

Experimental Determination of Cell Adhesion and
Proliferation Response to Substrata Thickness

by

Christopher M. Bruce

Submitted to the Department of Materials Science and Engineering in Partial
Fulfillment of the Requirements for the Degree of

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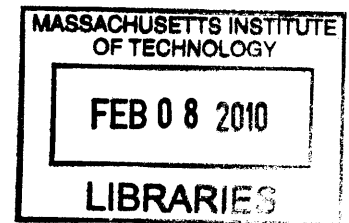
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ABSTRACT

Controlling cell behavior has been a primary goal for scientists, and physical interactions, specifically cell-surface interactions, have the potential to be a robust system for cell control. Much research has been conducted on the effect of substrata stiffness on cell behavior, but there has been no systematic study of the effect of varying substrata thickness, and its correlation to a substratum's effective stiffness that a cell feels. Furthermore, there have been differing views on what the critical thickness of a substrate is, above which there will be no difference in cell behavior.

An experimental study was carried out to determine the effects of substratum thickness on the behavior of cells adhering to polyacrylamide thin film gels functionalized with gelatin. Relatively compliant thin film gels, with an elastic modulus $E \sim 5$ kPa, were varied in thickness on stiff glass supports from ~ 75 nm to 60 microns. 3T3 fibroblast cells were seeded onto the gels to observe differences in behavior.

Observed cell behaviors were the projected area of the cells on the surface due to adhesive spreading and the rate of reduction of Alamar Blue dye, which correlates to the proliferation, or growth rate, of the cells. It was found that the cell area had a fairly well-defined power-law dependence on substrate thickness, while the gel thickness did not have a detectable effect on the rate of proliferation of the cells. Additionally, a theoretical model for thin film deflection was fit to the cell area data, and it described the area-thickness relationship well. By using the theoretical model, a critical thickness of $2.3 \mu\text{m}$ was identified over which average cell area would not change significantly. This critical thickness was found to be on the order of the reported length scale of focal adhesions in the cells, not the lateral dimensions of the cell.

These results are useful in establishing a practical lower limit of film thickness for normal cell behavior. Additionally, this relationship could be exploited as a way to control stem cell differentiation, cell size, cell motility, cell ligand density, and other cell behaviors.

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Introduction

Controlling cell behavior has been a primary goal for biologists, chemists and material scientists alike in recent history. In each field, advances have been made that allow for further control of both individual cells and cell populations. Chemistry in particular has been a primary way in which scientists choose to direct cell behavior, but physical interactions, specifically cell-surface interactions, also have the potential to be a robust system for cell control.

For adherent cells such as fibroblasts, smooth muscle cells, and mesenchymal stem cells, the surface to which a cell adheres provides a direct way to control cell behavior, not only with surface chemistry but also with the mechanical properties of the surface material. Mesenchymal stem cells have been shown to adopt functional behaviors or express tissue-specific proteins differently based on the stiffness of the substrate to which they adhere [1]. Similarly, cell spreading, cell migration and focal adhesion assembly have been shown to be affected by substrate stiffness [2-4].

Ultimately, a cell interacts with a surface by deforming the surface with traction forces applied through its focal adhesions, or localized adhesion sites on the cell membrane, and this is the only signal the cell has to “feel” the stiffness of the material. If a compliant film is made on top of a very stiff substrate such as glass, as the thickness of the film decreases into the length range of the size of the deflection the amount of deflection that occurs at the surface is constrained by the attachment to the underlying stiff substrate. This will make the effective stiffness felt by a cell on the surface greater than the actual stiffness of the thin film material.

Yang et al. have concluded that if the gel height is on the order of the lateral dimensions of the cell, then cells will be affected by the increased effective stiffness from the underlying substrate [5]. Additionally, they predict that far away from the site where a traction force is applied, there are significant differences in deflection between 1000 μm and 70 μm gels. However, this difference in deflection far away from the adhesion site would not be expected to affect cell behavior. Engler et al. have shown that smooth muscle cells show similar spreading on compliant polyacrylamide thin films of 70 μm and 5 μm , but show significantly different spreading on films with thicknesses of 70 μm and 0.5 μm [1]. This implies that there is a critical thickness between 5 μm and 0.5 μm where the cell can begin to feel the underlying stiff substrate through the soft thin gel film, or alternatively, where the deflections that the cell makes on the surface of the soft thin film – at the adhesion site – are affected by the underlying stiff substrate. This length scale is much smaller than the lateral dimensions of the cell, so it was concluded that the critical thickness would be on the order of the size of focal adhesions that cells use to attach to the surface and move across it, which are on the order of 1 μm [6].

Current research in the Van Vliet Lab for Material Chemomechanics at MIT models the deflection of a thin film on a rigid substrate due to a shear stress applied to the surface by the focal adhesions of a cell, and the deflection is termed as a function of the ratio of film height to the radius of the focal adhesion [7]. This model maps the effect of the thickness of a film on the deflection that can be made at the surface, where the cell would apply the force. According to this model, the deflection at the surface for a certain traction force will decrease by 10% once the gel thickness has decreased to 1.7 times the diameter of the focal adhesion, and it will decrease by 50% once the gel thickness has

decreased to 0.24 times the diameter of the focal adhesion [7]. If one pinpoints this 10% change as the threshold for significant change in surface deflection, then this gel height of 1.7 times focal adhesion diameter can be identified as a critical film thickness above which surface stiffness-controlled cell behavior should not change significantly.

The effects of varying substrate modulus over a wide range have been studied extensively, but research studying the effects of varying substrate thickness is lacking. In particular, no one has systematically probed into the effects of thin film thickness over a range of values on the order of focal adhesions. Furthermore, little research has been done on the effect of film thickness, or effective film stiffness, on the proliferation rate of cells. By studying the effects of varying substrate thickness over a range that spans the length scale of focal adhesions, we should be able to uncover the critical thickness above which cells are not affected by stiff underlying substrates and confirm or contradict the theoretical model that has been created in the Van Vliet group. If a strong relationship is found, this could be useful as a practical lower limit of film thickness for normal cell behavior and also as a way to control stem cell differentiation, cell size, cell proliferation, cell motility, cell ligand density, and other cell behaviors.

Experimental Methods

Polyacrylamide gel thin film synthesis

Polyacrylamide (PAA) thin gel films up to thicknesses of 60 μm were made on glass coverslips, according to the well-established protocol of Wang and Pelham, using 40% acrylamide solution and 2% bis solution (Bio-Rad Labs, Hercules, CA) as a crosslinker [8]. Polystyrene beads (Sigma-Aldrich, St. Louis, MO) of diameter 0.3 μm ,

0.6 μm , 1.1 μm , 3 μm and 6 μm were used to control thickness in the thinner gels by adding them to the acrylamide-bis solution to make a solution that was $\sim 0.1\%$ solids. Then during polymerization the coverslips were clamped together using C-clamps. For gels over 6 μm in thickness, droplet volume was used to control the thickness. In addition, a very thin gel sample was made by clamping coverslips together during polymerization without any beads to control the thickness.

Functionalizing the surface with type I collagen and UV radiation, as in the Wang and Pelham protocol, was not used so that the radiation would not promote more crosslinking in the gel, affecting its stiffness after polymerization. Gelatin (denatured collagen) and a carbodiimide-mediated procedure were used to covalently attach the gelatin to the PAA surface instead [9]. Gelatin (Becton Dickinson, Sparks, MD) was dissolved in PBS at a concentration of 1 mg/mL, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma-Aldrich, St. Louis, MO) was added in a concentration of 50 mM. Within 5 minutes of adding the EDC to the solution, the PAA gels were covered with the gelatin-EDC solution for 2 hours, and then rinsed gently with purified water multiple times. Gels were always stored in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Cambrex, Walkersville, MD) at 4°C for no more than 2 weeks.

Thin film characterization

It was found that the polystyrene beads compressed during clamped polymerization, resulting in thin gel film thicknesses less than the height of the beads that were in solution. Therefore, gel heights were not taken to be the same as the beads in

solution and were separately verified using AFM (Molecular Imaging, now Agilent, Santa Clara, CA; Si₃N₄ pyramid-shaped cantilevers with spring constant $k = 0.03 \text{ N/m}$ from Veeco, Camarillo, CA) height profiles over scratches or other edges of the gel film. The thicker gel film heights were found using calibrated optical microscope (Olympus IX81, Center Valley, PA) through focusing on the gel surface and the glass-gel interface.

Gel stiffness was determined using AFM indentation. Indents were made of $\sim 1 \text{ }\mu\text{m}$ on gels thicker than $50 \text{ }\mu\text{m}$ so that the thickness of the gel would not affect the stiffness measurement. Force-distance indentation curves were analyzed to determine Young's elastic modulus, E , using Igor Pro analysis software (WaveMetrics, Portland, OR), and a data fit corresponding to a conical/pyramidal indenter geometry (power law relationship where the power was fixed at 2) [10].

Cell culture and proliferation assays

The cells used in this study were NIH 3T3 fibroblasts (ATCC: CRL-1658). Cells were cultured in DMEM (Gibco/Invitrogen, Carlsbad, CA) with 5% bovine calf serum (HyClone, Logan, UT), seeded at a surface density of $5,000 \text{ cells/cm}^2$, and passaged at 80% confluence. Experiments were done in duplicate, and cells were seeded onto the PAA gels in plain media. After 24 hours, the coverslips containing the gels were transferred to new wells containing the normal media plus 5% Alamar Blue (AbD Serotec/MorphoSys, Kidlington, UK). Pictures of the cells were taken at 10x magnification with a phase contrast optical microscope (Olympus IX51, Center Valley, PA) immediately after transfer, 24 hours after seeding. Cells were also seeded onto PAA gels with no collagen functionalization to ensure that there was no adhesion to the

polyacrylamide itself. Alamar Blue samples were taken before immersing the coverslips, immediately afterwards, and at later intervals of 4, 12, 24, 36, 48 and 72 hours after seeding the cells. Control experiments were also done to ensure that the reduction of the Alamar Blue by the PAA gel itself was negligible.

Observation and Analysis

Cell area was analyzed using both Adobe Photoshop and ImageJ to identify the individual areas of a population of cells. Care was taken to only measure the area of cells that were not touching others, which would inhibit cell growth through confluence. Statistical analysis was done on the results to verify that the cell area distribution was single-peaked and not problematic in any way as well as to characterize the cell population at each gel thickness.

Alamar Blue samples were analyzed by taking the difference of the absorbance at 570 nm and 600 nm using UV-vis spectrometry (Cary 50 Bio by Varian, Walnut Creek, CA)). This value plateaus at a similar value for all cell densities after an amount of time, but the rate at which the absorbance difference value changes differs according to cell population size. This rate is approximately linear, and linear fits were made to the data for early time periods to determine the cell population. Control data was acquired by seeding cell populations at different densities in 5% Alamar Blue solutions, observing the rate at which the Alamar Blue was reduced, counting the cells after completion of the control experiment and then correlating the rate of Alamar Blue reduction to the cell numbers.

Results

PAA gel thickness

The thicknesses of the polyacrylamide gels were found using two methods. For thicker gels (greater than 6 μm in thickness), calibrated through focusing was used, and for thinner gels AFM surface profiles were used to find the gel thickness. The thicker gels followed very close to thickness estimates calculated from the volume of acrylamide-bis solution used during polymerization, so these estimates were taken to be valid. Finding the thickness of thinner gels proved more difficult because the beads used to control height compressed when they were clamped down during polymerization. The beads appeared to deform both plastically and elastically since their measured height was found to be less than the original diameter and since the final gel height was thinner than the final bead height. Presumably, the gels and beads were the same height under compression while the gel was polymerizing, and once the system was unloaded the beads rebounded elastically to some extent but the gel did not since it polymerized from liquid to gel while compressed. This compression effect made the gels much thinner than the expected height of the polymer beads (Figures 1 and 2), but more accurate analysis of the effects of thickness on adherent cells was allowed since the true thickness was known.

bead diameter (μm)	0.3	0.6	1.1	3.0	6.0
gel height (μm)	0.075*	0.15	0.6	0.8*	1.1

Figure 1. Table of bead diameters used to control gel thickness and the resulting gel thicknesses. * denotes that this height was not measured directly, but was estimated from the data trend.

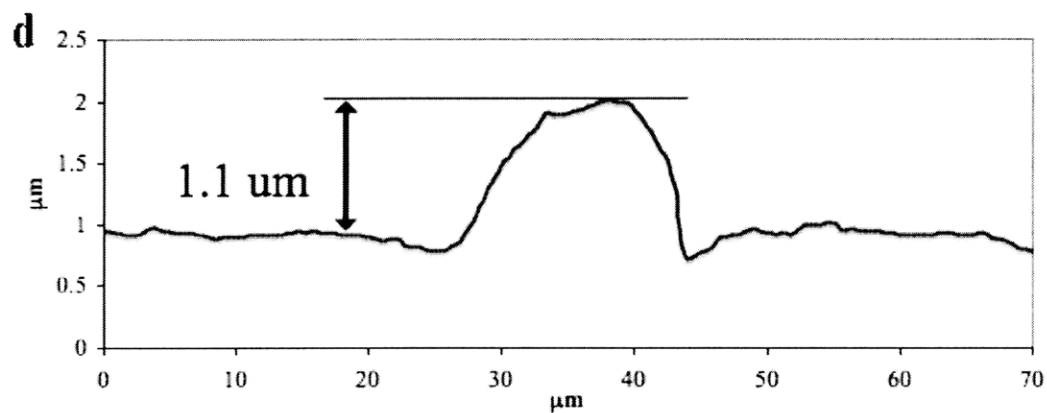
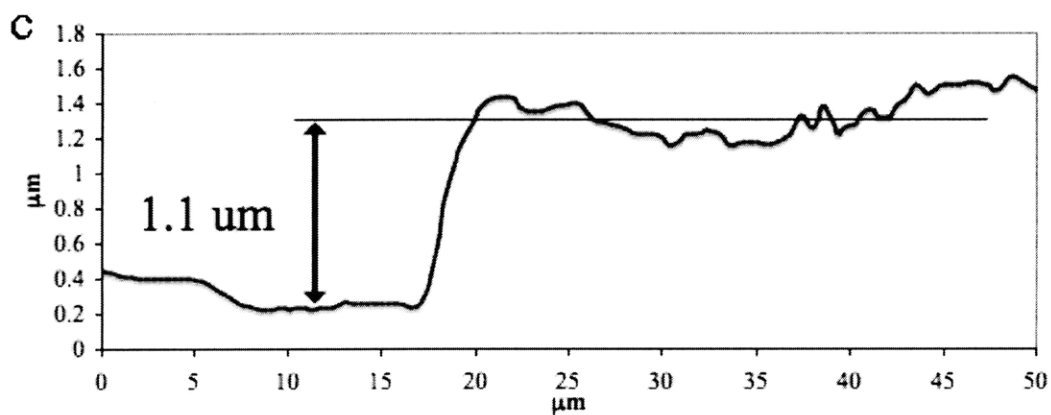
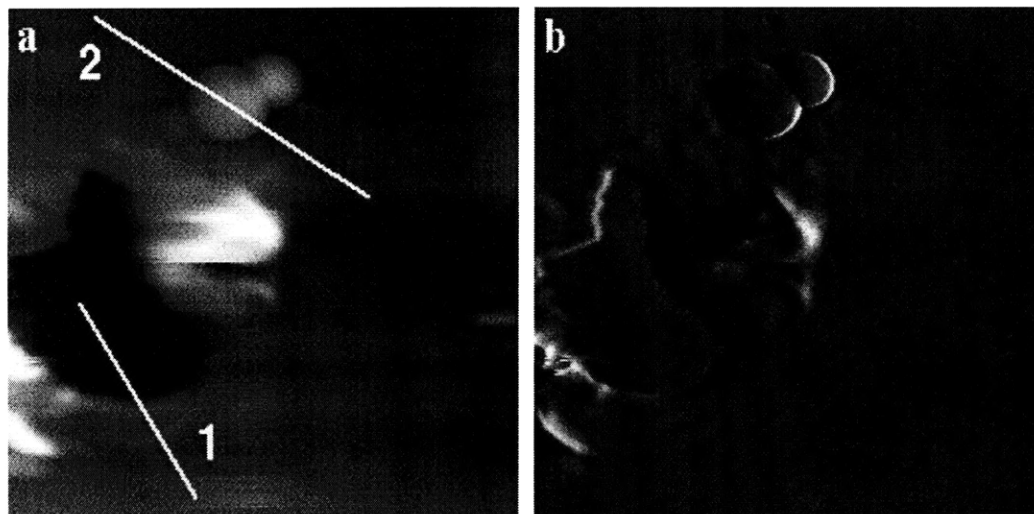


Figure 2. a) AFM height image of gel made with 6 μm -diameter polystyrene beads. 2 beads and an area of bare glass can be seen. b) AFM deflection image of same area. c) Height profile of line (1) in image (a), a gel height of 1.1 μm can be seen. d) Height profile of line (2), showing the bead is only 1.1 μm above the gel height for a total bead height of 2.2 μm despite the original 6 μm size. The bead also has a larger measured width than expected, even considering the probe tip's pyramidal geometry.

Imaging the edge or scratches of gels was very difficult due to the compliance of the gels. At many points the gel would delaminate from the glass substrate due to the force of the AFM tip scanning over it, and this produced unreadable results. Because of this, some gel thicknesses were not be measured directly, and their heights were interpolated from other measurements. However, enough clear height profiles were obtained of different gel thicknesses to allow confidence in these estimates.

Gel thickness measurements were also taken before and after attaching gelatin to the PAA surface to see if this changed the effective gel thickness. The difference in thickness before and after gelatin attachment was less than the standard deviation of the height measurements, so it was concluded that the gelatin did not make a significant difference in gel film height.

PAA gel stiffness

Indentations were made using the AFM on 60 μm thick gels to verify their stiffness. Thick gels were used so that the stiff glass substrate would not interfere with the measurement and a thin film correction would not have to be made. Indentation curves were analyzed using a model for a conical indenter geometry. Many indentations were made, and an average stiffness of 5.60 kPa with a standard deviation of 1.06 kPa was found. A typical indentation curve and its corresponding fit can be seen in Figure 3. The indentation model fits very well with the data with a slight deviation at the highest indentation depths. This is due to the effects of the glass support and the compression of the thin film affecting the measurement.

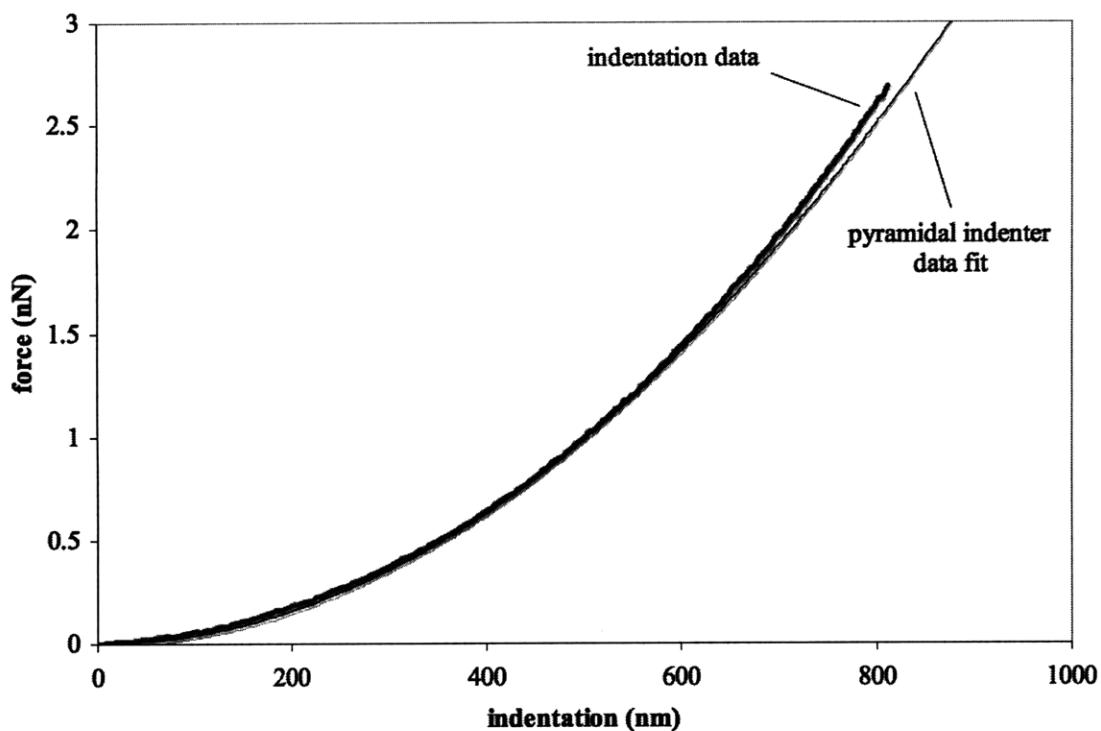


Figure 3. AFM indentation curve of thick PAA substrate. Data was analyzed using a fit for a conical indenter geometry. For this particular sample, the Young’s modulus was calculated to be 6.6 kPa, although the average stiffness of the gels was 5.60 ± 1.06 kPa.

Cell spreading and thin film deflection model

Adhesive cell spreading was measured by acquiring phase contrast images of the cells 28 hours after seeding onto the gels. A variety of cell shapes were observed, with cells on thinner gels tending to spread out and extend more, while cells on thicker gels tended to stay more spherical, with fewer dendritic protrusions.

Two methods were used to quantify the projected cell area A_c accurately. One approach used the software program ImageJ (National Institutes of Health, Bethesda, MD) and an algorithm to enhance the contrast of and sharpen the image of the cells

against the background. Then the software was used to detect the outline of the cell and quantify the substrata-contact area covered by each cell. The second method was very

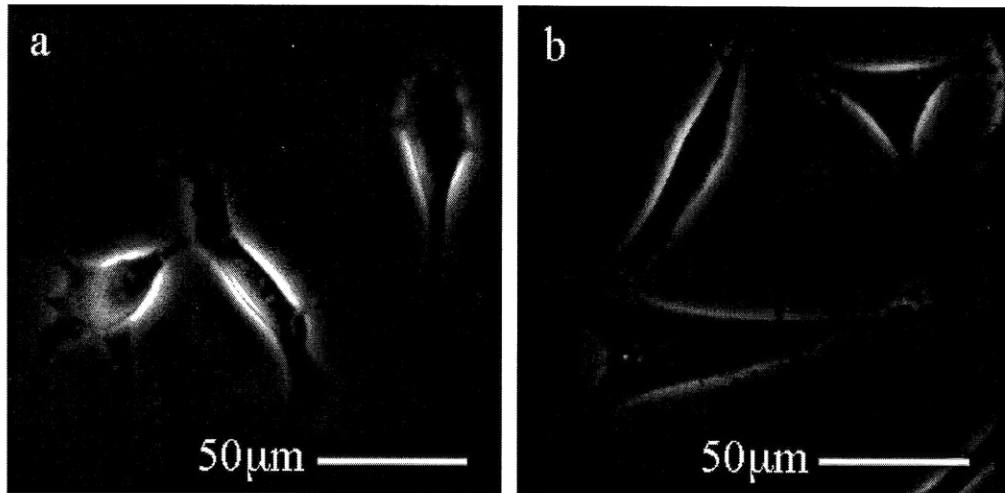


Figure 4. a) Cells adhering to a gel film of $\sim 30 \mu\text{m}$ thickness. These cells have a relatively small contact area with the substrata, and show less spreading on the surface than those adhering to a $0.075 \mu\text{m}$ thick gel (b) of the same composition and processing

similar, but was conducted in Adobe Photoshop using an outlining tool. The methods produced different values for cell area at each gel thickness, but each dataset followed the same trend qualitatively and quantitatively, with a consistent magnitude of offset between A_c datasets.

Furthermore, multiple experiments were done to measure the projected area of adherent cells. For the separate experiments, there were also offsets in the data similar to those between the two analysis methods, but this was due to natural variation in average cell size instead of differences in analysis technique. These differences in cell area between experiments and measurement method caused a rather wide range of cell area values at each gel thickness. However, despite the differences in values, the trend of the

data was consistent across experiments and analysis methods, indicating that there was a strong correlation between gel film thickness and the projected area of adherent cells.

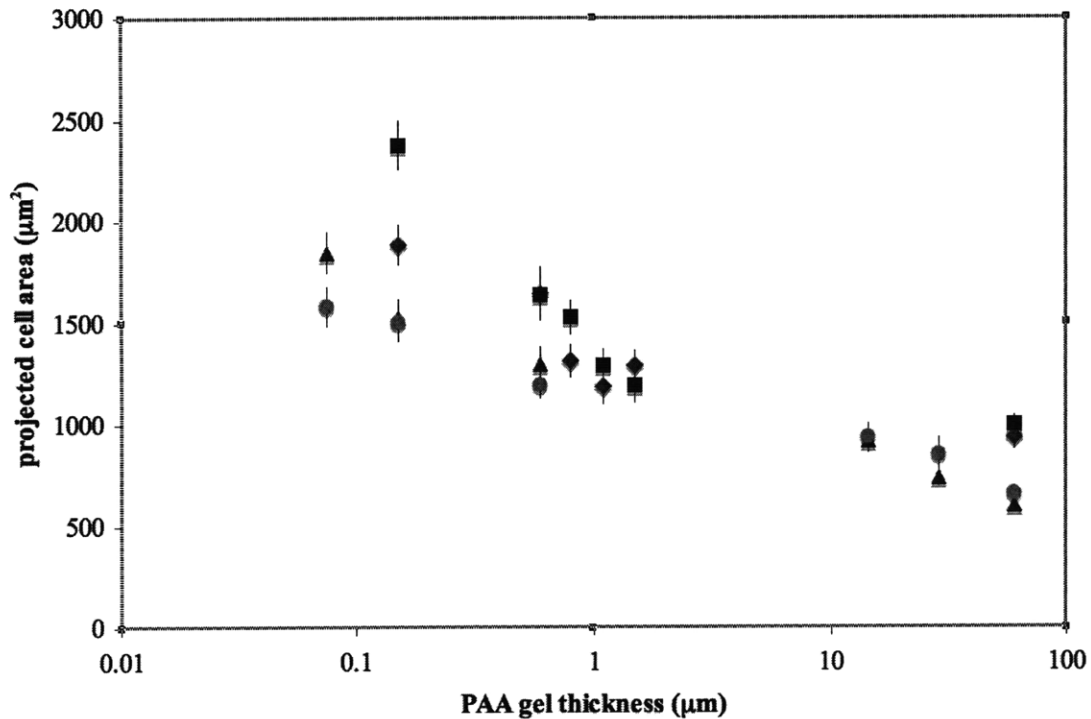


Figure 5. Projected cell area vs. gel film thickness (note logarithmic scale). ◆ and ● used the Photoshop analysis method and came from the first and second rounds of experiments, respectively. ■ and ▲ used the ImageJ protocol and also came from the first and second experiments, respectively. Data points are averages and error bars are \pm standard error.

To try and account for this variation in A_c values, the cell area measurements were normalized by the average cell area over the range of the corresponding replicate experiment, i.e. the average A_c of all the square-shaped data points. This normalization led to a much tighter range of cell area values, enhancing the trend that was observed in the original data – as gel thickness increased, the average cell area decreased. Not only did the cell area decrease, but it also closely followed the Van Vliet group’s theoretical model for surface traction induced deflection of compliant thin films (Figure 6).

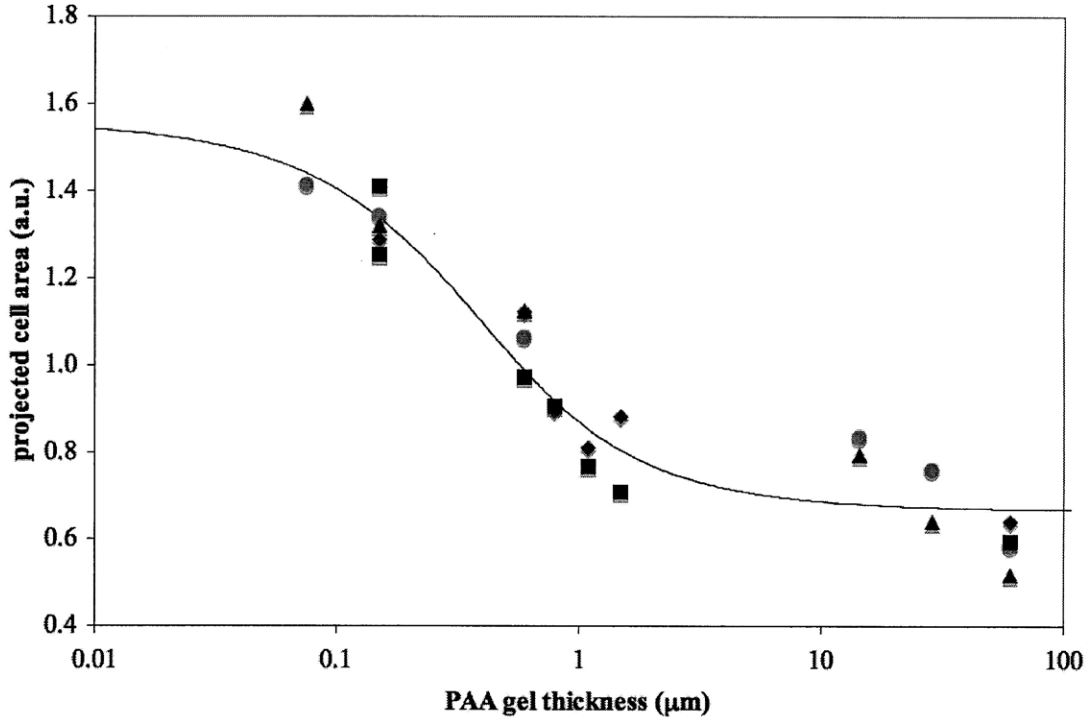


Figure 6. Normalized cell area $A_c/\langle A_c \rangle$ in arbitrary units shown as a function of gel thickness (note logarithmic scale). Normalization achieved by dividing the A_c data point by the average of the projected cell areas for the specific data set, i.e. diamonds or squares. Data points are the same as in Figure 5. The line is the prediction of the model created by the Van Vliet group for a focal adhesion of radius $0.7 \mu\text{m}$, fit to the data. $R^2 = 0.8857$.

This theoretical model is based on the deflection of a thin, compliant film attached to a rigid substrate, due to a shear stress applied over a circular area on the surface [7].

The circular area corresponds to a molecular focal adhesion. According to the model, the

shear stress at the focal adhesion will cause a maximum surface deflection of $u_B U\left(\frac{h}{a}\right)$,

where $u_B = \frac{Ta(2-\nu)}{2\mu}$ is the deflection of an infinitely thick compliant substratum, with

no effect from a rigid underlying substratum, and $U\left(\frac{h}{a}\right)$ is the adjustment factor, ranging

from 0 to 1, that adjusts the maximum deflection u_b according to the thickness of the compliant film. Here, T is the applied shear stress, a is the radius of the focal adhesion which is assumed to have circular dimensions, ν is Poisson's ratio of the gel film, μ is the shear modulus of the gel film, and h is the thickness of the gel film. The deflection adjustment is dependent on the ratio of the film thickness to the focal adhesion size, not just the specific magnitude of gel film thickness.

If the film is assumed to be incompressible, with a Poisson's ratio of 0.5, then U can be written as

$$U\left(\frac{h}{a}\right) = \frac{1}{3a} \left(\frac{h^2}{\sqrt{a^2 + h^2}} + 3\sqrt{a^2 + h^2} - 4h \right). \quad (1)$$

This expression increases monotonically from 0 to 1, and approaches 1 asymptotically. However, in these studies cell area was measured instead of surface deflections, so when applied to the cell area data the curve was fit using the focal adhesion radius and the range from maximum to minimum projected area as fitting parameters to find the curve of best fit. This led to an R^2 value of 0.8857 using a focal adhesion radius of 0.7 μm , a value within the expected range of focal adhesion size [6]. If this expression is used to approximate the data, then a critical thickness above which A_c will vary by less than 10% is found at 2.3 μm for this cell and thin film system.

This theoretical model can be compared to a simple power-law fit of the normalized cell area data. If this is done, a relatively good fit is still achieved with an R^2 value of 0.8318, but it does not fit the data as well as the thin film deflection model nor does it have any theoretical basis or reflect the physical limits of the system, i.e. it does not predict cells to not be affected by the substratum thickness above a critical thickness and asymptotically approach a certain value for projected area.

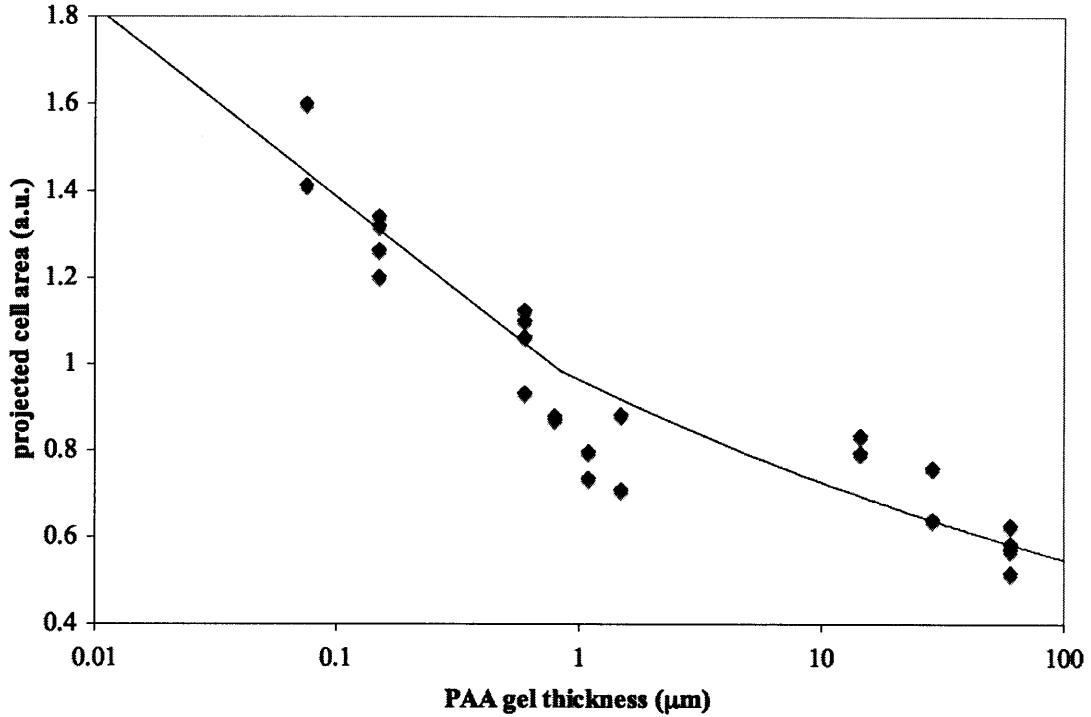


Figure 7. Normalized projected cell area plotted against thickness (note logarithmic scale), with a power-law fit. The power fit describes the data fairly well, with an R^2 value of 0.8318, but not as well as the thin film deflection model (Figure 6).

Cell area data was also checked to make sure that the distribution of A_c was single-peaked and that there were no irregularities such as a bimodal distribution of cell areas or a significant amount of outlying data points. To determine this, histograms were made of the distribution of cell area data for each gel thickness. The bin sizes for each histogram were determined using Scott's method [11]. This method prescribes a bin width of $h = \frac{3.5s}{\sqrt[3]{n}}$, where s is the standard deviation of the data set and n is the number of data points. No irregularities were found, and the cell area distributions were single-peaked. Examples of these distributions can be seen in Figure 8.

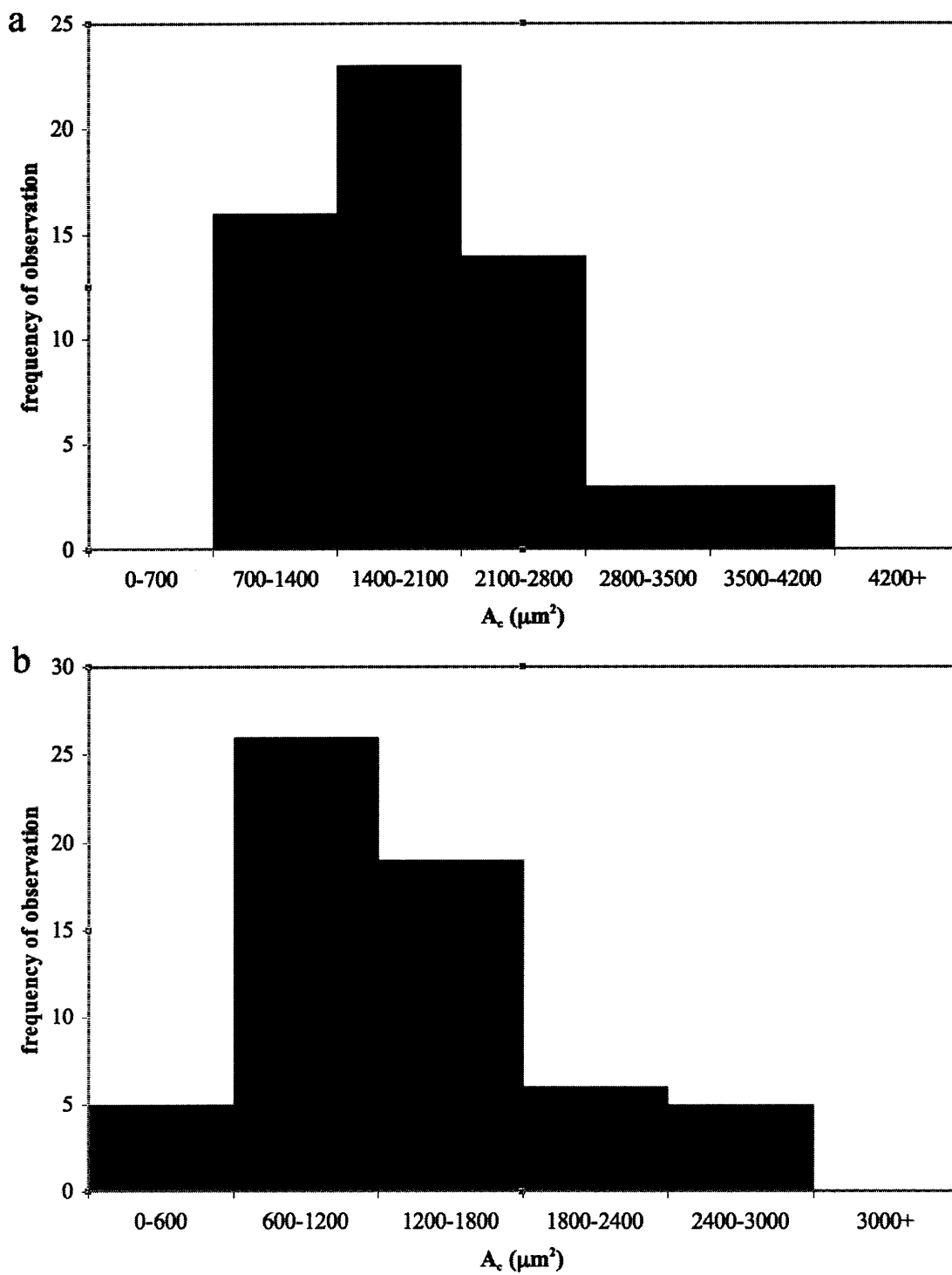


Figure 8. Histograms of cell area measurements, showing monomodal distributions. a) 0.15 μm gel thickness; b) 0.8 μm gel thickness.

Cell proliferation

The dye Alamar Blue was used to measure cell proliferation. When Alamar Blue is added to media that contains cells, the cells reduce the Alamar Blue, changing the media color from purplish-blue to pink. This is quantified by measuring the absorbance at wavelengths of 570 nm and 600 nm. Initially, when the media is purple, the difference between the absorbance at the two wavelengths will be at a minimum. Over time, as the media changes to a pink color, the difference in absorbance will increase until it

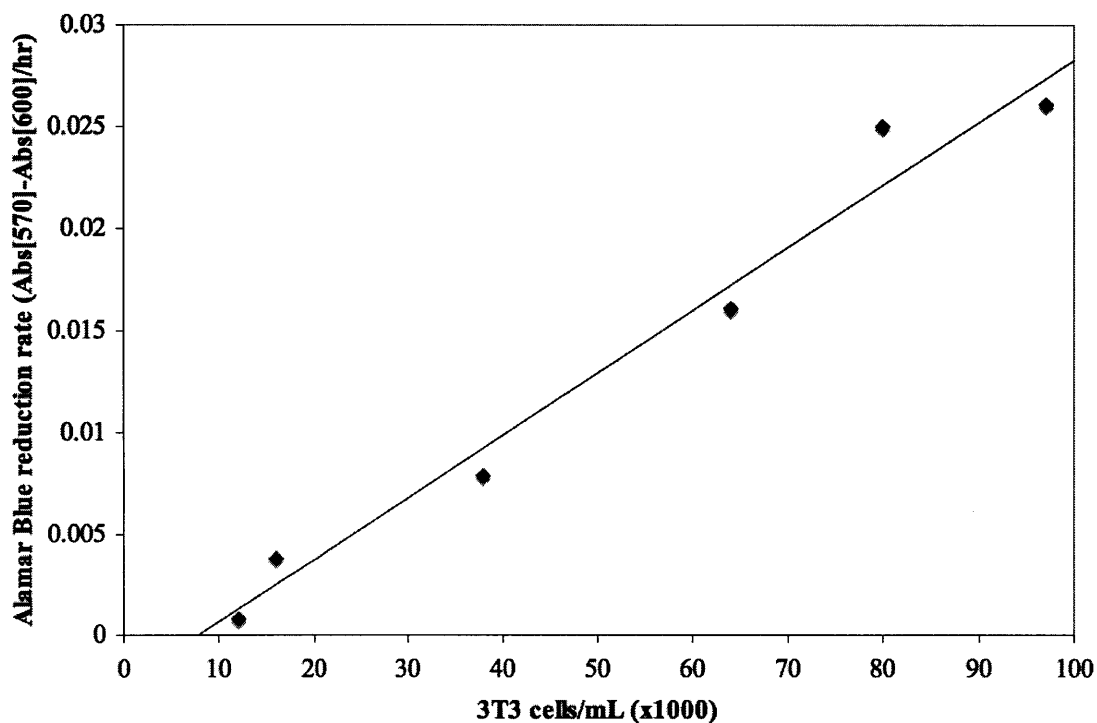


Figure 9. Reduction rate of Alamar Blue plotted against cell concentration on polystyrene. A linear relationship was found, and this relationship was used to correlate Alamar Blue reduction rate with cell population in other experiments. $R^2 = 0.9747$.

eventually plateaus. The rate of change of the difference in absorbance at the two wavelengths, or the rate of color change, will increase with cell population because there will be more cells to reduce the Alamar Blue. Therefore, once the relationship between

cell population and Alamar Blue reduction rate is found (Figure 9), Alamar Blue can be used as a way to measure the size of cell populations, and hence the rate at which a cell population has grown over a period of time. Here control experiments to correlate Alamar Blue reduction rate and cell number were done on plain polystyrene instead of PAA gels, but it was found that a PAA gel without adherent cells did not reduce Alamar Blue significantly by itself.

Cells were seeded onto the gel surfaces, then allowed to attach over 24 hours. After 24 hours, the gels were then put in the media containing Alamar Blue and measurement of dye reduction was initiated as $t = 0$. Two sets of experiments were done,

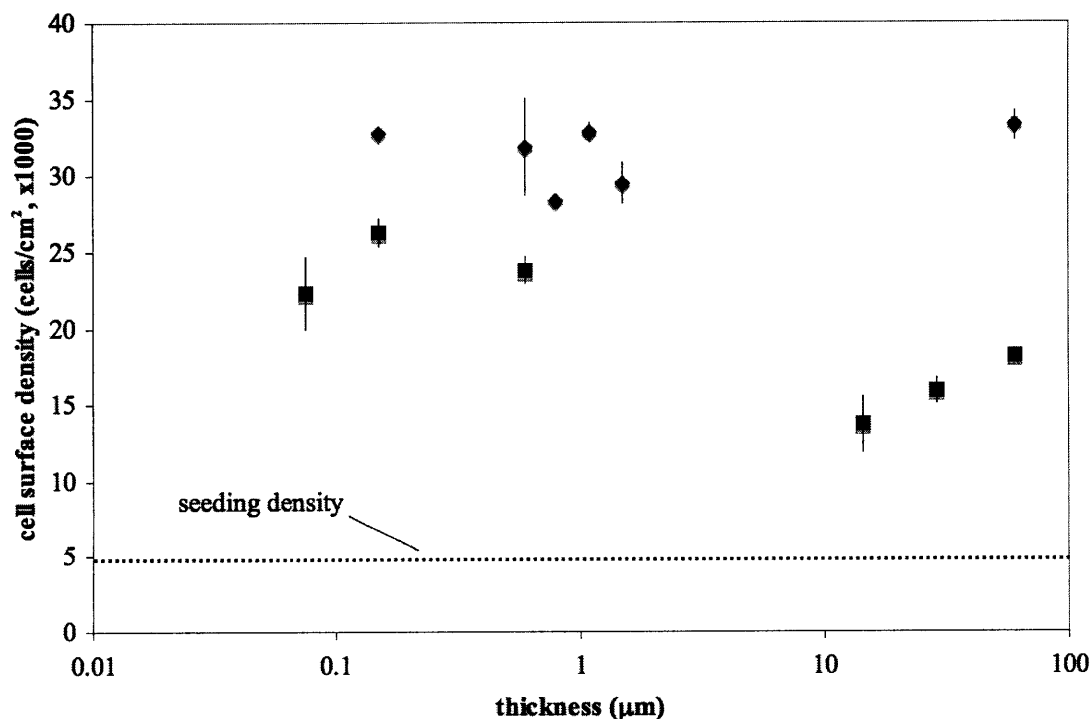


Figure 10. Cell density after 48 hours vs. gel thickness (note logarithmic scale). No detectable trend was found in the cell proliferation data between cell population after 60 hours and gel thickness. Control data from Figure 9 was used to convert Alamar Blue reduction rate to cell density. Data points are averages; error bars are \pm standard error between duplicate samples. \blacklozenge = trial 1; \blacksquare = trial 2. Cell densities did increase significantly over the seeding density, showing that cells were indeed proliferating.

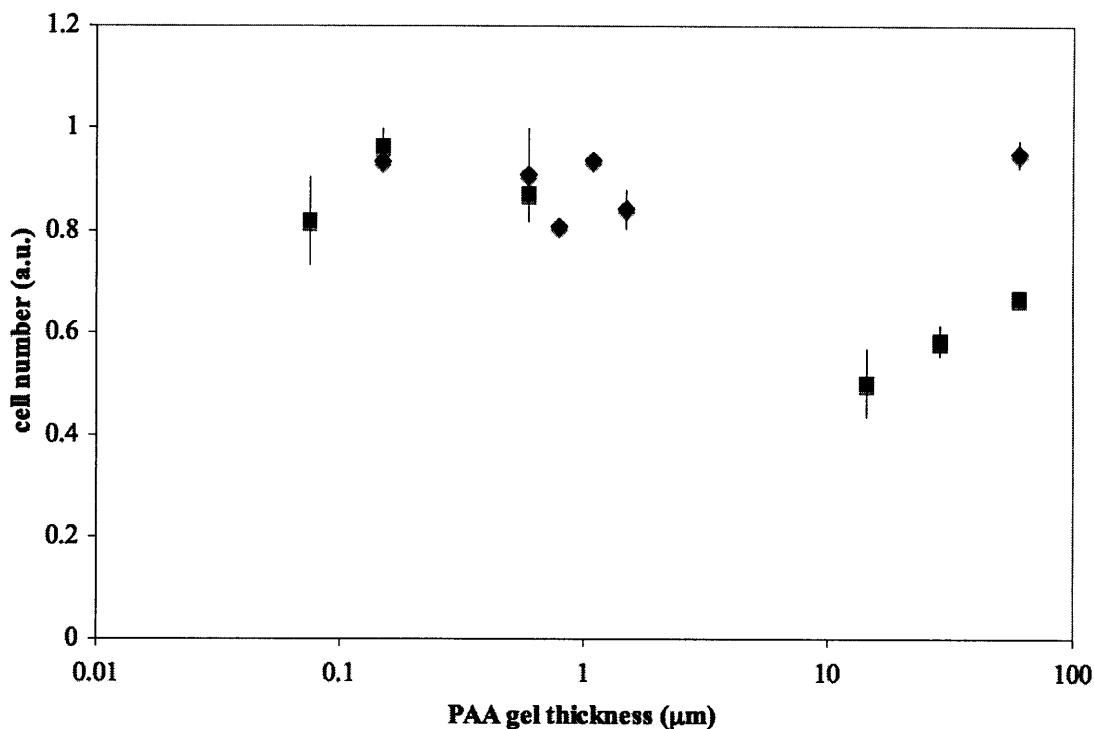


Figure 11. Cell number vs. gel thickness (note logarithmic scale). Normalization achieved by dividing cell number by the maximum cell number in the trial, i.e. the maximum value for diamond data points. Data points and error bars are same as Figure 10. There was still no detectable trend in the data after normalization.

and there was an offset in Alamar Blue reduction magnitudes between experiments similar to the projected cell area data. Therefore a normalized dataset was created by dividing each data point by the maximum value in that data point's trial, i.e. each data point in the first experiment was divided by the value of the largest data point in that experiment. Ultimately, no relationship was found between cell proliferation and gel thickness. The proliferation rate data that was obtained did not show any significant trend or correlation with gel thickness. While it is still possible that there is a relationship between proliferation and the gel thickness, or apparent stiffness, the variation within and among these experiments was too high to draw any conclusions. This variation would

come from inaccuracies in the seeding density of cells or small differences between cell culture experiments in proliferation due to other conditions such as media composition, nonsynchronization of cell cycles, inherent cell growth rates, etc.

Discussion

Thin gel film synthesis procedure

While other methods for creating thin gel films of specified height were explored, including spin coating PAA and spin coating a PDMS mold for PAA polymerization, using beads of specified heights proved to be the most practical method; however, by using softer polystyrene beads instead of harder beads such as borosilicate, the beads were able to compress permanently due to clamping pressure during polymerization.

This does provide an interesting advantage by allowing one to gauge the amount of pressure used to compress the beads, and hence allowing one to get a continuum of gel heights with this method. If one were to measure the applied pressure during clamping and polymerization by using a calibrated spring or a pressure gauge, then a systematic way for creating a thin gel film of any thickness could be created. If the beads were rigid though, then only a gel thickness of the bead height could be achieved. However, the compressibility of the beads does add another level of complexity to creating the thin gel films. Additionally, it also creates the requirement to separately verify the thickness of the gels since their height is not strictly bound to the bead height, which also adds some uncertainty to the experimentation.

AFM for thin gel film characterization

AFM measurements were used to characterize the thin gel films with moderate success. While the AFM provides a great tool to measure surfaces on the small scales of these films and the ability to do so in the fluid environments that are required by hydrated gels, many difficulties arise in practice. Much of this is due to the extreme nature of the thin hydrogels. Measuring such a compliant layer of such thin thickness is very difficult, despite the sensitivity of the AFM. In practice, the gels will often delaminate from the glass substrate under the pressure of the contact mode AFM probe tip. This can complicate thickness measurements and also disrupt the integrity of the gel. Furthermore, it is difficult to obtain a large sample size to accurately describe the gel thicknesses in detail using this method. It is possible that ellipsometry or some other method may provide a more ideal way to measure thickness, but this is also limited in the sense that it must be done on reflective surfaces and it is unclear what level of resolution and accuracy can be achieved through this method.

For gel stiffness measurements though, AFM indentation is very well suited for the task. Wang and Pelham's measurements for the Young's moduli of their gels have been largely discarded as inaccurate [8,4], but the AFM-enabled indentation provides a very precise way to measure stiffness, especially on the small depths and thicknesses appropriate for these gels.

Cell spreading

A very clear relationship between adhesive cell spreading as quantified by A_c and gel thickness h was found. This relationship could be described by either a power-law

equation or the theoretical thin film deflection model that has been described briefly here. Between these two options, the latter seems more appropriate not only because it is theoretically based on the mechanics of the films and the signals that the cell uses to determine its behavior, but also because it is more intuitive. The theoretical model, due to its form, accounts very well for the decreasing change in $A_c(h)$ as the film gets thicker, and asymptotically approaches the limit of a cell spreading on an infinite gel substrate. While the power-law fit accounts well for the data within the experimental range, it is questionable how it will hold up at the extremes of gel thickness.

The inherent inaccuracy of working with biological systems should also be mentioned. In these experiments, cell area was measured and a clear relationship between the average cell area and gel thickness was found, which leads to the correlations and conclusions described in this paper. However, when observing a population of cells there is always unavoidable variance. In these experiments there were always cells on a very thin gel that were smaller than some cells on a very thick gel, and there were cells on thick gels that were bigger than some cells on very thin gels, even though the averages obeyed a well defined trend. The consequence of this is that it may be very difficult to use gel thickness or effective stiffness to get a very well defined response out of a population of cells. While on average the cells may respond correctly, there may be great of variance in behavior, depending on the range of thickness or stiffness that can provide the desired results. Therefore, it is important to report and consider the distribution of the observed parameters, here, A_c and proliferation rate, as a function of the control parameter, here, h and the effective gel stiffness.

Cell proliferation

No correlation was found between gel thickness and cell proliferation or growth rate. This may be due to inaccuracies in the initial cell number seeded onto the gels, variations in gel area from sample to sample, inaccuracies in absorbance measurement or other unidentified factors. However, regardless of these possible sources of error, if there is indeed a trend to be found and it was obscured by the relatively small amount of variation in this experimental data, then the amount of control over cell proliferation that one can have by varying gel thickness is certainly limited. In other words, gel thickness and the associated effective gel stiffness do not strongly affect proliferation rates of 3T3 fibroblast cells.

Conclusion

Thin polyacrylamide gel thin films of varying thickness were synthesized and characterized in terms of thickness and elastic moduli, and 3T3 fibroblast cells were seeded onto these substrata. After observation, a strong dependence on film thickness was found for the projected area of adherent cells. On the other hand, no relationship was found between gel thickness and the rate of cell proliferation. The cell area-gel thickness relationship can be defined by a power-law or by a new theoretical model created in the Van Vliet group at MIT. These experimental data serve to verify the applicability of this model. Furthermore, using this model, a critical gel film thickness of 2.3 μm was found over which the gel thickness should have negligible effect on projected cell area.

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