Chapter 13

Micropatterning Cell Adhesion on Polyacrylamide Hydrogels

Jian Zhang, Wei-hui Guo, Andrew Rape, and Yu-li Wang

Abstract

Cell shape and substrate rigidity play critical roles in regulating cell behaviors and fate. Controlling cell shape on elastic adhesive materials holds great promise for creating a physiologically relevant culture environment for basic and translational research and clinical applications. However, it has been technically challenging to create high-quality adhesive patterns on compliant substrates. We have developed an efficient and economical method to create precise micron-scaled adhesive patterns on the surface of a hydrogel (Rape et al., Biomaterials 32:2043–2051, 2011). This method will facilitate the research on traction force generation, cellular mechanotransduction, and tissue engineering, where precise controls of both materials rigidity and adhesive patterns are important.

Key words Cell adhesion, Cell geometry, Substrate rigidity, Mechanotransduction

1 Introduction

Cell–cell interactions involve both the release of chemicals and the generation of mechanical forces; the latter includes forces transmitted through both cell–cell junctions [1] and cellular adhesions to the elastic extracellular matrix (ECM). Mechanical forces applied by contractile cells adhered to the ECM, termed traction forces, may propagate much more rapidly and efficiently than chemical signals to affect distant cells that adhere to the same matrix [2]. These mechanical signals are known to regulate a range of cellular activities including migration, proliferation, apoptosis, and differentiation [3–10]. In addition, adherent cells also use traction forces to probe physical properties of the environment such as rigidity and topography [11], such that long-lasting cell–cell interactions may be mediated by modifying physical characteristics of the ECM.

Due to their optical transparency, cost-effectiveness, ease of preparation, and controllable elasticity over the physiological range, polyacrylamide (PAA) gels have become widely used as a tool for measuring cellular traction forces and probing cellular responses to

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mechanical cues [12]. The lack of passive protein adsorption on PAA further allows the control of cell shape, spreading size, and migration by conjugating defined surface areas with specific ECM ligands. In principle, patterning of PAA may be achieved by microcontact printing ECM proteins onto a gel surface that has been chemically activated with agents such as the hetero-bifunctional crosslinker sulfo-SANPAH [12]. However, the deformability of both the stamp and the gel surface makes this approach poorly reproducible when applied to PAA [13, 14] or polydimethylsiloxane (PDMS; [15]). Alternatively, the surface of a hydrogel may be micropatterned at a high resolution using a combination of photochemistry and confocal laser scanning optics [16]; however, this approach is inefficient for patterning large surface areas.

We have developed a simple and efficient method for conjugating proteins to the surface of PAA hydrogels at a high resolution [17]. The method takes advantage of the extensive glycosylation of many ECM proteins, which contain vicinal diols that may be chemically activated with sodium m-periodate to form two aldehyde groups after a ring-opening reaction [13, 18]. The aldhehyde groups in turn react with polymerizing acrylamide to incorporate the ECM proteins into the PAA gel structure. In addition to the compatibility with a micropatterning procedure as described below, this simple approach is easily repeatable and requires much less time than previously reported methods (30 min compared to up to 4 h).

There are many potential applications of micropatterned hydrogels. Constraining cell shape proved useful in traction force measurements. Since traction forces vary with cell shape [15, 19, 20], eliminating the variability of cell shape reduces the standard deviation and allows the detection of previously obscure differences. More importantly, this method enables researchers to assess relative influences of cell shape and substrate rigidity on such processes as differentiation, apoptosis, and proliferation. For the study of cell migration, this method may also be used to constrain cells to take defined shapes and paths. Combinatorial manipulations of multiple parameters on micropatterned PAA will likely lead to new advances in both cell biology and tissue engineering.

2 Materials

2.1 Activation of Coverslips

1. Freshly prepared Bind-silane working solution. Mix 950 μl of ethanol (95 %; ACS/USP grade) and 50 μl of acetic acid with 3 μl of Bind-silane (Sigma-Aldrich, St. Louis, MO, USA).
2. Coverslips (45 mm × 50 mm, No. 1).
3. Diamond-tip pen.
4. Bunsen burner.

2.2 Photolithography

1. SPR-220.3 positive photoresist (MicroChem, Newton, MA, USA).
2. Coverslips (45 mm × 50 mm, No. 2).
3. Spin coater (e.g., Spin Processor from Laurell, North Wales, PA, USA). An inexpensive low-speed tabletop centrifuge may be modified to hold coverslips for spin coating [21].
4. Heating block or plate with precise temperature control for 115 °C.
5. UV source for i-line (365 nm). An inexpensive UV station with relatively uniform exposure may be constructed by mounting a high flux UV LED (Opto Technology Inc, Wheeling, IL, USA) over an orbit shaker where the photoresist-coated coverslip is placed. The setup is shown in [21].
6. Photomask with the desired pattern. Glass photomasks (e.g., from Advance Reproductions Corp, North Andover, MA, USA) are required for a resolution of pattern better than 10 μm. Otherwise, the mask may be ordered as an inexpensive transparency film (e.g., from CAD/Art Services, Bandon, OR, USA).
7. Microposit Developer MF-319 (MicroChem).
8. Glass Petri dishes.
9. Orbital shaker in a chemical fume hood.

2.3 Preparation of PDMS Stamps

1. Sylgard 184 Silicone Elastomer Kit, including Base and Curing Agent (Dow Corning Corporation, Midland, MI, USA).
2. Balance and weighing boats.
3. Heating block or incubator with precise temperature control for 70 °C.
4. Vacuum.
5. Disposable beakers.

2.4 Activation of ECM Proteins

1. Gelatin or ECM protein, such as collagen or fibronectin.
2. Sodium m-periodate (Sigma-Aldrich).

2.5 Micropatterning of Polyacrylamide Hydrogels

1. Glass coverslips (25 mm × 25 mm, No. 1).
2. Acrylamide.
3. Bisacrylamide.
4. HEPES: 200 mM, pH 8.5.
5. Nitrogen gas.
6. Ammonium persulfate (APS), freshly prepared 10 % (w/v) solution.
7. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).

8. Razor blades.
9. (For traction force microscopy): Fluorescent latex beads of different colors, such as Fluoresbrite carboxy microspheres, 0.2 μm, Yellow/Green (Polysciences, Warrington, PA, USA), and FluoSpheres carboxylate-modified microspheres: 0.1 μm, blue fluorescence (350/440) (Molecular Probes, Eugene, OR, USA).

3 Methods

3.1 Activation of Coverslips for the Bonding of PAA

1. Mark one side of the 45 mm × 50 mm, No.1 coverslip with a diamond-tip pen. Pass the coverslip over the inner flame of a Bunsen burner with the marked side facing the flame. The plasma in the flame increases the hydrophilicity of the glass surface. Allow the coverslips to cool to room temperature.
2. In a fume hood, apply approximately 30 μl of Bind-silane working solution onto the flamed side of coverslips and smear it evenly with the pipet tip. Remove excess Bind-silane with Kimwipes. Allow the Bind-silane to react for 3 min.
3. Rinse treated coverslip surfaces with ethanol and wipe with Kimwipes to remove any residual Bind-silane solution. Allow to air-dry (*see* **Note 1**).

3.2 Preparation of PDMS Stamps

1. Pass coverslip (45 mm × 50 mm, No. 2) over the inner flame of a Bunsen burner with the marked side facing the flame. Allow the coverslip to cool to room temperature.
2. In a fume hood with a spin coater set at 5,000 rpm for 30 s, spread 180 μl positive photoresist SPR-220 uniformly across the flamed side of the coverslip.
3. Bake the coverslips at 115 °C for 90 s on a heating block. Allow the coverslips to cool to room temperature.
4. Place the photomask over the coverslip and expose the assembly to 365 nm UV light. The exposure depends on the intensity of the light source. Using a high flux UV LED at a distance of 3 cm from the coverslip on an orbital shaker, the optimal exposure is around 45 s (*see* **Note 2**).
5. Bake the coverslip at 115 °C for 90 s on a heating block. Allow the coverslip to cool to room temperature.
6. Immerse the coverslip in Microposit Developer MF-319 in a glass Petri dish, placed on an orbital shaker inside a chemical fume hood, for approximately 45 s. Optimal timing and mixing conditions are affected by the exposure condition and should be controlled carefully (*see* **Note 3**).

7. Rinse the coverslip extensively in deionized water and allow to air-dry. The pattern on SPR-220, which serves as the molding for PDMS stamps, should be visible (*see* **Note 4**).
8. Weigh out approximately 5 g of Sylgard 184 Silicone Elastomer Base, add 1/10 volume (v/w) of the curing agent, and mix thoroughly. Degas for 30 min using house vacuum to remove air bubbles.
9. Incubate the coverslip covered with Sylgard at 70 °C on a heating block or in an incubator for at least 1 h.

3.3 Activation of ECM Proteins

1. Dilute gelatin to a final concentration of 0.1 % (w/v) in PBS, or Type I collagen to a final concentration of 0.01 % (w/v) in 50 mM sodium acetate (pH 4.5), or fibronectin to a final concentration of 0.001 % (w/v) in PBS.
2. While mixing, slowly add solid sodium *m*-periodate to the protein solution to reach a final concentration of 3.6 mg/ml. Allow the reaction to proceed for 30 min at room temperature (*see* **Note 5**).

3.4 Micropatterning of Polyacrylamide Gels

1. Prepare the PDMS stamp, activated protein, and Bind-silane-activated glass coverslips as described above (Subheadings 3.1, 3.2, and 3.3).
2. Pass a 25 mm × 25 mm coverslip over the inner flame of a Bunsen burner. Allow the coverslip to cool to room temperature.
3. Pipet approximately 200 μl of the activated protein solution onto the surface of the stamp, incubate at room temperature for 30 min, then remove excess solution by blowing with a stream of nitrogen gas.
4. Prepare the acrylamide solution with the desired concentrations of acrylamide and bisacrylamide in 10 mM HEPES. Typically 5 % (w/v) acrylamide and 0.1 % (w/v) bisacrylamide are used for the measurement of traction stress of fibroblasts. Degas with house vacuum for 20 min, as previously described [12].
5. Press the stamp against the 25 mm × 25 mm coverslip for 5 min (*see* **Note 6**).
6. Add 0.006 volumes of 10 % APS and 0.004 volumes of TEMED to the acrylamide solution and mix quickly and briefly to initiate the polymerization reaction.
7. Pipet 30 μl of the polymerizing acrylamide solution onto a Bind-silane-activated coverslip.

8. Without any delay, remove the stamp from the 25 mm × 25 mm coverslip and place the coverslip, stamped side facing down, on the polymerizing acrylamide solution.
9. Let the acrylamide polymerize to completion for 15–20 min at room temperature.
10. Peel off the top coverslip carefully with a razor blade. Cover the hydrogel surface immediately with PBS to prevent drying (*see* **Notes** 7 and **8**).

3.5 Preparation of Micropatterned Polyacrylamide Substrates for Traction Force Microscopy

1. Prepare the coverslip stamped with activated gelatin (or other ECM proteins) as described above (Subheading 3.4).
2. Prepare and degas the acrylamide solution as described above (Subheading 3.4).
3. Add 0.006 volume of 0.1 % (w/v) gelatin to the acrylamide solution. This is required for obtaining a uniform distribution of beads (*see* **Note 9**).
4. Add a dilution of 0.0008 volume of 0.2 μm (*see* **Note 10**) fluorescent latex beads into the acrylamide solution. Mix well and incubate at room temperature for 2.5 min (*see* **Note 11**).
5. Add 0.006 volume of 10 % (w/v) APS, 0.004 volume of TEMED, and 0.002 volume of 0.1 μm fluorescent beads of a different color from that used in **Step 4** (optional, *see* **Note 12**). Mix rapidly and pipet 30 μl on the surface of Bind-silane-activated coverslip.
6. Immediately place the stamped coverslip, patterned side down, on the acrylamide solution (*see* **Note 13**). Let the acrylamide polymerize to completion for 15–20 min at room temperature. Peel off the top coverslip carefully with a razor blade. Cover the hydrogel surface immediately with PBS to prevent drying (*see* **Note 14**).

4 Notes

1. Activated coverslips may be stored in a desiccator at room temperature for at least 3 months.
2. Tight contact between the photomask and photoresist is critical if the light source is not well collimated. A simple method involves placing the photomask over the coverslip and sandwiching them between two pieces of glass plates with paper clamps to ensure tight contact. In addition, it is critical to make sure that the patterned side on the photomask is facing the coverslip. For illumination with a less-than-uniform light source, the assembly of photomask and coverslip may be

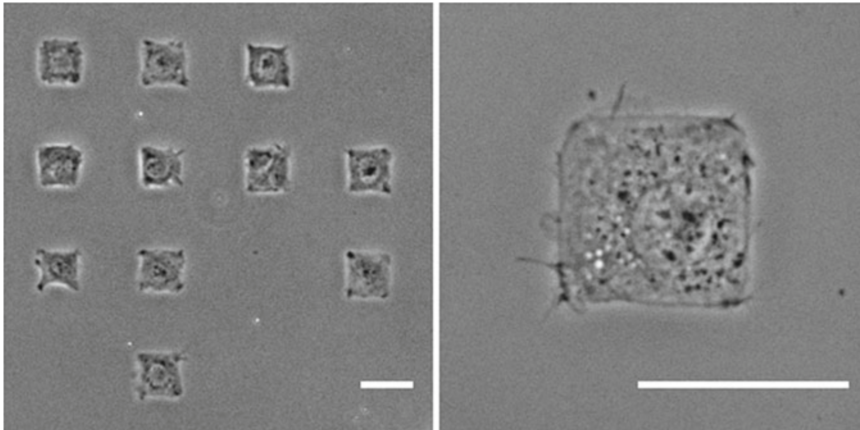

Fig. 1 MC3T3-E1 cells on PAA hydrogel patterned as a *square* with gelatin. Scale bar, 50 μm

placed on an orbit shaker [21]. Rotation at about 70 rpm during exposure creates a uniform average illumination across the surface. The exact exposure time depends on the optical condition and must be calibrated for each setup.

3. Developing in tetramethylammonium hydroxide (TMAH; 2.45 % in 0.1 % Triton X-100) generates results comparable to those using Microposit Developer MF-319.

4. To generate polyacrylamide substrates uniformly conjugated with ECM, simply use a clean coverslip to make a flat, patternless PDMS stamp.

5. Treatment with periodate causes vicinal diols in the sugar moieties of ECM proteins to undergo a ring-opening reaction, forming two aldehyde groups [13, 18], which are capable of copolymerizing with acrylamide, thus directly incorporating the ECM protein into the hydrogel. After the reaction, periodate can be removed with dialysis or a spin column. Activated proteins may be stored at −20 °C or −80 °C for several months.

6. The contact between the stamp and the coverslip should be even to ensure efficient transfer of the patterned protein. Even contact may be achieved by gently rolling a pencil back and forth over the PDMS stamp in two criss-cross directions. The quality of contact may be checked by looking at the glass–PDMS interface from different angles. Colorful interference fringes appear at the interface if the contact is good.

7. ECM-micropatterned hydrogel substrate should be sterilized using UV irradiation before inoculation with cells (Fig. 1).

8. Non-glycosylated proteins may be patterned using a similar procedure. The protein is stamped onto the coverslip as described above without activation. Acrylamide solution is prepared with the incorporation of acrylic acid-*N*-hydroxysuccinimide, which reacts with protein lysine residues, at a final concentration of 0.05 mg/ml.
9. We have found that fluorescent latex beads became concentrated onto protein conjugated areas during acrylamide polymerization, allowing easy visualization of the pattern but creating problems for the measurement of substrate strain for traction force microscopy. Addition of gelatin to the acrylamide solution prevents this concentration, suggesting electrostatic interactions as the cause of bead concentration. Proteins other than gelatin, e.g., BSA, may also be used to block beads concentration.
10. This beads concentration worked well to generate an optimal surface bead density for traction force microscopy (i.e., high enough to provide a good resolution while allowing the resolution of individual beads). The concentration may be adjusted according to the requirement of the experiment.
11. The incubation allows the protein (gelatin) in the solution to bind to the beads. Too short of an incubation provides insufficient blocking and causes beads to accumulate under protein conjugated areas. Conversely, too long an incubation causes beads to be excluded from protein conjugated areas, likely due to the repulsive electrostatic interactions. Blocking time may be influenced by protein and beads concentration, and needs to be adjusted accordingly.
12. Adding fluorescent beads of a different color without incubation causes these beads to concentrate in protein conjugated areas and facilitate the observation of pattern. Their use is optional. Alternatively the micropattern may be visualized using fluorescently labeled ECM protein.
13. The time for placing the stamped coverslip to the acrylamide solution is approximately 4 min after it is mixed with the first fluorescent beads. We have found that this overall incubation time works well for the current condition (*see* **Note 11**).
14. With this method, fluorescent beads with pre-incubation show a homogeneous distribution (Fig. 2a, b), while the beads without incubation reveal the micropattern (Fig. 2c, d).

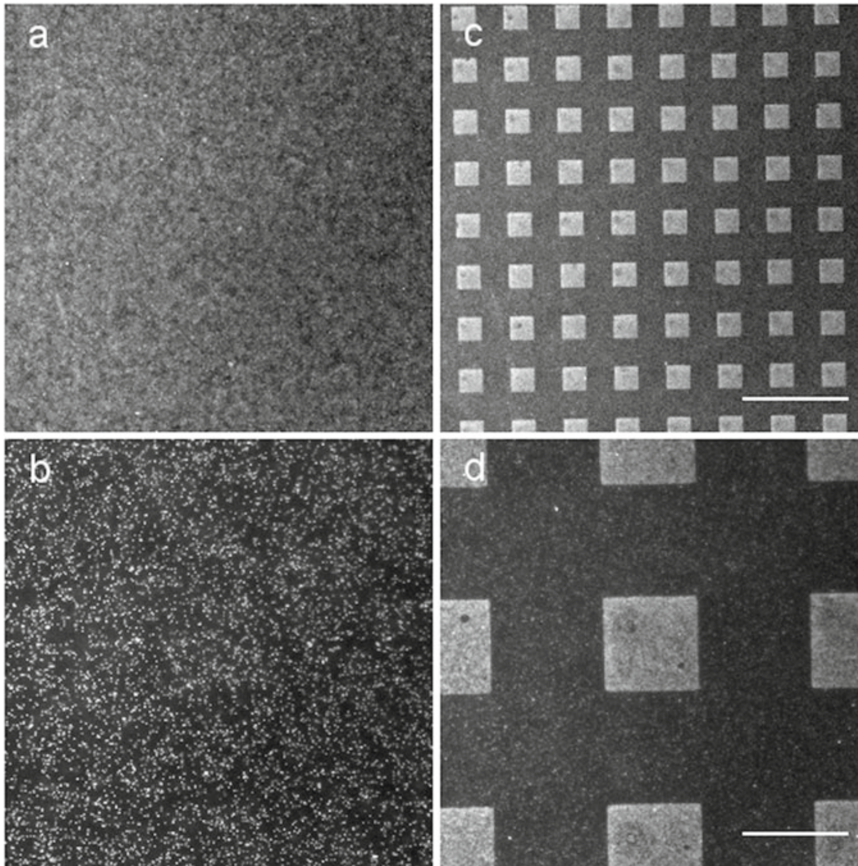

Fig. 2 Distribution of fluorescent beads embedded in the polyacrylamide hydrogel. Beads pre-incubated with protein show homogeneous distribution, suitable for traction force microscopy (**a**, **b**). In contrast, beads added immediately before gel polymerization concentrate in ECM-patterned areas, suitable for visualizing the pattern (**c**, **d**). Scale bar, 200 μm (**a**, **c**), 50 μm (**b**, **d**)

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References

1. Tambe DT, Corey-Hardin C, Angelini TE et al (2011) Collective cell guidance by cooperative intercellular forces. Nat Mater 10:469–475
2. Janmey PA, Miller RT (2011) Mechanisms of mechanical signaling in development and disease. J Cell Sci 124:9–18
3. Chen CS, Mrksich M, Huang S et al (1997) Geometric control of cell life and death. Science 276:1425–1428
4. Singhvi R, Kumar A, Lopez G et al (1994) Engineering cell shape and function. Science 264:696–698
5. Dike L, Chen C, Mrksich M et al (1999) Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. In Vitro Cell Dev Biol Anim 35: 441–448

6. McBeath R, Pirone DM, Nelson CM et al (2004) Cell shape, cytoskeletal tension, and rhoa regulate stem cell lineage commitment. Dev Cell 6:483–495
7. Kilian KA, Bugarija B, Lahn BT et al (2010) Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci 107:4872–4877
8. Wang H-B, Dembo M, Wang Y-L (2000) Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. Am J Physiol Cell Physiol 279:C1345–C1350
9. Engler AJ, Sen S, Sweeney HL et al (2006) Matrix elasticity directs stem cell lineage specification. Cell 126:677–689
10. Pelham RJ, Wang Y-l (1997) Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci 94:13661–13665
11. Rape A, Guo W-H, Wang Y-L (2011) Responses of cells to adhesion-mediated signals: a universal mechanism. In: Wagoner JA, Harley BAC (eds) Mechanobiology of cell-cell and cell-matrix interactions. Springer, USA, pp 1–10
12. Wang Y-L, Pelham RJ Jr (1998) Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells. In: Richard BV (ed) Methods in enzymology. Academic, New York, pp 489–496
13. Damljanovic V, Lagerholm BC, Jacobson K (2005) Bulk and micropatterned conjugation of extracellular matrix proteins to characterized polyacrylamide substrates for cell mechanotransduction assays. Biotechniques 39:847–851
14. Guo W-h, Wang Y-I (2007) Retrograde fluxes of focal adhesion proteins in response to cell migration and mechanical signals. Mol Biol Cell 18:4519–4527
15. Wang N, Ostuni E, Whitesides GM et al (2002) Micropatterning tractional forces in living cells. Cell Motil Cytoskeleton 52:97–106
16. Vignaud T, Galland R, Tseng Q et al (2012) Reprogramming cell shape with laser nanopatterning. J Cell Sci 125:2134–2140
17. Rape AD, Guo W-h, Wang Y-l (2011) The regulation of traction force in relation to cell shape and focal adhesions. Biomaterials 32:2043–2051
18. Fischer B, Thomas KB, Dorner F (1998) Collagen covalently immobilized onto plastic surfaces simplifies measurement of von Willebrand factor-collagen binding activity. Ann Hematol 76:159–166
19. Li F, Li B, Wang Q-M et al (2008) Cell shape regulates collagen type I expression in human tendon fibroblasts. Cell Motil Cytoskeleton 65:332–341
20. Reinhart-King CA, Dembo M, Hammer DA (2003) Endothelial cell traction forces on RGD-derivatized polyacrylamide substrata. Langmuir 19:1573–1579
21. Guo W-H, Wang Y-l (2011) Micropatterning cell-substrate adhesions using linear polyacrylamide as the blocking agent. Cold Spring Harb Protoc 2011(3):prot5582