velocity drops to ~86 nm/sec and individual steps become apparent. The mean size of steps may be determined by taking the power spectrum of the autocorrelation function of the record.[18,31,36] This spectrum (Fig. 7B, inset) displays a prominent peak at a spatial frequency of 0.121 nm^{-1}, corresponding to a step size of 8.26 nm for this particular record. This value—*which involves no elastic corrections*—is identical, within experimental error, to the kinesin step size of 8.3 ± 0.2 nm estimated in earlier work using a fixed trap, to which a 19% adjustment for series compliance had been applied.[18] The force clamp enables us, for the first time, to acquire stepping data for kinesin molecules subjected to high loads over distances as great as 200–300 nm, a feat previously impossible with a stationary trap.

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[39] Preparation of a Flexible, Porous Polyacrylamide Substrate for Mechanical Studies of Cultured Cells

By YU-LI WANG and ROBERT J. PELHAM, JR.

Introduction

Although cell culture is traditionally performed on plastic or glass substrates, cells in a multicellular organism live under a substantially different environment: they adhere to mechanically flexible tissues or basement membranes and are surrounded by fluid and nutrients. Accumulating evidence indicates that physical parameters such as mechanical forces,[1,2] flexibility,[3] fluid shear,[4,5] and media accessibility[6] can have profound effects on cell growth and differentiation.

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A number of flexible and/or porous substrates have been developed over the years, including silicone rubber,[7] collagen matrix,[8,9] fibrin clots,[10] and nucleopore filters.[6] Besides providing a more physiologic environment for cell culture, flexible substrates have been used for testing the effects of mechanical forces on cells[11] and for measuring traction forces that cells exert on the substrate.[7,12] The latter has been most elegantly done with polymerized films of silicone fluid. In the original version developed by Harris and co-workers,[7] the film covers a layer of silicone fluid and wrinkles on application of force, much like the response of a water bed. Although it is difficult to perform precise quantitative measurements, the magnitude of forces can be estimated based on the extent of wrinkling. This method has recently been improved by embedding particles in weakly polymerized, nonwrinkling films[12] (which allow a more precise measurement of forces based on the movement of beads[13]), and by using improved materials that allow systematic control of the flexibility.[14]

We have recently developed a culture substrate based on polyacryamide sheets coated with extracellular matrix proteins. Although polyacrylamide-based substrates have been around for some time,[15] their full potential has not yet been realized. The material has a number of favorable features. First, it has a nearly ideal mechanical property: it deforms in proportion to applied forces over a wide range, and recovers completely and instantaneously on the release of force. Second, it allows systematic and reproducible control of the flexibility of the substrate, by changing the relative concentration of acrylamide and bisacrylamide. Third, its excellent optical quality permits the observation of both immunofluorescence staining and microinjected fluorescent analogs at a high magnification. Fourth, the substrate uses specific extracellular matrix (ECM) molecules as the ligand for cell adhesion, while polyacrylamide itself shows no detectable interaction with the cell surface. Fifth, the porous nature of the polyacrylamide gel allows the penetration of media and provides a more physiologic environment for cell culture, particularly for epithelial cells.[6] We have used the substrate for testing the responses of cells to flexibility and to deformation forces,

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and for measuring traction/retraction forces that cells apply to the substrate during locomotion.

Preparation of Activated Glass Surface

Although the polyacrylamide substrate can be applied to an unprepared glass surface, it adheres poorly to glass and floats off easily. Thus for most experiments it is necessary to covalently attach the polyacrylamide sheet to the glass surface by chemical activation of the glass surface following the procedure of Alpin and Hughes.[16]

Materials and Reagents

Diamond-tipped pen
Coverslips (No. 1, 45 × 50 mm; Fisher Scientific, Pittsburgh, PA)
0.1 *N* NaOH
3-Aminopropyltrimethoxysilane (Sigma, St. Louis, MO)
Phosphate-buffered saline (PBS)
0.5% Glutaraldehyde in PBS (prepared by diluting 1 part of 70% stock solution, Polysciences, Inc., Warrington, PA, with 140 parts of PBS)

Procedure

1. Pass coverslips briefly through the inner flame of a Bunsen burner.
2. Smear a small volume of 0.1 *N* NaOH across the surface of the coverslip with a Pasteur pipette and air dry the coverslip. The surface should be covered with a film of dried NaOH.
3. Gently mark the treated surface with a diamond-tipped pen. This facilitates the identification of the proper side of coverslips in later steps.
4. Smear a small volume (~200 μl) of 3-aminopropyltrimethoxysilane evenly on the marked side of the glass surface with a Pasteur pipette. Allow the coverslip to sit horizontally for 4–5 min.
5. Cover the treated side of the coverslip with distilled H_2O and let sit for 5–10 min. When the surface is clear, rinse with distilled H_2O from a squirt bottle and soak in distilled H_2O for 5–10 min with gentle agitation.
6. Transfer the coverslips, marked side up, into petri dishes and cover the surface with 0.5% glutaraldehyde in PBS. Incubate at room temperature for 30 min.

[16] J. D. Alpin and C. Hughes, *Anal. Biochem.* **113,** 144 (1981).

7. Wash the coverslip extensively with multiple changes of distilled H_2O on a shaker and then let air dry vertically. The treated coverslips may then be used for gel attachment up to 48 hr after preparation.

Preparation of Polyacrylamide Sheets

Materials and Reagents

Coverslips, circular (No. 1, 22 mm diameter; Fisher)
Acrylamide (Bio-Rad, Hercules, CA), 30% w/v
N,N'-Methylene bisacrylamide (BIS, Bio-Rad), 2.5% w/v
Ammonium persulfate (Bio-Rad), 10% w/v
N,N,N',N'-Tetramethylethylenediamine (TEMED, Bio-Rad)
50 m*M* HEPES, pH 8.5
Fluorescent latex beads, 0.2-μm FluoSpheres, carboxylate-modified (Molecular Probes, Eugene, OR), for measuring traction forces only

Procedure

1. Mix acrylamide, BIS, and distilled H_2O to obtain a desirable concentration. For example, we most often use 10% acrylamide/0.26% BIS to prepare relatively stiff substrates, and 10% acrylamide/0.03% BIS for highly flexible substrates and for measuring traction forces. At 10% acrylamide, the BIS concentration can be varied between 0.025% and 0.4%. Too low a concentration generates viscous fluids instead of flexible solids. Too high a concentration makes the substrate opaque.
2. Add fluorescent latex beads to the mixture if the substrate is to be used for measuring traction forces. The beads are first sonicated briefly in a bath sonicator and 1/125 volume is added to the acrylamide mixture.
3. Degas the acrylamide/BIS solution.
4. Add 1/200 volume of 10% ammonium persulfate and 1/2000 volume of TEMED. Immediately pipette 10–25 μl of the solution onto the activated coverslip surface and cover with a 22-mm coverslip. Turn the coverslip assembly upside down if the solution contains fluorescent beads.
5. When acrylamide polymerizes (10–30 min), carefully remove the 22-mm coverglass and wash the gel with 50 m*M* HEPES on a shaker.

Activation of the Polyacrylamide Surface and Conjugation with Type I Collagen

Cultured cells adhere poorly to the polyacrylamide surface. To provide a physiologic adhesive surface for cell culture, the surface has to be coated

with adhesion molecules. An easy way to conjugate proteins to the polyacrylamide surface is to use a photoactivatable heterobifunctional reagent. Sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (sulfo-SANPAH) contains at one end a succinimidyl ester group, which reacts with lysine ε-NH_2. The other end is a phenylazide group that, on photoactivation, reacts nonspecifically with many chemically inert molecules including water and polyacrylamide. The following procedure uses sulfo-SANPAH to activate the polyacrylamide. Then type I collagen (or other adhesion molecules) is allowed to bind covalently to the activated surface.

Reagents

1 m*M* sulfo-SANPAH (Pierce Chemicals, Rockford, IL) in 50 m*M* HEPES, pH 8.5
50 m*M* HEPES, pH 8.5
Type I collagen (Amersham Life Science, Arlington Heights, IL), diluted to 0.2 mg/ml with 50 m*M* HEPES

Procedure

1. Drain fluid off the surface of the polyacrylamide gel. Carefully pipette 200 μl of 1 m*M* sulfo-SANPAH onto the surface.
2. Expose the surface to the UV light of a 30-W germicidal lamp at a distance of 6 in. for 5 min. Remove the darkened sulfo-SANPAH solution and repeat the photactivation procedure.
3. Wash the polyacrylamide sheet with two changes of 50 m*M* HEPES (pH 8.5), 15 min each, on a shaker.
4. Layer a 0.2 mg/ml solution of type I collagen on the substrate and allow to react overnight at 4° on a shaker.
5. Wash the gels with PBS. Mount the coverslip with the gel onto an appropriate culture chamber[17] and sterilize with UV irradiation.
6. Before plating cells, soak the gels for 30–45 min in culture medium at 37°.

Characterization of the Polyacrylamide Substrate

The thickness of the substrate can be estimated based on the initial volume of the acrylamide solution applied to the coverslip and the area of the gel. The actual thickness, which can be measured by focusing a microscope from the glass surface up to the gel surface, is affected by the

[17] N. M. McKenna and Y-L. Wang, *Methods Cell Biol.* **29,** 105 (1989).

acrylamide/BIS concentration and the degree of swelling/shrinkage of the gel in media. The protocol above gives a gel approximately 70 μm in thickness. The concentration of matrix proteins on the surface can be measured by a radioimmunoassay. Briefly, the surface is reacted with primary antibodies against the coated protein, then with an iodinated secondary antibody. The gel is then peeled off the coverslip with a razor blade and radioactivity counted.

The flexibility of polyacrylamide sheets can be characterized both macroscopically and microscopically. The former is performed by preparing gels 0.75 mm in thickness, using the apparatus for casting mini gels for gel electrophoresis. Strips of the gel 70 × 30 mm in size are then fixed at one end with binder clips attached to a horizontal bar or at the edge of a bench, and stretched vertically by attaching known weights (most conveniently by using binder clips) to the opposite free end (Fig. 1). Young's modulus, which measures the elasticity of materials, can then be calculated based on the original length (l), the change in length (Δl), the substrate's cross-sectional area (A), and the applied force ($F_\perp$; 1 g of weight applies 980 dynes of force), according to the equation: $Y = (F_\perp/A)/(\Delta l/l)$.

Microscopic compliance of the surface, which is affected by both

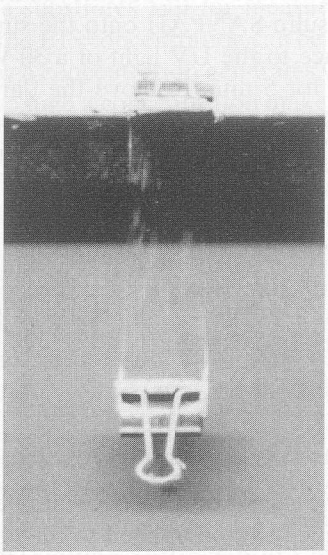

FIG. 1. Measuring the Young's modulus of polyacrylamide substrates. A 70- × 30- × 0.75-mm piece of polyacrylamide substrate is suspended from a laboratory bench and is deformed using binder clips as weights. The change in length is used to calculate Young's modulus.

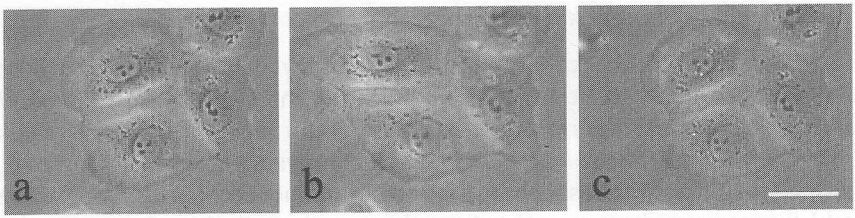

FIG. 2. Application of external mechanical forces to individual NRK epithelial cells. A microneedle is used to deform a 10% acrylamide/0.26% BIS substrate. (a) Before application of force, (b) during deformation, and (c) recovery of the cell and substrate after the release of tension. Bar = 10 μm.

Young's modulus and shear modulus, can be measured by deforming the gels horizontally with a flexible glass microneedle attached to a micromanipulator, essentially as described by Lee and co-workers.[12] Deformation of the gel is measured as the translocation of the needle tip, assuming that there is no slippage of the tip on the gel surface. The force applied by the needle is a function of needle deformation, which is measured by subtracting the distance of needle movement recorded by the micromanipulator with the distance of tip movement. The same needles are then calibrated by measuring their deformation after applying known submilligram weights.[12] The compliance is calculated as micrometer deformation per Newton applied force.

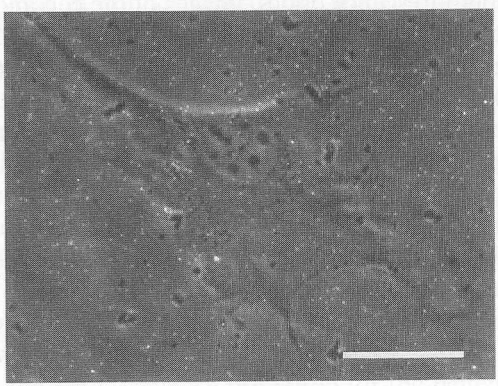

FIG. 3. Simultaneous visualization of 3T3 fibroblasts and 0.2-μm fluorescent latex beads embedded in a 10% acrylamide/0.03% BIS substrate. Movement of the beads is used as a means of measuring traction forces generated during cell locomotion. Bar = 10 μm.

Application of the Polyacrylamide Substrate

The morphology of cells cultured on polyacrylamide substrates can be studied with phase optics or with fluorescence microscopy after labeling/microinjecting with fluorescent probes. Using dry objective lenses, there is no significant degradation of the image by the polyacrylamide gel. However, at high magnifications using 63× or 100× oil immersion lenses, both phase and fluorescence images suffer from decreased resolution, due to the increased distance between the cell and the objective lens, and the spherical aberration of the lens. High-quality images can be obtained with long working distance, water-immersion lenses designed for laser confocal scanning microscopy.

We have used the polyacrylamide substrates in several types of studies.[3] First, it is clear that the mechanical property of the substrate, controlled by the acrylamide/BIS concentration, has profound effects on the motility, growth, and differentiation of cultured cells. A second application is to study the responses of cells to external deforming forces, which occur frequently within a multicellular organism. A microneedle mounted on a micromanipulator is poked into the gel ~50–100 μm from the cell, then dragged for a defined distance. Using a 10% acrylamide/0.26% BIS gel, the deformation of the substrate propagates for ~100–200 μm and causes the cell to deform by up to 30% (Fig. 2). While deforming forces can be applied in many ways, the present approach facilitates observations of the immediate responses of living cells to defined and easily regulated forces.

The third application of the polyacrylamide substrate is to measure traction forces during cell locomotion, using highly flexible substrates with fluorescent particles embedded inside. By simultaneously illuminating cells for phase and epifluorescence optics, images of the cell and beads immediately underneath can be recorded in the same image (Fig. 3). Simple time-lapse recordings readily show movements of the beads (i.e., changes in traction forces) during the protrusion/retraction of cultured fibroblasts. Moreover, by taking images of the beads before and after trypsinizing cells, a vectorial map can be generated that shows the global distribution of forces exerted on the substrate. Thus, by combining these applications, we expect the present substrate to provide not only culture conditions that mimic the physiologic environment, but also powerful means for mechanical manipulations and measurements of living cells.