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Effects of Micro-Features on Cell Detachment from Poly(N-isopropylacrylamide) Coated Polydimethylsiloxane Membranes

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Honors Project 4200:497-002

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Abstract

The tested hypothesis was that features on polydimethylsiloxane (PDMS) surfaces coated with a poly(N-isopropylacrylamide)/aminopropyltriethoxysilane or pNIPAAM/APTES thin film would accelerate cell detachment than the film coated on a unfeatured surface. This project tested samples with features generated by molds, wrinkling, and sandpaper roughened substrates. Surface feature generation methods were limited to mechanical means, and characterized by microscopy and strain rates. 50/50 mixtures of 1.5 wt.% pNIPAAM/ APTES were used to coat thin films (30-40 nm) on PDMS membranes by spin-coating, and the coated membranes were thermally annealed to chemically graft pNIPAAm/APTES on the membrane and their thermo-responsive property was assessed by water contact angle at a temperature above and below the transition temperature of pNIPAAm (i.e., 32°C). The contact angle was found to be 47.7° at 25°C and 95° at 40°C, illustrating the thermo-responsivity was achieved. . They were then seeded with human mesenchymal stem cells and incubated at 37 °C until cells reached confluence, after which, they were cooled to room temperature to allow cell detachment. The fastest detachment results came from surfaces wrinkled using a 1-dimensional strain of 0.5, with wavelengths and depth of features at the micron scale. On these surface, the first group of cells detached in 12 min , and all cells detached in 22 min, as compared to 14 min and 44 min for pNIPAAm/APTES on featureless membrane. The featured membranes were shown to significantly improve cell detachment, by allowing water to flow through channels, created by wrinkling, below the cells to accelerate pNIPAAm film hydration, hence speeding up cell detachment.

Executive Summary

Problem

Currently in industry there is a need for surfaces that allow cells to grow quickly and healthily, and after their incubation period is complete to detach them quickly without damaging the cells. The current cell harvesting process involves many steps that may cause damage to the cells being grown and can be time and labor intensive. This project sought to improve cell detachment time, with ease of use and deployment.

Results

The critical results from this project include the determination that the average depth of features that can be generated on PDMS using strains of 0.3-0.5 include a depth of 50-200nm and a width of 2-5μm. These features do not significantly increase the surface area available for cell growth, but do reduce the detachment times. UpCell™, a commercial surface meant to allow for rapid detachment, had a detachment time of 20mins for the first cell, with incomplete cell detachment even after 75mins and shaking the dish. One dimensional stretching done with Sample O, the best sample for cell detachment, which was coated with the thermo-responsive polymer, poly(*N*-isopropylacrylamide) or pNIPAAm, had the first cell (shown on a screen, with an area of 1280 μm x 970 μm) detached in 12mins and with all cells being fully detached 22mins after cooling, and no cells remaining on the surface after the dish being gently shook at 30 mins. For pNIPAAm coated PDMS membrane without features, the first cell detachment took 14mins with full detachment not occurring until 44 mins.

Coating the PDMS surface drastically changed the surface chemistry due to the added thermo-responsive materials. Uncoated PDMS without features had a water contact angle of 60.5° at 22°C and 74.4° at 45°C. The coated surface had a contact angle of 47.7° at 22°C and 95°

at 45°C showing the changes from a hydrophilic surface at low temperatures to a hydrophobic one at temperatures higher than the lower critical solution temperature (LCST) of pNIPAAm.

Conclusions

This project showed that adding surface features will accelerate cell detachment from pNIPAAm coated surfaces, and the features can be easily created by using one-dimensional stretching of the PDMS membrane prior to coating pNIPAAm. The thermoresponsive nature of the pNIPAAM/APTES matrix was shown to improve cell detachment at room temperature, and to be favorable for cell attachment and growth at incubation temperatures. The hydrophobic and hydrophilic nature of the surface was clearly demonstrated at different temperatures, and performs better than the pNIPAAm coated PDMS membrane without features. Cell growth and detachment was shown to be better than current commercial available products.

Implications

Skills that have been developed as a result of this experiment include general lab skills, preparation of chemical solutions, and preparation of polymer sheets. New use of equipment was also learned, including optical microscopes, IFM, SEM, spin-coater, plasma chambers, and contact angle goniometer. These skills can be used in future lab settings, and also help me to better understand good lab practice in the future. Other skills that have been developed include better problem solving skills, confidence interpreting experimental results, and how to interpret results in the light of background knowledge. Design of and execution of experiments under a program sponsor is also a new set of skills that will be useful in the future as well.

The results obtained from these experiments can benefit society by improving process using live culture cells. This can be in disease research, biological research, and vaccine production. These forms of research directly impact society by improving medicine and

informing decisions on how to improve everyday life. There are also educational advantages to spending less time on labor intensive cell detachment methods, and spending more time studying, creating, and interpreting new experimental results.

Recommendations

Future research should include determination of optimal solution concentrations for cell detachment, and improved methods for large scale production of the PDMS surface. Using concentrations other than 1.5% in ethanol should be conducted, along with at ratios other than 50/50 pNIPAAM to APTES. Performing more precise testing in both one and two dimensions should be conducted to obtain results at a wider range of conditions. Experiments using a variety of cell types could also improve knowledge of how different cells would interact with these surfaces.

Advice for future students would include begin their projects early, and leaving plenty of time to repeat experiments where failures, mistakes, or poor conditions lead to undesired results. Having a thorough background knowledge in the field of choice, along with an understanding of previous research is critical to understand the research that is being conducted. Work closely with the project sponsor to receive regular assistance and updates, along with collecting detailed project notes along the way.

Introduction

In the process of cell manufacturing, cells grown on a surface are usually removed using an enzyme such as Trypsin. This process requires several steps such as aspirating of the culture medium, washing cells with Phosphate-buffered saline (PBS), adding trypsin and incubating the cells in the presence of trypsin, terminating the action of trypsin with more protein containing medium, collecting the cell suspension, and centrifuging the suspension to remove the trypsin containing supernatant, before the cells can be harvested. As such the process is manual and takes time, also, trypsin can remove some of the desired proteins and protein-protein interactions, hence functions of the cells and in more severe case can cause damage to the cells during this period. Furthermore, there is a potential for contamination to cells during trypsinization. In recent years, the use of stimuli responsive surfaces, especially thermo-responsive (TR) surfaces, to eliminate the use of Trypsin in cell harvesting has attracted great interests. However, there are still many issues associated with these TR techniques. Two main issues are the detachment rate, which could be in hours, and the detachment temperature that in some cases has to be at ~ 4°C.

Our research group has been investigating and developing simple methods for creating TR surfaces, mainly poly(N-isopropylacrylamide) or pNIPAAm based surfaces, for faster (< 30 minutes) cell detachment at room temperature[1,2,3,4] pNIPAAm is a thermoresponsive polymer that shows a lower critical solution temperature (LCST) of 32°C in water. This means it is hydrophilic (soluble in water) at temperatures below 32°C and hydrophobic (insoluble in water) above this temperature. When cells are incubated close to 37°C, the hydrophobic surface allows cells to attach and proliferate. Once cells are grown to a certain confluency, cooling this coating below 32°C changes the pNIPAAm so that it attracts water, which then flushes the cells from the surface so that they can detach. Our surfaces allow the cells to detach from 5 to 30 minutes

without the need of trypsin or mechanical scraping. However, our current approaches use rigid materials, such as glass or silicon wafer, as the supporting substrates for the TR polymers, which have potential limitations (e.g., chipping off, creating sharp debris) in cell manufacturing.

This research seeks to expand on our current method by utilizing an elastic membrane made of polydimethylsiloxane (PDMS) as the substrate to reduce the concerns of substrate breakage, as well as adding features to the substrate to promote cell attachment and accelerate water penetration and subsequent hydration of the grafted pNIPAAm coating, hence allowing an even faster (within minutes) cell detachment. In particular, this research seeks to introduce wrinkling and onto the PDMS surface that the pNIPAAm is being coated to. PDMS surface has a chemical similarity to glass due to its backbone siloxane groups (Si-O-Si) that are similar to silica (SiO_x) in glass and silicon wafer.

Introducing wrinkling and features to this surface will improve cell growth by better mimicking the natural surfaces that cells traditionally grow on. Some features will be approximately 10-20 microns in height, as compared to a typical cell size of 10s to 100 microns. These wrinkles will be characterized for how they can be produced, their shape, frequency, size, and distribution. The effect that they have on the pNIPAAm coating process and cell growth and cell detachment will also be evaluated.

Background

In 2016, Newby, Brink, and Alghuniam published a study describing a method for immobilizing pNIPAAm on surfaces using an APTES network[2]. The APTES and pNIPAAm solutions are combined in ethanol at a 50/50 ratio at 1.5 wt% of total solution. They were then spin-coated onto an oxidized glass slide and cured at 160°C for 1-3 days. This created an interpolymer network between the pNIPAAm and APTES, where the APTES molecules polymerized and crosslinked with the pNIPAAm and silica oxide (or glass) surface. These interpolymer chains immobilized the pNIPAAm allowing it to be chemically bonded to a surface, and still retain the thermoresponsive properties. This method was replicated for this experiment, using a PDMS surface rather than glass. The chemically similar PDMS and glass surfaces allowed this process to remain effective.

The PDMS surfaces themselves were wrinkled using a method detailed by Yang, in which their team stretched PDMS sheets and oxidized them using oxygen plasma while under stress[5]. One-dimensional and two-dimensional stretching methods were used to obtain features in both 1 and 2 dimensions on the surfaces at the microscale. When the strain was released after oxidizing the surface, a buckling of the thin hardened surface occurred while the soft PDMS underneath returned to its original shape. This same method was also utilized to create micro-scale features in these experiments.

Characterization equipment used for this experiment included optical microscopy, infinite focus microscope (IFM), scanning electron microscope (SEM), and a water contact angle goniometer. This equipment was used to image and examine the PDMS surfaces after features had been imposed, and take measurements of the size and depth of the features that had been

generated. The goniometer was used for measuring water contact angle and effects that temperature and the thermoresponsive surface had on the surface energy interactions with water.

Methods

Preparation of PDMS Sheets

Flat featureless PDMS sheets are created by mixing two liquid reagents (Dow Corning's Sylgard® 184 elastomer and its curing agent) at a ratio of 10:1 using 10grams of base for 1gram of curative (base elastomer:curing agent), the mixture (~ 8 g) was poured into a polyethylene 100mm diameter petri dish and cured at room temperature for 48hrs. This process of producing and curing the PDMS produces a very smooth surface that is devoid of defect, presuming no air bubbles will be introduced into the reagents during the mixing procedure.

Production of Surface Features

Features were produced by three primary methods, surface wrinkling, plastic molds, and substrate surface treatment. Surface wrinkling was done by stretching the PDMS sheets on a glass slide and measuring the strain rates of the sheets. They were then oxidized under high intensity air plasma, under vacuum, for 10mins to oxidize the PDMS surface to form a thin layer of silica. The second method, plastic molds, were conducted using prepared 3D printed molds to generate large surface features, ~1mm across. The PDMS was poured over the molds and allowed to cure for 48hrs. The substrate surface treatment was conducted using polished aluminum as a substrate. The aluminum was sanded 10 times, in a uniform direction with #600 and #120 sandpaper using the method described in Shimizu[7].

Preparation and Application of pNIPAAM and APTES Coating

Solution was prepared by separately dissolving pNIPAAM and APTES at 1.5% by mass seperately in ethanol. After the pNIPAAM and APTES dissolved entirely into their respective solutions, they were combined to form a 50/50 APTES/pNIPAAM solution at 1.5%. Solutions were prepared fresh prior to each usage and used to spincoat the PDMS surface at 2000 rpm for

60 seconds under vacuum. They were then placed in an oven at 160°C for 48hrs to allow the coating to cure.

Surface Analysis

Surfaces were examined under two optical microscopes, an Olympus upright microscope (OM) (model IX 71) and an infinite focus microscope (IFM, model xxx). ImageJ and the xxx were used, respectively, to measure the depth of the surface features from the OM and IFM images.

Cell Seeding and Cell Detachment

3ml of ~150 human mesenchymal stem cells (hMSCs) in hMSC growth medium were seeded in a 35mm dish containing **pNIPAAm/APTES immobilized** PDMS samples **that had been** thoroughly rinsed with cold DI water**,** and allowed to incubate at 37 °C (>LCST of pNIPAAm or 32 °C) for 2-3 days until the cells reached confluence. The dishes were placed on the microscope stage and the warm medium (>32 °C) was allowed to cool to 22 °C. The cell detachment process was followed via a microscope-video system and using a 10X phase objective. A sequence of images were captured with a preset time interval (5 s, 10 s, 20 s or 30 s) until the attached cells were detached. The samples were shaken after 30-75mins depending on the samples, and observations were made about any remaining cells. Comparisons were made between UpCell™ and the pNIPAAM/APTES coated PDMS membranes.

Contact Angle

Water contact angle was measured using a contact angle goniometer (Model 100-00 from ramé-hart, inc). Advancing and static contact angles were measured on the oxidized PDMS membranes and PDMS membranes coated with pNIPAAm/APTES at both 22°C and 45°C to

compare hydrophilic and hydrophobic surface responses. ImageJ was used to measure the contact angles using recorded images.

Results

Note that these experiments were only conducted a single time, and thus no statistical data could be collected on the results. This is most notable in the cell detachment portion, where these results may have been a one-time occurrence. This was only a preliminary study to determine which direction to pursue further.

Strain Rates

Table 1 Shows the strain rates for stretching each of the samples. Samples labeled with a subscript X or Y denote 2D stretching in the X and Y directions respectively. Samples with no subscript were stretched one dimensionally.

Sample	Before Stretching (mm)	After Stretching (mm)	Strain
A	17	25	0.471
B	15	21	0.400
Cx	20	26	0.300
Cy	19	24	0.263
D	24	32	0.333
Ex	15	18	0.200
Ey	14	20	0.429
F	17	26	0.529
G	24	32	0.333
H	23	34	0.478
Ix	23	29	0.261
Iy	19	23	0.211
Jx	23	31	0.348
Jy	23	32	0.391
K	27	42	0.556
L	28	44	0.571
M	22	31	0.409
N	20	30	0.500
O	20	30	0.500
P	20	32	0.600
Qx	23	34	0.478
Qy	22	27	0.227
Rx	21	24	0.143
Ry	21	25	0.190

PDMS Surfaces

Many surfaces were analyzed using an optical and IFM microscope. The images will be displayed at various zooms and the images taken using IFM will be paired with measurements of the depth of the features. Largest depth of features extended to 2 μm.

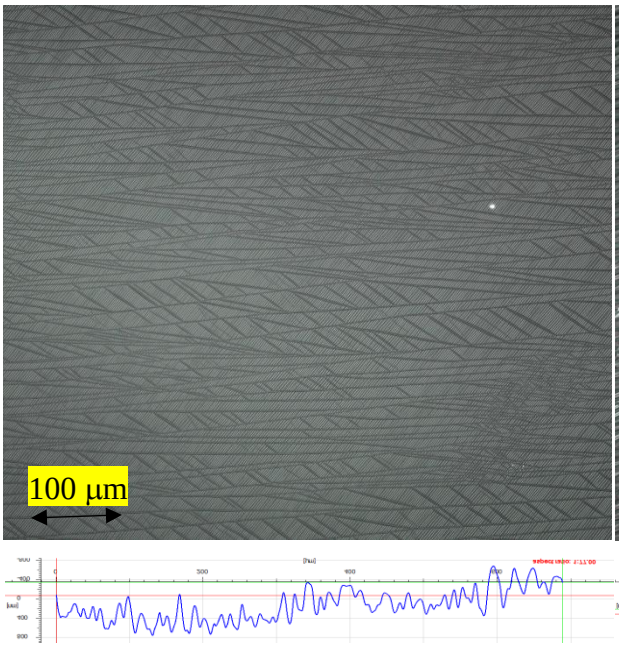

Figure 2 Sample D at 20x under IFM with depth analysis.

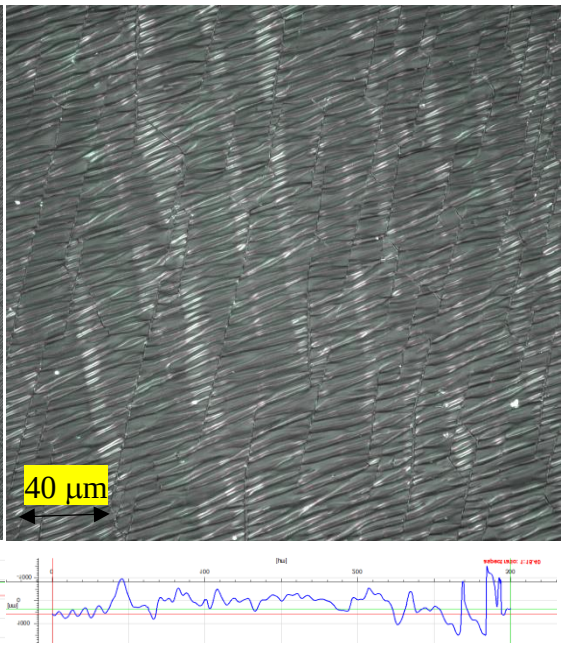

Figure 1 Sample G at 50x under IFM with depth analysis

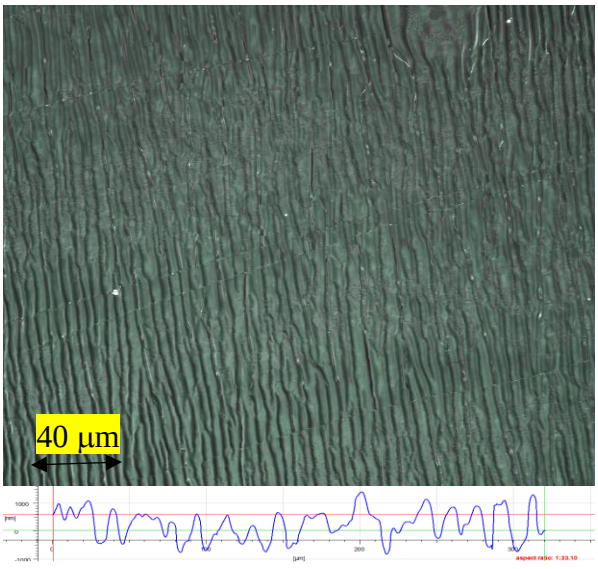

Figure 3 Sample H at 50x under IFM with depth analysis.

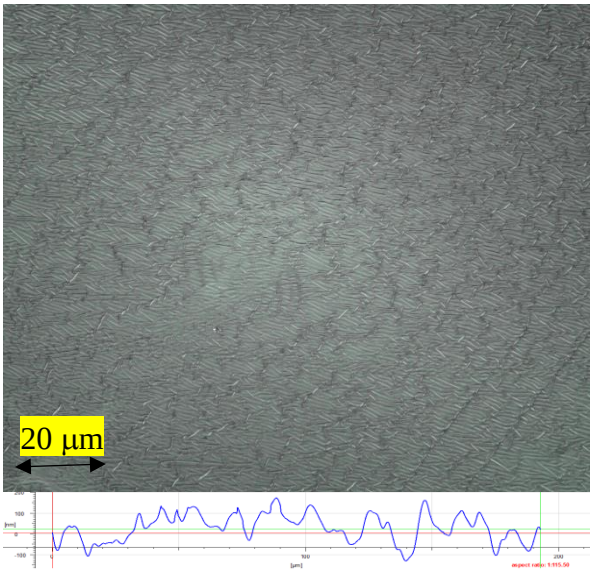

Figure 4 Sample J at 100x under IFM with depth analysis.

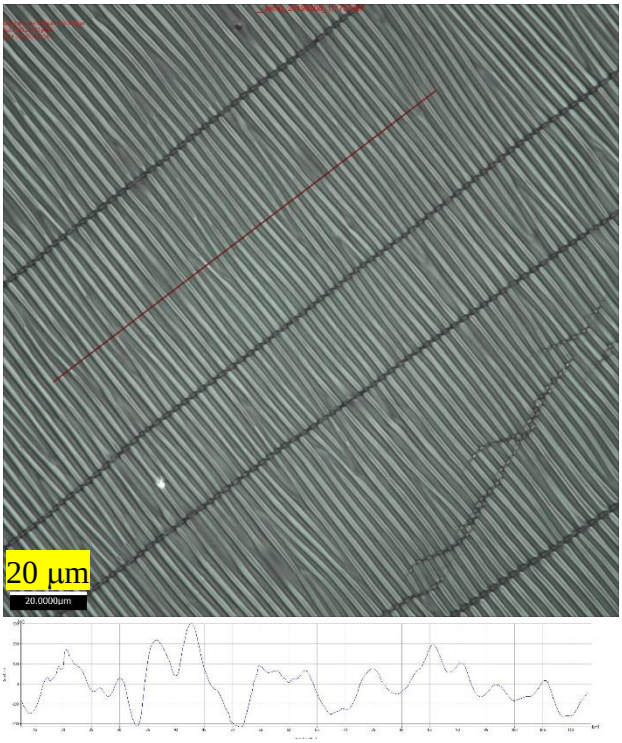

Figure 8 Sample M at 100x under IFM with depth analysis.

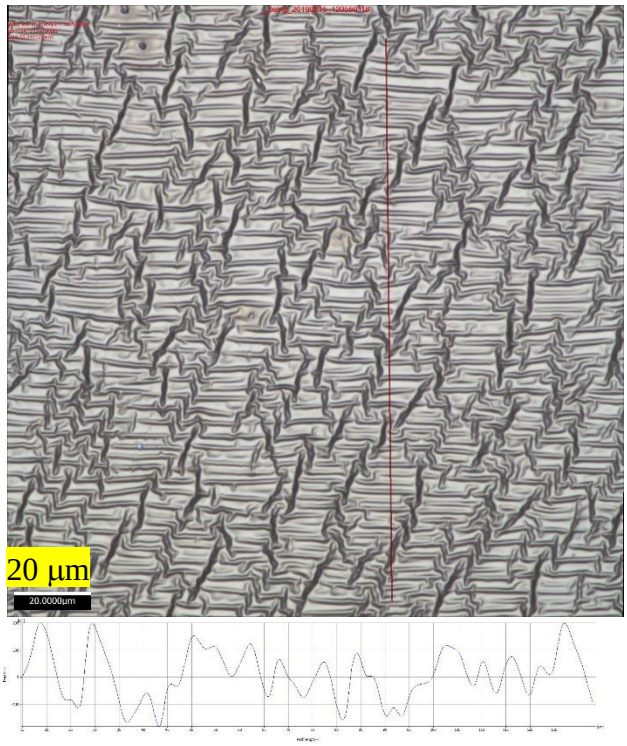

Figure 6 Sample R at 100x under IFM with depth analysis.

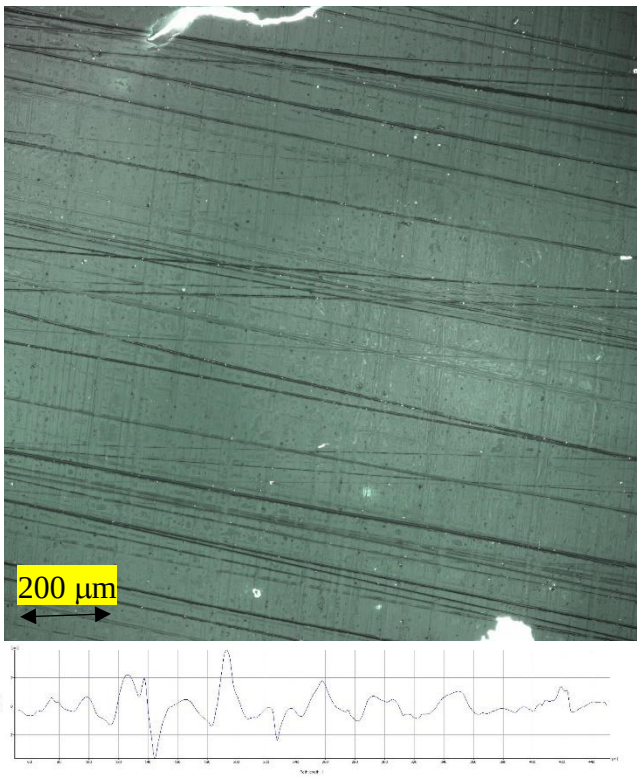

Figure 7 Sample S 10x under IFM with depth analysis.

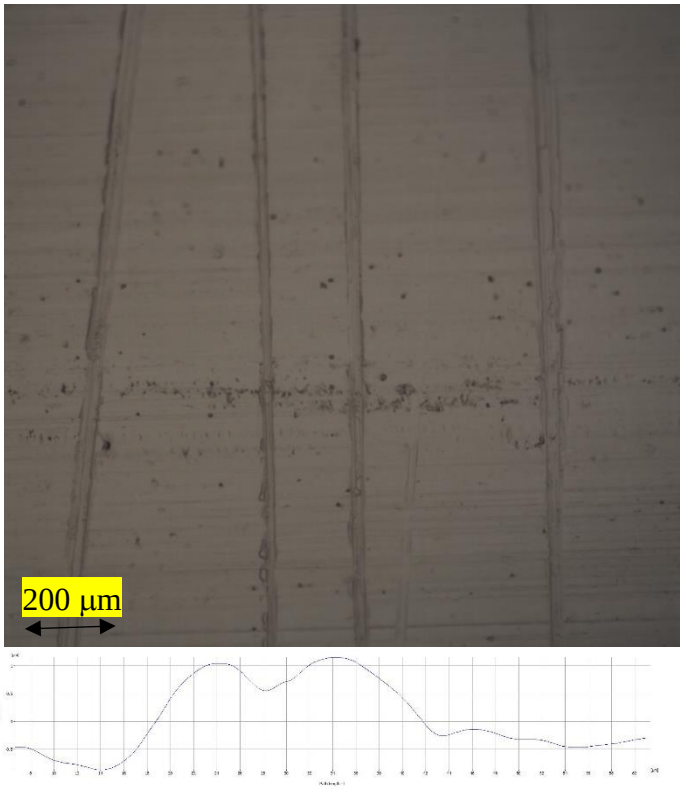

Figure 5 Sample T at 20x under IFM with depth analysis along red line.

Contact Angle

Table 2 Shows the measurements of advancing and static contact angles at two temperatures of coated and uncoated PDMS (oxidized by air plasma) samples.

Contact Angle		22°C	45°C
PDMS	Adv1	44.3°	59.8°
	Adv2	57.0°	72.7°
	Static	74.4°	60.5°
PDMS+ APTES/pNIPAAM	Adv1	67.0°	95.7°
	Adv2	59.0°	95.0°
	Static	47.7°	95.0°

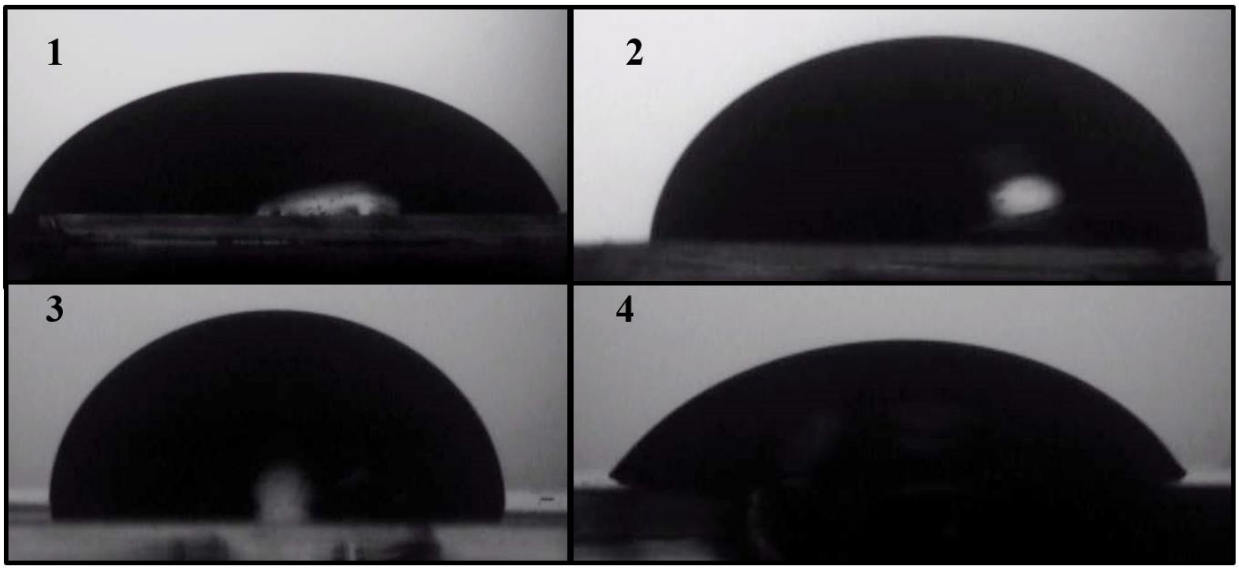

Figure 9 Shows water droplets on the PDMS surfaces at various conditions. (1) Uncoated PDMS at 45°C (2) Uncoated PDMS at 22°C (3) PDMS Coated with APTES/pNIPAAM at 45°C (4) PDMS Coated with APTES/pNIPAAM at 22°C

Cell Detachment

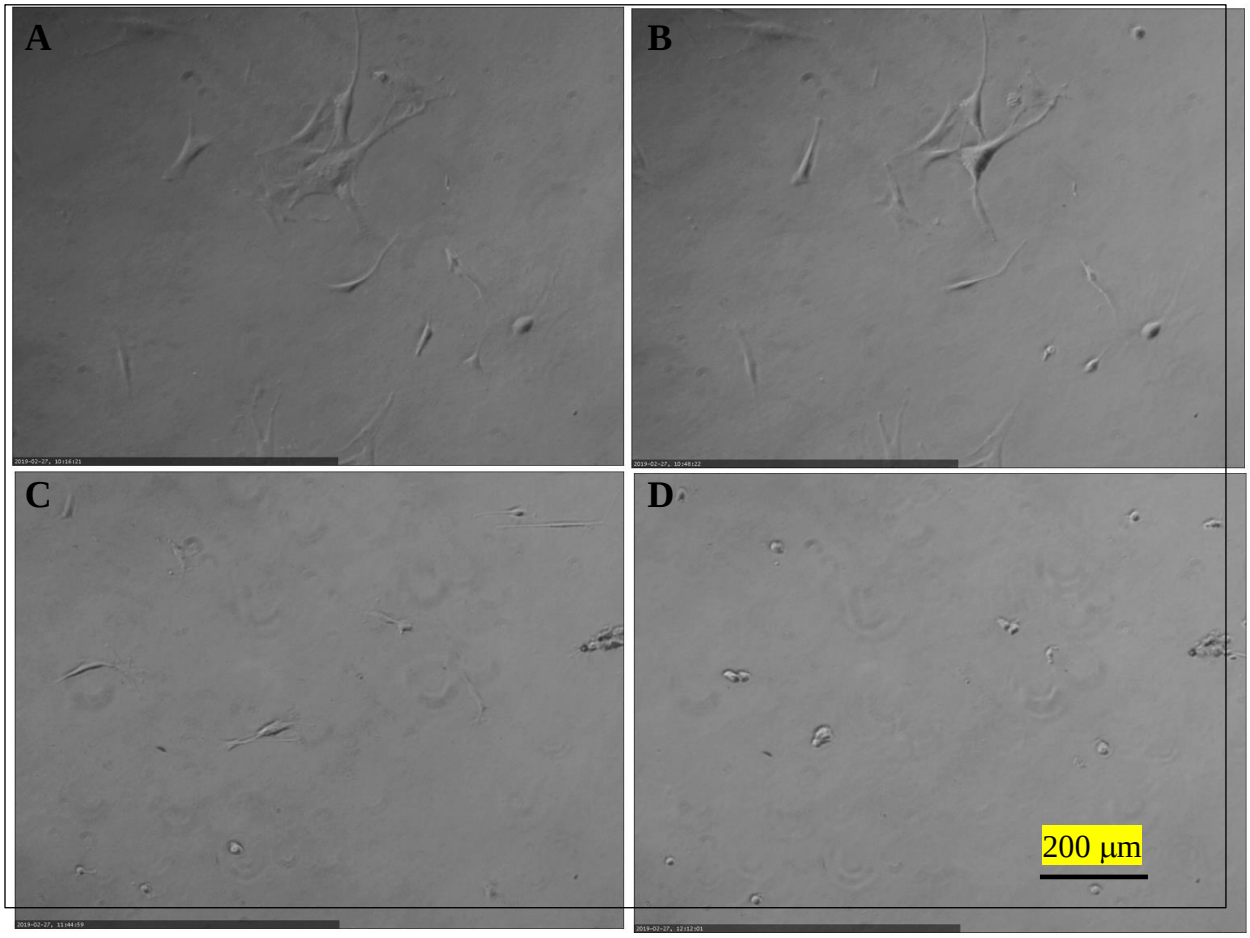

Figure 10 Shows two surfaces before and after cell detachment. (A) is UpCell™ at 4 minutes, compared to (B) which is UpCell at 75mins. (C) Shows Sample O at 4mins and (D) Is Sample O at 30 mins.

Table 3 Shows the cell detachment data for 9 samples.

Cell Detachment Data	UpCell	Control	Sample O	Sample R	Sample X	Sample Y	Sample Uc	Sample Vc	Sample Wc
Begin to Detach	19	10	10	20	29	12	14	15	22
First Cell Complete Detachment	26	14	12	26	34	15	N/A	19	30
Time for All Cells to Detach	>75	>75	22	>75	>75	>75	>75	>75	>75
Do cells remain prior to shaking?	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Time for Shaking	75	44	30	50	55	57	67	55	75
Do Cells Remain After Shaking?	Yes	No	No	Yes	No	No	Yes	Yes	Yes

Discussion

The PDMS sheets were strained according to the values found in **Table 1** prior to being cured under the UV radiation. The UV light creates a hardened outer surface according to Yang, which then forms small surface wrinkles upon release of the strain by unclipping the surface [4]. After analysis under optical and IFM microscopes, the images shown in the PDMS Surfaces section detail the different surfaces at various zoom rates. This shows the type of features that were generated from stretching in 1&2 dimensions.

Figure 2 displays sample D and shows significant surface cracking, and multiple generations of cracks along the surface of the material. This cracking occurred after the surface was UV cured and the strain was released from the material. This PDMS surface was likely hardened to the point of creating cracking patterns once the strain was released.

In **Figure 1** sample G was shown at 50x, and is a surface that was spincoated with pNIPAAM/APTES, creating the shimmering surface effects seen in the images. The surfaces displayed similar cracking to sample D, but also had wavy surface features as well, likely from the coated portion of the surface. Both surfaces were stretched with an identical strain rate (0.333), indicating that this visual change is only due to the changes in the surface treatment.

Figure 3 displays sample H at 50x, and shows a similar shimmering effect to sample G due to the surface coating. The features are likeways more wavy in nature than their uncoated surface counterparts. These wavy features replace the cracking of the uncoated surfaces likely due to the lower stiffness of the cured polymer surface, over its brittle counterparts.

Figure 4 displays sample J, which was stretched in 2D with ~equal strain in both dimensions as shown in **Table 1**. As discussed in Yang[4], 2 dimensional stretching with equal

strain can create small features, such as the ones shown in **Figure 4** where the features are nearly indiscernible even at high zoom rates, and have very little depth. The pattern is nearly a random two-dimensional system of peaks and troughs as well.

Figure 8 displays sample M at a 100x zoom rate, showing the smallest generation of surfaces features that appear between the cracking shown in lower zooms such as on Sample H in **Figure 2**. These highly ordered features are significantly smaller than cellular scales, with widths of 3-5 μm and depths of 150-300 nm. **Figure 6** has a very similar categorization of features, however it was stretched in 2D and coated with pNIPAAM/APTES. The channels and features show a more random distribution, with very similar width and depths to sample M.

Figure 7 and **Figure 5** show the surfaces prepared using the sanded aluminum surface. The features are much deeper than when using the stretching method, giving ridges sticking up at a distance of 1-2 μm. Due to the molded nature of these surfaces, they create a series of high ridges, rather than oscillating high and low sections. These features are easily visible at 20x and can also be seen with the naked eye with relative ease.

The contact angle of the pure PDMS surface and the coated surface were measured at room temp (22°C) and elevated temperatures (45°C). At low temperatures, the pNIPAAM become hydrophilic, and at elevated temperatures (such as those used for cell incubation) the surfaces are hydrophobic. These results can be seen clearly in **Figure 9** and in **Table 2.** These drastic changes in the hydrophobicity of the surfaces is hypothesized to be the primary mechanism for improved cell detachment.

Looking at the cell detachment data in **Error! Reference source not found.**, the commercially available surface, UpCell™, takes 19min for the first cell to detach, and after 75

mins has cells remaining on the surface, even after shaking the surface and viewing to see what remains. Contrasted to Sample O, the best performing sample, where the first cell detaches after 10mins, and all cells on this surface were detached after 22 mins. Shaking was still conducted and no cells remained. Sample R performed worse than Sample O and the control sample as well.

This is hypothesized to be due to the detachment mechanism of the samples, where the lower temperatures make the surface hydrophilic, thus flushing the water underneath the cells and lifting them off of the surface. Sample O is hypothesized to outperform Sample R because it was stretched in 1 dimension, giving linear channels for the water to travel through to get underneath the cell and remove it. Sample R was stretched in 2 dimensions, giving a more random distribution of features, not allowing the water to penetrate beneath the cells as readily.

Samples X and Y created their features using sanded aluminum substrate to mold the PDMS surface. These features did not perform as well, likely due to the larger size of the features, and their raised nature as well. Since they are spread further apart as well, they do not add a lot of benefits to the surface at the cellular scale.

Samples Uc, Vc, and Wc were created using a plastic 3D printed mold. These surfaces had significant cellular detachment issues due to the features being too large. The cells would partially detach, but the sections inside of the features would remain stuck there and not detach, even after shaking the samples.

Overall, the PDMS surface coated with the pNIPAAM/APTES solution, using a one dimensional stretching method seems to be the best performing surface for cellular detachment. Using strain rates from 0.3-0.5 seem to be the best performing surface to create features large

enough to channel water, but small enough not to allow the cells to get stuck within the features themselves. These features are also generated in parallel lines, allowing water to be channeled underneath the cells when the temperature changes significantly enough for the surface to go from hydrophobic to hydrophilic. Since these experiments were only conducted one time, statistical data hasn't been accumulated sufficiently to determine if this process is repeatable. Further work is being conducted to pursue one-dimensional strains further, but results have not yet been analyzed. Recommendations for future work have been made in these areas.

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