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In Situ Mechanical Interferometry of Matrigel™ Films

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Abstract

Many biological materials and cell substrates are very soft (Young's modulus < 500 Pa) and it is difficult to characterize their mechanical properties. Here we report local elasticity of the surface layers of Matrigel™ films used for cell culture. We used a new measurement technology, Mechanical Imaging Interferometry, to obtain point mechanical measurements over micron-sized areas. The median values of 650 Pa +/- 400 Pa (# measurements, n=50), determined by the Hertz contact model, agree well with bulk measurements, however on the micro-scale the films were heterogeneous and contained regions distinctly stiffer than average (1-2 kPa). The first measurement of yield strengths of 170 Pa +/- 100 Pa (n=43) indicate that Matrigel™ films deform plastically at stress levels of similar scale to cell tractional forces.

Introduction

In cell culture, substrate properties, both chemical and physical, have a significant effect on the growth, maintenance and morphology cells, and are a critical variable in the morphogenic behavior of adherent cells such as fibroblasts, neurons and hepatocytes. This is due in part to soluble and bound signaling proteins contained in substrate coatings and in part to the physical micro-architecture of the films themselves 1. Recent studies indicate that substrate stiffness has a determining influence on cultured cells in some cases 2. However, biological materials and cell substrate coatings are typically very soft and adhesive, and it is difficult to characterize their mechanical properties 3-5. Further, the mechanistic linkage between substrate mechanical properties and cell behavior is only partially understood and the effect of a specific substrate coating on a specific cell type must be determined empirically 6.

A variety of natural polymers are used as films to coat plastic or glass cell culture dishes, thereby presenting a preferred surface to the cells as they bind. These include fibronectin, collagens, vitronectin and poly-lysine, to name a few. Among the most widely used cell culture substrates is Matrigel™ film⁷. It is a natural secretion of cultured Engelbreth-Holm-Swarm mouse tumor cells and consists primarily of the natural biopolymers laminin, collagen IV and entactin, as well as trace amounts of various protein growth factors. It is thought to closely mimic the natural extracellular matrix (ECM) secreted by adherent cells.

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Some cell types form the appropriate morphology and gene expression patterns on Matrigel™ film much more readily than on other surfaces. As a result, Matrigel™ film is used widely in toxicology studies, neuronal cell culture, angiogenesis assays and tumor invasion assays.

Despite their technological importance, Matrigel™ films as they exist in cell culture conditions (salt buffer, pH 7-8 and 37 °C), have not been well characterized mechanically. Bulk measurements using traditional rheometry techniques indicate that Matrigel™ bulk gel is soft, having a Young's modulus < 500 Pa, but films used in cell culture are typically applied and aged in a fashion that can substantially change their physical properties vs. the bulk 7-10. Also, bulk measurements cannot capture the micro-heterogeneities that are critical to the interaction with cells.

In this Letter we present novel measurements of Matrigel™ films performed in situ, under typical cell culture conditions. We used a new and unique optical measurement technology, Mechanical Imaging Interferometry, to obtain point mechanical measurements over micron-sized areas 11. With this method we also profiled the three-dimensional distribution of material in the film, on a microscopic scale. We discuss the implications of these measurements for understanding the morphology and mechanical behavior of cells cultured on these films.

Experimental Section

Preparation of Matrigel™ Films

Measurements were performed in simulated cell culture conditions, i.e. under electrolyte buffer at physiologic temperatures. Films were prepared according to the “thin coating method”, using Growth Factor Reduced Matrigel™ (BD Bioscience) at a concentration of 8.2 mg per mL 9. All handling and storage was as suggested by the manufacturer. The films were coated onto the bottom of a cylindrical observation dish at a density of 1 μ L per 30 mm², allowed to dry at room temperature for one hour and then hydrated under phosphate buffered saline (PBS buffer, pH 7.4: 140 mM NaCl₂, 4.3 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄) for one hour at 37 °C before observation. The observation chamber was sealed to prevent fluid evaporation over the course of the experiment.

Mechanical Imaging Interferometry

This method has been described in detail elsewhere 11; we will summarize it here briefly. The system is an interferometric microscope, based on a modified Veeco NT 9300 Optical Profiler, that images the film through liquid, and tracks the position of spherical, magnetic microreflectors resting at the film-liquid interface. It is in principle an optical microscope with a 20X 0.28NA Michelson interference objective that allows for the observation of not only lateral features with typical optical resolution (1.16 μ m for the 20x objective) but also height dimensions of reflective objects below the scale of one nanometer. The Michelson interferometer is composed of a beam splitter, reference mirror and compensating fluid cell to adjust for optical path differences induced by fluid in the observation chamber. During each measurement, the objective head is scanned vertically from the surface to a height of 40 microns above the surface, such that each point in the volume passes through focus. The interferometer is aligned so that the interference intensity distribution along the vertical scanning direction has its peak (best fringe contrast) at approximately the best focus position. The vertical-axis position of each microreflector is determined as the location of the coherence peak within the scan. By measuring microreflectors of known height fixed to a solid substrate in liquid, we determined that the z-axis measurement repeatability was < 20 nm.

The experimental geometry is depicted in Fig. 1a. Spherical, ferromagnetic microreflectors ~8 microns in diameter are placed at the Matrigel™ film-liquid interface under phosphate buffered saline (PBS), pH 7.4. Microreflectors are indented into the film by magnetically applying a series of known forces, from 0.37 nN to ~2 nN, and from this force-indentation data the local mechanical stiffness is determined. The microreflectors were roughly the same size as a mammalian cell, 8-10 microns in diameter.

As shown in Fig. 1b, three types of images are obtained: a bright field microscopic image of the film (left); a three-dimensional profile of the microreflectors as they rest on the film (middle); and an optical thickness image of the film itself, which corresponds to the material density at every pixel (right). The interferometric height profile includes only the microreflector since the film-liquid interface is minimally reflective. Optical thickness is calculated from the relative phase shift in the incident light as it propagates through the transparent film. Optical thickness determined by interferometry is a well established technique to quantitatively and non-invasively measure local material density in transparent samples.¹² In Fig. 1b the optical thickness image shows the film and a step edge where there is no film and the underlying silicon substrate appears in blue. In this image, apparent height corresponds to increased optical path length due to the higher index of refraction of the film versus the surrounding media. The microreflector is opaque and does not appear in the optical thickness image.

Figure 1c shows a 3D rendering of two optical thickness images. On the left, a region of film bordered by bare silicon substrate on either side. On the right, a continuous region of film with a scratch to expose the underlying substrate. These images do not depict the true surface roughness, rather they show the inhomogeneity in material density within the film at each point.

Microreflectors

Elemental nickel microspheres (2-10 μm dia.) were obtained from Duke Scientific as a dry powder. For each experiment, approximately 0.1 mg of powder was mixed with 1 mL of buffer media or buffer as described below. Smaller diameter particles were removed by sedimentation, resulting in a dilute suspension with size distribution ~8-10 μm . Magnetic force was applied to the microreflectors using a cylindrical rare earth magnet oriented axially along the vertical direction below the test chamber. The magnet was positioned with a feedback controlled motorized micrometer, allowing better than $\pm 5\%$ relative precision in the control of the magnetic force applied to a microreflector. Magnetic forces were calibrated with a ferromagnetic-tipped microcantilever array, having a known spring constant and magnetic moment.

Results and Discussion

Five separate films were prepared. Their thickness varied between 10 and 20 microns, and was roughly uniform over an individual field of view, 300×230 microns. The films were transparent in the bright field images with a slight granularity 5-20 microns in diameter (Fig 1 b,c). Optical thickness images revealed that this granularity corresponded to regions of higher material density, present either as bumps on the film surface or denser inclusions. While our measurements did not probe the chemical nature of these structures, we speculate that they are regions of the material that gelled with a different density or that they are some component of the Matrigel, which is a mixture of various polymers, that precipitated within the gel.

Interestingly, the optical thickness of the films decreased over the course of several hours. An example of this is seen in Fig. 2a. The same region was measured at the start of the

experiment and after twenty four hours. The film is sealed under buffer, at 37 °C during this interval. The cross sectional profiles, on the right, show that the optical thickness of the film has decreased from ~25 nm to ~18 nm in 24 hours. This indicates a loss of material and possibly shrinkage of the film itself. The material density, as measured by optical thickness, decreased by roughly 20% over this time. At a different location on the film, the absolute height of two microreflectors tracked over 24 hours also slowly decreased though slightly less on a percentage basis, ~10%-20% (Fig. 2b). Together, these data suggests a gradual shrinking in thickness of the film, possibly caused by diffusion of material into the surrounding liquid. We rule out a change in ionic strength as the cause of the film shrinkage because the chamber was sealed during the experiment. This result indicates that the mechanical structure of Matrigel™ film remains in flux during the time when freshly seeded cells typically bind and equilibrate (hours)⁹. Local measurements of the elasticity of the film were made by applying a series of increasing steps of force to the microreflectors and recording their displacement into the film. In each cycle, a constant force was applied for 80 seconds and then removed for 80 seconds, allowing the microreflector to rebound. The range of forces varied from 0.37 nN to ~2.0 nN. A typical time series of microreflector indentations is given in Fig. 3a. In the first five cycles the film exhibited a mostly elastic behavior, returning to the same equilibrium position under no load. However, after the fifth cycle, indicated by (*), the microreflector failed to recover to the previous no-load position, indicating the onset of plastic deformation. Over fifty individual microspheres were measured this way over five different film samples. The films behaved elastically at force less than ~1.0 nN, with the microspheres returning to the same equilibrium position between force steps. At forces above ~1.0 nN, the microspheres began to “sink” into the film in a non-recoverable fashion. We interpret this as a transition from purely elastic to elastic/plastic behavior at higher forces and strains.

The films also displayed a “delayed elasticity” or viscoelastic creep response that was pronounced at higher forces (> 1.0 nN). This was observed as a slow drift in the equilibrium position of the indented microsphere under load. At forces less than 1 nN this effect was negligible (less than 10% of the total indentation). A full characterization of the viscoelastic properties of these films is ongoing.

The Young's modulus (E) at each location was calculated using the Hertz model for a spherical indenter ¹³:

$$E=0.75 \sqrt{\frac{9F^2}{16R\delta^3}} \quad (1)$$

Where F is the force applied to the microsphere, R is the sphere radius, δ is the deflection into the film, and the Poisson's ration for the film is 0.5. For these calculations, the indentation depths ($\delta < 0.2 R$) and film thickness (thickness > $\times R$) were sufficient to satisfy validity of the Hertz model.

A typical distance versus force plot is given in Fig. 3b. The indentation curves fit the Hertz model well at the lower force levels ($R^2 > 95\%$), while at higher forces (indicated by * in Fig.3b) the indentations were larger than would be predicted from the model, indicating that the film became more compliant.. This is consistent with the postulated onset of plasticity at larger forces.

Our calculations using the Hertz contact model do not include the effect of adhesive forces between the microsphere and the film. There is no doubt a modest adhesive contribution to the probe-film interaction that could be more accurately explored by collecting data lower force levels than used here (< 0.2 nN), and applying an adhesive contact model appropriate

for soft materials such as the Johnson-Kendall-Roberts (JKR) model¹⁴. However, the quality of the Hertz fits over an extended force range (~0.3-1.5 nN) suggest that the Young's moduli derived from the Hertz and JKR models would not be significantly different within error.

The film in all cases was very soft, as seen in the histogram of individual measurements given in Fig. 3c. The median Young's modulus was 650 Pa, which is as soft as or softer than the elastic moduli measured for mammalian cells, by a variety of techniques (roughly 500 Pa to 20 kPa), growing on harder substrates¹⁵. A small fraction of the point measurements were substantially larger than the median, ranging from 1-2 kPa. The indentation curves for these data points were not unusual and we conclude that the film contains a minority of "stiffer" regions. These may correspond to the granular regions observed in the optical thickness profiles.

The maximum contact pressure, P_{max} , experienced by the film under a single microsphere, at a specific force F , can be calculated from the Hertz model, given by 13:

$$P_{max} = \left(\frac{6F(E/0.75)^2}{\pi^3 R^2} \right)^{1/3} \quad (2)$$

This corresponds to the pressure at the point on the very bottom of the microsphere.

For each microsphere we calculated the value of P_{max} at the force level which first induced plastic yield. As shown in Fig. 3d, plastic yield occurred at values of $P_{max} \sim 100$ to 400 Pa, with a median value of 170 Pa. In order to compare this yield stress with the tractional forces produced by cells, we calculate the maximum value of shear stress, which is given by $\tau_{max} = 0.31 P_{max}$, and occurs in the film just below the point of maximum pressure¹³. The maximum shear stress at the onset of plastic behavior in these films ranged from ~30 to 125 Pa.

Significantly, adherent cell types can exert tractional stresses in excess of 200 Pa, a level well above that required to induce plastic yield in these films¹⁶. Studies have shown that stiff substrates induce proliferation of cell-surface adhesion complexes, and result in tensioning of the cell's core cytoskeleton, among other effects; in some cell types this behavior is associated with an undifferentiated phenotype². The ability of the substrate to resist strain as the cell increases its tractional force would therefore be an important feature of this process. Our measurements indicate that Matrigel™ films are easily deformed at force levels achievable by live cells. Also, rather than stiffening with increased strain, as is in some biopolymer gels, Matrigel™ film yields at low forces and becomes effectively softer². The Matrigel™ films' inability to support structural tension may partly explain its ability to promote a differentiated phenotype in some cells.

Conclusions

Local measurement of very soft materials (Young's modulus < 500 Pa) is extremely challenging³. We used a new measurement technology, Mechanical Imaging Interferometry, to obtain point mechanical measurements of soft Matrigel™ films, a widely used substrate matrix material, over micron-sized areas. We found that the median elastic modulus values of 600 Pa +/- 400 Pa (# of measurements, n=50) agree well with bulk measurements, however on the micro-scale the films were mechanically heterogeneous and contained regions distinctly stiffer than average (1-2 kPa). The first measurement of yield strengths of 170 Pa +/- 100 Pa (n=43) indicate that Matrigel™ films deform plastically at stress levels of similar scale to cell tractional forces. We also profiled the three-dimensional

distribution of material in the film, on a microscopic scale. The films decreased in material density and shrank in height by ~ 20% over 24 hours, indicating the mechanical structure remains in flux over the timescale which freshly seeded cells bind and equilibrate (hours).

Acknowledgments

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Figure 1. (a).
Experimental geometry.

Figure 1. (b). White light (left) interferometric (middle) and optical thickness (right) images of an 8 micron microreflector (white arrow) on a 12 micron thick Matrigel™ film in PBS buffer.

Figure 1. (c).
3D renderings of optical thickness images.

Figure 2. (a).
Optical thickness images take 24 hours apart.

Figure 2. (b).
The absolute height of two microreflectors tracked over 24 hours (same sample, different location for Fig 2a.)

Figure 3. (a).

A force-distance curve showing the deflection of an 8 micron dia. nickel microreflector into a 15 micron-thick Matrigel™ film under a series of increasing forces (0.37 nN to 1.45 nN).

Figure 3. (b).

The corresponding force-distance measurements fitted to the Hertz contact model for a spherical indenter, from which the film's elastic modulus was calculated.

Figure 3. (c).

A histogram of the individual measurements of Young's modulus, pooled from five different samples. Each count represents the measurement of an individual microsphere in the elastic regime before onset of plastic deformation.

Figure 3. (d). Histogram of the corresponding maximum pressure exerted by the spherical indenter on the film at the yield point calculated for data in Fig 3 (c).