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Regenerating the cell resistance of micromolded PEG hydrogels

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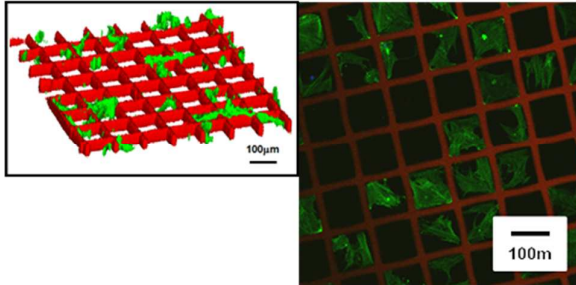
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Graphical Abstract text

Micromolded hydrogels lose non-fouling properties and enable cell adhesion and migration on the gel (left). We develop a method to regenerate the cell resistance of the gels resulting in robust resistance to cell adhesion (right).



Abstract

Polydimethylsiloxane stamp materials used during soft lithography undermine the non-fouling behaviour of bio-inert PEG-based hydrogels, resulting in increased protein adsorption and cell adhesion and migration on the gel. This previously unreported phenomenon undermines the function of lab on a chip devices that require the device to be bio-inert, and slows the implementation of promising micromolding and imprinting methods for 3D culture and commercial cell culture systems. We illustrate that the degree of cell adhesion and protein adsorption to the gels correlates with the amount of residual stamp material remaining at the hydrogel interface after fabrication. After identifying this previously unreported phenomenon, we screened multiple polymer cleaning/fabrication techniques in order to maintain/restore the non-fouling properties of the gels including PDMS curing and extraction, use of other common soft lithography stamp materials, post-fabrication cleaning of the hydrogels, and changing the composition of the hydrogel. The optimal solution was determined to be incorporation of reactive sites into the hydrogel during micromolding followed by grafting of PEG macromers to these sites post-fabrication. This treatment resulted in micromolded hydrogels with robust cell resistant properties. Broadly, this work identifies and solves a previously unreported problem in hydrogel micromolding, and specifically reports the development of a cell culture platform that when combined with video microscopy enables high-resolution *in situ* study of single cell behaviour during *in vitro* culture.

Key words: Cell adhesion, colony forming unit assay (CFU), soft lithography, PDMS, hydrogel

1. Introduction

Micromolding of non-fouling hydrogels is a commonplace strategy for generating micropatterned cell culture substrates and microfluidic devices for lab- and organ- on-a-chip applications¹⁻⁶. These devices are meant to create geometries to pattern/confine/route cells and/or biomolecules against a background that resists protein adsorption and cellular attachment^{1-3, 5, 7-11}. They are often fabricated through soft lithography by creating an elastomeric stamp with the inverse pattern, filling the features of the stamp with a hydrogel precursor solution and crosslinking the solution through UV-initiated free radical polymerization. Upon demolding, a hydrogel with the desired micropatterning is produced^{1-4, 9, 11}. Hydrogel devices are most commonly fabricated from poly(ethylene glycol) (PEG) due to its non-fouling properties, the commercial availability of monomers, ease of fabrication, and long history of use in the biomaterials community^{1-4, 9, 11}.

The most common soft lithography stamp material is crosslinked polydimethylsiloxane (PDMS) (usually Sylgard 184) due to its flexible and elastomeric nature, low surface energy which facilitates demolding, and its ability to make conformal adhesions to an underlying substrate^{1-3, 6, 9, 11-15}. In most previously published protocols the non-fouling hydrogels are micromolded with the PDMS stamp and it is assumed that the non-fouling character of the hydrogel is retained^{1-3, 6}. In this contribution however, we determine that contact with PDMS and other common stamp materials used in soft lithography drastically undermines the non-fouling properties of the bio-inert hydrogels by contaminating the hydrogel surface with residual stamp material that enables protein adsorption and cell adhesion and migration. Such changes to the interfacial properties of the hydrogel have detrimental effects on the function of lab-on-a-chip devices which require a bio-inert background, and can yield incorrect conclusions in patterned hydrogel studies that purport the examination of isolated cell behaviour.

Specifically, we are interested in generating this hydrogel microwell array platform in order to improve upon the colony forming unit (CFU) assay for adherent cells such as mesenchymal stem cells (MSCs) found in bone marrow aspirate. Currently, bone marrow aspirate is plated on 2D tissue culture plastic and the adherent cells are cultured in medium that promotes the specific survival and proliferation of the stem cells. After 2-3 weeks of culture, colonies of MSCs will appear. The total number and size

of the colonies provides researchers the ability to estimate the number of stem cells in the original bone marrow sample as well as the proliferative capacity of the cells¹⁶⁻¹⁸. However, the traditional CFU assay has several limitations: 1) These cells are highly mobile thus during multiple weeks of culture one could envision cell migration that could result in multiple colonies arising from the progeny of a single stem cell, and 2) neighbouring colonies will often merge resulting in difficulty in accurately assessing the number of colonies and limiting the culture to a relatively low threshold of cell plating densities. For these reasons we have developed a hydrogel microwell array platform that will isolate individual cells and their progeny during culture. When this platform is combined with video microscopy it guarantees an accurate count of stem cell colonies and enables the high-resolution measurement of the proliferation capacity of individual cells. Since we are particularly interested in using this platform to improve the CFU assay of MSCs in bone marrow aspirate, we chose to utilize adult bone marrow-derived and expanded MSCs as the model cell in this work. The PDMS-molded hydrogel microwell arrays in this research were initially unable to confine individual MSCs during culture thus any data collected from this microwell array platforms – or others produced using similar protocols purporting isolated cell behaviour – was subject to skepticism. Furthermore, micromolded platform have recently been developed to generate highly defined cell microenvironments (or niches) by functionalizing non-fouling hydrogels with specific biomolecules¹⁹⁻²¹. However, one could envision similar sorption events occurring on these materials thus exposing cells to unexpected molecules on the substrate surface and thus undermining conclusions drawn from these studies.

Since PDMS micromolding is a well established, easily accessible, and user-friendly technology, our goal in this research was to identify methods that maintain or regain the cell resistant properties of the micromolded non-fouling hydrogels. To do so we explored four possible strategies:

1. Systematically vary PDMS processing to minimize transfer of stamp material during hydrogel fabrication,
2. Use other common stamp materials as an alternative to PDMS,
3. Employ strategies to prevent stamp material transfer during hydrogel fabrication or remove residual stamp material after hydrogel fabrication, and
4. Use other gel chemistries that may retain non-fouling character despite micromolding.

Herein, we are the first to report the unwanted stamp/hydrogel interactions that occur during the microfabrication of bio-inert hydrogels, and we identify a technique that successfully regenerates the non-fouling behaviour of the micromolded hydrogels.

2. Results

2.1. PDMS stamping enables cell adhesion to non-fouling PEG hydrogels

Hydrogel microwell arrays were produced through micromolding using PDMS stamping according to previously reported fabrication protocols^{2,3,11}. The microwell arrays were fabricated on polystyrene that was exposed to argon gas plasma, a treatment that results in the formation of peroxides and hyperoxides on the polymer surface and then enables surface grafting of molecules through free radical induced polymerization²²⁻²⁵. In this work, the reactive sites were used to covalently couple the hydrogels microwell arrays to the underlying polystyrene substrate.

During microwell array fabrication a PDMS stamp patterned with a micropillar array was brought into contact with the polystyrene substrate so that the tops of the pillars made conformal adhesions with the underlying polystyrene. A monomer precursor solution was perfused into the stamp via capillary action, and the hydrogel precursor solution was cured through exposure to UV light. Upon demolding we are left with a hydrogel microwell array covalently linked to the underlying polystyrene substrate. Due to the conformal adhesion of the stamp with the polystyrene, monomer solution was excluded from these regions, resulting in a microwell array with exposed polystyrene floors separated by PEG-hydrogel walls. The original hydrogel formulation we used in this study is abbreviated PEG(1000) and is a gel system which has previously been published as non-fouling (refer to the Experimental section for details on polymer composition)³. Since relatively hydrophobic species such as oestrogen have been shown to accumulate at PDMS interfaces and be absorbed into the stamp material we used Irgacure 2959 as the free radical initiator as it is the one of the most hydrophilic and cytocompatible²⁶. In this work, square microwells with a footprint of 100x100 μm , a wall width of 20 μm , and a wall height of 40 μm were fabricated. The fabrication procedure is schematically shown in Figure 1A. These hydrogel arrays are robustly stable showing no delamination for greater than two weeks in cell

culture conditions.

Cell migration between wells of the microwell array was observed using time-lapse video microscopy (Figure 2A). From the two dimensional phase contrast images we could not verify if cells were climbing over the PEG walls or under the walls at sites of poor substrate/wall adhesion, or a combination of the two. Confocal microscopy was used to better assess cellular migration between microwells. Figure 2B presents three confocal z-slices. The first slice ($z=0\ \mu\text{m}$) is at the polystyrene interface, the second slice ($z=25\ \mu\text{m}$) is approximately halfway up the wall, and the third slice ($z=50\ \mu\text{m}$) is in a focal plane above the height of the PEG(1000) wall. The hydrogel walls (red) show no overlap with the actin cytoskeleton of the cells (green) at the $z=0\ \mu\text{m}$ and $z=25\ \mu\text{m}$ z-slices. However, at the $z=50\ \mu\text{m}$ slice we observe co-localization of the green and red fluorescent signal indicating that cells are on top of the hydrogel walls. Figure 2C presents a confocal z-stack of aMSCs in the hydrogel microwell arrays where we observe cells spanning multiple microwells. No cells were observed to migrate under the walls at sites of poor substrate/wall adhesion. Furthermore, cells were observed settling and adhering directly to the top of the hydrogel walls during seeding, and the cells present on top of the walls were often observed migrating *along* the length of the walls for hundreds of microns before entering into a new well. These data indicate that cells are migrating between microwells by adhering to and migrating on the PEG(1000) hydrogel walls. However, by changing the microwell array fabrication process we were able to fabricate micromolded hydrogels with robust non-fouling properties that eliminated the undesired cell migration between wells as observed in Figure 2D. In this manuscript we first focus on determining the mechanism that enables cell adhesion to these otherwise non-fouling gels and then identify a strategy to regenerate the non-fouling properties of the micromolded hydrogels.

Cell adhesion to and migration along the PEG(1000) hydrogels that enabled inter-well cell migration was an unexpected phenomena due to previous reports illustrating the non-fouling properties of PEG(1000) gels³. We first sought to confirm that PEG(1000) hydrogels were resistant to aMSC adhesion, and then ascertain the mechanism allowing cellular adhesion to and migration along the walls. We developed a surrogate screening assay where cells are seeded onto planar hydrogel slabs that were contacted with the stamp material of interest during photopolymerization. Upon seeding

with the aMSCs, these planar surfaces provide a platform to quickly and easily assess cell/hydrogel interactions. The fabrication strategy to generate the planar hydrogels is schematically shown in Figure 1B.

In our first set of cell adhesion experiments the planar hydrogel slabs were fabricated in contact with three different materials: glass coverslips, Teflon, and PDMS/80/4/4 (the numbers following PDMS describe the cure temperature of the stamp, the cure time, and the extraction time. For instance, PDMS/80/4/4 was cured at 80°C for 4 hours followed by 4 hours of extraction, 2 hours in hexane followed by 2 hours in ethanol. PDMS/120/48/48 would refer to PDMS that was cured at 120°C for 48 hours and then extracted for 48 hours, 24 hours in hexane followed by 24 hours in ethanol). Hydrogels fabricated in contact with an inert argon gas atmosphere acted as the negative control while tissue culture polystyrene (TCPS) and polystyrene which has been argon plasma treated, contacted with a blank PDMS stamp, and UV irradiated (to mimic the floor of hydrogel microwell arrays) were used as positive controls. aMSCs incubated on TCPS adhered and spread, and have the classic aMSC spindle-shape morphology (Figure 3A). Similarly, the aMSCs incubated on polystyrene that has been treated to mimic the floor of the microwells showed similar adhesion characteristics and spindle-shape cell morphology (Figure 3B). Furthermore, cells were able to grow to confluence on these materials, illustrating their cytocompatibility. Hydrogel slabs photopolymerized against argon gas, glass, and Teflon showed essentially no cell adhesion indicating that this hydrogel is resistant to the adhesion of aMSCs. Any cells that may have been loosely adherent to these surfaces had a rounded morphology indicating poor cell/material interactions (Figure 3C). Cells incubated on the planar hydrogel slabs that were contacted with PDMS/80/4/4 during photopolymerization were able to adhere more readily than to the negative control; however, instead of adhering as discrete cells they adhered as cell clusters to the hydrogel and had only a partially spread morphology (Figure 3D). As observed through time-lapse video microscopy, the cells adhered as clusters because their rate of adhesion to the hydrogel from suspension was slower than the rate at which they formed cell/cell junctions with neighbouring cells resulting in the formation of suspended cell clusters before adhesion to the hydrogel.

This clustering behaviour made assessing the degree of cell adhesion to the hydrogels difficult as the

cell clusters varied widely in size; some clusters contained a very large number of cells while others contained only one or two. Therefore, when quantifying cell adhesion to the planar hydrogel slabs we chose to quantify it as "adherent cell clusters per micrograph." This is an appropriate rubric to measure cell adhesion to the planar hydrogels, as it provides a reliable method to quickly assess cell/material interactions. Furthermore, our ultimate goal in this research is to find strategies to maintain/restore the non-fouling behaviour of the gels, i.e. making a gel which shows essentially no cell cluster attachment as observed in the case of the negative control, a goal which this rubric can measure. Furthermore, after using the planar hydrogel slab experiments to identify possible strategies for maintaining/regenerating the cell resistant properties of the hydrogels, promising techniques can be used to fabricate hydrogel microwell arrays to see if they are successful at eliminating cell migration within the hydrogel microwell arrays.

Quantification of adherent cell clusters to hydrogel slabs is presented in Figure 3E. Gels contacted with argon gas during gelation adhered essentially no cells, and cell adhesion to glass- and Teflon-contacting gels was not significantly different. Gels contacted with PDMS/80/4/4 during photopolymerization show a statistically greater population of adherent cell clusters than the other conditions. These data indicate that the gels can be photopolymerized in contact with some solid interfaces and still retain their cell resistant properties suggesting that the cell adhesion to the micromolded PEG(1000) hydrogels is due to some unique characteristic of the PDMS versus the other contact materials explored in this set of experiments.

2.2. PDMS processing reduces but does not eliminate cell adhesion to planar hydrogels

We hypothesize that PDMS species are transferring to the hydrogel interface during micromolding resulting in a hydrogel surface contaminated with PDMS, and this surface contamination is what is enabling cell adhesion. If PDMS contamination of the hydrogel interface is enabling cell adhesion to the gel, additional PDMS processing may reduce or eliminate this unwanted behaviour; therefore, we cured the PDMS at increased temperatures for longer periods of time in order to drive the crosslinking reaction further to completion and extracted the stamps for longer times in order to more fully remove unreacted species^{23, 26-31}.

PDMS materials were cured under four different conditions: 80°C for 4 hours, 120°C for 48 hours, 150°C for 48 hours, and 180°C for 48 hours. All stamps were clear and colourless after curing except 180°C-cured stamps were yellow in colour indicating thermal degradation. The 180°C-cured stamps were not used for further testing. The stamps were then extracted for various lengths of times in good solvents to remove unreacted species²⁸. After extraction PDMS/80/4/4 stamps lost approximately 2 wt% while PDMS/80/48/48 lost $4.45 \pm 0.10\%$, PDMS/120/48/48 lost $4.08 \pm 0.24\%$, and PDMS/150/48/48 lost $3.81 \pm 0.16\%$ of their mass. Each mean is statistically different indicating that increased curing time/temperature reduces the amount of extractables in the PDMS; however, all PDMSs contain ~4 wt% of extractables regardless of cure temperature/time. To ensure that the 48-hour extraction protocol was removing all extractables from the stamps, the extraction was repeated two more times. The stamps did not lose statistically significant additional amounts of mass during the second and third extraction, indicating that all extractables that could be removed with this protocol were removed within a single round of extraction.

Planar PEG(1000) hydrogel slabs were fabricated in contact with the PDMS/80/48/48 and PDMS/150/48/48 featureless stamps their surface composition was probed through XPS analysis. These data are compared to PEG(1000) slabs that were fabricated in contact with an inert argon gas atmosphere as control. Survey scans of PDMS-contacted hydrogels identified carbon, oxygen, and silicon species at the hydrogel interface (Figure 4A). The most prominent peaks are the C1s and O1s peaks that arise from the PEG hydrogel. As the hydrogel is non-conducting, charge correction was used adjust the C1s peak to the literature-reported value of 286.5 eV³². After charge correction, the smaller peaks present in the survey scan closely align with published values of the placement of PDMS peaks corroborating the idea that residual stamp material is contaminating the surface of the PEG hydrogel³². Figure 4B shows an enlarged region of the survey scan's Si2p peak, and it can be seen that the amount of silicon at the surface varies with the material that the gel was contacted with during fabrication. Specifically hydrogels that were contacted with the less cured PDMS/80/48/48 materials have the largest peak, hydrogels contacted with PDMS/150/48/48 have a smaller peak, and the argon contacted materials have a small yet non-zero Si2p peak. These data indicate that the quantity of silicone on the hydrogel surfaces varies with the processing of the PDMS stamps, and that larger cure times and thorough extraction results in less PDMS contamination of the hydrogel.

Interestingly there are small silicon peaks on the gels that were fabricated in contact with an inert argon atmosphere (controls). We believe this PDMS was deposited from the vapour phase during the lyophilization step from the PDMS O-ring or the silicone grease used to seal the vacuum desiccator during transport as has been reported elsewhere³³⁻³⁵. High-resolution XPS spectra were collected and analysed to calculate the composition of the hydrogel surface, and it was determined that hydrogel surfaces polymerized in contact with PDMS/80/48/48 contained 6.35 ± 0.15 % silicon and hydrogel surfaces polymerized in contact with PDMS/150/48/48 contained 3.9 ± 0.09 % silicon.

Planar hydrogel slabs were fabricated in contact with the different PDMSs, and aMSCs adhered to all planar hydrogel slabs that contacted PDMS during photopolymerization regardless of stamp processing (Figure 4A-C). However, the degree of cell spreading observed on the PDMS/150/48/48-contacting gels was less than on the other test surfaces indicating poorer cell/material interactions³⁶. The adhesion of cell clusters to these hydrogels was quantified (Figure 4D). Gels contacting PDMS/80/4/4 and PDMS/80/48/48 stamps showed the highest degree of cell adhesion, which was statistically greater than all other treatments. These data indicate that cell adhesion to slabs can be minimized - but not eliminated - by PDMS processing, and that highly cured/extracted stamps result in hydrogels to which fewer cells clusters can adhere.

To determine if cell adhesion to these hydrogels is due to the inability of the gels to resist protein deposition the hydrogel surfaces were exposed to a solution of fluorescently tagged fibronectin, an ECM protein which is commonly used as a growth surface for aMSCs during culture. From these studies we observed that gels fabricated in contact with PDMS/80/48/48 adsorbed ~65% of the protein which was adsorbed by the TCPS positive control. However, as the stamps were cured and extracted for prolonged times less protein adsorption was observed on the gels. In fact, the PDMS/150/48/48-contacted hydrogels shows no statistically significant increase in protein deposition over the negative control which may explain the minimal spreading exhibited by cell clusters adhering to the PDMS/150/48/48-contacted gels (Figure 4E). Although smaller quantities of fibronectin adsorbed to the PDMS/150/48/48, the increased curing resulted in substantially stiffer gels that did not form sufficient conformal adhesions to the underlying substrate. The microwells produced using

the PDMS/150/48/48 gels had a thin PEG skin on the bottom of the wells making the arrays unfit for this application (data not shown). Therefore, the PDMS/150/48/48 stamps were not pursued further.

These data strongly suggest that the interwell cell migration observed in the hydrogel microwell arrays is caused by the deposition of hydrophobic PDMS species to the PEG(1000) hydrogel interface during photopolymerization that then enables protein adsorption and cell adhesion and migration on the hydrogel. Furthermore, curing the PDMS at higher temperatures and extracting for longer times reduces but does not eliminate cell attachment to the hydrogels.

2.3. Exploring strategies to maintain/restore the non-fouling properties of planar PEG-hydrogels

In this section we examine several different treatments in an attempt to maintain/restore the non-fouling properties of the gels. We look at using alternative stamp materials, the addition of a soluble mold release agent to the hydrogel precursor solution, sonication of the gels post-fabrication, and variations to the hydrogel chemistry.

Although PDMS is by far the most commonly used stamping material, other materials have also been reported in the literature such as polyurethane-based Norland Optical Adhesives (NOA 60) and perfluoropolyethylethers such as Fluorolink (FL)³⁷⁻⁴¹. To assess if these other stamp materials provide an acceptable alternative for the micromolding of non-fouling hydrogels, cell adhesion experiments to PEG(1000) hydrogel slabs produced in contact with featureless NOA 60 and FL stamps were performed.

Cell cluster adhesion of aMSCs was seen on the NOA 60- and FL-contacting hydrogels, indicating that these materials are also not suitable for the micromolding of non-fouling hydrogels when used as stamps. Cell adhesion results are quantified in Figure 5A. It was found that neither the NOA 60- nor the FL-exposed gels had statistically fewer cell clusters attached than the PDMS-contacted samples, and all treatments resulted in gels which adhered a statistically larger population of cells than the negative control. Furthermore, the NOA 60 and FL stamps are stiffer and less able to form conformal adhesion to the underlying substrate resulting in poorer replication of patterning during micromolding.

Therefore, we continued working with PDMS materials and began exploring methods for preventing the changes in the hydrogel surface chemistry or regenerating the desired properties post-fabrication.

Mold release agents are commonly used in the polymer processing field as low molecular weight additives which facilitate detachment of the polymer after the molding procedure. We sought to employ a soluble mold release agent, sodium dodecyl sulfate (SDS), that one could envision self assemble along the hydrophobic PDMS mold during perfusion of the precursor solution and blocking the transfer of PDMS oligomer to the PEG(1000) hydrogel surface. After fabrication the excess SDS could be removed by extracting the gel in ethanol and water resulting in a contaminant free hydrogel interface. Sodium dodecyl sulfate was added to the polymer precursor solution at concentrations between 0 and 5 wt% based on the mass of the PEG(1000) macromer. At low SDS concentrations (≤ 1 wt%) no changes in cell cluster adhesion was observed. Interestingly, when 2% SDS was added we observed the opposite trend; we saw increased cell cluster adhesion to the gels. One possible explanation for this behavior is that although the SDS did accumulate at the PDMS interface it actually helped solubilize any PDMS oligomer at the interface thus facilitating its transfer to the hydrogel. At 5% SDS we observed the expected drop in cell adhesion to the gels, and the cell attachment was not statistically different than the negative control (Figure 5B). Based on these data, we will later attempt to regenerate the non-fouling capacity of the hydrogel microwells arrays by adding 5% SDS to the monomer solution.

PEG(1000) hydrogel slabs were also sonicated in an attempt to remove physically adsorbed contaminants from the gel surface post-fabrication. Gels were sonicated in absolute ethanol, 50 vol% ethanol in DI water, or 100% DI water. Less polar solvents were not used as they would damage the polystyrene substrate. Planar hydrogels sonicated in either 50% ethanol or DI water showed no statistical difference in their cell adhesion as compared to the negative control. These data indicate that sonication for prolonged periods of time in a solution that contains water regenerates the cell resistant properties of the PEG(1000) gels (Figure 5C), and therefore we will later sonicate hydrogel microwell arrays in an attempt to restore their non-fouling properties.

Last, we explored if varying the chemical composition of the hydrogel could generate a material that

retained its non-fouling behaviour despite being exposed to PDMS during gelation. Five gel systems were explored as an alternative to the PEG(1000) hydrogel: a PEG hydrogel with an average molecular weight of 2000 between crosslinks (PEG(2000)), a PEG hydrogel copolymerized with a PEG-monomethacrylate in order to generate a brush structure (co10), a PEG hydrogel copolymerized with methacrylic acid and then grafted with PEG-monoamine (g10), a sulfobetaine hydrogel (SB), and a polyacrylamide hydrogel (PA) (refer to the Experimental section for details on hydrogel synthesis). In order to be useful as micromolded devices, gels must both retain their shape after fabrication and possess the desired non-fouling properties. To assess the moldability of these gels they were fabricated into hydrogel microwell arrays and equilibrated in a cell culture environment. Microwell walls fabricated from PEG(2000) exhibited a large degree of waviness illustrating that this system is not useful for the fabrication of hydrogel microwell arrays. Therefore, this gel system was not used in cell culture studies. When more than 10 vol% of the co-monomers were added to the co10 or g10 copolymers these micromolded hydrogels also exhibited visible waviness indicating that the 10 vol% of co-monomer is the maximum that can be incorporated. The amount of crosslinker used to generate the SB and PA hydrogels is the minimum amount that can be used before these micromolded gels also begin to show visible waviness.

Planar hydrogel slabs of co10, g5, g10, SB, and PA were fabricated in contact with both PDMS/80/48/48 and PDMS/150/48/48 and the cell cluster adhesion was assessed (Figure 5D). Interestingly, the direct incorporation of the monomethacrylate molecules (the co10 hydrogel) resulted in an increase in cell cluster adhesion to the planar hydrogel slabs. The SB and PA hydrogels showed similar amounts of cell adhesion as the PEG(1000) gels indicating that these alternative gel system are equally susceptible to the changes in cell resistance upon micromolding. However, the g5 and g10 gels exhibited drastically reduced cell cluster adhesion resulting in hydrogel slabs that showed no statistical difference in cell adhesion compared to the negative control. From these data we will produce microwell arrays using the g5 and g10 polymer chemistries in order to test if this technique restores the bio-inert properties of the micropatterned gels.

2.4. Regenerating the non-fouling properties of 3D micromolded PEG hydrogels

By exploring aMSC adhesion to planar PEG hydrogel slabs we have identified three techniques that maintaining/regenerating the non-fouling behavior of 2D planar PEG-based hydrogels: 1) incorporation of 5% SDS as a mold release agent, 2) sonication in 50% ethanol or pure DI water for 2 hours, and 3) grafting PEG(750)amine to carboxylic acid groups in the hydrogel post-fabrication (the g5 and g10 gels). However, it is necessary to verify that these approaches also prevent cell adhesion to the gels which are micromolded into a three dimensional structure.

aMSCs were seeded into microwell arrays fabricated using five different conditions as described in Figure 6. Using time-lapse and confocal microscopy we observed interwell cell migration in all arrays except the array fabricated from the g10 hydrogel (Figure 6A-C). Cells seeded into the g10 microwell array showed excellent confinement to their wells, no cell migration along the length of the walls was seen, and no interwell cell migration was observed (Figure 6C). Using time-lapse video microscopy the degree of interwell cell migration was quantified as a "cell migration parameter" which is defined as the number of interwell cell migration events/100 cells/hr, and the results from this study are presented in Figure 6D. We observed that all micromolded systems have unacceptably high levels of interwell cell migration except the array which was fabricated from the g10 hydrogel using a PDMS/120/48/48 stamp; no interwell cell migration was observed in this system indicating that we have generated a hydrogel which retains its cell-resistant character despite being micromolded in contact with a PDMS stamp during fabrication, and confinement of cells was confirmed through video microscopy for at least 10 days in culture when the experiment was terminated.

We then utilized the cell-resistant microwell arrays along with time-lapse video microscopy to analyse the real-time behaviour of individual MSCs and their progeny for 48 hours in culture (Figure 7). In Figure 7A (left panel) we see a well that contained a single cell at $t = 0$ and that the same well contained four cells at $t = 48$ hours (right panel). The behavior of 50 wells with single occupants at $t = 0$ were tracked for 48 hours. The number of cells in each of these 50 wells was quantified at the end of the experiment and compiled into the histogram presented in Figure 7B. From these data we see large variability in the proliferation potential of the individual MSCs. 50% of the cells did not proliferate during the course of the experiment while a small number of cells underwent multiple population doublings resulting in wells containing up to 6 cells at the end of the experiment. Furthermore, using

time lapse microscopy we tracked the proliferation kinetics of these cells over the 48 hour experiment and the data for 10 of these cells which again illustrates heterogeneity in the single cell proliferative potential of the MSCs (Figure 7C).

3. Discussion

In this research we fabricated high aspect ratio and densely packed hydrogel microwell arrays where the microwells have well bottoms of an exposed and cell adhesive polystyrene separated by walls of PEG(1000) hydrogel. This lab on a chip device was intended to isolate individual cells and their progeny during culture thus enabling real time and high-resolution study of individual cells in culture, especially rare cell types such as stem and progenitor cells present in complex cell mixtures. However, the migration of cells between microwells undermines the function of this and many other micromolded devices that require a bio-inert background. Furthermore, our observations illustrate a previously unreported challenge on how to micromold non-fouling hydrogels so that they retain their cell resistant properties.

PEG hydrogels have been illustrated to undergo oxidation reactions that result in loss of the non-fouling character thus spurring on the development of more stable non-fouling materials such as zwitterionic polymers, mixed-charge polymers, and the poly(2-methyl-2-oxazoline) polymers⁴²⁻⁴⁷. Some may argue that it is PEG degradation that is enabling the cell migration in this system. However, the oxidation of the PEG gels does not pose a problem over relatively short *in vitro* experiments such as what are described here^{42, 47}. Furthermore, the cells were able to adhere to these gels immediately upon seeding indicating that oxidation of the gel was not a main contributor to cell adhesion to these gels.

After determining that the interwell cell migration occurs solely by cells adhering and migrating on the hydrogel we developed the surrogate assay of cell adhesion to planar slabs fabricated in contact with different materials to quickly and easily assay how the stamp material affects the adhesion of aMSCs to the PEG(1000) hydrogels. In the first set of experiments the cell cluster adhesion to hydrogels fabricated in contact with glass, Teflon, and PDMS/80/4/4 was assessed while gels that contacted an inert argon gas atmosphere during gelation acted as the experimental control. Glass was selected as

it is a relatively hydrophilic and non-leaching material while Teflon was selected as it is a highly crystalline polymer that should also be largely non-leaching. Furthermore, Teflon and PDMS have similar hydrophobicities of approximately 109° as measured by static contact angle measurements⁴⁸.⁴⁹ We discovered that there is something unique about the PDMS that resulted in changes to the gel's surface properties as it was the only treatment that resulted in up regulation of cell cluster adhesion to the PEG(1000) hydrogel.

Cell attachment to biomaterial surfaces is mediated by adsorbed proteins that contain cell binding domains that enable cell adhesion. The non-fouling properties of PEG gels – along with many other hydrogels such as the zwitterionic (ex. sulfobetaine, SB) and polyacrylamide (PA) hydrogels – are linked to the hydrophilicity of the gels, that result in a surface to which it is thermodynamically unfavourable for protein adsorption from aqueous environments and thus resistant to cell attachment⁵⁰. One could envision hydrophobic PDMS species transferring to the hydrogels during micromolding and that this surface contamination undermines the bio-inert properties of the PEG hydrogel, thus allowing protein adsorption and cell adhesion to the otherwise non-fouling material. One could envision multiple ways by which PDMS could transfer to the hydrogel interface during micromolding. Here we postulate four mechanisms, although the authors feel that the first mechanism is the most likely. First, PDMS contains low molecular weight oligomer that is not covalently coupled to the polymer network^{26-30, 51, 52}. These low molecular weight species can migrate to the polymer interface, leach from the polymer, or transfer to other surfaces upon contact^{26, 29, 31, 51, 53, 54}. Although the presence of these low molecular weight species in PDMS is known, many soft lithography protocols do not take steps to rigorously remove the oligomer, and assume that the non-fouling properties of the gels will be maintained through the microfabrication process^{1-3, 6, 11, 13-15}. It is feasible that such low molecular weight PDMS oligomer is being physically adsorbed onto or entangled in the surface of the hydrogel network during gelation. Second, PDMS is highly permeable on the molecular level. This high diffusivity of compounds in PDMS could result in the penetration of PEG macromer into the stamp during perfusion that is later incorporated into the hydrogel network upon gelation. During the demolding process this entanglement could result in pieces of the PDMS stamp being pulled from the stamp resulting in a hydrogel surface that is again physically contaminated with PDMS. Third, the chemistry used to cure the PDMS is not orthogonal to the chemistry used to cure the hydrogel. The

curing of the PDMS stamp is performed with a platinum catalysed hydrosilylation reaction involving the addition of Si-H groups across carbon-carbon double bonds, and the hydrogel is fabricated through free radical polymerization of terminal PEG methacrylate groups. If unreacted PDMS precursor components are present at the interface of the PDMS stamp during hydrogel curing this could result in covalent coupling of PDMS species to the hydrogel interface. Last, it is feasible that more than one of these phenomena may be occurring simultaneously.

Through XPS analysis we discovered that residual PDMS was present at the hydrogel interface after gelation, and that the presence of PDMS correlates strongly with protein adsorption to the gels and cell cluster adhesion. Furthermore, increasing the cure time/temperature of the PDMS and thorough extraction of the stamp material helped minimize - but not eliminate - cell cluster adhesion to the gels.

PDMS processing alone was not sufficient to eliminate the undesired cell adhesion to the PEG(1000) hydrogels. Therefore, we screened other methods of maintaining/regenerating the non-fouling character of the micromolded gels: 1) using alternative stamp materials (NOA 60 and FL), 2) blocking the deposition of PDMS oligomer to the hydrogel during fabrication by adding the internal mold release agent SDS, 3) removing the residual PDMS after fabrication through sonication, and 4) altering the composition of the hydrogel. Through these experiments we identified three techniques which produced planar hydrogel slabs which did not show a statistically significant increase in cell cluster adhesion: 1) addition of 5 vol% SDS to the monomer precursor solution, 2) sonication in either 50% ethanol in DI water or 100% DI water for 2 hours, and 3) using a post-fabrication grafting chemistry attach PEG macromers onto the gel surface (the g5 and g10 gel composition).

It is important to note that there are reports in the literature of other methods of changing the surface properties of PDMS stamps such as plasma treatment of the stamp surface to create a glassy layer to impede the diffusion of PDMS oligomer to the interface or treating the stamp with a chlorosilane to prevent to prevent sticking of the PDMS to the PEG hydrogel.^{55, 56} However, the authors chose not to pursue such PDMS modifications because the formation a glassy layer on the surface of the stamp would result in a stiffer material that would not form the necessary conformal adhesion with the underlying substrate thus resulting in well bottoms coated with PEG hydrogel. Furthermore, the

silanization of the stamp would introduce other hydrophobic small molecules to the interface that may also transfer the hydrogel.

The three strategies that maintained/regenerated the planar hydrogel's cell resistant properties were used to fabricate hydrogel microwell arrays to see if the results translated to gels which have been micromolded into a three dimensional device. The behaviour of aMSCs in the arrays was captured using time-lapse video microscopy and the interwell cell migration was quantified. We determined that the addition of SDS did not reduce the interwell cell migration of cells in the array likely due to the much higher surface area of the micropatterned PDMS stamp in comparison to the featureless stamp used to fabricate planar hydrogel slabs. The hydrogel microwell arrays were also sonicated in either 50% ethanol in DI water and 100% DI water. The planar hydrogel slabs were sonicated for 2 hours; however, we observed detachment of the hydrogel arrays from the polystyrene substrate after only 10 minutes of sonication in either fluid. Interwell cell migration was observed in arrays that were sonicated for 10 minutes illustrating that this is not a suitable cleaning protocol for the microwell arrays fabricated in this research.

The optimum strategy for regenerating the cell resistant properties of micromolded hydrogels was to use the alternative hydrogel chemistry (the g10 hydrogel composition) where 10% methacrylic acid was added to the polymer precursor solution thus resulting in a hydrogel that contained carboxylic acid functionality. By utilizing carbodiimide chemistry we covalently functionalized the micromolded gel with PEG-monoamine macromers, a technique that has been shown to improve the non-fouling properties of surfaces^{57, 58}. It has been previously illustrated that both the length of the grafted PEG chains as well as their packing density influence the non-fouling properties of the resulting materials. We chose to graft the hydrogel with PEG macromers with a molecular weight of 750 Da as molecules in this molecular weight range have been successfully used previously. Furthermore, we chose to explore two grafting densities (5% and 10%) as the grafting density has been shown to affect the biological properties of PEG-modified surfaces^{42, 59}. When the PEG macromers were covalently linked to the surface of the hydrogel post-fabrication we created a micro-fabricated device capable of robust cell confinement. Although the PDMS contamination is likely still present on the hydrogel, this post-grafting protocol appears leads to surfaces that masks the presence of the PDMS and is

resistant to cell adhesion for at least 10 days in *in vitro* culture.

To be useful as a platform of the CFU assay, this microwell array platform must not only allow researchers to isolate a cell and its progeny for prolonged periods during *in vitro* culture, but also enable the researchers to quantify the number of cells per well at the end of the experiment and track individual cell behaviour throughout the course of the experiment. Using time-lapse video microscopy we tracked the behaviour of 50 cells for 48 hours (Figure 7). At the beginning of the experiment the cells were the sole occupants of their respective wells while at the end of the experiment many of the cells had undergone cellular division. However, the cells showed striking heterogeneity in their proliferative potential. A staggering 50% of the cells did not divide during the 48 hour experiment while we observed multiple cells undergo two or more population doublings during this time (Figure 7B and 7C). These data closely correspond to literature stating that many MSCs during *ex vivo* culture exit the cell proliferation cycle, and that a few highly proliferative cells within the larger population are responsible for the bulk of the cellular proliferation. However, this previously published analysis was performed on unconfined and highly motile cells, and thus required the use of complex cell tracking software.⁶⁰ Due to the cellular confinement present in this platform we were able to collect similar data without the need of tracking software and computer programming knowledge making this technology much more useful in biological laboratories. Furthermore, we have illustrated that this microwell array platform possesses the necessary property of collecting high-resolution single cell behaviour during culture necessary for use in the CFU assay.

These results illustrate a previously unrecognized technical knowledge gap in how to appropriately micromold non-fouling hydrogels in order to retain their bio-inert properties. Furthermore, we present a user-friendly and straightforward technique for overcoming this challenge, and greatly improve the performance of non-fouling lab on a chip systems produced through micromolding. Not only is this technology of critical importance for the isolation of migratory cell types as illustrated here, but has broader impact in the field of micropatterned cell culture devices. For instance, recent research has utilized such micropatterned substrates to engineer the cellular microenvironment by decorating the non-fouling background of the device with specific biomolecules¹⁹⁻²¹. However, these data imply that if the substrates are fabricated through micromolding the adsorption of molecules from solution could

present cells with other binding ligands casting doubt on the results drawn from these experiments that claim a fully defined cell adhesion surface.

4. Experimental

4.1. Silicon master fabrication

Glass photomasks with the desired patterns were purchased from Infinite Graphics Pte Ltd (Singapore). Nanyang Technological University's Microfabrication Centre produced silicon masters. Briefly, a cleaned silicon wafer was spin-coated with the SU-8 3025 negative photoresist (MicroChem, Boston, MA) and patterned by photolithography. In this work all silicon masters are patterned with an array of square microwells of specific dimensions: the footprint of the square wells is 100x100 μm , the spacing between wells is 20 μm , and the height of the features is 40 μm . The silicon master was silanized with (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (Gelest, Morrisville, PA) under vacuum overnight. Silicon masters were stored in a dust free container until use.

4.2. Stamp fabrication and processing

Glass and Teflon. Glass coverslips and Teflon were used as featureless stamps to produce planar hydrogel slabs. Glass coverslips were cleaned by first hand washing in a 2% micro-90 soap solution; sonicated for 10 minutes each in a 2% micro-90 soap solution, 1M NaOH solution, and DI water; rinsed with DI water; dried with a stream of argon gas; and used immediately. Teflon was prepared in the same manner except it was sonicated in 95% ethanol instead of 1M NaOH.

Sylgard 184. Polydimethylsiloxane (PDMS) stamps were fabricated using the Sylgard 184 kit (Dow Corning, Midland, MI) by mixing a 10:1 weight ratio of the pre-polymer:curing agent. The two liquids were manually mixed for five minutes to ensure homogenization of the solution, put under vacuum for 30 minutes to remove air bubbles, poured onto either a micropatterned master to produce a stamp with the desired features or a flat master to produce a featureless stamp, and cured for the desired time (either 4 or 48 hours) at the desired temperature (80, 120, 150, or 180°C). After curing the PDMS, stamps were cut to size and extracted for the desired length of time in hexane followed by ethanol (either 2 hours in each solvent or 24 hours in each solvent).

Norland Optical Adhesives. Norland Optical Adhesive 60 (NOA 60, Norland Products, Cranbury, NJ) was poured onto a flat master to create featureless stamps and cured by exposure to UV light (365nm, 14 mWatts/cm²) for 2 hour. After curing, the stamps were cut to size and extracted hexane followed by ethanol for 24 hours each.

Fluorolink. Fluorolink AD 1700 (FL, Acota, Shrewbury, UK) fluoroacrylate macromer solution was mixed with 4 wt% of Darocur 1173 liquid free radical initiator (BASF Performance Chemicals, Germany) by vortexing. The solution was then poured onto a flat master to create featureless stamps and cured using UV initiated free radical polymerization (365nm, 14 mWatts/cm²) for 10 minutes. After curing the stamps were cut to size and extracted for 24 hours in chloroform and then 24 hours ethanol.

The quantity of oligomer that was extracted from the stamp materials was quantified by weighing an ~0.2g sample of stamp material before and after the desired extraction protocol. The percent mass loss was calculated by subtracting the mass of the extracted material from the original mass, dividing the difference by the original mass, and multiplying by 100%. A total of five samples were extracted per treatment (n=5).

All stamps were transferred into an argon-filled glove box the day before fabrication in order to de-oxygenate the stamps.

4.3. Sulfobetaine methacrylate monomer synthesis

The sulfobetaine methacrylate monomer (SBMA) was synthesized by reacting propane sultone (Sigma–Aldrich, Milwaukee, WI) and dimethylamino ethyl methacrylate (Sigma–Aldrich) at equimolar concentrations in acetone as previously described⁴³. The concentration of each reagent was 0.1 M and the reaction proceeded for 2 days at ambient conditions. The SBMA precipitated out of the reaction solution as a white solid, which was collected by filtration. The filter cake was washed three times with 100 mL of acetone to remove unreacted species. The product was dried under vacuum and stored at 4 °C.

4.4. Hydrogel fabrication

All hydrogels were produced in an argon filled glove box using both de-oxygenated solutions and stamps.

Hydrogel microwell arrays. Polystyrene (PS) substrates were cleaned by sonication for 10 minutes in each 2% micro-90 soap solution, absolute ethanol, and DI water. Substrates were then rinsed with DI water and dried in a stream of argon. PS is then placed on the lower electrode of a March PX 500 parallel plate plasma etcher (Nordson March, Conrad, CA) (13.56 MHz RF power) and exposed to argon gas plasma at 300 Watts (0.43 Watts/cm² electrode) and 20% flow rate (475 to 485 mTorr) for 10 minutes and then exposed to the ambient environment for 5 minutes before being transferred into the glove box. Inside the glove box the PDMS stamp was immediately placed on the polystyrene and gently tapped down to ensure conformal adhesion between the pillars of the stamp and the PS substrate. 15 μ L of the desired monomer solution was pipetted along the edge of the stamp without allowing the pipette to touch the stamp. The monomer solution was drawn into the features of the stamp by capillary action. Upon complete perfusion the sample was cured through exposure to 365nm UV light (10-12 mWatts/cm²) for 5 minutes. The UV light was first passed through an IR filter to prevent overheating of the sample. After curing, the stamp was gently peeled from the substrate to reveal a hydrogel microwell array that was covalently attached to the underlying PS substrate. The hydrogel was submerged in ethanol for 2 hours to extract unreacted species, ethanol was aspirated and replaced with PBS, and samples were stored in a cell culture incubator overnight to equilibrate before beginning cell experiments. Hydrogel microwell arrays used in fluorescence or confocal microscopy experiments contained 0.003 wt% Polyfluor 570 (methacryloxyethyl thiocarbamoyl rhodamine B, Polysciences) based on the mass of the monomer to enable fluorescent visualization of the gel.

Hydrogel slabs. Freestanding and planar hydrogel slabs were produced by adding 350 μ L of the desired hydrogel precursor solution to the well of a 12 well plate. The fluid covered 1mm pre-cleaned polystyrene spacers that were placed along the edge of the well. The desired stamp material was placed on top of the precursor solution and was supported by the spacers enabling good contact between the stamp and precursor solution and ensuring the production of planar gels with uniform

thickness. The planar slabs were cured, extracted, and equilibrated in the same manner as the hydrogel arrays.

Hydrogel formulations. Several different hydrogel formulations were used in this research. The PEG(1000) hydrogel was fabricated from a 20 vol% solution of polyethylene glycol dimethacrylate ($M_n = 1000$) (Polyscience, USA) in 70% ethanol using 14mg/mL Irgacure 2959 as photoinitiator (BASF, Germany). The PEG(2000) hydrogel was polymerized from the same precursor solution as the PEG(1000) except that the solution contained 20 vol% polyethylene glycol dimethacrylate ($M_n = 2000$) (Sigma-Aldrich) instead of the 1000 Da macromer. The co10 hydrogel was polymerized from the same precursor solution as the PEG(1000) with an additional 10 vol% of polyethylene glycol monomethacrylate ($M_n = 475$) (Sigma-Aldrich) based on the volume of PEG(1000) macromer. The g10 hydrogel was polymerized from the same precursor solution as the PEG(1000) gel with an additional 10 vol% of methacrylic acid (Sigma-Aldrich) based on the volume of the PEG(1000) macromer. After fabrication and extraction the acid groups in the g10 hydrogels were reacted with equimolar amounts of EDC (Sigma-Aldrich) and polyethylene glycol monoamine ($M_n = 750$) (Sigma-Aldrich) in ethanol overnight to covalently couple the PEG-amine to the acid groups through the formation of an amide bond. The SB hydrogels were fabricated using 750 mg/mL sulfobetaine methacrylate monomer and 100 mg/mL pentaerythritol tetraacrylate crosslinker in 70% ethanol. The polyacrylamide hydrogels were fabricated using 200 mg/mL acrylamide (Sigma-Aldrich) and 45mg/mL bisacrylamide (Fisher Scientific, USA) in 70% ethanol. For both SB and PA hydrogel synthesis 14mg/mL Irgacure 2959 was used as photoinitiator (BASF, Germany).

4.5. XPS characterization of hydrogel interface

Planar hydrogel slabs were dehydrated through lyophilisation using an Alpha 2-4 LSC lyophilizer (CHRiST, Germany) for 12 hours. Upon removal from the lyophilizer the samples were transported to the XPS equipment in a vacuum desiccator and analysed immediately. XPS analyses were performed at A*Star's Institute of Materials Research and Engineering using a Theta Probe small spot XPS analyser (Thermo Fisher) or MIT's Centre for Materials Science and Engineering (CMSE) using a Versaprobe II. Survey scans were used to identify species at the hydrogel interface followed by high-resolution scans to identify the composition of the surface. Since the hydrogel is not conductive,

charge correction was utilized to position the C1s peak at 286.45 eV according to previously published data³².

4.6. Hydrogel blocking/cleaning protocols

SDS incorporation. Some hydrogel precursor solutions contained sodium dodecyl sulphate (SDS) as a mold release agent. The quantity of SDS added to the precursor solutions is presented as weight % based on the weight of PEG(1000) macromer in solution. Residual SDS was removed by extraction the gel in both ethanol and PBS.

Sonication. Hydrogel slabs were placed in the wells of a 12 well plate, submerged in the desired liquid (either ethanol, DI water, or a mixture of the two), and sonicated for 2 hours in an Elmasonic S30H bath sonicator (Elma Hans Schmidbauer, Germany) with the sonication liquid being changed every 30 minutes. Microwell arrays were sonicated for 10 minutes.

4.7. Protein adsorption to hydrogels

The top surfaces of planar hydrogel slabs and tissue culture polystyrene (positive control) were incubated with a 5 µg/mL solution of Hilyte 488 Fibronectin (Cytoskeleton, Denver, CO) in PBS for 1 hour in cell culture conditions. The hydrogel slabs were rinsed 3 times with PBS to remove unabsorbed protein and submerged in 1 mL of PBS in a 2 well chamber slide. The top surfaces of the gels were imaged for green fluorescence emission using a Leica DMI 6000B microscope with a collection time of 3 seconds per image. The fluorescence intensity of five non-overlapping images from each sample was measured using ImageJ image processing program (NIH, Bethesda, MD), and four samples per condition were analysed (n=20). Hydrogel slabs and tissue culture polystyrene that were not exposed to the fluorescently tagged protein were imaged in the same manner to assess the autofluorescence of these materials. The intensity of the autofluorescence was subtracted from the intensity of the protein-adsorbed samples in order to remove signals that arise due to autofluorescence. Data were normalized to the TCPS positive control.

4.8. Cell culture

Passage 2 human adult mesenchymal stem cells (aMSCs) were obtained from Lonza (Basel,

Switzerland) and cultured according to manufacturer instruction. Briefly, cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% MSC qualified FBS (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). Cells were plated in T-75 flasks at 500 cells/cm², incubated in a humidified environment at 37°C and 5% CO₂, and medium was changed every 2-3 days. Passage 2 cultures were expanded up to a maximum of 70% confluence, lifted with TrypLE Express (Life Technologies) and cryopreserved in growth medium with 10% DMSO and 30% MSC qualified FBS to create a stock of cells. For cell experiments, cells from the stock were thawed and cultured according to the above protocol to obtain the desired number of cells, lifted using TrypLE Express, counted with a haemocytometer, centrifuged, old media was aspirated, and cells were diluted to the desired density through the addition of fresh media. Passages 3 through 5 were used for cell experiments.

4.9. Cell seeding

Immediately before cell seeding the hydrogel slabs and microwell arrays were UV sterilized for 30 minutes and incubated with a 5 µg/mL solution of fibronectin for 1 hour and rinsed 3 times with PBS. Hydrogel microwell arrays were seeded at a density of 7x10³ aMSCs/cm² and hydrogel slabs were seeded at a density of 2.5x10⁴ cells/cm².

4.10. Cell adhesion to planar hydrogel slabs

After the 24 hours of incubation, medium along with any unattached cells were aspirated from the culture. The non-adherent cells were re-plated on TCPS and incubated for 24 hours to ensure that the cells retained adhesion capacity as an indication of maintained cell viability. The hydrogels were transferred to wells in a new 12 well plate and covered with pre-warmed medium. Cell attachment to the hydrogel interface was immediately assessed with phase contrast microscopy at 10x magnification. Adherent cells clusters were counted and data are presented as the number of cell clusters per image. Five randomly selected and non-overlapping images were taken per hydrogel, and four hydrogels of each treatment were observed (n=20). Sulfobetaine slabs were opaque so adherent cells were first stained with Cell Tracker Green (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and imaged using fluorescence microscopy.

4.11. Cell migration in hydrogel microwell arrays

Hydrogel microwell arrays that were seeded with aMSCs were allowed to incubate for 24 hours to allow firm adhesion of the cells in the array. The arrays were then transferred to the incubation chamber of the Leica DMI 6000B microscope that was pre-equilibrated to cell culture conditions. Arrays were imaged at 10x for 15 hours at 10 minute time intervals. Images were combined into time lapse videos through the use of ImageJ. The inter-well migration of 50 randomly selected cells from each of four non-overlapping regions was assessed during the 15 hour video (n=200).

4.12. Tracking individual cell behaviour in hydrogel microwell arrays

Hydrogel microwell arrays that were seeded with aMSCs were allowed to incubate for 24 hours to allow firm adhesion of the cells in the array. The arrays were then transferred to the incubation chamber of the Leica DMI 6000B microscope that was pre-equilibrated to cell culture conditions. Arrays were imaged at 10x for 48 hours at 10 minute time intervals. Images were combined into time lapse videos through the use of ImageJ. 50 wells were identified that had a single MSC occupant. These wells were tracked for the duration of the experiment, and the time of each cell proliferation event was recorded for each cell.

4.13. Confocal imaging of cells in hydrogel microwell arrays

48 hours after seeding, microwell arrays containing aMSCs were fixed in 4 % formaldehyde for 15 minutes at room temperature, cells were permeabilized by exposure to TritonX (0.5%) for 10 min, and stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA) and DAPI (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Confocal microscopy was performed on a Zeiss LSM 780 (Carl Zeiss, Germany).

4.14. Statistical analysis

Data is presented as mean \pm standard deviation. Multiple means were compared with an analysis of variance (ANOVA) test to determine if any pairs of means were statistically different. If the ANOVA indicated statistical difference then the Tukey-Kramer Honestly Significant Difference Test was used to determine which of the pairs of means were statistically different. Pairs of means are marked with "ns" are not statistically different from the negative control, a PEG(1000) hydrogel fabricated in

contact with an inert argon gas atmosphere.

5. Conclusions

We have identified that micromolding of non-fouling PEG hydrogels through soft lithography using PDMS, Norland Optical Adhesive 60, and Fluorolink undermines the cell resistant properties of the gels that in turn undermines the function of non-fouling lab on a chip devices. We explored a wide range of techniques in an attempt to maintain/restore the cell resistant character of the gel and identified the optimal strategy: post-fabrication grafting of a PEG-brush structure to the walls through the use of reactive sites incorporated through co-polymerization. This work addressed a previously unreported problem in micromolding non-fouling hydrogels and will greatly improve the performance of cell resistant lab on a chip devices. Furthermore, these data illustrate that this platform possesses the necessary properties of cell isolating/tracking needed to be used for the CFU assay.

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7. References

- 1 K. B. Lee, Y. H. Jung, Z. W. Lee, S. Kim, I. S. Choi, *Biomaterials* **2007**, *28*, 5594-600 DOI 10.1016/j.biomaterials.2007.08.036.
- 2 H. Kim, R. E. Cohen, P. T. Hammond, D. J. Irvine, *Advanced Functional Materials* **2006**, *16*, 1313-1323 DOI 10.1002/adfm.200500888.
- 3 H. C. Moeller, M. K. Mian, S. Shrivastava, B. G. Chung, A. Khademhosseini, *Biomaterials* **2008**, *29*, 752-63 DOI 10.1016/j.biomaterials.2007.10.030.
- 4 M. C. Lensen, P. Mela, A. Mourran, J. Groll, J. Heuts, H. Rong, M. Moller, *Langmuir : the ACS journal of surfaces and colloids* **2007**, *23*, 7841-6 DOI 10.1021/la7007683.
- 5 P. Kim, H. E. Jeong, A. Khademhosseini, K. Y. Suh, *Lab on a Chip* **2006**, *6*, 1432-1437 DOI 10.1039/B610503c.
- 6 N. Y. Lee, Y. K. Jung, H. G. Park, *Biochemical Engineering Journal* **2006**, *29*, 103-108 DOI 10.1016/j.bej.2005.02.025.
- 7 P. J. Yoo, *Methods in molecular biology* **2011**, *671*, 147-60 DOI 10.1007/978-1-59745-551-0_8.
- 8 W. G. Koh, M. Pishko, *Sensors and Actuators B-Chemical* **2005**, *106*, 335-342 DOI 10.1016/j.snb.2004.08.025.
- 9 D. H. Kim, J. Park, K. Y. Suh, P. Kim, S. K. Choi, S. Ryu, S. Park, S. H. Lee, B. Kim, *Sensors and Actuators B-Chemical* **2006**, *117*, 391-400 DOI 10.1016/j.snb.2005.11.051.
- 10 R. K. Iyer, L. L. Y. Chiu, M. Radisic, *Journal of Biomedical Materials Research Part A* **2009**, *89A*, 616-631 DOI 10.1002/jbm.A.32014.
- 11 K. Y. Suh, J. Seong, A. Khademhosseini, P. E. Laibinis, R. Langer, *Biomaterials* **2004**, *25*, 557-563 DOI 10.1016/S0142-9612(03)00543-X.
- 12 D. Qin, Y. Xia, G. M. Whitesides, *Nature protocols* **2010**, *5*, 491-502 DOI 10.1038/nprot.2009.234.
- 13 J. Wu, I. Wheeldon, Y. Guo, T. Lu, Y. Du, B. Wang, J. He, Y. Hu, A. Khademhosseini, *Biomaterials* **2011**, *32*, 841-8 DOI 10.1016/j.biomaterials.2010.09.026.
- 14 J. Fukuda, A. Khademhosseini, Y. Yeo, X. Yang, J. Yeh, G. Eng, J. Blumling, C. F. Wang, D. S. Kohane, R. Langer, *Biomaterials* **2006**, *27*, 5259-67 DOI 10.1016/j.biomaterials.2006.05.044.
- 15 J. M. Karp, J. Yeh, G. Eng, J. Fukuda, J. Blumling, K. Y. Suh, J. Cheng, A. Mahdavi, J. Borenstein, R. Langer, A. Khademhosseini, *Lab on a Chip* **2007**, *7*, 786-794 DOI 10.1039/B705085m.
- 16 A. K. Majors, C. A. Boehm, H. Nitto, R. J. Midura, G. F. Muschler, *J Orthop Res* **1997**, *15*, 546-57 DOI 10.1002/jor.1100150410.
- 17 N. A. Marcantonio, C. A. Boehm, R. J. Rozic, A. Au, A. Wells, G. F. Muschler, L. G. Griffith, *Biomaterials* **2009**, *30*, 4629-38 DOI 10.1016/j.biomaterials.2009.05.061.
- 18 G. F. Muschler, H. Nitto, C. A. Boehm, K. A. Easley, *J Orthop Res* **2001**, *19*, 117-25 DOI 10.1016/S0736-0266(00)00010-3.
- 19 M. P. Lutolf, R. Doyonnas, K. Havenstrite, K. Koleckar, H. M. Blau, *Integr Biol (Camb)* **2009**, *1*, 59-69 DOI 10.1039/b815718a.
- 20 M. P. Lutolf, P. M. Gilbert, H. M. Blau, *Nature* **2009**, *462*, 433-41 DOI 10.1038/nature08602.
- 21 M. Roccio, S. Gobaa, M. P. Lutolf, *Integr Biol (Camb)* **2012**, *4*, 391-400 DOI 10.1039/c2ib00070a.
- 22 C. Wang, J. R. Chen, *Applied Surface Science* **2007**, *253*, 4599-4606 DOI 10.1016/j.apsusc.2006.10.014.
- 23 S. Rayatpisheh, P. Li, M. B. Chan-Park, *Macromolecular bioscience* **2012**, *12*, 937-45 DOI 10.1002/mabi.201100477.
- 24 I. L. J. Dogue, N. Mermilliod, R. Foerch, *Nuclear Instruments & Methods in Physics Research Section B-Beam Interactions with Materials and Atoms* **1995**, *105*, 164-167.
- 25 N. Inagaki, S. Tasaka, Y. Goto, *Journal of Applied Polymer Science* **1997**, *66*, 77-84 DOI 10.1002/(Sici)1097-4628(19971003)66:1<77::Aid-App9>3.0.Co;2-L.
- 26 K. J. Regehr, M. Domenech, J. T. Koepsel, K. C. Carver, S. J. Ellison-Zelski, W. L. Murphy, L. A. Schuler, E. T. Alarid, D. J. Beebe, *Lab on a Chip* **2009**, *9*, 2132-2139 DOI 10.1039/B903043c.
- 27 D. T. Eddington, J. P. Puccinelli, D. J. Beebe, *Sensors and Actuators B-Chemical* **2006**, *114*, 170-172 DOI 10.1016/j.snb.2005.04.037.
- 28 J. N. Lee, C. Park, G. M. Whitesides, *Analytical Chemistry* **2003**, *75*, 6544-6554 DOI 10.1021/Ac0346712.
- 29 D. J. Graham, D. D. Price, B. D. Ratner, *Langmuir : the ACS journal of surfaces and colloids* **2002**, *18*, 1518-1527 DOI 10.1021/La010059z.
- 30 X. He, D. S. Dandy, C. S. Henry, *Sensors and Actuators B-Chemical* **2008**, *129*, 811-817 DOI 10.1016/j.snb.2007.09.087.
- 31 K. Glasmaster, J. Gold, A. S. Andersson, D. S. Sutherland, B. Kasemo, *Langmuir : the ACS journal of surfaces and colloids* **2003**, *19*, 5475-5483 DOI 10.1021/la026558x.

- 32 G. Beamson, D. Briggs, *High resolution XPS of organic polymers : the Scienta ESCA300 database*. Editor, Wiley, Chichester England ; New York, **1992**, pp. 295 p.
- 33 P. Cong, T. Kubo, H. Nanao, I. Minami, S. Mori, *Tribology Letters* **2005**, *19*, 299-309 Doi 10.1007/S11249-005-7447-3.
- 34 Y. S. Lo, N. D. Huefner, W. S. Chan, P. Dryden, B. Hagenhoff, T. P. Beebe, *Langmuir* **1999**, *15*, 6522-6526 Doi 10.1021/La990371x.
- 35 M. T. Timko, Z. H. Yu, J. H. Kroll, J. T. Jayne, D. R. Worsnop, R. C. Miake-Lye, T. B. Onasch, D. Liscinsky, T. W. Kirchstetter, H. Destailats, A. L. Holder, J. D. Smith, K. R. Wilson, *Aerosol Science and Technology* **2009**, *43*, 855-865 Doi 10.1080/02786820902984811.
- 36 T. A. Horbett, in *Biomaterials Science An Introduction to Materials in Medicine* 3rd ed., ed. by B. D. Ratner, A. S. Hoffman, F. J. Schoen, J. E. Lemons, Elsevier, Oxford, UK, **2013**, pp. 394.
- 37 J. P. Rolland, R. M. Van Dam, D. A. Schorzman, S. R. Quake, J. M. DeSimone, *Journal of the American Chemical Society* **2004**, *126*, 2322-2323 Doi 10.1021/Ja031657y.
- 38 M. C. Lensen, P. Mela, A. Mourran, J. Groll, J. Heuts, H. T. Rong, M. Moller, *Langmuir* **2007**, *23*, 7841-7846 Doi 10.1021/La7007683.
- 39 A. Priola, R. Bongiovanni, G. Malucelli, A. Pollicino, C. Tonelli, G. Simeone, *Macromolecular Chemistry and Physics* **1997**, *198*, 1893-1907 DOI 10.1002/macp.1997.021980618.
- 40 Y. H. Kim, J. Park, P. J. Yoo, P. T. Hammond, *Advanced Materials* **2007**, *19*, 4426-+ DOI 10.1002/adma.200700979.
- 41 J. Park, Y. S. Kim, P. T. Hammond, *Nano Letters* **2005**, *5*, 1347-1350 Doi 10.1021/NI050592p.
- 42 D. E. Heath, S. L. Cooper, *Journal of biomedical materials research. Part A* **2010**, *94*, 1294-302 10.1002/jbm.a.32811.
- 43 D. E. Heath, S. L. Cooper, *Acta Biomaterialia* **2012**, *8*, 2899-2910 DOI 10.1016/j.actbio.2012.03.052.
- 44 R. Hu, G. Z. Li, Y. J. Jiang, Y. Zhang, J. J. Zou, L. Wang, X. W. Zhang, *Langmuir* **2013**, *29*, 3773-3779 Doi 10.1021/La304708b.
- 45 C. Jin, Y. L. Jiang, T. Qian, Q. R. Han, B. X. Wang, J. Shen, *Chemical Journal of Chinese Universities-Chinese* **2013**, *34*, 205-209 Doi 10.7503/Cjcu20120430.
- 46 L. Mi, M. T. Bernards, G. Cheng, Q. M. Yu, S. Y. Jiang, *Biomaterials* **2010**, *31*, 2919-2925 Doi 10.1016/J.Biomaterials.2009.12.038.
- 47 B. Pidhatika, M. Rodenstein, Y. Chen, E. Rakhmatullina, A. Muhlebach, C. Acikgoz, M. Textor, R. Konradi, *Biointerphases* **2012**, *7*, Artn 1 Doi 10.1007/S13758-011-0001-Y.
- 48 G. C. S. Collins, A. C. Lowe, D. Nicholas, *European Polymer Journal* **1973**, *9*, 1173-1185 Doi 10.1016/0014-3057(73)90120-1.
- 49 A. Mata, A. J. Fleischman, S. Roy, *Biomedical microdevices* **2005**, *7*, 281-93 10.1007/s10544-005-6070-2.
- 50 B. D. Ratner, A. S. Hoffman, in *Biomaterials Science: An Introduction to Materials in Medicine*, 3rd ed., ed. by B. D. Ratner, A. S. Hoffman, J. S. Frederick, J. E. Lemons, Academic Press, Waltham, MA, **2013**, pp. 241-7.
- 51 L. J. Millet, M. E. Stewart, J. V. Sweedler, R. G. Nuzzo, M. U. Gillette, *Lab on a Chip* **2007**, *7*, 987-94 10.1039/b705266a.
- 52 J. A. Vickers, M. M. Caulum, C. S. Henry, *Analytical Chemistry* **2006**, *78*, 7446-7452 Doi 10.1021/Ac0609632.
- 53 L. Yang, N. Shirahata, G. Saini, F. Zhang, L. Pei, M. C. Asplund, D. G. Kurth, K. Ariga, K. Sautter, T. Nakanishi, V. Smentkowski, M. R. Linford, *Langmuir : the ACS journal of surfaces and colloids* **2009**, *25*, 5674-5683 Doi 10.1021/La804272n.
- 54 X. J. Wang, M. Ostblom, T. Johansson, O. Inganas, *Thin Solid Films* **2004**, *449*, 125-132 DOI 10.1016/j.tsf.2003.10.153.
- 55 N. Sniadecki, C. Chen, *Methods in Cell Biology* **2007**, *83*, 313-28.
- 56 H. Shiku, T. Saito, C. Wu, T. Yuasukawa, M. Yokoo, H. Abe, T. Matue, H. Yamada, *Chemistry Letters* **2006**, *35*, 234-5.
- 57 X. F. Yuan, D. Fabregat, K. Yoshimoto, Y. Nagasaki, *Colloids and Surfaces B-Biointerphases* **2012**, *92*, 25-29 Doi 10.1016/J.Colsurf.2011.11.013.
- 58 S. Zanini, M. Muller, C. Riccardi, M. Orlandi, *Plasma Chemistry and Plasma Processing* **2007**, *27*, 446-457 Doi 10.1007/S11090-007-9094-Y.
- 59 R. Michel, S. Pasche, M. Textor, D. G. Castner, *Langmuir* **2005**, *21*, 12327-12332 Doi 10.1021/La051726h.
- 60 M. Whitfield, W. Lee, K. Van Vliet, *Stem Cell Research* **2013**, *11*, 1365-77.

8. Figure captions

Abstract Figure. Micromolded hydrogels lose non-fouling properties enabling cell adhesion and migration on the gel (left). We develop a method to regenerate the cell resistance of the gels enabling robust cell confinement (right).

Figure 1. Schematics of hydrogel microwell array and hydrogel slab fabrication: (A) fabrication of hydrogel microwell arrays through placement of micropatterned PDMS stamp on peroxide-activated polystyrene; perfusion of monomer solution; UV crosslinking; and stamp demolding, and (B) fabrication of featureless polymer slabs through placement of polystyrene spacers into the well of a 12-well plate; addition of monomer solution; insertion of a featureless stamp which is supported by spacer and contacts the monomer solution; UV crosslinking; and stamp demolding.

Figure 2. aMSC adhesion and migration on PEG(1000) hydrogel microwell arrays enabling cell migration between microwells. Panel A shows frames from a time-lapse movie at $t = 0$, $t = 20$, and $t = 80$ minutes. The arrow points to a cell that migrates between wells. Panel B shows a gallery of confocal z-slices where the actin cytoskeletons of the cells sit in a focal plane above that of the microwell walls. Panel C shows a 3D confocal reconstruction showing cells spanning multiple wells in the hydrogel microwell array by crossing over the PEG(1000) hydrogel walls. Panel D illustrates that through appropriate optimization of the microfabrication protocol we regenerated the cellular resistance of the gels resulting in arrays where no interwell cell migration was observed.

Figure 3. Representative images of aMSCs adhering to different biomaterials: (A) TCPS, (B) polystyrene treated to mimic the floors of the microwells, (C) PEG(1000) hydrogel photopolymerized in contact with an argon gas atmosphere, and (D) PEG(1000) hydrogel photopolymerized in contact with PDMS/80/4/4. Panel E shows a quantification of cell cluster adhesion to these biomaterial interfaces. In all graphs data is presented as mean \pm standard deviation. Cell cluster adhesion to data labeled with "ns" are not statistically different from the argon-gas exposed control surfaces.

Figure 4. Surface analysis of PEG(1000) gels fabricated in contact with different PDMSs and the interaction of aMSCs with these surfaces. (A) The XPS survey spectra of a PEG(1000) hydrogel that contacted PDMS/80/48/48 during polymerization, and (B) a zoom in of the Si2p peaks from the survey spectra showing varying amounts of Si on the hydrogel surface based on the PDMS it contacted during synthesis. Panels C-E show representative images of cell clusters adhering to PEG(1000) hydrogel interfaces fabricated in contact with: (C) PDMS/80/48/48, (D) PDMS/120/48/48, and (E) PDMS/150/48/48. Panel F shows a quantification of cell cluster adhesion PEG(1000) hydrogels. Panel G shows a quantification of fluorescently tagged fibronectin adsorption to PEG(1000) hydrogels.

Figure 5. Quantification of aMSC cluster adhesion to planar hydrogel slabs assess different strategies for maintaining/regenerating the cell resistance of stamp-contacted hydrogels: (A) PEG(1000) gels in contact with the alternative soft lithography stamp materials NOA 60 and FL, (B) the addition of SDS to the PEG(1000) precursor solution as an internal mold release agent where gels are fabricated in contact with PDMS/120/48/48 stamps, (C) sonication of PEG(1000) hydrogels fabricated in contact with PDMS/120/48/48 stamps in various dilutions of ethanol in DI water, and (D) the use of alternative gel chemistries. In all studies the control condition was PEG(1000) hydrogels polymerized in contact with an inert argon gas atmosphere.

Figure 6. aMSC adhesion and migration on hydrogel microwell arrays fabricated through various strategies using a PDMS/120/48/48 stamp: (A) PEG(1000) hydrogel containing 5% SDS, (B) PEG(1000) hydrogel sonicated for 10 minutes in 50% ethanol in DI water, and (C) g10 hydrogel. Panel D shows a quantification of inter-well cell migration of cells in hydrogel microwell array fabricated under various conditions.

Figure 7. Using the hydrogel microwell arrays and video microscopy to assess single cell behavior during culture: (A) Arrows point to a well containing a single cell at $t = 0$ hrs (left) and the same well that contains four cells at $t = 48$ hrs, (B) a histogram that compiles the frequency of well occupancy (cells/well) at $t = 48$ hours of 50 wells that had a single occupant at $t = 0$, and (C) Proliferation kinetics of wells that had only a single occupancy at $t = 0$ hrs.

9. Figures

Figure 1.

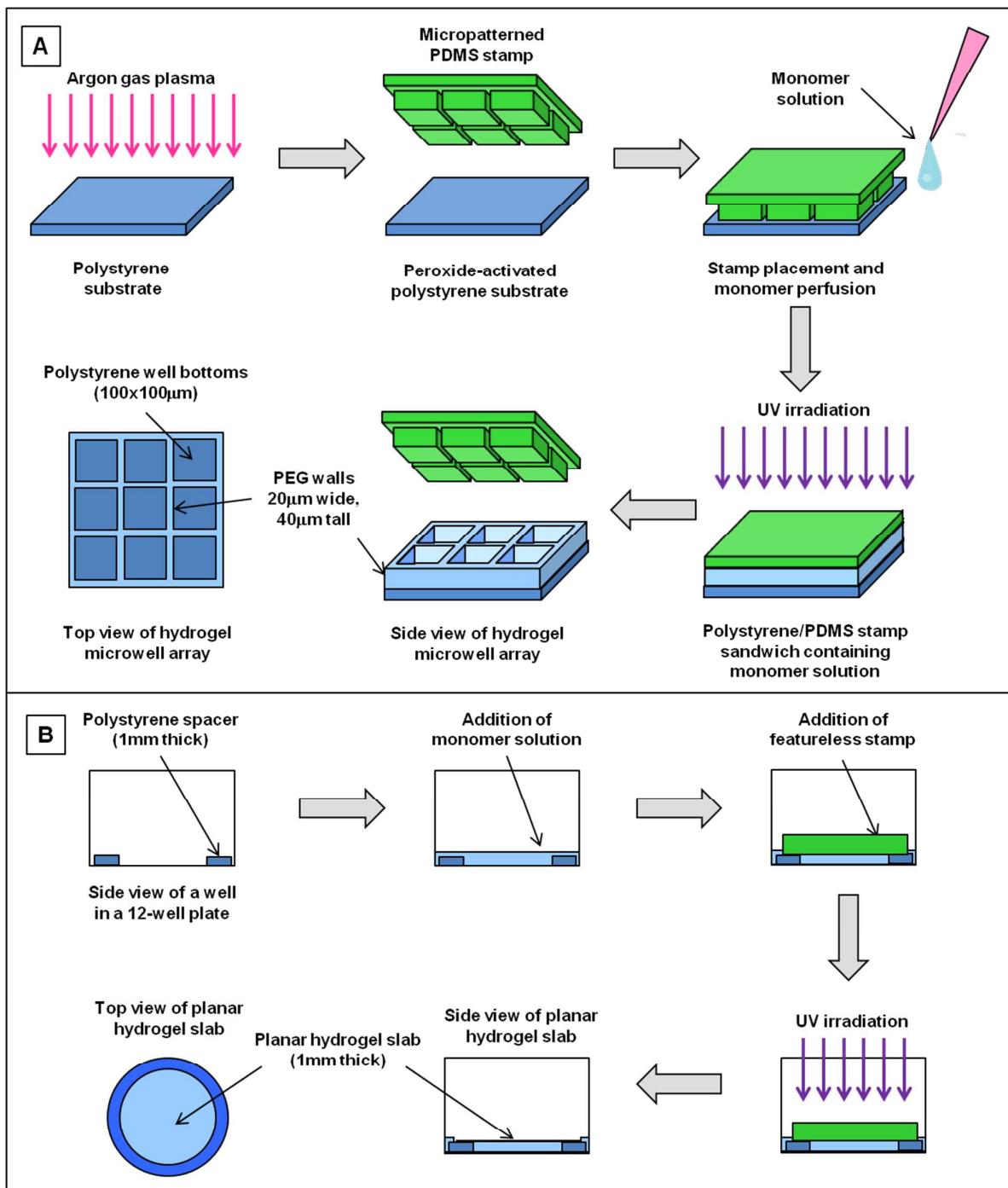


Figure 2.

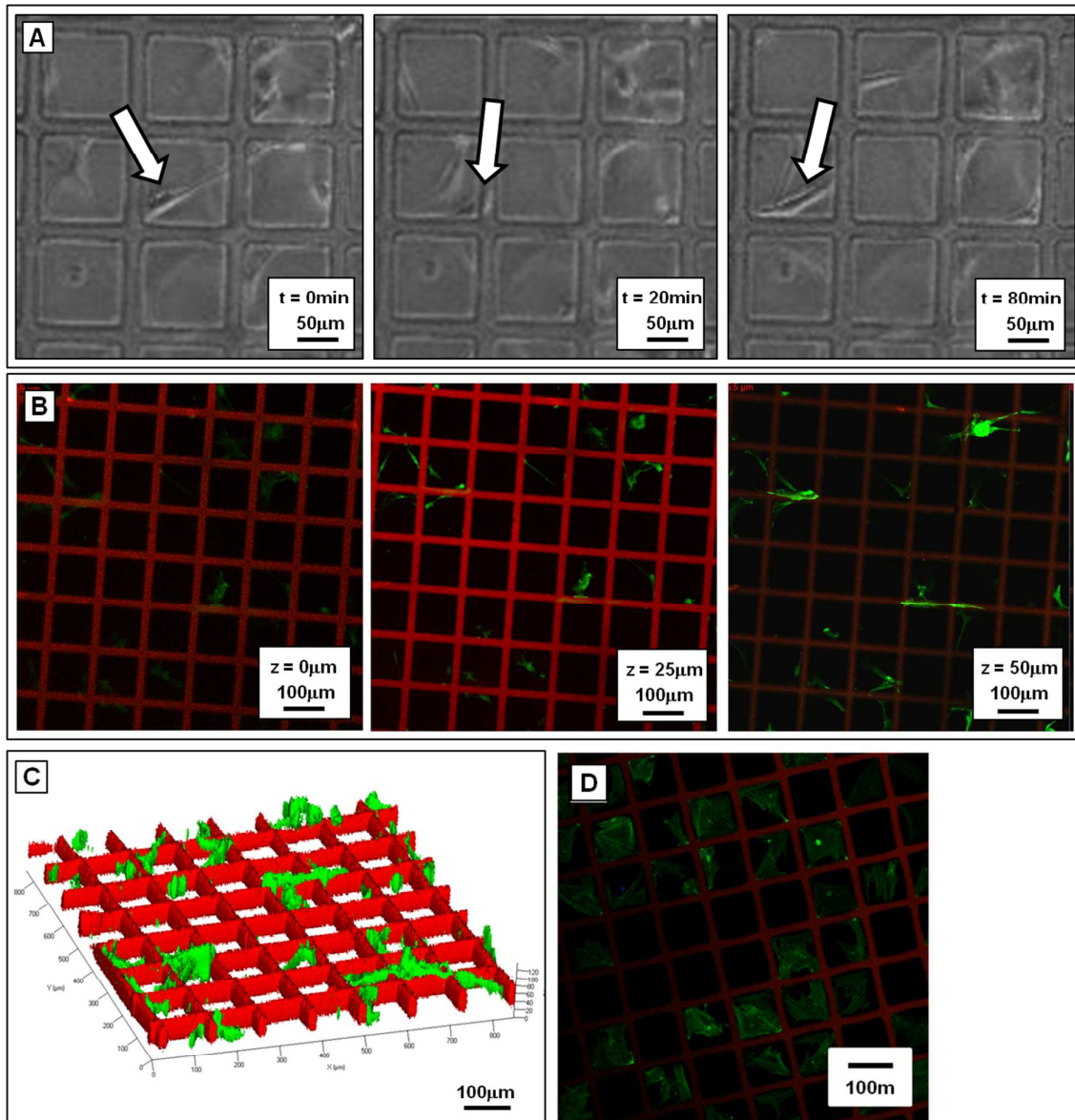


Figure 3.

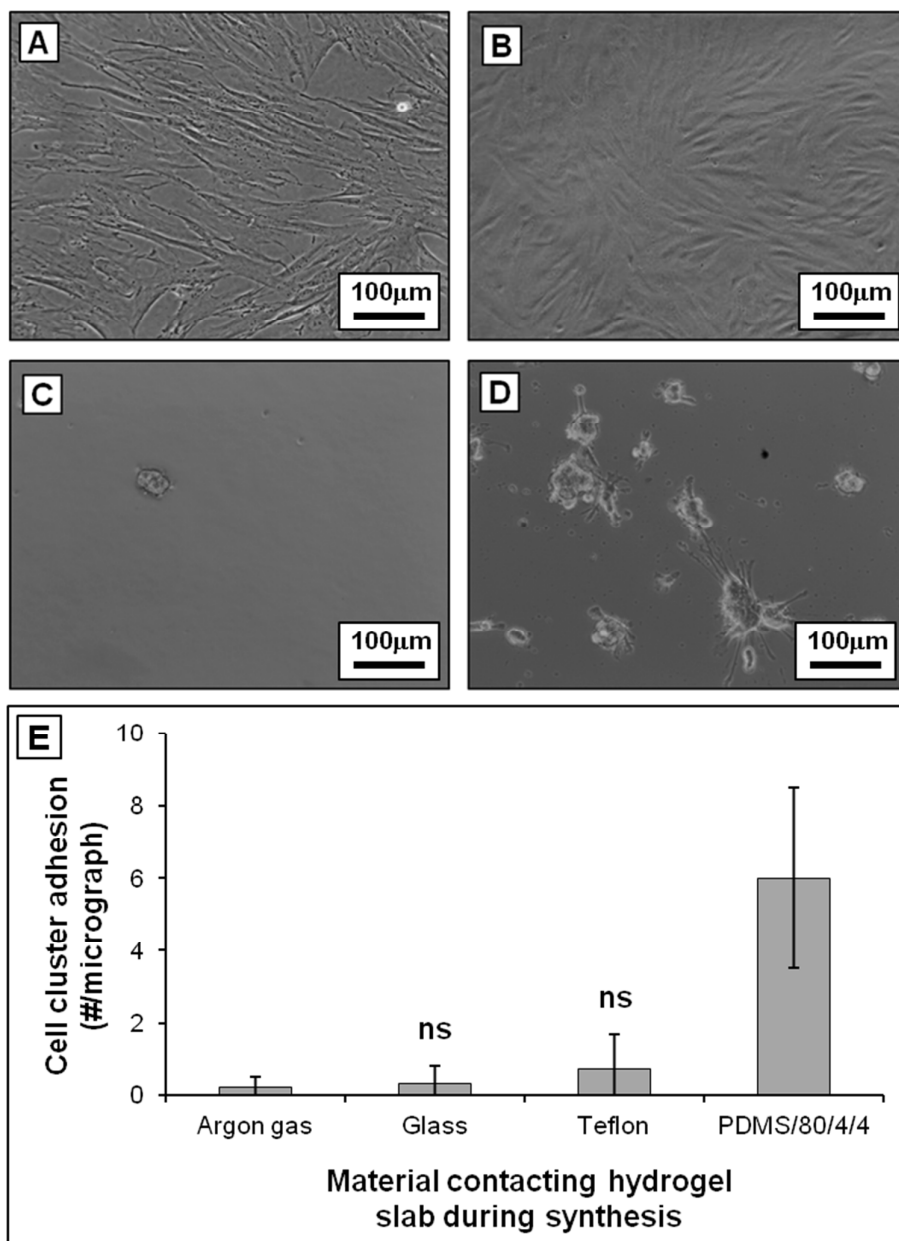


Figure 4.

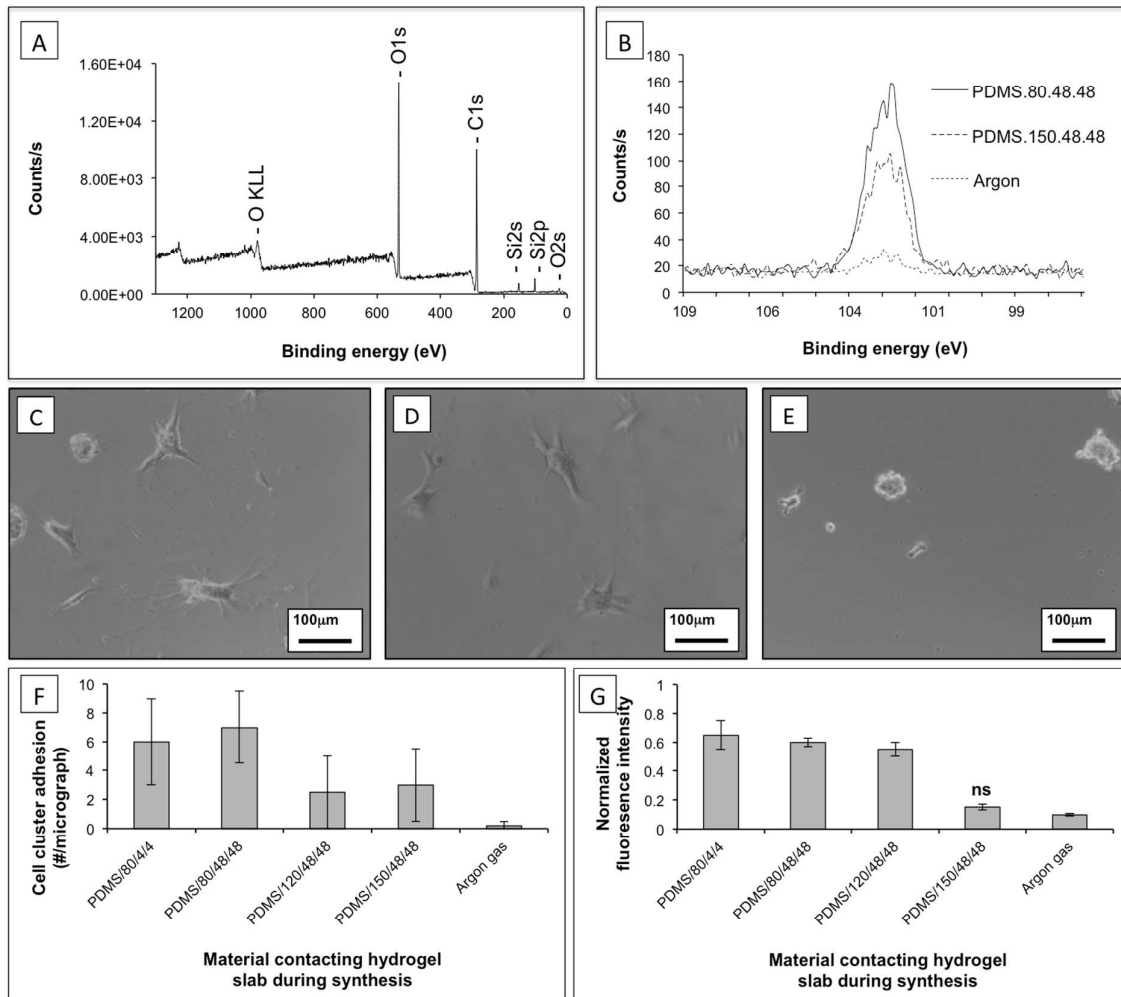


Figure 5.

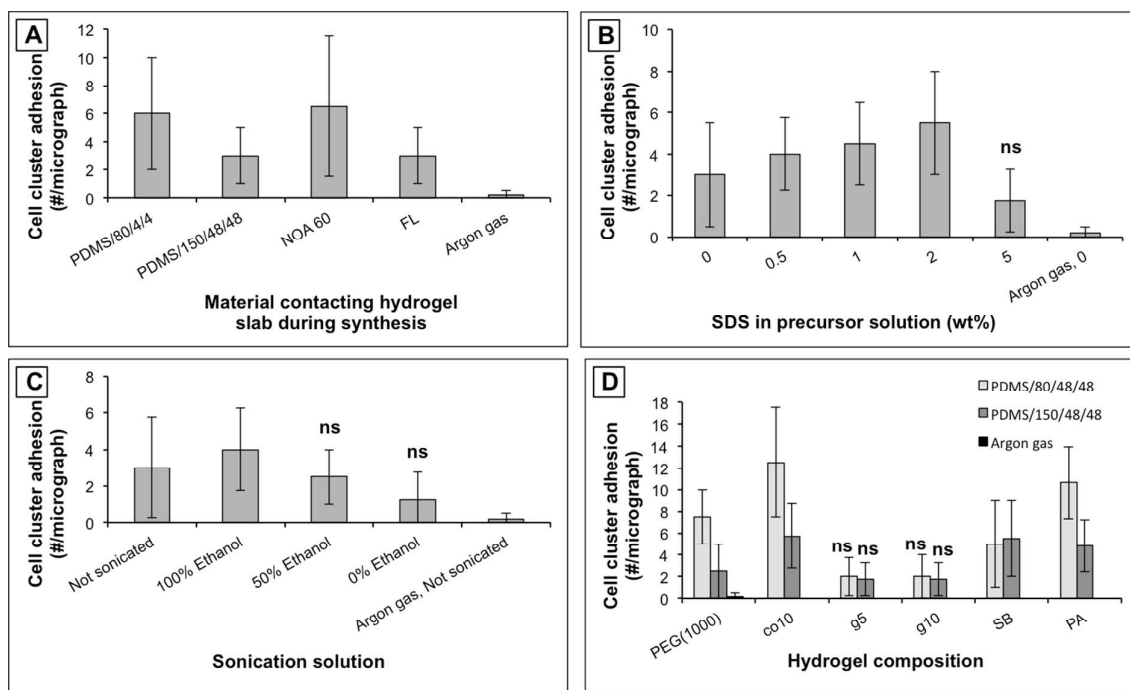


Figure 6.

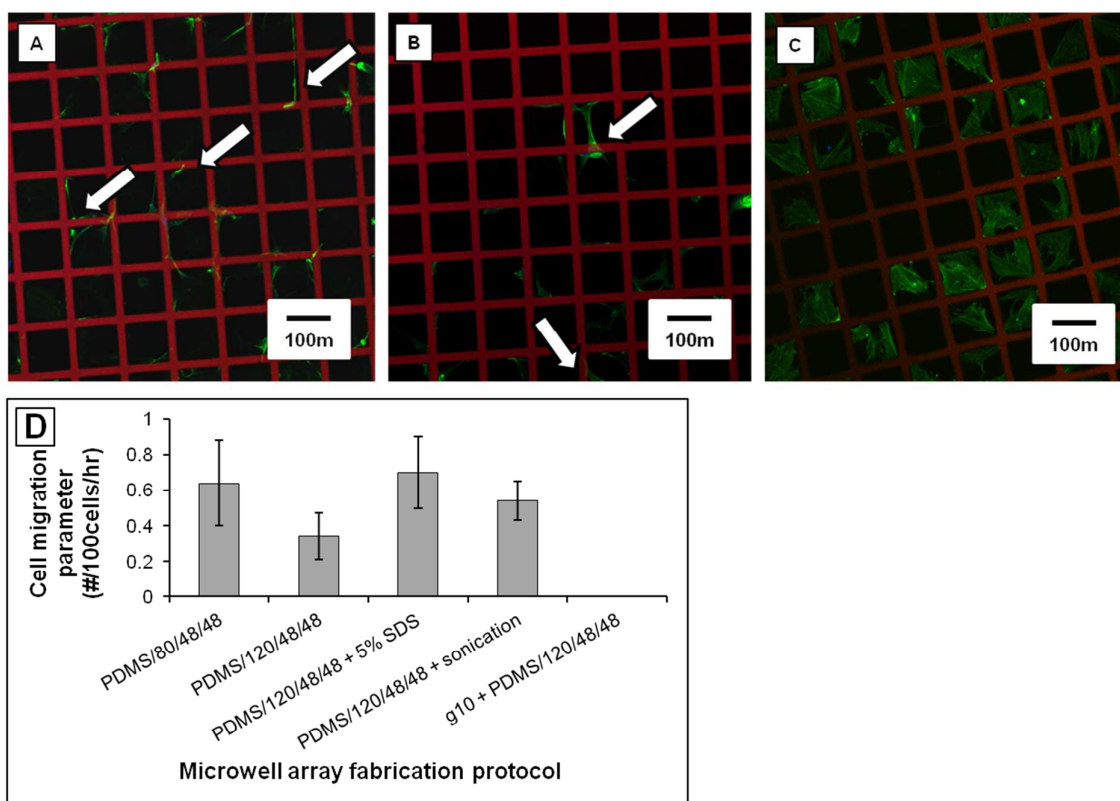


Figure 7.

