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Chapter 17: Mechanical deformation of cultured cells with hydrogels

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Abstract

Polyacrylamide hydrogels can be used to culture cells in a range of stiffness that can closer mimic physiological environments. Changes in environmental stiffness have been documented in conditions such as fibrosis, cancer, and aging. In this chapter, we describe a method in which we pour gels directly into multiwell-plates using a plastic support that covalently binds to the polymerizing hydrogel. The hydrogel is then crosslinked to calf skin collagen using a crosslinker. The result is a thick hydrogel, scalable to any size plate, which covers the entire surface of the well with no edge effects. The gels can be routinely assembled and are easily reproducible. These scaffolds are used as *in vitro* models to study fibroblast reaction to variation in environmental stiffness.

Keywords

Hydrogels; Fibroblasts; Polyacrylamide; Stiffness; Multiwell; Cell culture

1. Introduction

Cells embedded in an extracellular matrix make connections with and exert force on their environment. The makeup and physical properties of the matrix in turn influence the cell shape and its ability to spread. Cells in a fragmented matrix attempt to make connections and exert force, but collapse in the absence of the intact matrix [1–3]. These cells are rounded in shape and have a reduced capacity to spread or stretch, leading to functional differences in the cell signaling pathways and extracellular matrix production [4–8]. Cells grown on polyacrylamide hydrogels with varying degrees of crosslinking can mimic an environment in which cells are able to spread or stretch to varying degrees [9,10]. “Stiffer” hydrogels, in which there is a higher ratio of bis-acrylamide to acrylamide are resistant to deformation by cells, and result in larger, spread out, or stretched cells. “Softer” hydrogels, in which there is a lower ratio of bis-acrylamide to acrylamide deform easily and result in a collapsed cell phenotype in which cells are smaller, rounded, and unable to stretch or spread out. The hydrogels are easy to prepare, reproducible, and have the ability to be tuned to a wide range of stiffness.

We use an adaptation of a multiwell polyacrylamide hydrogel protocol [10] in which the gels are poured directly into the well, eliminating the need for dehydration and rehydration of the gels. The gels can easily be scaled up or down to fit the size of any plate or multiwell plate.

2. Materials

2.1. Plate preparation

1. Cell culture plates. Plates of any well size can be used. We will describe this protocol with 6 well-plates (just scale the given volumes up or down to account for volume changes if you need to use larger- or smaller-sized plates).
2. PolyAcrylamide Gel (PAG) film, *i.e.* sheets of transparent flexible polyester film designed to support polyacrylamide gels (we use 138 mm × 158 mm sheets of GelBond® PAG film for acrylamide gels from Lonza). This film covalently bonds with acrylamide monomers during the polymerization process resulting in a polyacrylamide gel that remains permanently adhered to the film. Protect from light.
3. Silicone elastomer kit (we use Sylgard® 184 from Dow Corning). Supplied as a two part kit. When liquid components are thoroughly mixed, the mixture cures to a flexible silicone elastomer that cures at a constant rate regardless of sectional thickness and without exothermal heat production.
4. Paraffin film such as parafilm®: Use quadruply-folded film to cut discs to the size of the well. These discs will be referred to as “paraffin coverslips” below.
5. Aluminum Foil

2.2. Polyacrylamide hydrogels

1. 40% (w/v) acrylamide and 2% (w/v) bis-acrylamide solutions. Store at 4 °C.
2. 10% (w/v) ammonium persulfate solution is prepared fresh each time, using ultrapure water (*see* Note 1).
3. N, N, N, N-Tetramethyl-ethylenediamine (TEMED) can be stored at room temperature (*see* Note 2).
4. Forceps
5. Shaker plate

2.3. Crosslinking and coating

1. Long-arm crosslinker that contains an amine-reactive ester and a photoactivatable nitrophenyl azide (we use Sulfo-SANPAH (sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)-hexanoate) from ThermoFisher Scientific): Prepare a 50 mg/ml stock solution in DMSO. Snap freeze 40 µl aliquots in liquid

¹Ammonium persulfate powder should be stored desiccated at room temperature and is good for one year.

²TEMED is toxic and may cause respiratory irritation and should only be used under a fume hood.

nitrogen, and store at -80°C until use. Just before use, dissolve 40 μl aliquots in 1.96 ml of ultrapure water (*see* Note 3).

2. Sterile phosphate buffered saline (PBS), pH 7.4. The solution can be made, purchased concentrated, or ready to use. PBS is used for solution preparation and washing of gels. The total amount needed will depend on the well size of the gels.
3. Type I collagen stock solution (we use calf skin collagen from Elastin Products Company). Prepare a 0.1 mg/ml working solution fresh each time in PBS.
4. 1% (w/v) Bovine serum albumin (BSA) solution in PBS; sterilize *via* 0.22 μm filtration. Excess solution can be stored at 4°C for subsequent use.

2.4. Additional reagents and equipment

1. Biosafety cabinet (tissue culture hood) with ultraviolet (UV) germicidal light
2. UV lamp for the crosslinking of Sulfo-SANPAH (*see* Note 4)
3. Degassing chamber
4. Fume hood
5. Cell culture medium (we use Minimum Essential Media (MEM)- α containing 10% (v/v) Fetal Bovine Serum (FBS) for primary fibroblasts), and standard cell culture reagents (such as sterile trypsin, PBS, etc.)
6. Standard cell culture utensils (sterile pipettes, tubes, etc.)

3. Methods

3.1. Plate preparation

1. Cut some PAG film to the size of the wells you will be using (here, 6 well-plate = 34 mm-diameter discs). It may be helpful to print and cut a paper template beforehand. Take care to expose to as little light as possible. Pre-cut film can be stored away from light for future use (*see* Note 5).
2. Cut paraffin coverslips to the same size as the PAG films. Press the cut coverslips flat on the benchtop until use (*see* Note 6).
3. Prepare the silicone elastomer in the fume hood: Cut the tip off of a 1 ml pipet tip and pipette 1 ml of elastomer base and 100 μl of elastomer curing agent (10:1 ratio) in a disposable receptacle (we use the top of an old cap from a 50 ml conical tube). Mix the elastomer thoroughly with a pipette tip.

³Work quickly with Sulfo-SANPAH. Try to reconstitute, add to wells, and transfer to UV source within 10 minutes.

⁴Sulfo-SANPAH can be crosslinked using a wide range of UV wavelengths and intensity [12]. Efficiency of crosslinking should be determined experimentally for each lamp. For our lab purposes, we use a UVB lamp (i.e. $\sim 280\text{--}315\text{ nm}$) with an output of 120 $\text{mJ}/\text{cm}^2/\text{min}$ for 15 minutes.

⁵For 96-well plates, a high quality 1-hole punch can be used to cut the PAG film. We use the “crop a dille” hole-punch (available at most craft stores) with 96-well plates that have a 0.34 cm growth area.

⁶Ensure that there is no air caught between any of the parafilm layers, so that they remain flat.

4. Add approximately 100 μ l of elastomer mix to the bottom of each well. Using a droplet of water, determine which size of the pre-cut PAG film is hydrophobic, and place the dry film hydrophobic side down into the well. Push the film down into the silicone elastomer until the elastomer is spread evenly under the entire surface of the film. Repeat for the remainder of the wells.
5. Wrap the plates in aluminum foil to protect the light-sensitive PAG film during curing of the elastomer. Place wrapped plates and leftover mixed elastomer in a dry 37 °C incubator to cure. Curing usually takes at least 4 hours and can be prolonged overnight. Use the leftover mixed epoxy to validate curing time.
6. Glued plates can be used right away or stored at least a month at room temperature in the dark for later use.

3.2. Polyacrylamide hydrogels

1. Prepare the acrylamide solutions corresponding to different stiffness by mixing 40% acrylamide, 2% bis-acrylamide, water, and 10% APS solution according to the stiffness of gel needed, and using the volumes detailed in Table 1 (*see Note 7*). [table 1 near here]
2. Place the mixed solutions in a degassing chamber for at least 30 minutes.
3. Transfer the solutions to the fume hood and add 5 μ l of TEMED for every 10 ml of polyacrylamide solution, one solution at a time.
4. Immediately transfer to wells, adding enough solution to cover the hydrophilic PAG film (previously glued and cured) at the bottom of the well. The thickness of the gel does not matter, as long as the entire well is covered (*see Note 8*). Keep leftover solution as a polymerization control.
5. Right after pouring, cover each hydrogel with a paraffin coverslip, taking care to avoid bubbles (*see Note 9*).
6. Allow the gels to polymerize at room temperature. Different percent gels will polymerize at different rates. Use any leftover solution to observe if gel is fully polymerized. Gels may be left overnight to polymerize and coated in the morning.

3.3. Crosslinking and coating

1. Remove paraffin coverslips using forceps (*see Note 10*).
2. Quickly prepare working solution of crosslinker as described in section 2.3 (*see Note 3*).

⁷Alternate ratios of acrylamide and bis-acrylamide can be used. However, surface creasing of the gel can occur when bis-acrylamide percentage is lower than 0.028% (w/v). A lower stiffness gel can still be achieved by lowering acrylamide percentage [13]. Surface creasing will negatively affect cell shape and attachment.

⁸We use 2 ml acrylamide solution for one well of a 6-well plate.

⁹If a bubble is formed, simply use thin forceps to lift one edge of the paraffin film and slowly re-lower the paraffin coverslip.

¹⁰Paraffin coverslips may be reused if they are still in good condition.

3. Cover hydrogels with crosslinker solution. Add enough solution to completely cover the gel (we use 1 ml per well of a 6-well plate).
4. Transfer plate to UV lamp for crosslinking and expose to appropriate dose of UV light (*see* Note 4).
5. Aspirate and discard crosslinking solution and wash 3 times (2 ml per well of a 6-well plate) for 20 minutes each with PBS under moderate shaking.
6. Discard the last rinsing solution, add 2 ml of fresh PBS to each well, and transfer to a tissue culture hood with germicidal light. Close the hood and turn on the light for 2 hours to sterilize the plate.
7. Under sterile conditions, aspirate PBS, and add sterile collagen solution to each well (we use 1 ml per well of a 6-well plate). Ensure that the solution is evenly spread over the entire gel. Incubate for 2 hours at room temperature.
8. Aspirate and discard the collagen solution and wash three times with sterile PBS.
9. Aspirate and discard PBS, and add sterile BSA solution. Incubate for 1 hour at room temperature.
10. Aspirate and discard BSA solution and wash three times with sterile PBS. Hydrogels are now ready to be used. Alternatively, PBS can be added to each well and plates can be stored at 4 °C for 3 days.

3.4. Plating cells

1. Trypsinize active primary fibroblast cultures and prepare a 100,000 cells/ml cell suspension using routine culture techniques [11] (*see* Note 11).
2. Plate cells in wells at no more than 70% confluency using medium that contains FBS using typical well volumes for the plate size used, *i.e.* 2 ml/well for a 6 well-plate (see Notes 12 and 13). Cells plated on stiff gels will continue to divide and remain viable indefinitely. Collapsed cells grown on the softest gels (2.39 kPa) are good for 3 days, before they begin to lose viability.
3. Follow cells over time: Biochemical analysis can be performed after directly applying an RNA/DNA/protein extraction solution to the hydrogel, incubating, and harvesting. Histological analysis can be performed using routine staining techniques and an inverted microscope.

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¹¹This cell concentration is the one we use with primary fibroblasts. Cell concentration may need to be adjusted when working with other cell types.

¹²If cells on soft gels are plated at a high confluency, they can form attachment to each other and stretch out instead of remaining collapsed. FBS-containing medium can be removed and replaced with serum-free medium once cells have attached.

¹³Attachment to the hydrogels is not only a function of the stiffness of the hydrogel, but also the concentration of crosslinker used to bind the collagen [14].

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Figure 1. Primary human fibroblasts grown on different stiffness polyacrylamide hydrogels for 24 hours in MEM α containing 10% FBS and observed with a phase contrast microscope and 10X objective.

Table 1
Reported Young's modulus and various ratios of acrylamide to bis-acrylamide for different stiffness gels [10].

Young's modulus (kPa)	% Acrylamide	% bis-Acrylamide	ml 40% Acrylamide	ml 2% bis-Acrylamide	ml H ₂ O	μl 10% APS
2.39 *	5%	0.03%	1.25	0.15	8.60	50
10.64	5%	0.1%	1.25	0.50	8.25	50
29.14	8%	0.1%	2.00	0.50	7.50	50
66.01	8%	0.25%	2.00	1.25	6.75	50
112.25	12%	0.25%	3.00	1.25	5.75	50

* This modulus is extrapolated from reported data (see Note 7)