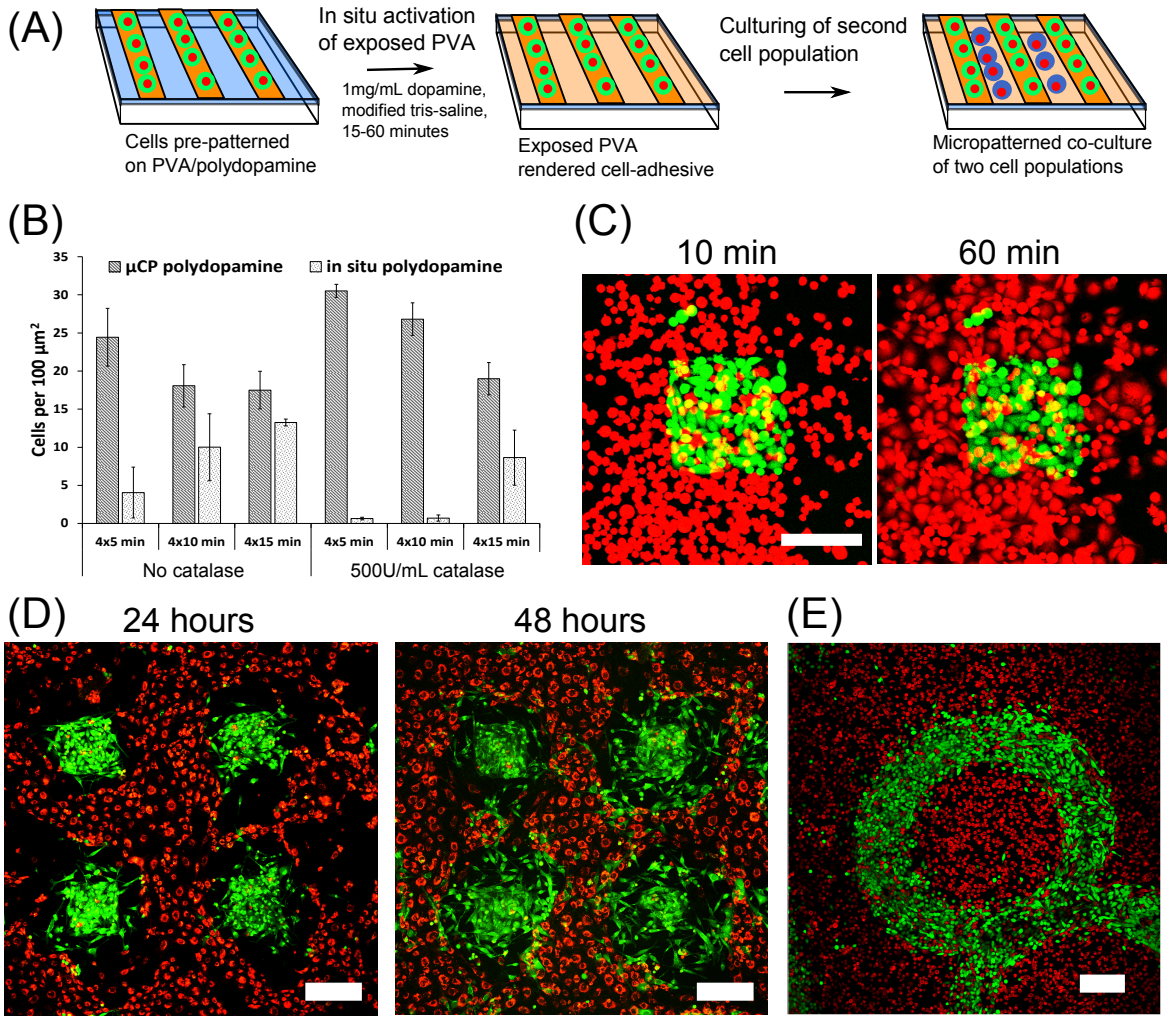

Figure 5: (A) The co-culture process starts with patterned cells, then modifies the cell-repellent PVA before adding the second cell population, which will attach onto the newly available areas. (B) The surface modification strength of different dopamine deposition treatments, as measured by the ratio between the number of cells per unit area on microcontact printed polydopamine and *in situ* deposited polydopamine. Catalase gave significantly ($p<0.05$) lower activation for 4x5 and 4x10 minute treatments. (C) After *in situ* activation samples pre-patterned with HeLa cells (green, calcein-AM labeled), HUVEC cells (red, calcein red-orange-AM labeled) quickly adhere and spread on the newly available areas outside the HeLa cell patterns. Scale bar 100 µm. (D) Longer-term observation after 24 and 48 hours of co-culturing of HeLa cells (green, calcein-AM labeled) and HUVEC cells (red, calcein red-orange-AM labeled) shows how the HeLa cells spread outward from their initial patterns, while the HUVEC cells simultaneously recede, although also forming a tight endothelial network surrounding the HeLa cells. Scale bar 200 µm. (E) Arbitrary patterns of co-cultures, even of the same cell type, can be made using this technique. Here shown by a co-culture of patterned HeLa cells (green cells, calcein-AM labeled) and HeLa cells seeded after *in situ* dopamine modification of the surrounding PVA (red cells, CellTracker Red labeled). Scale bar 200 µm.

of up to about 75% of the cell density on microcontact printed polydopamine.

Model patterned co-cultures systems were produced using dissimilar cells types (HeLa and HUVEC, Figure 5C,D) and the same cell types (differentially labeled HeLa cells, Figure 5E). HUVEC cells seeded after *in situ* activation of samples with patterned HeLa cells rapidly adhered (within 60 minutes) to the areas newly rendered cell adhesive Figure 5C. A time-lapse of the 2 first hours of co-culturing can be seen in Supplementary Movie S1. In this example a 4x15 minute *in situ* activation was used, and although this was shown to give a certain cytotoxicity after 24 hours, the observed HeLa cell patterns remained quite viable and dense, as indicated by the live cell-staining with calcein-AM (green). HeLa-HUVEC co-cultures were also observed 24 and 48 hours after the co-culturing was initiated by HUVEC seeding (Figure 5D). After 24 hours a clear interaction between the cells could be observed, with the HUVEC cells somewhat receding from the areas with HeLa cells compared to the attachment after 2 hours, and the HeLa cells expanding out from their initially defined patterns. At 48 hours the HeLa cells were spreading further, while the HUVEC cells formed a dense network surrounding the expanding HeLa cells, but with little overlap between the cell types. Note that even at 48 hours, the patterned HeLa cells that were the initial cell population for the co-culture experiment remain viable, as indicated by the retainment of the calcein-AM (green fluorescence) loaded prior to co-culturing.

To further demonstrate that this method of co-culturing can produce arbitrary patterns of cells without any requirements for differential adhesive properties among cell types, two differentially labeled HeLa cell populations were co-cultured (Figure 5D). The first population was cultured on microcontact-printed patterns as before, and after an *in situ* polydopamine deposition, the second population was introduced and grew overnight. Large scale patterns could be maintained with high cell densities of each cell type, while successfully segregating the cell populations. Smaller scale patterns could also be used, although cell migration between the different areas caused mixing of the cell populations over time, a phenomenon more noticeable for smaller patterns (Figure S9).

4. Discussion

In this paper we show that by using off-the-shelf chemicals and simple fabrication strategies it is possible to provide tools for advanced cell culture experiments such as cell confinement, cell migration, cell-cell interactions and co-culture studies. Hydrogels formed from a spin-cast PVA film immersed in culture medium is an excellent and stable cell repellent material, and when PVA is tightly attached to the underlying substrate, it was shown to be stable under culture conditions. Since the cell repellent material is a hydrogel film and not a monolayer (such as PLL-g-PEG) as typically used in other cell patterning approaches [61], the surface behaviour is less dependent on the chemistry of the underlying substrate. In comparison with similar systems the use of a hydrogel film allows longer anti-fouling stability and more versatility with concern to substrate type

[48].

In our system polydopamine has three functions. Firstly it is deposited on the culture substrate to decrease the contact-angle, facilitating spin-coating, and to increase the adhesion of the PVA film. Secondly, thin polydopamine patches are microcontact printed onto PVA, providing stable cell adhesive areas. Finally, it can be deposited onto the PVA surface in cell compatible conditions, allowing for activation of the cell repellent surface.

Using polydopamine as an adhesion layer allows PVA immobilization on nearly any surface, as polydopamine deposits on most materials [42]. Additionaly, the chemistry involved is mild and does not require the use of toxic compounds, such as glutaraldehyde [36]. We were able to generate patterns on polystyrene, PDMS, PET, glass and aclar. The last material is often used to grow cells prior to embedding for electron microscopy, which potentially could open up new possibilities for correlative imaging of patterned cells with optical and electron microscopy [62]. As PVA does not contain functional groups such as amines or thiols which are known to bind covalently with polydopamine[42], the bonding at the interface between PVA and polydopamine is not known. It is plausible that non-covalent interactions such as hydrogen bonding could play a role, as PVA is known to strongly interact through hydrogen bonds [63]. In a study on polydopamine/PVA mixed films this was also indicated as a possible mechanism of interaction between polydopamine and PVA [49]. Further study is needed to determine the details of the PVA-polydopamine interaction both in the case of microcontact printing and deposition from solution.

Microcontact printed polydopamine was stable on the surface of PVA hydrogel films, confining HeLa cells in defined patterns for up to 3 weeks, and without any alterations other cell types (PC3 and HUVEC) could be patterned in the same way. It is widely described that polydopamine can also be relatively easily modified further by binding to functional molecules [56, 64] and proteins [43, 65] through amine and thiol groups. As demonstrated with PEI in this work, such modifications can provide necessary modifications for certain cell types, without the need to develop new printing processes for each molecules. Polydopamine is not only a convenient platform for further modifications, but was in fact necessary to provide sufficient adhesion of e.g. PEI to the anti-fouling PVA hydrogel. Direct patterning of proteins and polyelectrolytes has been shown to be possible on PEG-silane monolayers or on custom-made PEG/methacrylate brush polymers [**?**, **?**], but in our work both PEI and PLL were quickly rinsed away on the PVA hydrogels. Thus through microcontact printing of polydopamine a range of biomolecules could potentially be presented in a micropatterned arrangement on the anti-fouling hydrogel surface without altering the hydrogel chemistry.

In situ deposition of polydopamine was demonstrated as method to render PVA cell adhesive under cell compatible conditions. Switching a surface from generally cell repellent (i.e. cell repellent to most cell types) to cell adhesive typically requires external triggers such as temperature [66, 67] or electrical fields [68], mechanical handling [25, 69], extensive prior chemical alteration of the cell patterning materials [70] or complex

infrastructure such as printing robotics [71]. The presented method is therefore rather simple, in that only pipetting of solutions is involved and not depended on the type of co-cultured cells, as long as they are adherent. By tuning the deposition time, the cell adhesiveness of the polydopamine-modified PVA could be modified, providing further options for future studies. Polydopamine is adhesive for a range of cell types as shown in this an other work[72, 73, 74, 45].

Our approach has certain limitations that should be investigated further. It must be noted that only the first cell population is precisely patterned, while the second cell population will fill in the entire surrounding area not occupied by the first cell population after *in situ* polydopamine deposition. Further, cell migration will degrade the spatial localization of the two cell populations as the experiment progresses, as the entire surface is rendered cell adhesive. For certain applications, such as automated high-throughput single cell screening where the interaction between two cell types at pre-defined locations are of interest, this presents a challenge, and a focus of development should be the increased spatial control of both cell populations. However, since large scale cell-cell interactions and cell migration is often a phenomena one wishes to study in co-culture systems [23], this is not necessarily a significant limitation.

The cytotoxicity of *in situ* dopamine deposition, likely mediated by the formation of reactive oxygen species (ROS) during dopamine oxidation[60] is a concern. By reducing the incubation time and by introducing catalase as a reactive oxygen species scavenger we have significantly reduced the cytotoxicity as measured by the MTT assay. However, it is not entirely clear what the effect of catalase is on the *in situ* deposition of polydopamine. Polydopamine appears to form in solution, however a significant reduction in cell adhesion was observed on *in situ* modified PVA in the presence of catalase. Further investigations into the deposition of polydopamine in the presence of catalase are underway in our lab.

A second factor to consider is that *in situ* deposited polydopamine likely not only adheres to the PVA, but also coats the initial cell population. Polydopamine has been used previously to encapsulate live yeast cells [75] by polydopamine deposition in solution, which somewhat reduced the yeast cell viability and growth rate, although higher dopamine concentrations ($2\,\mathrm{mg\,mL^{-1}}$) and longer deposition times (3 hours) where used. On the other hand, no cytotoxicity was observed when myoblasts ingested polydopamine-coated liposomes[76] or when MDCK cells were exposed to polydopamine-coated Fe_2O_3 nanoparticles [77]. Polydopamine also bears a structural resemblance to melanin [78], which can be phagocytosed and degraded through lysosomal degradation by macrophages in human retinas [79]. Although these examples are not necessarily directly comparable, it appears that it is the oxidative processes, and potentially reactive intermediates involved in the formation of polydopamine, rather than the polydopamine itself that induces cytotoxicity.

The level of cytotoxicity was low enough that it did not prevent the generation of well defined co-cultures at shorter (a few hours) or longer (at least 48 hours) times. Although outside the scope of this study, it is likely that further optimizing of

factors such as dopamine concentration, deposition time, solution refreshing and medium composition will lead to further reduced cytotoxicity while still enabling functional and versatile co-cultures. In our work it was observed that the HeLa cells spread outward and the HUVEC cells somewhat retracted from the HeLa cells, consistent with other reports on HeLa/HUVEC co-culture [80]. Still, for any biological assays, cytotoxicity and other possible effects on cell biology must be assessed carefully for the cell types under study.

5. Conclusion

A system combining the cell repellent properties of poly(vinyl alcohol) and the cell adhesive properties of polydopamine was demonstrated. It was shown that polydopamine could be patterned onto dry poly(vinyl alcohol) films by microcontact printing. The patterns remained stable even when the hydrogel was hydrated, in turn enabling long-term patterned cell growth of HeLa, HUVEC, PC3 and HEK293 cells. The system was also compatible with plasma activated bonding, making it possible to integrate with PDMS devices for patterning of cell growth inside microfluidic channels. In addition to micro-contact printing, solution deposition of polydopamine was shown to activate initially cell repellent PVA, allowing cell attachment. A modified cell-compatible polydopamine deposition solution was used for *in situ* "switching" of PVA from cell-repellent to cell adhesive also during cell culturing. Finally, by combining microcontact printed polydopamine with *in situ* polydopamine deposition, a proof-of-concept patterned co-culture consisting of HUVEC and HeLa or HeLa and HeLa was demonstrated. It was demonstrated that this form of micropatterned co-cultures can allow the systematic and high-throughput investigation of interactions between different cell types.

The possibility for patterned cell cultures and patterned co-culture were realized through simple chemistry with low-cost commercially available materials and a minimum of infrastructure. These type of simple and readily available systems could allow non-specialized labs access to more advanced cell culturing methods without the need to invest in complex equipment or involved chemical procedures.

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References

[1] Maureen A. Walling and Jason R E. Shepard. Cellular heterogeneity and live cell arrays. *Chem. Soc. Rev.*, 40(7):4049–4076, Jul 2011.

[2] Martin L. Yarmush and Kevin R. King. Living-cell microarrays. *Annu Rev Biomed Eng*, 11:235–257, 2009.

[3] Krisztián Papp, Zoltán Szittner, and József Prechl. Life on a microarray: assessing live cell functions in a microarray format. *Cellular and Molecular Life Sciences*, 69(16):2717–2725, 2012.

[4] Hoyoung Yun, Kisoo Kim, and Won Gu Lee. Cell manipulation in microfluidics. *Biofabrication*, 5(2):022001, June 2013.

[5] Jorge I Rodríguez-Dévora, Bimeng Zhang, Daniel Reyna, Zhi-Dong Shi, and Tao Xu. High throughput miniature drug-screening platform using bioprinting technology. *Biofabrication*, 4(3):035001, June 2012.

[6] R. Singhvi, A. Kumar, G. P. Lopez, G. N. Stephanopoulos, D. I. Wang, G. M. Whitesides, and D. E. Ingber. Engineering cell shape and function. *Science*, 264(5159):696–698, Apr 1994.

[7] S. Huang, C. S. Chen, and D. E. Ingber. Control of cyclin d1, p27(kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol Biol Cell*, 9(11):3179–3193, Nov 1998.

[8] Shohei Yamamura, Hiroyuki Kishi, Yoshiharu Tokimitsu, Sachiko Kondo, Ritsu Honda, Sathuluri Ramachandra Rao, Masahiro Omori, Eiichi Tamiya, and Atsushi Muraguchi. Single-cell microarray for analyzing cellular response. *Anal. Chem.*, 77(24):8050–8056, Dec 2005.

[9] Kenneth L. Roach, Kevin R. King, Basak E. Uygun, Isaac S. Kohane, Martin L. Yarmush, and Mehmet Toner. High throughput single cell bioinformatics. *Biotechnol Prog*, 25(6):1772–1779, 2009.

[10] Jian Tang, Rong Peng, and Jiandong Ding. The regulation of stem cell differentiation by cell-cell contact on micropatterned material surfaces. *Biomaterials*, 31(9):2470–2476, Mar 2010.

[11] Michael T. Yang, Jianping Fu, Yang-Kao Wang, Ravi A. Desai, and Christopher S. Chen. Assaying stem cell mechanobiology on microfabricated elastomeric substrates with geometrically modulated rigidity. *Nat Protoc*, 6(2):187–213, Feb 2011.

[12] Drew M Titmarsh, Huaying Chen, Ernst J Wolvetang, and Justin J Cooper-White. Arrayed cellular environments for stem cells and regenerative medicine. *Biotechnology Journal*, 2012.

[13] Kristopher A Kilian, Branimir Bugarija, Bruce T Lahn, and Milan Mrksich. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proceedings of the National Academy of Sciences*, 107(11):4872–4877, 2010.

[14] Wei Song, Hongxu Lu, Naoki Kawazoe, and Guoping Chen. Adipogenic differentiation of individual mesenchymal stem cell on different geometric micropatterns. *Langmuir*, 27(10):6155–6162, 2011.

[15] Xingyu Jiang, Derek A. Bruzewicz, Amy P. Wong, Matthieu Piel, and George M. Whitesides. Directing cell migration with asymmetric micropatterns. *Proc Natl Acad Sci U S A*, 102(4):975–978, Jan 2005.

[16] Girish Kumar, Carlos C. Co, and Chia-Chi Ho. Steering cell migration using microarray amplification of natural directional persistence. *Langmuir*, 27(7):3803–3807, Apr 2011.

[17] P. Kingshott and H.J. Griesser. Surfaces that resist bioadhesion. *Curr. Opin. Solid State Mater. Sci.*, 4(4):403–412, 1999.

[18] R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber, and G. M. Whitesides. Patterning proteins and cells using soft lithography. *Biomaterials*, 20(23-24):2363–2376, Dec 1999.

[19] Tai Hyun Park and Michael L. Shuler. Integration of cell culture and microfabrication technology. *Biotechnol. Prog.*, 19(2):243–253, 2003.

[20] B. Lom, K. E. Healy, and P. E. Hockberger. A versatile technique for patterning biomolecules onto glass coverslips. *J. Neurosci. Methods*, 50(3):385–397, Dec 1993.

[21] A. Folch, B. H. Jo, O. Hurtado, D. J. Beebe, and M. Toner. Microfabricated elastomeric stencils for micropatterning cell cultures. *J. Biomed. Mater. Res.*, 52(2):346–353, Nov 2000.

[22] C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, and D. E. Ingber. Geometric control of cell life and death. *Science*, 276(5317):1425–1428, May 1997.

[23] H. Kaji, G. Camci-Unal, R. Langer, and A. Khademhosseini. Engineering systems for the

generation of patterned co-cultures for controlling cell–cell interactions. *BBA: Gen. Subjects*, 1810(3):239–250, 2011.

[24] Sangeeta N Bhatia, Martin L Yarmush, and Mehmet Toner. Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3t3 fibroblasts. *J. Biomed. Mater. Res.*, 34(2):189–199, 1997.

[25] Cheul H. Cho, Jaesung Park, Arno W. Tilles, Franois Berthiaume, Mehmet Toner, and Martin L. Yarmush. Layered patterning of hepatocytes in co-culture systems using microfabricated stencils. *Biotechniques*, 48(1):47–52, Jan 2010.

[26] K. Y. Lee and D. J. Mooney. Hydrogels for tissue engineering. *Chem Rev*, 101(7):1869–1879, Jul 2001.

[27] Jeanie L Drury and David J Mooney. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*, 24(24):4337–4351, 2003.

[28] Ferdinand Brandl, Florian Sommer, and Achim Goepferich. Rational design of hydrogels for tissue engineering: impact of physical factors on cell behavior. *Biomaterials*, 28(2):134–146, 2007.

[29] Christie M Hassan and Nikolaos A Peppas. Structure and applications of poly (vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods. In *Biopolymers· PVA Hydrogels, Anionic Polymerisation Nanocomposites*, pages 37–65. Springer, 2000.

[30] Nikolaos A Peppas and Shauna R Stauffer. Reinforced uncrosslinked poly (vinyl alcohol) gels produced by cyclic freezing-thawing processes: a short review. *Journal of Controlled Release*, 16(3):305–310, 1991.

[31] N.A. Peppas. *Hydrogels in Medicine and Pharmacy: Polymers*. Hydrogels in Medicine and Pharmacy. CRC Press, 1987.

[32] NA Peppas and D. Tennenhouse. Semicrystalline poly (vinyl alcohol) films and their blends with poly (acrylic acid) and poly (ethylene glycol) for drug delivery applications. *STP Pharma Sci*, 14(4):291–297, 2004.

[33] Jennifer R. Du, Sigrid Peldszus, Peter M. Huck, and Xianshe Feng. Modification of poly(vinylidene fluoride) ultrafiltration membranes with poly(vinyl alcohol) for fouling control in drinking water treatment. *Water Res.*, 43(18):4559–4568, Oct 2009.

[34] DA Barrett, MS Hartshorne, MA Hussain, PN Shaw, and MC Davies. Resistance to nonspecific protein adsorption by poly (vinyl alcohol) thin films adsorbed to a poly (styrene) support matrix studied using surface plasmon resonance. *Anal. Chem.*, 73(21):5232–5239, 2001.

[35] B. E. Rabinow, Y. S. Ding, C. Qin, M. L. McHalsky, J. H. Schneider, K. A. Ashline, T. L. Shelbourn, and R. M. Albrecht. Biomaterials with permanent hydrophilic surfaces and low protein adsorption properties. *J Biomater Sci Polym Ed*, 6(1):91–109, 1994.

[36] Thomas Peterbauer, Johannes Heitz, Michael Olbrich, and Steffen Hering. Simple and versatile methods for the fabrication of arrays of live mammalian cells. *Lab. Chip*, 6(7):857–863, Jul 2006.

[37] Andrew D. Doyle. Generation of micropatterned substrates using micro photopatterning. *Curr Protoc Cell Biol*, Chapter 10:Unit 10.15, Dec 2009.

[38] Chao-Min Cheng and Philip R. LeDuc. Micropatterning polyvinyl alcohol as a biomimetic material through soft lithography with cell culture. *Mol. Biosyst.*, 2(6-7):299–303, Jun 2006.

[39] B.E.B. Jensen, M.H. Alves, B. Fejerskov, B. Städler, and A.N. Zelikin. Surface adhered poly (vinyl alcohol) physical hydrogels as tools for rational design of intelligent biointerfaces. *Soft Matter*, 8(17):4625–4634, 2012.

[40] ML Pourciel, J. Launay, W. Sant, V. Conédéra, A. Martinez, and P. Temple-Boyer. Development of photo-polymerisable polyvinyl alcohol for biotechnological applications. *Sensor Actuat B: Chem*, 94(3):330–336, 2003.

[41] N. Kawazoe, L. Guo, M.J. Wozniak, Y. Imaizumi, T. Tateishi, X. Zhang, and G. Chen. Adipogenic differentiation of mesenchymal stem cells on micropatterned polyelectrolyte surfaces. *J. Nanosci. Nanotechnol.*, 9(1):230–239, 2009.

[42] Haeshin Lee, Shara M. Dellatore, William M. Miller, and Phillip B. Messersmith. Mussel-inspired surface chemistry for multifunctional coatings. *Science*, 318(5849):426–430, Oct 2007.

[43] H. Lee, J. Rho, and P.B. Messersmith. Facile conjugation of biomolecules onto surfaces via mussel adhesive protein inspired coatings. *Adv. Mater.*, 21(4):431–434, 2008.

[44] Martin E. Lynge, Rebecca van der Westen, Almar Postma, and Brigitte Städler. Polydopamine–a nature-inspired polymer coating for biomedical science. *Nanoscale*, 3(12):4916–4928, Dec 2011.

[45] S.H. Ku, J.S. Lee, and C.B. Park. Spatial control of cell adhesion and patterning through mussel-inspired surface modification by polydopamine. *Langmuir*, 26(19):15104–15108, 2010.

[46] Hsiu-Wen Chien, Wei-Hsuan Kuo, Meng-Jiy Wang, Shiao-Wen Tsai, and Wei-Bor Tsai. Tunable micropatterned substrates based on poly(dopamine) deposition via microcontact printing. *Langmuir*, 28(13):5775–5782, Apr 2012.

[47] Kang Sun, Lusheng Song, Yunyan Xie, Dingbin Liu, Dong Wang, Zhuo Wang, Wanshun Ma, Jinsong Zhu, and Xingyu Jiang. Using self-polymerized dopamine to modify the antifouling property of oligo(ethylene glycol) self-assembled monolayers and its application in cell patterning. *Langmuir*, 27(10):5709–5712, May 2011.

[48] Kang Sun, Yunyan Xie, Dekai Ye, Yuyun Zhao, Yan Cui, Fei Long, Wei Zhang, and Xingyu Jiang. Mussel-inspired anchoring for patterning cells using polydopamine. *Langmuir*, 28(4):2131–2136, 2011.

[49] Y. Zhang, B. Thingholm, K.N. Goldie, R. Ogaki, and B. Städler. Assembly of poly (dopamine) films mixed with a non-ionic polymer. *Langmuir*, 2012.

[50] L. Hosta-Rigau, B.E.B. Jensen, K.S. Fjeldsø, A. Postma, G. Li, K.N. Goldie, F. Albericio, A.N. Zelikin, and B. Städler. Surface-adhered composite poly (vinyl alcohol) physical hydrogels: Polymersome-aided delivery of therapeutic small molecules. *Adv. Healthc. Mater.*, 2012.

[51] Dong Qin, Younan Xia, and George M Whitesides. Soft lithography for micro-and nanoscale patterning. *Nature protocols*, 5(3):491–502, 2010.

[52] Yanan Yue, Fan Jin, Rui Deng, Jinge Cai, Yangchao Chen, Marie Lin, Hsiang-Fu Kung, and Chi Wu. Revisit complexation between dna and polyethylenimineeffect of uncomplexed chains free in the solution mixture on gene transfection. *Journal of Controlled Release*, 155(1):67–76, 2011.

[53] R. A. Durst and B. R. Staples. Tris-tris-hcl: a standard buffer for use in the physiologic ph range. *Clin Chem*, 18(3):206–208, Mar 1972.

[54] Johannes Schindelin, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, Curtis Rueden, Stephan Saalfeld, Benjamin Schmid, Jean-Yves Tinevez, Daniel James White, Volker Hartenstein, Kevin Eliceiri, Pavel Tomancak, and Albert Cardona. Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9(7):676–682, Jul 2012.

[55] David Neas and Petr Klapetek. Gwyddion: an open-source software for SPM data analysis. *Central European Journal of Physics*, 10:181–188, 2012.

[56] Hsiu-Wen Chien and Wei-Bor Tsai. Fabrication of tunable micropatterned substrates for cell patterning via microcontact printing of polydopamine with poly(ethylene imine)-grafted copolymers. *Acta Biomater*, 8(10):3678–3686, Oct 2012.

[57] Jamil El-Ali, Peter K Sorger, and Klavs F Jensen. Cells on chips. *Nature*, 442(7101):403–411, 2006.

[58] F. Bernsmann, V. Ball, F. Addiego, A. Ponche, M. Michel, J.J.A. Gracio, V. Toniazzo, and D. Ruch. Dopamine- melanin film deposition depends on the used oxidant and buffer solution. *Langmuir*, 27(6):2819–2825, 2011.

[59] E. Herlinger, R.F. Jameson, and W. Linert. Spontaneous autoxidation of dopamine. *J. Chem. Soc., Perkin Trans. 2*, (2):259–263, 1995.

[60] M.V. Clement, L.H. Long, J. Ramalingam, and B. Halliwell. The cytotoxicity of dopamine may be an artefact of cell culture. *J. Neurochem.*, 81(3):414–421, 2002.

[61] Gabor Csucs, Roger Michel, Jost W Lussi, Marcus Textor, and Gaudenz Danuser. Microcontact printing of novel co-polymers in combination with proteins for cell-biological applications.

Biomaterials, 24(10):1713–1720, 2003.

[62] Gavin E Murphy, Kedar Narayan, Bradley C Lowekamp, Lisa M Hartnell, Jurgen AW Heymann, Jing Fu, and Sriram Subramaniam. Correlative 3d imaging of whole mammalian cells with light and electron microscopy. *J. Struct. Biol.*, 176(3):268–278, 2011.

[63] Hongbin Li, Wenke Zhang, Weiqing Xu, and Xi Zhang. Hydrogen bonding governs the elastic properties of poly (vinyl alcohol) in water: single-molecule force spectroscopic studies of pva by afm. *Macromolecules*, 33(2):465–469, 2000.

[64] Kyungtae Kang, Insung S. Choi, and Yoonkey Nam. A biofunctionalization scheme for neural interfaces using polydopamine polymer. *Biomaterials*, 32(27):6374–6380, Sep 2011.

[65] Ryosuke Ogaki, Dines T. Bennetsen, Ilko Bald, and Morten Foss. Dopamine-assisted rapid fabrication of nanoscale protein arrays by colloidal lithography. *Langmuir*, 28(23):8594–8599, Jun 2012.

[66] M. Hirose, M. Yamato, O. H. Kwon, M. Harimoto, A. Kushida, T. Shimizu, A. Kikuchi, and T. Okano. Temperature-responsive surface for novel co-culture systems of hepatocytes with endothelial cells: 2-d patterned and double layered co-cultures. *Yonsei Med J*, 41(6):803–813, Dec 2000.

[67] M. Yamato, O. H. Kwon, M. Hirose, A. Kikuchi, and T. Okano. Novel patterned cell coculture utilizing thermally responsive grafted polymer surfaces. *J Biomed Mater Res*, 55(1):137–140, Apr 2001.

[68] Muhammad N Yousaf, Benjamin T Houseman, and Milan Mrksich. Using electroactive substrates to pattern the attachment of two different cell populations. *Proc. Natl. Acad. Sci. USA*, 98(11):5992–5996, 2001.

[69] Dylan Wright, Bimalraj Rajalingam, Selvapraba Selvarasah, Mehmet R Dokmeci, and Ali Khademhosseini. Generation of static and dynamic patterned co-cultures using microfabricated parylene-c stencils. *Lab on a Chip*, 7(10):1272–1279, 2007.

[70] Paula M Mendes. Stimuli-responsive surfaces for bio-applications. *Chemical Society Reviews*, 37(11):2512–2529, 2008.

[71] Feng Xu, Jonathan Celli, Imran Rizvi, Sangjun Moon, Tayyaba Hasan, and Utkan Demirci. A three-dimensional in vitro ovarian cancer coculture model using a high-throughput cell patterning platform. *Biotechnology journal*, 6(2):204–212, 2011.

[72] Seonki Hong, Keum Yeon Kim, Hwang Jin Wook, Sung Young Park, Kang Dae Lee, Dong Yun Lee, and Haeshin Lee. Attenuation of the in vivo toxicity of biomaterials by polydopamine surface modification. *Nanomedicine*, 6(5):793–801, 2011.

[73] Sook Hee Ku and Chan Beum Park. Human endothelial cell growth on mussel-inspired nanofiber scaffold for vascular tissue engineering. *Biomaterials*, 31(36):9431–9437, 2010.

[74] Sook Hee Ku, Jungki Ryu, Seon Ki Hong, Haeshin Lee, and Chan Beum Park. General functionalization route for cell adhesion on non-wetting surfaces. *Biomaterials*, 31(9):2535–2541, 2010.

[75] Sung Ho Yang, Sung Min Kang, Kyung-Bok Lee, Taek Dong Chung, Haeshin Lee, and Insung S Choi. Mussel-inspired encapsulation and functionalization of individual yeast cells. *Journal of the American Chemical Society*, 133(9):2795–2797, 2011.

[76] Rebecca van der Westen, Leticia Hosta-Rigau, Duncan S Sutherland, Kenneth N Goldie, Fernando Albericio, Almar Postma, and Brigitte Städler. Myoblast cell interaction with polydopamine coated liposomes. *Biointerphases*, 7(1-4):1–9, 2012.

[77] Jingyu Si and Hua Yang. Preparation and characterization of bio-compatible fe¡ sub¿ 3¡/sub¿ o¡ sub¿ 4¡/sub¿@ polydopamine spheres with core/shell nanostructure. *Materials Chemistry and Physics*, 128(3):519–524, 2011.

[78] Jürgen Liebscher, Radosław Mrówczyński, Holger A Scheidt, Claudiu Filip, Niculina D Hădade, Rodica Turcu, Attila Bende, and Sebastian Beck. The structure of polydopamine–a never ending story? *Langmuir*, 2013.

[79] Peter Gouras, Kristy Brown, Lena Ivert, and Martha Neuringer. A novel melano-lysosome in the

retinal epithelium of rhesus monkeys. *Experimental eye research*, 93(6):937–946, 2011.

[80] Hirokazu Kaji, Takeshi Yokoi, Takeaki Kawashima, and Matsuhiko Nishizawa. Controlled cocultures of hela cells and human umbilical vein endothelial cells on detachable substrates. *Lab Chip*, 9(3):427–432, 2009.