14

Double-Hydrogel Substrate as a Model System for Three-Dimensional Cell Culture

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Summary

When cultured on two-dimensional surfaces, most adherent cells show profound differences from those in their native habitats. In addition to chemical factors, it is likely that both physical parameters, such as substrate rigidity, and topographical factors, such as the asymmetry in integrin anchorage, play a major role in the differences. We have designed a simple culture system that provides flexible, adhesive substrates for both dorsal and ventral cell surfaces. Fibroblasts in this system show the spindle or stellate morphology found in native tissues. The ease of preparation, versatility, and optical quality of this model system should greatly facilitate the understanding of cellular behavior and functions in vivo.

Key Words: Cell adhesion; tissue engineering; cell mechanics; hydrogel; polyacrylamide.

1. Introduction

Decades of advances in cell culture have greatly improved the exploration into cellular functions. However, most conventional cell cultures are performed on surfaces of glass or charged polystyrene. Unlike tissues, these materials are stiff and nonporous and impose a striking dorsal–ventral asymmetry in terms of receptor anchorage. Although the simplicity and economy of the approach are directly responsible for the rapid advances in cell biology, it has long been recognized that cells in such cultures show many profound differences from those in vivo. For example, fibroblasts in native tissues are spindle or stellate in shape, with few prominent lamellipodia, stress fibers, or focal adhesions. However, these structures appear within a few hours of plating on a flat polystyrene or glass surface. Many types of cells also lose their differentiated characteristics

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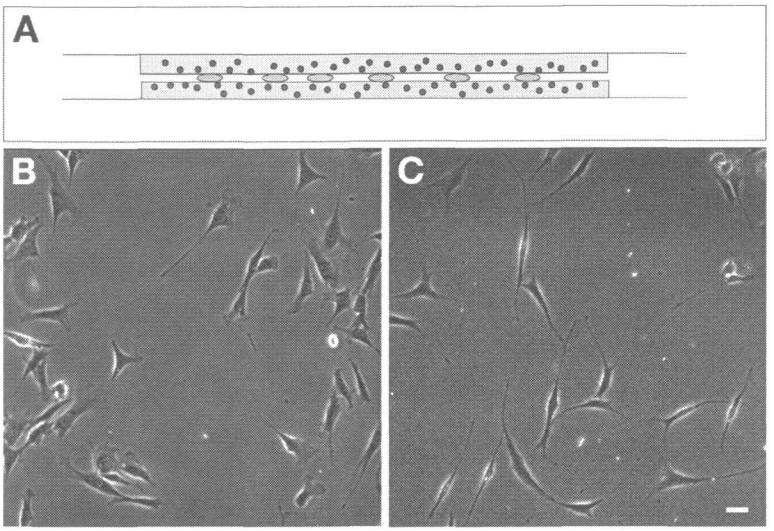

Fig. 1. The double-hydrogel culture system and its effects on fibroblast morphology. **(A)** Cells are cultured between two sheets of extracellular matrix coated polyacrylamide, each about 75 μm in thickness and embedded with 0.5 μm red (top gel) or green (bottom gel) fluorescent microbeads. **(B)** Fibroblasts on fibronectin-coated single hydrogels maintain typical two-dimensional morphology. **(C)** Fibroblasts in the double-hydrogel culture become highly elongated and lack lamellipodia. Bar = 30 μm. (Modified from **ref. *8***.)

and start proliferation. These differences have become an increasing concern to cell biologists as the attention is shifting from the basic growth and motile behavior toward physiological functions.

Over the years a number of model systems have emerged that attempt to address the various shortcomings of the conventional Petri dish system. For example, porous membranes have been used to maintain the polarization of epithelial cells ***(1–3)***, whereas three-dimensional (3D) collagen and Matrigel matrices have been developed to promote the native stellate morphology of fibroblasts and acinus morphogenesis of breast epithelial cells ***(4–7)***. However, few existing model systems provide optimal conditions for light microscopy, and some of them, such as Millipore or Nucleopore membranes, provide very limited similarities to tissues.

In this chapter we describe a model 3D culture system that possesses several unique properties. Cells are cultured between thin, flexible, porous sheets of hydrogels coated with defined matrix proteins. The system has an optical quality comparable to that of two-dimensional cultures for imaging such structures as the cytoskeleton and adhesion complexes in live cells (**Fig. 1A**). In addition, the system allows easy control of parameters including the type and concentration

of extracellular matrix proteins and mechanical properties such as compliance. Although the method was originally designed to address morphological differences between cells in two-dimensional cultures and in native tissues or artificial matrices *(8)*, it may find wide applications in other areas such as gene expression, cancer invasion, and tissue engineering.

2. Materials

2.1. Activated Cover Slips

1. No.1 glass cover slips of suitable dimension for culture chamber (*see* **Note 1**).
2. 22-mm Round cover slips.
3. 0.1 *N* NaOH.
4. 3-Aminopropyltrimethoxysilane (Sigma-Aldrich, St. Louis, MO) stored at 4°C.
5. 0.5% Gluteraldehyde in phosphate-buffered saline (PBS). Gluteraldehyde stocks (70%, Polysciences, Warrington, PA) are stored at 4°C and diluted in PBS immediately before use.
6. Bunson burner.
7. Forceps.
8. Pasteur pipets.

2.2. Polyacrylamide Hydrogels

1. Stock solution of acrylamide (40% w/v, Bio-Rad, Hercules, CA) stored at 4°C.
2. Stock solution of *N,N'*-methylene bisacrylamide (2% w/v, Bio-Rad) stored at 4°C.
3. Stock of 1 *M N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 8.5, filtered and stored at 4°C
4. *N,N,N,N'*-Tetramethylethylenediamine (TEMED; Bio-Rad) stored at 4°C.
5. 10% Ammonium persulfate (Bio-Rad), stored desiccated at room temperature.
6. Fluorescent latex beads, 0.5 or 0.2 μm in diameter (Fluospheres, Molecular Probes) stored in the dark at 4°C (*see* **Note 2**).
7. Two pairs of fine-tipped forceps.

2.3. Conjugation of Proteins to the Hydrogels

1. Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate, also known as sulfo-SANPAH (Pierce Chemical, Rockford, IL) (*see* **Note 3**). This product is light sensitive and should be stored desiccated at –20°C and dissolved immediately before use.
2. Dimethylsulfoxide (DMSO) stored desiccated at room temperature.
3. 50 m*M* HEPES, pH 8.5, solution should be filtered and stored at 4°C and warmed to room temperature before use.
4. UV light source with 302-nm bulbs (VWR no. 21476-0101). Use appropriate protection because UV light can burn skin and damage eyes.
5. The protein of interest, typically fibronectin, collagen, or laminin (although peptides and antibodies have also been successfully conjugated) (*see* **Note 4**).
6. PBS, filtered and stored at room temperature.

2.4. Cell Culture and Media

1. Cells should be healthy and seeded onto the substrate under normal growth conditions at 5000 cells per 22-mm substrate (*see* **Note 5**).
2. If cells are to be transiently transfected, they should not be transfected on the hydrogels, but on conventional polystyrene dishes, and reseeded onto the hydrogels following recovery for an appropriate period of time (*see* **Note 6**).
3. An important control requires that a protein-free hydrogel be used for the top substrate. This substrate should not be exposed to serum prior to sandwiching.

2.5. Assembly of the Double-Hydrogel Substrate

1. Diamond-tip pen.
2. Stainless steel weight of approx 30 g.
3. A square piece of glass 2 cm × 2 cm × 5 mm, 4–5 g.
4. Sterile Pasteur pipets.

3. Methods

The double-hydrogel substrate involves sandwiching cells between two single-layer polyacrylamide hydrogels, which were described in detail for traction force detection *(9)*. The culture system provides cells with a flexible, porous support as in native tissues and with anchorage sites for integrins on both ventral and dorsal surfaces. We have previously shown that the top substrate makes initial contact with the thickest part of the cell, above the nucleus, which is sufficient to induce an elongated morphology in fibroblasts and a reduced number of actin stress fibers and focal adhesions (**Fig. 1B,C**), typical of fibroblasts in vivo and in collagen gels *(5–7)*. Individual extensions exert forces on either the bottom or the top substrate, but not both, because of their limited thickness *(8)*.

The double-hydrogel substrate offers several advantages over other 3D model systems as described in **Heading 1.**, including the ability to control the flexibility, porosity, and matrix protein coating. Because the cells do not penetrate into the gel, images may be collected from a limited number of focal planes without confocal laser scanning optics. The high optical quality offers excellent resolution for structures in live cells such as focal adhesions. However, these advantages also impose constraints to cell shape and migration, and the culture environment is not entirely isotropic as for cells embedded in collagen or Matrigel gels.

3.1. Activation of Cover Slips

Sheets of polyacrylamide hydrogels are cast between two cover slips. The bottom sheet is cast between a large cover slip (e.g., 45 × 50 mm, no. 1), which is to be mounted into a culture chamber, and a small 22-mm circular cover slip.

The top sheet may be prepared similarly, and then trimmed out of the large cover slip. The large cover slips need to be chemically activated to allow polyacrylamide to attach covalently; otherwise the gel will slide off during handling.

1. Clean the surface of the large cover slips and mark the side to be activated with a diamond pen.
2. Hold the cover slip to be activated with a pair of forceps and pass across the inner flame of a Bunson burner with the marked surface facing down to make the surface hydrophilic.
3. Smear a small volume of 0.1 *N* NaOH over the flamed surface of the cover slips using a Pasteur pipet. Allow the surface to air-dry.
4. Working in a fume hood, use a Pasteur pipet to smear the marked surface of the cover slips with 3-aminopropyltrimethoxysilane and incubate for 5 min.
5. Rinse the cover slips extensively with distilled water until the glass is clear.
6. Working in a fume hood, prepare a 0.5% solution of gluteraldehyde in PBS and pipet sufficient solution to cover the surface of the cover slips. Incubate for 30 min.
7. Wash the cover slips extensively in distilled water until the activated surface is clear; allow to air-dry.
8. Activated cover slips can be stored in a desiccator at room temperature for many weeks.

3.2. Preparation of Polyacrylamide Hydrogel Sheets

1. Activated cover slips are mounted into chamber dishes with the activated side facing up (*see* **Note 7**).
2. Although a much smaller volume would suffice, for convenience a 5-mL polyacrylamide solution is prepared at a final concentration of 5% acrylamide, 0.1% *bis*-acrylamide, and 10 m*M* HEPES. Stock acrylamide and *bis*-acrylamide solutions are mixed with distilled water, 1 *M* HEPES pH 8.5, and 50-µL latex beads to achieve the desired concentrations (*see* **Note 8**). Beads of different colors are used for top and bottom substrates (*see* **Note 2**).
3. The mixture is then degassed for 20 min to ensure reliable polymerization.
4. During degassing, collect reagents, pipets, and cover slips for the subsequent steps in order to cast the gel immediately following induction of polymerization.
5. After degassing add 30 µL of freshly prepared 10% w/v ammonium persulfate, and gently mix by swirling the container.
6. Complete the activation by adding 20 µL of TEMED and gently mix.
7. Working quickly, place a 15-µL drop of activated acrylamide solution onto the center of the activated cover slip and gently place a 22-mm round cover slip on the drop.
8. Quickly invert the sample to allow the fluorescent microbeads to settle to the surface of the gel away from the large cover slip.
9. Allow the gel to polymerize for at least 30 min.
10. After polymerization is complete, turn the sample right-side up and flood the gel with 50 m*M* HEPES, pH 8.5, to reduce surface tension between the small cover slip and the gel.

11. Remove the small cover slip with a pair of fine forceps immediately before conjugating matrix proteins to the gel (*see* **Note 9**). The small cover slip may be left in place if the gel is to be stored at 4°C.

3.3. Conjugation of Matrix Proteins to the Hydrogel

1. Prepare a fresh solution of 1 m*M* sulfo-SANPAH by first dissolving the solid into DMSO at 5% (w/v) concentration. Slowly add 50 m*M* HEPES, pH 8.5, while vortexing vigorously (*see* **Note 10**).
2. Cover the surface of the gel with 300 µL of solution and expose to UV light at a distance of 2.5 in. below the lamp tube for 6 min (*see* **Note 11**).
3. Rinse the solution from the gel surface with one wash of 50 m*M* HEPES, pH 8.5, and repeat **step 2**.
4. Quickly rinse two to three times with 50 m*M* HEPES, pH 8.5, and immediately add the solution of the matrix protein. Allow the conjugation reaction to proceed at 4°C overnight.
5. Rinse gently with PBS to remove excess matrix proteins and store in PBS at 4°C until ready for culturing.

3.4. Preparing Substrates for Culture

1. Polyacrylamide hydrogels must first be "sterilized" and equilibrated with media before culturing. Quasi-sterilization is performed by placing the gels covered with minimal PBS in a culture hood under the UV germicidal light for 10–20 min.
2. Remove PBS, add culture medium, and place in a CO_2 incubator for 30–60 min to allow the gel to swell to a steady state (*see* **Note 12**).
3. Freshly trypsinized cells are seeded onto the substrates at approx 5000 cells per 22 mm substrate.
4. Cells are typically given several hours to overnight to attach and spread.

3.5. Assembly of the Double-Hydrogel Substrate

1. Top substrates may be carved out of a cover slip already mounted in a culture chamber or from an unmounted large cover slip placed over a supporting frame (*see* **Note 13**). Using a diamond-tip pen, press down firmly and trace along the perimeter of the 22-mm circular hydrogel. Although the entire circle does not always come out intact, fragments of the circle may work equally well.
2. Place the top substrate into a Petri dish containing medium and return to the incubator until needed.
3. In preparation for the sandwich assembly, collect the 30-g weight, square weighing glass, forceps, media, and Pasteur pipets in the culture hood. The bottom substrate containing attached cells and the top substrate are then brought to the hood.
4. Working quickly, use a Pasteur pipet under vacuum to aspirate as much media as possible from the bottom substrate and to remove any large dangling pieces of gel that might affect the contact between the top and bottom substrates.
5. Use a pair of forceps to pick up the top substrate from the Petri dish. Aspirate off excess media and any dangling pieces of gel with a Pasteur pipet.

6. Gently place the cover slip with the top substrate over the cells. Make sure that the gel is facing down. Carefully place the square weighing glass over the sandwich followed by the 30-g stainless steel weight for 30 s.
7. Add a small amount of medium such that the surface is just below the top of the square glass. Remove the metal weight with a pair of forceps. The square weighing glass stays on the sandwich.
8. Return the culture to the incubator or begin the experiment (*see* **Note 14**).

3.6. Observing the Live Culture Under the Microscope

Because the distance between the top and bottom substrates is affected by the presence of debris, it is important that this distance be verified in the region of observation.

1. Cells are scanned at a low magnification (e.g., using a ×10 phase objective lens). 3T3 fibroblasts take a spindle or stellate shape within 120 min of application of the top substrate.
2. Switch to a dry objective lens of a higher magnification such as ×40. The distance between the top and bottom substrate is judged by focusing first on fluorescent beads on the top surface of the bottom gel, using fluorescence optics. The filter cube of the microscope is switched to match the color of beads in the top substrate. The focal plane is then brought up slowly until beads on the bottom surface of the top gel just come into focus. The distance is then estimated from the angular travel of the focusing knob, using a conversion factor provided by the microscope manufacturer.
3. 3T3 cells show an elongated morphology and maintain their motility when the substrate is separated by 3–6 μm. Cells in regions of a greater substrate distance show no response in morphology to the top gel, whereas those in regions of a smaller distance show impeded motility.

4. Notes

1. A variety of culture chambers may be used. One simple design, as described previously *(9)*, is made of Plexiglas with a 35-mm-diameter hole drilled into the center. The No.1 cover slip measuring 45 × 50 mm (Fisher Scientific) is mounted using silicone high vacuum grease. An alternative design uses a disposable 60-mm polystyrene dish with a 35-mm hole drilled through the middle using a cup saw. A 45-mm round cover slip (Labor Optik GmBH, Friedrichshofen, Germany) is then adhered using an optical adhesive (Norland Optical Adhesive 71, Norland Products Inc., Cranbury, NJ).
2. Red and green microspheres give optimal results for distinguishing top and bottom substrates. However, when cells are labeled with a fluorescent probe, such as green fluorescent protein, both substrates may be labeled with red beads and distinguished by the level of focusing.
3. We have found that sulfo-SANPAH activity may vary between lots. An alternative method is to use a classical carbodiimide such as EDC (1-ethyl-3-[3-dimethyl

aminopropyl]carbodiimide-HCl) on a composite gel of polyacrylamide/polyacrylic acid. This method has been described previously *(9)*.

4. Proteins to be conjugated by sulfo-SANPAH should not be dissolved in Tris buffer because it competes with the protein for binding to sulfo-SANPAH. Tris buffer may be replaced easily by dialysis. Some proteins, such as laminin, maintain better functions if bound noncovalently and indirectly to the substrate via a layer of polylysine conjugated with sulfo-SANPAH to the polyacrylamide.
5. A lower cell density allows for easier analysis of the cells and eliminates complications that arise from cell–cell contacts. However, the morphological change does occur even at higher densities.
6. We have experienced very low efficiency of transfections and high rate of cell death when using lipid-based transfection methods on cells adhered to the hydrogels. Transfection using the Amaxa Nucleofector before seeding cells onto the hydrogel gives optimal results (Amaxa, Gaithersburg, MD).
7. It is easier to work with the hydrogels when the cover slips are mounted to chamber dishes at an early stage of preparation. However, it is also possible to cast the gels on the unmounted cover slips and prepare the chamber later.
8. For a typical preparation of a 5%/0.1% gel, we use the following recipe: 625 µL 40% acrylamide, 250 µL 2% *bis*-acrylamide, 50 µL 1 *M* HEPES, 50 µL Fluouspheres, 4025 µL distilled water.
9. Leave the small 22-mm cover slip on top of the polyacrylamide if the gel is to be stored without conjugation, to better preserve the surface of the gel. The gel may be left in 50 m*M* HEPES, pH 8.5, at 4°C. The small cover slip may be removed using two pairs of fine-tipped forceps: one for bracing an edge and the second for lifting the opposite edge.
10. Sulfo-SANPAH is unstable in solution and should be used immediately after preparation. The reagent has a marginal solubility and may be better handled by first dissolving the solid in a small volume of DMSO. HEPES solution at room temperature may then be added while vortexing vigorously.
11. Photoactivation should cause the sulfo-SANPAH solution to darken considerably, but the extent of darkening can be inconsistent between experiments and between different lots of the reagent.
12. The physical properties of hydrogels vary with temperature and osmolarity. Therefore, it is very important to acclimate the gels to the actual culture condition before use.
13. Instead of cutting the top substrate out of a large cover slip, it may be prepared with two pieces of round 22-mm cover slips. However, it is often difficult to maintain the flatness of the gel along the edge when cast with small cover slips. This causes problems in controlling the space within the sandwich.
14. Depending on the experimental design, cell elongation may be observed from rounded and freshly attached cells or from cells already spread on the bottom substrate. The cells reach a similar steady state as soon as 2 h after applying the top substrate, although we typically culture the cells in the sandwich overnight before observation.

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References

1. Meier, S. and Hay, E. D. (1975) Stimulation of corneal differentiation by interaction between cell surface and extracellular matrix. I. Morphometric analysis of transfilter "induction." *J. Cell Biol.* **66,** 275–291.
2. Mauchamp, J., Chambard, M., Verrier, B., et al. (1987) Epithelial cell polarization in culture: orientation of cell polarity and expression of specific functions, studied with cultured thyroid cells. *J. Cell Sci.* **8,** 345–358.
3. Cook, J. R., Crute, B. E., Patrone, L. M., Gabriels, J., Lane, M. E., and Van Buskirk, R. G. (1989) Microporosity of the substratum regulates differentiation of MDCK cells in vitro. *In Vitro Cell Dev. Biol.* **25,** 914–922.
4. Schmeichel, K. L. and Bissell, M. J. (2003) Modeling tissue-specific signaling and organ function in three dimensions. *J. Cell Sci.* **116,** 2377–2388.
5. Grinnell, F. (2003) Fibroblast biology in three-dimensional collagen matrices. *Trends Cell Biol.* **13,** 264–269.
6. Cukierman, E., Pankov, R., Stevens, D. R., and Yamada, K. M. (2001) Taking cell-matrix adhesions to the third dimension. *Science* **294,** 1708–1712.
7. Friedl, P. and Brocker, E. B. (2000) The biology of cell locomotion within three-dimensional extracellular matrix. *Cell. Mol. Life Sci.* **57,** 41–64.
8. Beningo, K. A., Dembo, M., and Wang, Y.-L. (2004) Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc. Natl. Acad. Sci. USA* **101,** 18,024–18,029.
9. Beningo, K. A., Lo, C.-M., and Wang, Y.-L. (2002) Flexible polyacrylamide substrata for the analysis of mechanical interactions at cell-substratum adhesions. *Methods Cell Biol.* **69,** 325–339.