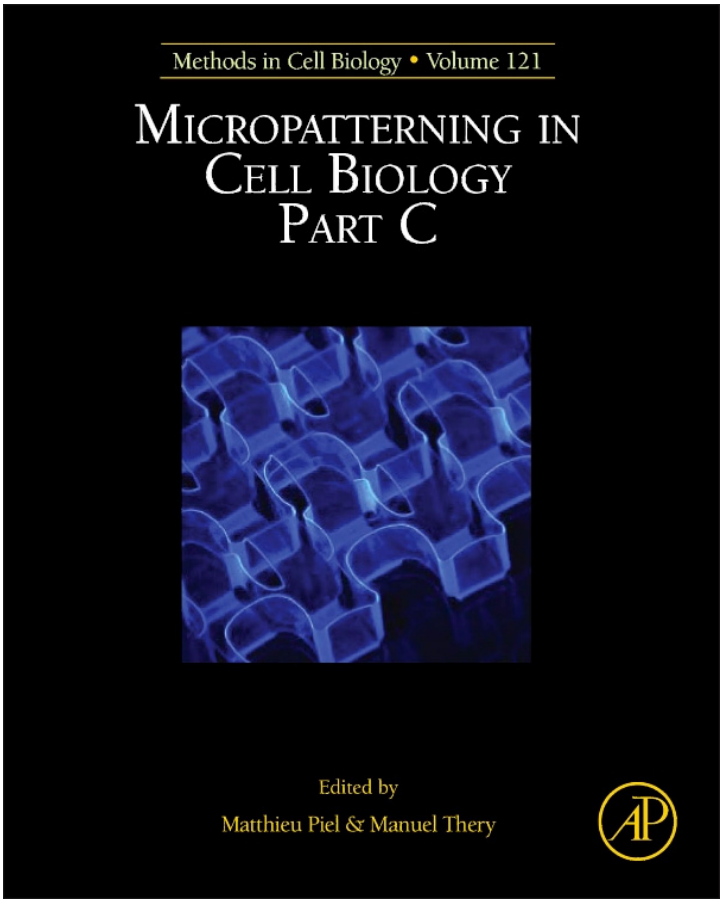

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CHAPTER

1 Preparation of a Micropatterned Rigid-Soft Composite Substrate for Probing Cellular Rigidity Sensing

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CHAPTER OUTLINE

Abstract

Substrate rigidity has been recognized as an important property that affects cellular physiology and functions. While the phenomenon has been well recognized, understanding the underlying mechanism may be greatly facilitated by creating a microenvironment with designed rigidity patterns. This chapter describes in detail an optimized method for preparing substrates with micropatterned rigidity, taking advantage of the ability to dehydrate polyacrylamide gels for micropatterning with photolithography, and subsequently rehydrate the gel to regain the original elastic state. While a wide range of micropatterns may be prepared, typical composite substrates consist of micron-sized islands of rigid photoresist grafted on the surface of polyacrylamide

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hydrogels of defined rigidity. These islands are displaced by cellular traction forces, for a distance determined by the size of the island, the rigidity of the underlying hydrogel, and the magnitude of traction forces. Domains of rigidity may be created using this composite material to allow systematic investigations of rigidity sensing and durotaxis.

INTRODUCTION AND RATIONALE

Micropatterning has been utilized during the past two decades to create microenvironments of defined geometry at a micron scale (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001). It allows systematic testing of specific features of the *in vivo* environment for their biological effects. By controlling the geometry of adhesion areas on glass, previous micropatterning studies have demonstrated the effects of cell shape and size on events such as apoptosis, proliferation, differentiation, and migration (Chen, 1997; Dike et al., 1999; McBeath, Pirone, Nelson, Bhadriraju, & Chen, 2004; Pouthas et al., 2008; Singhvi et al., 1994; Wang, Ostuni, Whitesides, & Ingber, 2002).

An important parameter that would benefit from micropatterning studies is substrate rigidity, which is known to cause profound cellular responses (Discher, Janmey, & Wang, 2005; Engler, Sen, Sweeney, & Discher, 2006; Pelham & Wang, 1997). Most studies of cellular rigidity sensing have relied on the use of either elastic polymers or bendable micropost arrays as the substrate. The former included polydimethylsiloxane (PDMS) and polyacrylamide, where the elasticity may be controlled over a wide range by altering the concentration of the crosslinker and/or the base material. The latter involved the preparation of PDMS pillars with bending moduli varied over a limited range by changing the diameter or height of the pillars (Tan et al., 2002; Trichet et al., 2012), or over a wider range by changing both the rigidity of PDMS used for pillar fabrication and dimension of the pillars (Sun, Jiang, Okada, & Fu, 2012).

Both elastic polymers and micropost arrays may be micropatterned by selective coating of the surface with adhesive proteins to control cell size, shape, and migration. Methods used include microstencils (Wang et al., 2002), microcontact printing (Théry & Piel, 2009), activation with deep UV exposure though a mask (Tseng et al., 2011), and microcontact printing of activated proteins on glass followed by transfer of the pattern to the elastic substrate (Rape, Guo, & Wang, 2011). What has been lacking, however, is a method to create micropatterns of mixed rigidity, given the importance of studying cellular responses at a rigidity interface such as in durotaxis (Lo, Wang, Dembo, & Wang, 2000). Previous methods to address durotaxis have involved the creation of a border of rigidity across the substrate surface (Trichet et al., 2012; Wang, Dembo, Hanks, & Wang, 2001), with serious limitations in the number cells that may be studied at the border.

Several methods have been developed for creating a rigidity interface across the substrate surface, including polymerization of hydrogels using photosensitive reactions and patterned UV illumination (Nemir, Hayenga, & West, 2010; Wong, Velasco, Rajagopalan, & Pham, 2003), and overlay of a thin hydrogel layer on micron-sized rigid topographic features (Choi et al., 2012; Gray, Tien, & Chen, 2003). Rigidity changes may also be created locally in real time using hydrogels formed with photo-labile crosslinkers (Frey & Wang, 2009; Kloxin, Benton, & Anseth, 2010). While these methods create fixed rigidity domains of similar adhesiveness, different questions may be addressed by creating small mobile islands of rigid adhesive materials grafted onto soft nonadhesive surfaces. In the initial study using this approach, we showed that long-range substrate strain between the islands dominates over local rigidity, in determining cellular responses (Hoffecker, Guo, & Wang, 2011).

To prepare such composite substrates, we take advantage of the ability to dehydrate polyacrylamide hydrogels, for the attachment of photoresist and micropatterning using photolithography, and subsequently rehydrate the hydrogel to regain the original elastic property. The polyacrylamide surface remains nonadhesive to cells, while the photoresist may be treated with extracellular matrix proteins to enhance cell adhesion. We present here a detailed method for generating this material, using only inexpensive equipment without a clean room.

1.1 MATERIALS

1. Coverslip (45 mm × 50 mm #1; Fisher Scientific, Pittsburgh, PA)
2. Diamond tip pen
3. Bunsen burner
4. Bind silane working solution: Mix 950 mL, 95% ethanol, and 50 mL of 95% glacial acetic acid, and add 3 μL of bind silane (γ-methacryloxypropyltrimethoxysilane; GE Healthcare, Waukesha, WI) to form the working solution
5. Ethanol (C_2H_5OH) 95%
6. Acrylamide solution (40% w/v; BioRad, Hercules, CA)
7. Bis-acrylamide solution (2% w/v; BioRad)
8. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; BioRad)
9. Ammonium persulfate aqueous solution (APS, 10% w/v; BioRad)
10. 10 × and 1 × phosphate buffered saline (PBS, pH 7.4)
11. Aqueous sucrose solution (50% w/v)
12. Rain-X
13. Coverslip (#2, 25 mm circular or square; Fisher Scientific)
14. Razor blade
15. Tweezers
16. Heating block set to 95 °C
17. SU-8 2002 (MicroChem Corp., Newton, MA)

18. SU-8 developer (MicroChem Corp.)
19. Spin coater
20. UV light source for crosslinking SU-8
21. Orbital shaker
22. Photomasks, on inexpensive transparency films for patterns larger than 10 μm or chrome plated lime glass for smaller features
23. Glass Petri dishes

1.2 METHODS

Our method involves the following steps: (A) Preparation of thin sheets of polyacrylamide of defined rigidity covalently bound to a glass coverslip for stability (Fig. 1.1A); (B) dehydration of the polyacrylamide sheet to allow grafting and

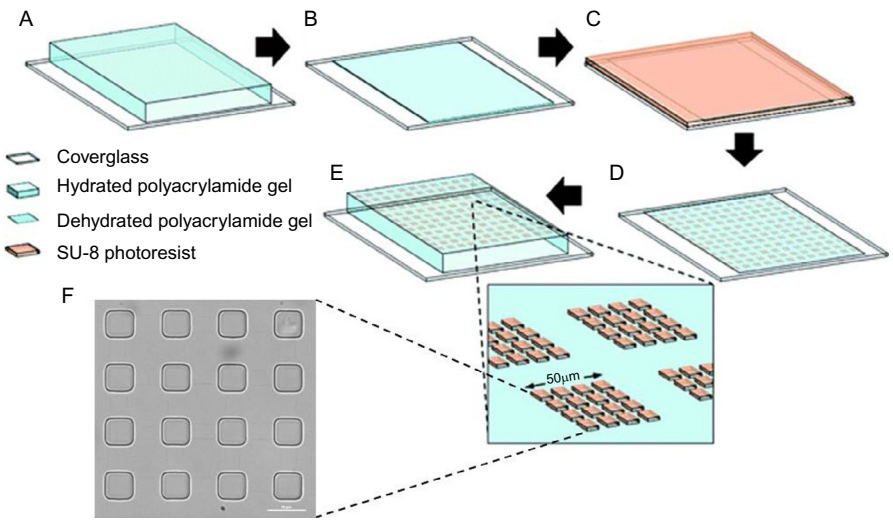

FIGURE 1.1

Schematic depicting the steps involved in composite substrate fabrication. (A) Polyacrylamide hydrogels are polymerized on bind-silane-activated glass coverslips. (B) Hydrogels are air dried to dehydrate and flatten the hydrogel before micropatterning. (C) A thin layer of SU-8 is spin coated on the dehydrated hydrogel surface. (D) The surface is patterned via UV exposure through a photomask containing the desired pattern and developed to remove unexposed regions of SU-8 leaving behind the desired pattern. (E) Rehydration of the polyacrylamide in PBS allows the hydrogel to reswell with the patterned SU-8 islands grafted to the surface. (F) Phase contrast image of the composite substrate consisting of a 4×4 array of small SU-8 islands on a polyacrylamide gel surface.

Reproduced from Hoffecker et al. (2011).

micropatterning of SU-8 using photolithography (Fig. 1.1B–D); (C) rehydration of the polyacrylamide sheet and coating of the surface of SU-8 to promote cell adhesion (Fig. 1.1E). The pattern of SU-8 grafted to the polyacrylamide gel may be easily seen using transmitted optics (Fig. 1.1F).

1.2.1 Preparation of polyacrylamide gel base

A thin sheet of polyacrylamide gel serves as an elastic base for anchoring rigid islands of SU-8 photoresist, such that translocation of the islands is determined by the rigidity of polyacrylamide, the size of the islands, and forces applied by cells to the adhesive islands. The sheet of polyacrylamide must be covalently bound to a glass surface during dehydration, when the hydrogel sheet would otherwise shrink and detach from the surface. Bonding is established by pretreating the glass surface with bind silane (γ-methacryloxypropyltrimethoxysilane), which reacts with glass through the trimethoxysilane moiety and copolymerizes with acrylamide through the methacryl moiety. For this procedure, one side of a coverslip (45 mm × 50 mm in our applications) is marked with a diamond tip pen for identification, the marked side is passed through the plasma of a Bunsen burner flame to render the surface hydrophilic, and 30 μL of bind silane working solution is spread evenly over the flamed side of the coverslip, in a fume hood, using a cotton swab. After 15 min at room temperature, when the surface has fully dried, the treated side is rinsed with 70% ethanol and allowed to air dry. These activated coverslips can be stored at room temperature in a desiccator for at least 3 months.

A solution of acrylamide and bis-acrylamide is then polymerized on the activated glass surface. A uniform sheet is formed by polymerizing a small volume underneath a top coverslip (25 mm circular or square in our applications). The use of a top coverslip also restricts oxygen exposure, which inhibits the polymerization of acrylamide. A very thin sheet, formed by using a volume of 0.03–0.04 μL/mm^2 surface area during polymerization, is desirable for compatibility with microscope optics. In addition, a thin gel barely rises above the glass surface after dehydration, which facilitates spin coating and tight contact with the photomask during photolithography (Section 1.2.2).

Removal of the top coverslip is facilitated by coating its surface with either Rain-X (to increase hydrophobicity) or 50% sucrose solution (to serve as a separation layer). Such treatment is essential to minimize the adhesion of the polyacrylamide surface to the top coverslip, which would otherwise introduce defects on the gel surface, particularly for soft gels, during the removal of the top coverslip. Surface defects in turn compromise the quality of micropatterning and interfere with microscopy. Rain-X solution is smeared over the coverslip and wiped away with a Kimwipe. The surface is then rinsed with distilled water and wiped until clear. It is important to ensure complete treatment of the surface with Rain-X to prevent any local adhesion of the coverslip to polyacrylamide.

To coat the surface with sucrose solution, one side of a top coverslip is marked with a permanent marker and the unmarked side is passed through the plasma of a Bunsen burner flame to render the surface hydrophilic and ensure an even coating of

sucrose solution. Approximately 100 μL of 50% w/v sucrose solution is then placed on the flamed side of the coverslip and spread over the entire surface. Uniform coating is achieved by placing the coverslip in a spin coater and spinning the sample at 5000 rpm for 15 s.

The rigidity of the polyacrylamide sheet is controlled by changing the concentration of acrylamide and/or bis-acrylamide (Pelham & Wang, 1997). Typically, calculated volumes of acrylamide, bis-acrylamide, and 0.1 mL of 10 × PBS are mixed with distilled water to reach a total volume of 1 mL and then placed in a sealed chamber under house vacuum to degas for 30 min. Skin contact with unpolymerized acrylamide should be avoided as it is a neurotoxin. Freshly prepared 10% w/v APS and TEMED are then added at a volume of 6 and 4 μL, respectively, and mixed by gentle tapping or pipetting. A 20 μL droplet of solution is pipetted immediately onto a bind-silane-activated coverslip and a Rain-X or sucrose-coated top coverslip is placed on top using a pair of fine tweezers, with the coated side facing the acrylamide solution. The acrylamide solution should spread uniformly underneath the top coverslip; if not, gently moving the coverslip with a pair of tweezers should help. When using a sucrose-coated top coverslip, the coverslip assembly should be turned upside down during polymerization to avoid the settling of sucrose into the acrylamide solution due to its higher density. The solution is then allowed to polymerize for at least 30 min at 25 °C.

Following polymerization, the sandwich is turned right side up and the top coverslip removed carefully. Rain-X treated coverslips are removed by flooding the surface of the coverslip with distilled water and waiting for at least 15 min to allow water to seep in. A razor blade is then used to lift the top coverslip very slowly off the polyacrylamide gel, with the gel staying submerged in water, to prevent the gel from cracking due to the strain. Sucrose-coated coverslips are easier to remove and are recommended for soft gels to avoid surface cracking. They may be removed by immersing the sandwich in hot distilled water in a Petri dish to dissolve the sucrose. The coverslip should release in 20–30 min. Following the removal of the top coverslip, the gel should be equilibrated with distilled water in a Petri dish for 30 min on a shaker. This helps prevent the formation of crystals during the subsequent drying.

The edges of the polyacrylamide gel often show a slight lip that could disrupt proper micropatterning. These may be removed using a razor blade to cut away ~1 mm along the edge while keeping the gel hydrated under distilled water. The gel is then rinsed with water to remove any bits of polyacrylamide gel and allowed to air dry overnight. Complete drying of the gel is essential for the subsequent coating with SU-8 in organic solvent and photolithography.

1.2.2 Micropatterning of the polyacrylamide surface with SU-8 photoresist

Once the polyacrylamide sheet is dry, the surface is micropatterned using SU-8, a negative photoresist that polymerizes upon UV exposure (Fig. 1.1). Photomasks with a desired pattern may be obtained on either plastic transparencies (for patterns larger

than 10 μm) or chrome plated lime glass (for high resolution patterns), from companies such as CAD/Art services (Bandon, OR) or Photo-Sciences (Torrance, CA), respectively, which accept CAD file formats such as dwt. Note that areas for SU-8 coverage should be clear, while areas in between should be masked.

Micropatterning is performed following standard photolithography procedures. The coverslip is first baked on a temperature-regulated heating plate for 1 min at 95 °C, to ensure that the gel surface is completely dry. After cooling to room temperature, approximately 300 μL of SU-8 photoresist solution is spread on the coverslip to cover the dehydrated gel surface. The coverslip is placed in a spin coater and spun at 5000 rpm for 20 s, then baked for 2–3 min at 95 °C and cooled to room temperature. Using SU-8 2002, this procedure should create a uniform layer ∼2 μm in thickness.

Contact exposure represents the most economical way to transfer the pattern from the photomask onto the SU-8 photoresist. The coverslip is placed on a platform stand underneath a UV light source with the SU-8 side facing up, overlaid with the photomask with the patterned side facing down, and covered with a piece of plate glass 3 mm in thickness that has an area matching that of the platform. Several binder clips are placed around the plate glass to clamp the plate glass to the stand to ensure tight contact between the photomask and the coverslip. The source of UV may range from an arc lamp with collimated optics for uniform exposure (OAI, San Jose, CA) to inexpensive 360-nm UV photodiodes (Jelight, Irvine, CA). The latter should be used in conjunction with an orbital shaker, which rotates the coverslip assembly underneath the light beam to achieve uniform exposure. The exposure time is dependent on both the power of the UV source and the distance between the lamp and the coverslip, and must be determined by trial and error (see Section 1.2.4, point 2). At a light power of 100 nJ/cm^2 at the sample, the optimal exposure time should be around 60–90 s.

After exposure, the coverslip is baked for 2–3 min at 95 °C and allowed to cool to room temperature. It is then immersed in the SU-8 developer in a Petri dish for 60–90 s with agitation, rinsed briefly with ethanol from a squirt bottle, and immersed in a separate Petri dish of ethanol for ∼30 s. After air drying, the micropattern should be easily visible under a microscope. There should be no speckles or films between the intended areas of SU-8, which indicate residual SU-8 due to incomplete development. In addition to potential problems with imaging, residual SU-8 may cause cell adhesion to an otherwise nonadhesive polyacrylamide surface. It may be removed by additional treatment with SU-8 developer, for approximately 10 s, and ethanol rinse as described earlier.

It is essential that all traces of SU-8 developer be removed from the hydrogel to mitigate the risk of cytotoxicity. For immediate use, the coverslip should be washed with PBS in a Petri dish for an hour with shaking, to allow both rehydration of the gel and removal of residual developer. Alternatively, the coverslips may be baked for 4 h at 95 °C to evaporate the residual developer and then stored in a dessicator at room temperature for up to 3 weeks. Repeated cycles of gel hydration and dehydration should be avoided as they can introduce microcracks on the gel surface.

1.2.3 Surface coating with extracellular matrix proteins and cell seeding

The gel of the composite substrate is rehydrated in PBS for approximately 1 h at room temperature and then sterilized under the UV of a biosafety cabinet for 15–20 min. SU-8 is known to have a biofouling surface (Voskerician et al., 2003), which passively adsorbs proteins from serum containing media. Thus, cells may attach to the SU-8 surface to some extent without treatment. Cell adhesion to SU-8 surfaces may be optimized by incubation with an extracellular matrix protein such as fibronectin, while the polyacrylamide surfaces should remain nonadhesive regardless of the incubation. A 20-min incubation at room temperature with the protein of interest (e.g. 10 μg/mL fibronectin in PBS) is usually sufficient for the promotion of cell adhesion.

Before plating cells, the composite substrate is equilibrated with culture media for at least 30 min in a CO_2 incubator. Once plated, cells should attach to areas occupied by SU-8 within 15 min, although a longer period may be required if the adhesive areas are small.

While the SU-8 pattern and adhered cells are easily visible at a low magnification with phase contrast or bright field optics, the use of high magnification oil immersion lens may be limited by the combined thickness of the hydrogel and photoresist. A lens of long working distance is essential, and better resolution may be achieved with a water immersion lens to avoid spherical aberration. It is also noteworthy that SU-8 emits autofluorescence when excited at 488 nm, therefore fluorophores with a long wavelength are preferred over probes such as Green Fluorescent Protein.

1.2.4 Troubleshooting

Although the present method may seem straightforward, problems may arise from the improper execution of a few crucial steps.

1. *Poor micropatterning*: After development, the micropattern may not look as expected. Poor contact between the photomask and SU-8 creates ill-defined borders of the micropattern. Debris on a dirty photomask, or beading of the edge of polyacrylamide or SU-8 (from poor spin coating) can create space between the photomask and SU-8. In addition, overexposure can cause SU-8 areas to appear larger than expected with poorly defined edges, while overdeveloping would cause SU-8 areas to appear smaller with rounded corners.
2. *Poor association of SU-8 with the polyacrylamide surface*: During development, the micropattern may become detached from the polyacrylamide surface. SU-8 is most likely underexposed or insufficiently heated after exposure. Underexposure prevents proper formation of crosslinks within SU-8 all the way down to the polyacrylamide surface, which grafts the photoresist to the gel surface and provides the resistance against the developer. Insufficient baking after UV exposure also prevents exposed regions of SU-8 from curing properly, while too

thick a layer of polyacrylamide may slow down heat conduction and require a longer period of baking.

3. *Adhesion of cells to supposedly nonadhesive areas of polyacrylamide*: When cells are plated they may attach in between SU-8 islands to the hydrogel surface. Most likely SU-8 is underdeveloped, which causes a thin film of unexposed SU-8 to remain on the surface of polyacrylamide. This residual film then adsorbs proteins and mediates cell adhesion. The problem may be rectified by additional treatment with SU-8 developer as noted in Section 1.2.2.
4. *Cracking of the gel beneath SU-8*: After rehydration there are cracks easily visible in the hydrogel surface between SU-8 islands. The most likely cause is adhesion of the top coverslip to the gel during removal, possibly due to incomplete coverage of the surface of top coverslip with Rain-X or sucrose solution. In addition to ensuring complete coverage, increasing the concentration of bis-acrylamide and decreasing the concentration of acrylamide may alleviate this problem by increasing gel stability while maintaining the same elastic modulus.
5. *Poor cell adhesion*: During plating the cells may not quickly adhere to the SU-8 islands. If plated cells fail to attach to SU-8 within 20–30 min, a likely cause is insufficient time of incubation with extracellular matrix proteins or serum containing medium.

1.3 DISCUSSION

A significant advantage of the procedure above is the use of inexpensive equipment, including high flux UV-LED as the light source in conjunction with an orbital shaker for uniform illumination (Guo & Wang, 2011). In addition, as pointed out by Tsai, Crosby, and Russell (2007), most biological applications can tolerate some defects in the micropattern, which allows photolithography to be performed without a clean room facility.

The effects of the above procedures on rheological properties of the polyacrylamide hydrogel have been assessed with sheets ~500 μm in thickness using a Bohlin Gemini Advanced Rheometer (Malvern Instruments Inc., NJ). No significant difference in shear modulus was found between untreated hydrogels and hydrogels subjected to the micropatterning procedure without UV exposure (thus removal of the entire layer of SU-8 during the development), suggesting that the process of micropatterning, including dehydration and rehydration, does not affect mechanical properties of the hydrogel.

To determine if cellular traction forces may cause small SU-8 islands to dislodge from the surface of polyacrylamide hydrogels, cells were allowed to adhere overnight to a regular array of islands on a soft hydrogel. While traction forces caused visible distortion of the pattern, the islands returned to their regular positions upon removal of the cell with trypsin (Fig. 1.2). This indicates that the SU-8 islands are well adhered to the polyacrylamide hydrogel, and that cellular traction forces do not cause slippage between the gel and islands.

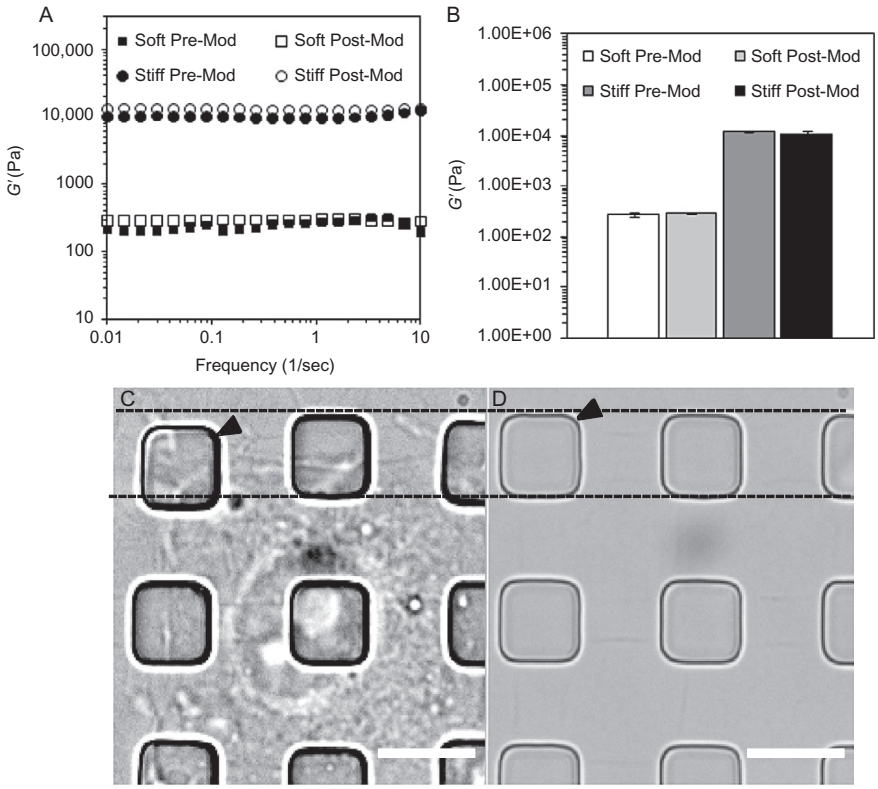

FIGURE 1.2

(A) The shear modulus of a stiff and soft hydrogel, before and after the micropatterning procedure without UV exposure, was measured using parallel plate rheometry at a constant strain of 0.1 as a function of frequency. As the frequency changes, the mechanical properties of the gel are not significantly changed before or after modification. (B) Average values for shear modulus of a stiff and soft gel show that there is no significant change in gel mechanical properties from the micropatterning step. Error bars represent standard error of the mean (SEM). (C) To assess the elastic recovery of the hydrogel, cells were cultured overnight on the patterned substrate. Displacement of the island is evident, yellow lines and arrow, from the force exerted on the island by the cell. (D) After removal of the cell with trypsin the island recovers to the original position evidenced by yellow line and arrow. This illustrates the elastic recovery of the gel and that there is no slippage of the island on the gel when force is exerted on the island.

Reproduced from Hoffecker et al. (2011).

Compared to homogeneous substrates, composite materials prepared with the present procedure are particularly advantageous for studying cellular responses to rigidity. Both the stiffness of the hydrogel base and the pattern of adhesive photoresist can be varied according to the experimental design. For example, the spatial resolution for cellular rigidity sensing has been assessed by varying the size of the island

and the distance between islands. The results demonstrated that long-range strains dominate cellular responses over local rigidity (Hoffecker et al., 2011; Trichet et al., 2012), although other approaches suggested that local rigidity may also play a role under some conditions (Ghassemi et al., 2012; Sun et al., 2012). Future applications of composite substrates may help dissect the mechanism of rigidity sensing at both spatial regimes.

Recent studies have demonstrated the importance of mechanical microenvironments in regulating cell physiology, differentiation, migration, and organization (Discher et al., 2005; Engler et al., 2006). Micropatterning methods are emerging as an important tool for engineering microenvironments *in vitro* to mimic the conditions *in vivo* or dissect the effects of specific parameters. The present method may be modified for different applications. For example, while cell adhesion is limited to the SU-8 surface following the present procedure, the entire surface including polyacrylamide may be rendered adhesive using activation reagents such as Sulfo-SANPAH (Pierce, cat. No. 22589, Rockford, IL). Cells would then probe the substrate continuously, rather than respond to the deformability between rigid SU-8 domains.

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