ARTICLE

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PEGDA Hydrogels With Patterned Elasticity: Novel Tools for the Study of Cell Response to Substrate Rigidity

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ABSTRACT: The ability of cells to migrate in response to mechanical gradients (durotaxis) and differential cell behavior in adhesion, spreading, and proliferation in response to substrate rigidity are key factors both in tissue engineering, in which materials must be selected to provide the appropriate mechanical signals, and in studies of mechanisms of diseases such as cancer and atherosclerosis, in which changes in tissue stiffness may inform cell behavior. Using poly(ethylene glycol) diacrylate hydrogels with varying polymer chain length and photolithographic patterning techniques, we are able to provide substrates with spatially patterned, tunable mechanical properties in both gradients and distinct patterns. The hydrogels can be patterned to produce anisotropic structures and exhibit patterned strain under mechanical loading. These hydrogels may be used to study cell response to substrate rigidity in both two and three dimensions and can also be used as a scaffold in tissue-engineering applications.

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KEYWORDS: substrate rigidity; substrate stiffness; PEGDA; hydrogel; patterning; photolithography

Introduction

In their natural environment, most cells are surrounded by a scaffold composed of extracellular matrix molecules. This scaffold provides sites for adhesion, mechanical and biochemical signals, and structural support. Many tissue-engineered constructs include a scaffolding or matrix material to fill some or all of these roles. An ideal material for use as a scaffold would be bioinert, adhesive to desired cell type(s), and capable of supporting long-term cell viability. The mechanical properties of the scaffold are also important, as the elasticity of the underlying matrix can affect cell spreading, proliferation, and even differentiation (this extensive body of work is reviewed in Nemir and West, in press; Peyton et al., 2007). Differential cell behavior in response to substrate mechanical properties may play a role in processes ranging from embryogenesis (Ingber, 2006) to the pathogenesis of disease states (Li et al., 2007; Paszek and Weaver, 2004).

Studies of cell responses to substrates with patterned or gradient rigidity have exclusively used polyacrylamide (Gray et al., 2003; Lo et al., 2000; Wong et al., 2003; Zaari et al., 2004) or poly(dimethylsiloxane) (PDMS) substrates (Gray et al., 2003; Saez et al., 2007). Both of these materials have serious limitations preventing their use for 3D encapsulation of cells. The acrylamide monomer is highly toxic (Xi et al., 2006), limiting the use of polyacrylamide gels to relatively short-term studies in two dimensions, while the processing parameters for PDMS substrates with patterned rigidity are incompatible with cellular encapsulation (Gray et al., 2003). There is therefore a need for a scaffold system with patterned rigidity with which one can investigate cell response to substrate stiffness in both two and three dimensions, as well as one which may be implanted for use in tissue-engineering constructs. This article will describe one such system.

Poly(ethylene glycol) (PEG) is a hydrophilic, water soluble, biocompatible polymer (Padmavathi and Chatterji, 1996; Ratner, 2004) that has been suggested for use in a variety of biomedical applications (reviewed in Tessmar and Gopferich (2007); Veronese and Mero (2008)). The polymer is poorly immunogenic, non-toxic at molecular weights above 400 Da, is readily cleared by the kidneys, and is approved by the FDA for internal consumption (Harris, 1992). The polyether backbone cannot be degraded by mammalian enzymes (Harris, 1992). Enzymatic degradation of a cross-linked polymer can therefore be initiated only by the

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controlled inclusion of degradable sequences at crosslinking sites (West and Hubbell, 1998).

The PEG molecule is neutral, highly mobile, and heavily hydrated in aqueous solution, with a large exclusion volume (Harris, 1992). These properties have been used to explain PEG's inherent resistance to protein adsorption when covalently crosslinked: the molecule has few sites for protein binding, its high mobility allows little time for proteins to form positive attachments, and the surrounding water molecules exclude other molecules from nearing the polymer surface (Harris, 1992). The resistance to protein adsorption allows PEG-based hydrogels to act as blank slates for cell adhesion, as they can be rendered selectively cell-adhesive by the addition of specific ligands but will otherwise not support the adsorbed protein layer that mediates cell attachment to most materials used in biological applications (Hern and Hubbell, 1998).

Substituting terminal hydroxyl groups with acrylates, forming poly(ethylene glycol) diacrylate (PEGDA), allows the polymer to be crosslinked to form a three-dimensional polymer network. While PEGDA may be crosslinked by a variety of methods, the use of photopolymerization is particularly versatile for use in tissue-engineering applications (Nguyen and West, 2002). Photopolymerization allows for spatial and temporal control of polymerization as well as formation of complex shapes (Hahn et al., 2006). With careful selection of an appropriate photoinitiator, crosslinking can occur under sufficiently mild conditions to permit encapsulation of living cells within the polymer matrix (Nguyen and West, 2002).

PEGDA hydrogels are highly tunable. The mechanical properties of the hydrogels can be controlled by varying the molecular weight or concentration of the polymer, with an increase in elastic modulus with increasing polymer concentration or decreasing polymer molecular weight (Al-Nasassrah et al., 1998; Gunn et al., 2005; Padmavathi and Chatterji, 1996). The mesh size and swelling ratio can be similarly controlled (Cruise et al., 1998), and the mechanical and biochemical properties can be varied independent of one another (Peyton et al., 2006).

In this article, we will describe the synthesis of PEGDA hydrogels with patterned elasticity. Due to their excellent biocompatibility and tunable physical properties, these hydrogels may be used to study cell response to substrate rigidity in both two and three dimensions and can also be used as a scaffold in tissue-engineering applications.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Synthesis of PEGDA

Poly(ethylene glycol) diacrylate (PEGDA) was prepared as previously described (DeLong et al., 2005). Briefly, 0.1 mmol/mL dry poly(ethylene glycol) (3.4 or 20 kDa; Fluka, Milwaukee, WI) was combined with 0.4 mmol/mL acryloyl chloride and 0.2 mmol/mL triethylamine in anhydrous dichloromethane under argon, stirring overnight. The resulting PEGDA was washed with K_2CO_3 (EMD, Gibbstown, NJ), dried with anhydrous $MgSO_4$ (Fisher Scientific, Fair Lawn, NJ), and precipitated in diethyl ether (Fisher Scientific), then filtered and dried in vacuo.

Synthesis of Acryloyl-PEG-RGDS

Heterobifunctional acryloyl-PEG-SCM (Laysan Bio, Arab, AL) was combined with RGDS (American Peptide, Sunnyvale, CA) in a 1:1.2 molar ratio and diisopropylethylamine in a 1:2 molar ratio in anhydrous dimethyl sulfoxide and allowed to react under argon overnight. The solution was then dialyzed against ultra pure water using a 2000 MWCO regenerated cellulose dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA), lyophilized, and stored at $-20^{\circ}C$ until use.

Preparation of PEGDA Hydrogels With Distinct Patterns of Elasticity

Polymer solution for base hydrogels was prepared by dissolving 0.1 g/mL 20 kDa PEGDA in HEPES-buffered saline (10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] and NaCl in ultra pure water, pH adjusted to 7.4) (HBS) with 10 μL/mL photoinitiator solution (2,2-dimethoxy-2-phenyl-acetophenone 300 mg/mL in *N*-vinylpyrrolidone). Solutions were sterile filtered, injected into molds made with glass slides separated by 1 mm spacers, and exposed to long-wavelength UV light (365 nm, 10 mW/cm^2) for 45 s. Hydrogels were placed in HBS until fully swollen, then placed into a sterile-filtered soak solution composed of 0.2 g/mL 3.4 kDa PEGDA in HBS with 10 μL/mL photoinitiator solution and were allowed to soak on a rocker table under argon overnight. Samples were removed from soak solution, rinsed briefly with HBS to remove solution from surface, and patterned for 1 min under UV light using a photomask printed on a transparency, forming an interpenetrating network of 3.4 kDa PEGDA within the 20 kDa base gel in only the areas exposed to UV light. Transparencies were placed ink-side up unless otherwise noted.

Striped samples were patterned with ~350 μm-wide 3.4/20 kDa stripes separated by 20 kDa stripes of equal (50% patterned), double (33% patterned), or quadruple (20% patterned) width. Control samples were prepared using clear (100% patterned) or uniformly black (0% patterned) photomasks. Samples were then placed in HBS overnight to allow uncrosslinked soak solution to diffuse out prior to mechanical testing. This process is shown pictorially in Figure 1. Samples used for compressive testing with atomic force microscopy (AFM) were patterned with ~400×600 μm^2 stiff rectangles against a soft background.

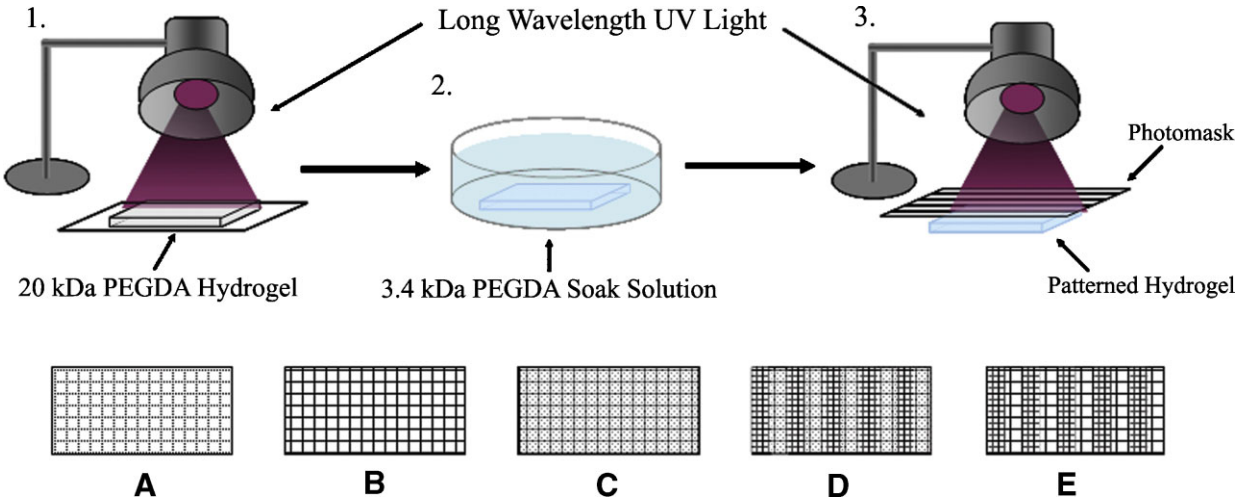

Figure 1. Schematic diagram of patterned hydrogel formation. First, 20 kDa PEGDA base hydrogel (0.1 g/mL) is crosslinked under UV light for 45 s (1, A to B). Next, base hydrogel is placed in soak solution containing 0.2 g/mL 3.4 kDa PEGDA + 10 μL/mL photoinitiator solution and allowed to soak overnight (2, B to C). The hydrogel is removed from soak solution, rinsed briefly, and placed under UV light for 1 min using a photomask to restrict crosslinking to patterned areas (3, C to D). Finally, hydrogel is soaked in HBS to allow uncrosslinked soak solution to diffuse out (D to E).

The length of the soaking step required for a uniform distribution of polymer soak solution within the base hydrogel was determined using confocal microscopy to visualize diffusion of 3 kDa dextran-fluorescein (Molecular Probes, Eugene, OR) within the base gel. To determine whether photoinitiator quenching during sample soaking might affect mechanical properties of the patterned hydrogels, hydrogel samples were removed from soak solution after various time points, crosslinked using a clear transparency as a photomask, and subjected to mechanical testing as described below.

Preparation of Hydrogels With Gradient Elasticity

Hydrogels with gradient elasticity were formed using a gradient maker (CBS Scientific Co., Del Mar, CA). The two reservoirs of the gradient maker contained 0.1 g/mL PEGDA (3.4 and 20 kDa, respectively) in HEPES-buffered saline (HBS, pH 7.40) mixed with 10 μL/mL photoinitiator solution and then sterilized via filtration (0.22 μm filter). Fluid flow between gradient reservoirs was controlled by a Teflon valve centered between the reservoirs and gradient outflow rate was controlled by a peristaltic pump. Mixing was maintained using a stir plate with a magnetic stir bar in each reservoir. The resulting polymer gradient was dripped into a rectangular glass mold and locked into place by crosslinking with long-wavelength UV light (365 nm, 10 mW/cm^2) (Fig. 2). Hydrogels were removed from molds and placed in HBS to swell prior to mechanical testing.

Preparation of Hydrogels for Cell Studies

Hydrogels with patterned rigidity used in cell studies were made as described in Figure 1, with the addition of 3 mM acryloyl-PEG-RGDS to the base 0.05 g/mL 20 kDa PEGDA

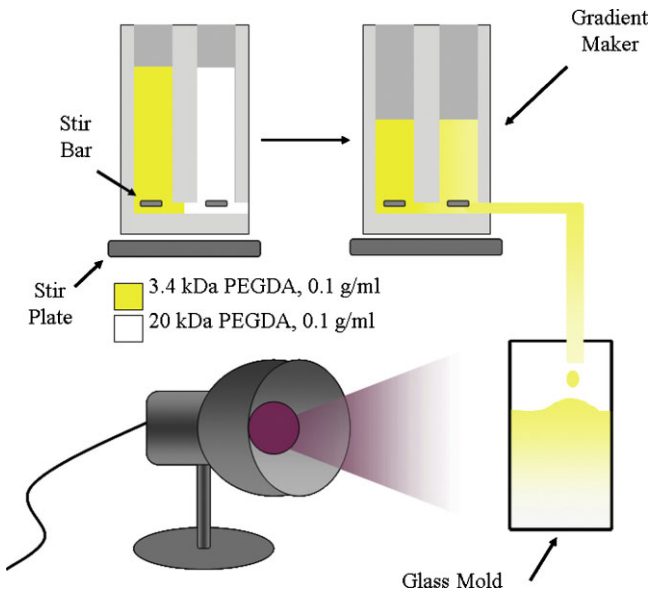

Figure 2. Schematic diagram of gradient hydrogel formation. Reservoirs of gradient maker are filled with solutions of PEGDA + photoinitiator. Fluid flow between reservoirs is controlled by a Teflon valve and gradient outflow is controlled by a peristaltic pump. Mixing is maintained using a stir plate with a magnetic stir bar in each reservoir. The resulting gradient is captured within a rectangular glass mold and crosslinked using long-wavelength UV light.

hydrogel solution. The soak solution contained 0.15 g/mL 3.4 kDa PEGDA. These hydrogels were patterned with stiff rectangles against a soft background, as for AFM.

Cell Culture

RAW 264.7 macrophages (ATCC, Manassas, VA) were cultured in DMEM (ATCC) with 10% v/v fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% v/v antibiotic solution (5,000 units penicillin + 5 mg streptomycin/mL) at 37°C and 5% CO_2. Media was changed every other day, and cells were passaged at ~80% confluence. Cells were seeded on patterned hydrogels at 50,000 cells/cm^2, and non-adherent cells were rinsed off after 4 h. Hydrogels were imaged after 24 and 48 h.

Visualization of Patterning

Patterns within the PEGDA hydrogels were discernable without the use of contrast agents due to differences in refractive index between stiff and soft regions. However, to distinctly visualize and image patterns, hydrogels were soaked in 10 mg/mL solutions of fluorescently labeled dextran of different sizes in HBS (dextran-fluorescein, 10, 40, 70, or 500 kDa; Molecular Probes). Hydrogels were removed from dextran solutions, rinsed in MilliQ water for 10 min then imaged on a fluorescence microscope (Zeiss Axiovert 135, Thornwood, NY; excitation at 436 nm, emission at 550 nm).

As a second method of visualizing hydrogel patterning, 1 mg/mL acrylated fluorescein (Sigma-Aldrich) was added to the soak solution. The acrylated fluorescein was then covalently immobilized within the polymer network during photocrosslinking, and uncrosslinked fluorescein was allowed to diffuse out prior to imaging. To confirm that patterning continued through the depth of the hydrogel, *z*-stack images of the patterned hydrogels were taken with 5 μm spacing between slices using a confocal microscope (Zeiss Live5, Thornwood, NY), and orthogonal projections through the hydrogel were produced using ImageJ software (NIH, Bethesda, MD).

In situations where fluorescent imaging was not available, patterned hydrogels were soaked overnight in HBS with 0.01% w/v cresyl violet acetate (Aldrich Chem. Co., Milwaukee, WI), which preferentially dyed stiff regions of the hydrogel. This method was utilized during mechanical testing using AFM as well as imaging of relative strain in stiff and soft areas of patterned hydrogels during tensile testing.

Characterization of Hydrogel Mechanical Properties

For hydrogels with patterned rigidity, dogbone-shaped samples ($N = 3$–4) were cut using a metal punch, measured using digital calipers, then subjected to tensile testing using an Instron Model 3340 materials testing device with a 10 N load cell (Norwood, MA). Dogbones were aligned with the long axis either parallel or perpendicular to the stripes. Instron Series IX/s software was used for system control and data acquisition. Uniaxial strain was applied at a rate of 6 mm/min and the force-elongation data collected was used to calculate average elastic modulus, defined as the slope of the linear portion of the stress-strain curve. Data analysis was performed in Microsoft Excel. Prior to mechanical testing, hydrogels with gradient elasticity were sliced into sequential 1.5–2 mm thick transverse sections along the length of the gradient. To evaluate uniformity of elasticity within each slice and to confirm a gradient of elastic modulus on one axis only, one gradient hydrogel was sectioned as described, then each section was divided into multiple rectangular specimens ($N = 4$). A thin piece of balsa wood was attached to each end of each specimen using a cyanoacrylate-based adhesive, and this wood was gripped during testing. To evaluate consistency of gradient formation between hydrogels, multiple hydrogels with gradient elasticity ($N = 4$) were sectioned, then dogbone-shaped samples were removed from each slice using a metal punch, measured, glued to balsa wood, and subjected to mechanical testing as described above. The use of cresyl violet acetate to dye the hydrogel did not significantly affect mechanical properties.

A Bioscope System AFM (Model 3A; Veeco, Santa Barbara, CA) mounted on an Axiovert 100 TV inverted optical microscope (Carl Zeiss, Jena, Germany) was used to obtain indentation force-depth curves for hydrogels patterned with rectangles. The bioscope system contains a Nanoscope IIIa controller and Nanoscope III 5.12 software. A glass probe holder and silicon sleeve allowed for testing in liquid. Hydrogels were mounted to a 60 mm Petri dish filled with 1.8 mL of deionized phosphate-buffered saline (PBS). Prior to hydrogel testing, cantilever deflection sensitivity was calibrated on a bare Petri dish bottom immersed in deionized PBS. A video camera (Model TM 34KC; Pulnix, Yokohama, Japan) was used to display real-time images during testing. AFM probes consisting of silicon–nitride cantilevers (spring constant 0.06 N/m) fused with a 5 μm diameter spherical glass bead (Novascan Technologies, Ames, IA) were allowed to repeatedly indent and retract, with the total displacement between 0 and 800 nm, starting 0.5 V from the surface of the hydrogels in force mode at 0.5 Hz. Each hydrogel was tested at multiple locations with about 150 force curves acquired per location and 512 data points per curve.

Analysis of AFM Data

Force curves were analyzed as in Trache et al. (2005), with some modifications. Cantilever deflection d (V) was determined from the laser position on the quadrant photodiode and was monitored as a function of piezo movement using:

$$\delta = z - d_s d$$

where δ is the hydrogel deformation or indention depth (nm), z the piezo displacement (nm), and d_s the deflection sensitivity (nm/V). The force derived from the cantilever deflection was calculated using:

$$f = k\, d_s d$$

where f is the applied force (nN) and k the cantilever spring constant (nN/nm). To calculate the apparent Young's modulus (E), we employed Sneddon's modification to the Hertz model for a spherical indentation of a flat, homogeneous, infinitesimal strains, and semi-infinite elastic material by a rigid probe (Sneddon, 1965).

$$f_{\text{sphere}} = k d_s d = 4/3(E/1 - \upsilon^2)\text{sqrt}(R)d^{3/2}$$

where υ is the Poisson ratio (assumed to be 0.5) and R the radius of the sphere. The simplifying assumptions may not readily apply for cells; however, given the small area of indentation by the spherical probe, relatively flat hydrogel surface (variation less than 750 nm as shown by height AFM images), linear force-indentation curve and greater material uniformity of hydrogels we choose to implement this model as a stiffness approximation method. The initial point of contact was first determined by fitting a bidomain polynomial algorithm to the raw AFM force curve as described in Costa (2006). We then used the slope to calculate the Young's modulus for each curve. Distributions for the measured values were averaged at each location.

Statistical Analysis

Results are reported as the mean ± standard deviation unless otherwise noted. The statistical significance of differences in mechanical properties between patterned hydrogels was determined using a two-tailed Student's t-test when comparing two groups or ANOVA with Tukey post hoc analysis when comparisons of multiple groups were required. Linear regression analysis and Pearson product moment correlation were performed on gradient hydrogel mechanical data. Differences were considered significant for $P < 0.05$. Statistical analysis was performed using Sigma Stat with Systat Software (San Jose, CA) and Microsoft Excel.

Results

Determination of Steady State

Mechanical testing of hydrogels removed from soak solution and crosslinked at various time points revealed that a mechanical steady state was reached after 4 h and lasted at least out to 96 h, the last time point collected (Fig. 3). Of note, samples that were soaked under room air rather than argon exhibited significant photoinitiator quenching and loss of mechanical properties at similar time points. Confocal microscopy using fluorescently labeled dextran as a model for PEGDA in the soak solution confirmed a uniform distribution of the soak solution within base hydrogels by 4 h.

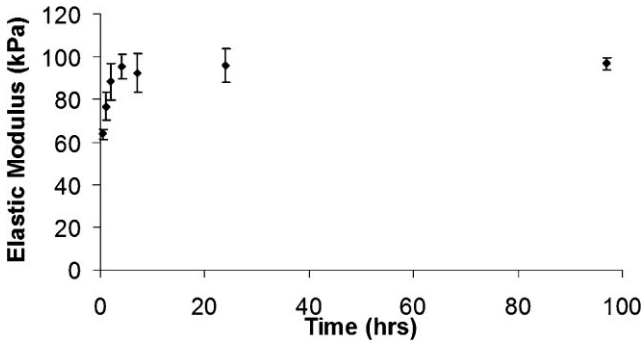

Figure 3. Determination of soak time. Variation in elastic modulus with soak time reached a steady state after 4 h. This steady state was maintained out to 96 h, the last time point collected. $N = 4$–5 at each time point.

Visualization of Patterning

To better visualize patterned hydrogels and to confirm that patterns made with 3.4 kDa PEGDA within a 20 kDa PEGDA hydrogel extended throughout the construct and were not limited to surface changes, hydrogels with elasticity patterned in stripes were soaked in solutions containing fluorescently labeled dextran with molecular weight ranging from 10 to 500 kDa. This strategy takes advantage of the fact that PEGDA hydrogel mesh size is closely related to the molecular weight of the polymer, with larger polymer molecular weight corresponding with larger mesh size and smaller polymer molecular weight corresponding to smaller. By visualizing the diffusion of dextran into the hydrogel, it is possible to see transitions in hydrogel mesh size. It was expected that only dextran of intermediate size would allow pattern visualization; above this size dextran would be excluded from all regions of the hydrogel, while below this size dextran would diffuse freely throughout the patterned and unpatterned regions.

Figure 4A shows an overlay of phase contrast and fluorescent images of a hydrogel patterned in stripes after soaking in a solution containing 70 kDa fluorescently labeled dextran. This pattern was not visible in hydrogels that had been soaked in 500, 40, or 10 kDa dextran. As expected, dextran is present only within the larger mesh size, softer areas of the hydrogel. This hydrogel was patterned with the transparency placed with the printed side in contact with the gel surface, which accounts for the surface patterning seen. Figure 4B shows a phase-contrast and fluorescent overlay of a hydrogel patterned with stiff rectangles against a soft background. Here, the fluorescent image shows location of acrylated fluorescein within stiff areas. Confocal imaging confirms that patterning continues throughout the thickness of the hydrogel (data not shown).

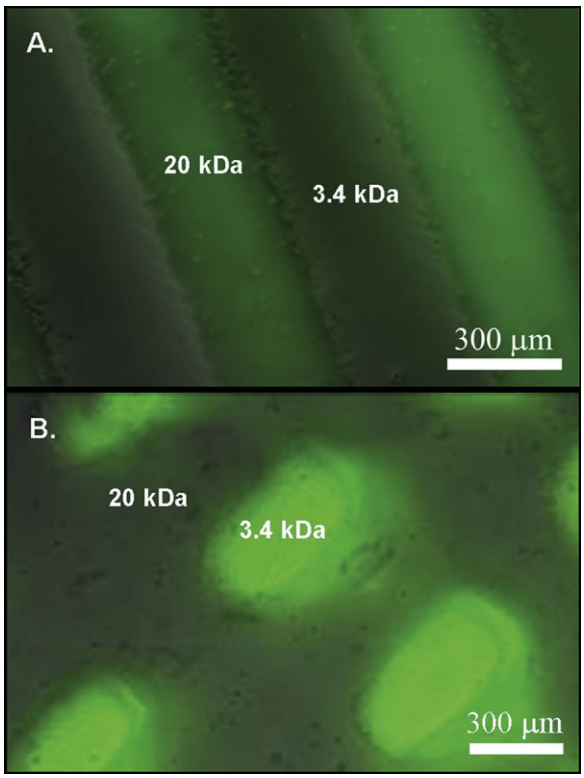

Figure 4. Visualization of patterning. Overlays of phase contrast and fluorescent images of hydrogels made using protocol shown in Figure 1. Interpenetrating patterns within hydrogels could be visualized by soaking patterned gel in a solution of 70 kDa dextran-fluorescein, which diffuses freely into the larger mesh size, 20 kDa stripes but is excluded from areas patterned with 3.4 kDa PEGDA (**A**). Alternatively, acrylated fluorescein can be included within the soak solution prior to patterning, then photocrosslinked into the polymer network only in areas of UV exposure (**B**).

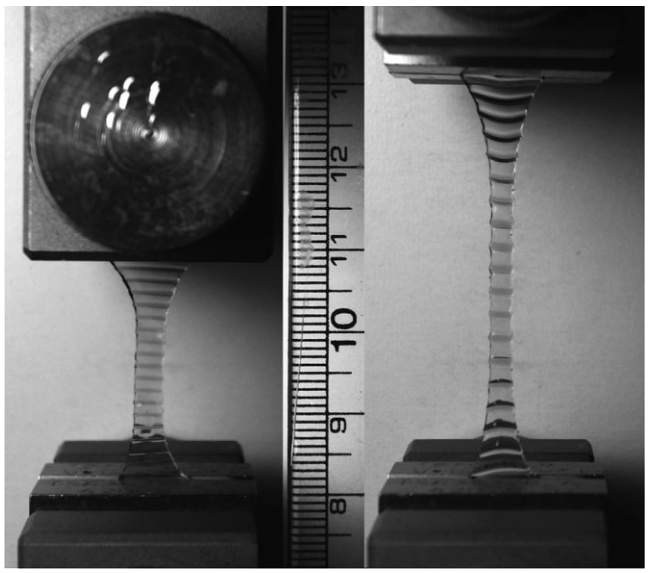

Figure 5. Differential strain within patterned hydrogel. Striped hydrogel subjected to tensile testing shows preferential strain in softer (clear) stripes, while stiffer (dark) stripes show minimal stretching.

Characterization of Hydrogels With Distinct Patterns of Elasticity

The bulk mechanical properties of hydrogels with elasticity patterned in stripes were determined as described in the Materials and Methods Section. By mechanically testing hydrogels both parallel and perpendicular to the stripe axis, it was possible to determine whether this type of patterning created anisotropy in the bulk material. We hypothesized that the elastic modulus parallel to the stripes, where strain would be limited by the less elastic 3.4 kDa stripes, would be higher than that perpendicular, where the more elastic 20 kDa stripes could freely stretch. When striped hydrogels dyed with cresyl violet acetate were placed under tension perpendicular to the stripe axis, there was a clear difference in strain between stiff and soft stripes (Fig. 5). This was not seen when tension was applied parallel to the stripe axes. Quantification of individual stripe elongation during mechanical testing revealed significantly more strain in soft stripes than in stiff stripes at high stresses ($P < 0.0002$).

As shown in Figure 6, there was a significant difference in directional modulus for 20% and 33% patterned hydrogels ($P < 0.02$). Differences in directional modulus did not achieve significance for 50% patterned hydrogels. This is likely due to the large deviation observed in the 50% patterned gels tested perpendicular to the pattern. The elastic modulus of patterned hydrogels showed a strong dependence on the spacing of patterned stripes and thus the percentage of the base hydrogel volume that was patterned

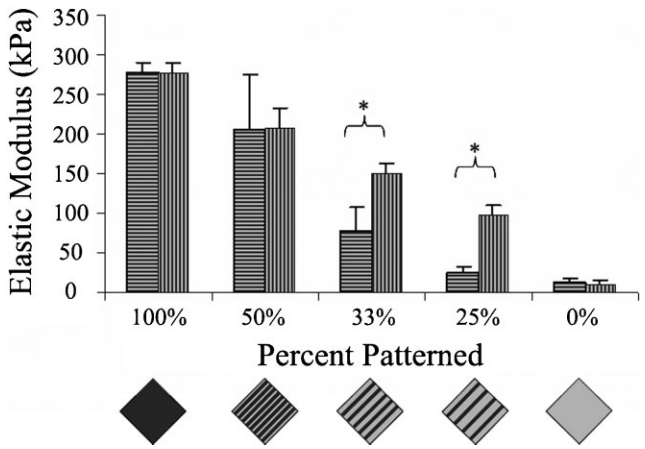

Figure 6. Striped hydrogels exhibit anisotropy. Hydrogels with rigidity patterned in stripes underwent tensile testing in directions parallel (vertical lines) and perpendicular (horizontal lines) to stripe axes. In hydrogels patterned with two soft stripes to every stiff stripe (33% patterned) or three soft stripes to every stiff stripe (25% patterned), there was a significant difference in effective elastic modulus with testing direction. This difference was not seen in entirely stiff hydrogels (100% patterned) nor in entirely soft hydrogels (0% patterned), as expected. $N = 3$–4 for each group. $^{*}P < 0.02$.

with 3.4 kDa PEGDA. All groups within each testing direction were statistically different from one another with $P < 0.01$.

Compressive testing of hydrogels using AFM confirmed a difference in modulus between patterned areas, with a modulus of 167.1 ± 20.6 kPa for crosslinked rectangles versus 92.4 ± 9.9 kPa for the softer areas between rectangles ($P < 0.02$).

Characterization of Hydrogels With Gradient Elasticity

Hydrogels with gradient elasticity were made as described in the Materials and Methods Section, sliced, and subjected to bulk tensile testing to evaluate their mechanical properties. Because we wished to create a gradient of elasticity on one axis only, the degree of variability within each slice of the gradient gel was of interest. To evaluate this, each slice from a single gradient hydrogel was divided into four specimens and subjected to tensile testing. The testing confirmed the formation of a gradient of elasticity (Fig. 7). Pearson's product moment correlation indicated a significant, positive correlation with a P-value of 1.093×10^{-81} and a correlation coefficient of 0.979.

Next, the degree of variability between multiple hydrogels made using the same protocol was assessed (Fig. 7). Again, Pearson's product moment correlation again indicated a significant, positive correlation with a P-value of 3.129×10^{-31} and a correlation coefficient of 0.974. Gradient hydrogels exhibited patterned swelling due to the gradient of mesh size formed during the crosslinking process. This is shown in Figure 8.

Patterned Hydrogels Reveal Influence of Rigidity on Cellular Behavior

In order to demonstrate the utility of PEGDA hydrogels with patterned rigidity for studying cellular behavior, we seeded

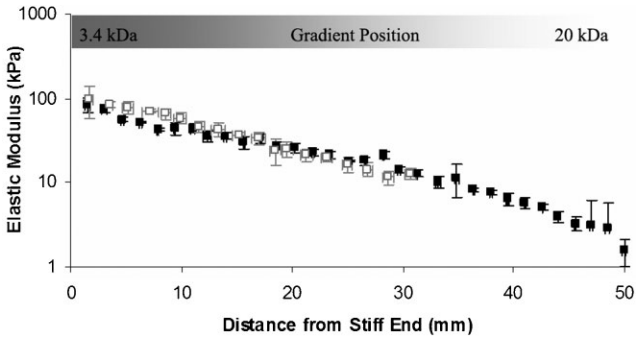

Figure 7. Variation in gradient hydrogel elastic modulus: filled squares demonstrate variation in elastic modulus within a single gradient hydrogel. Gel was divided into ~1 mm sections along the length of the hydrogel, then each section was further subdivided into four segments for mechanical testing. Empty squares show variation in elastic modulus between gradient hydrogels ($N = 4$). X-axis indicates distance from the stiff end of the gradient hydrogel for each slice tested. Pearson's product moment correlation: $P < 1 \times 10$–30.

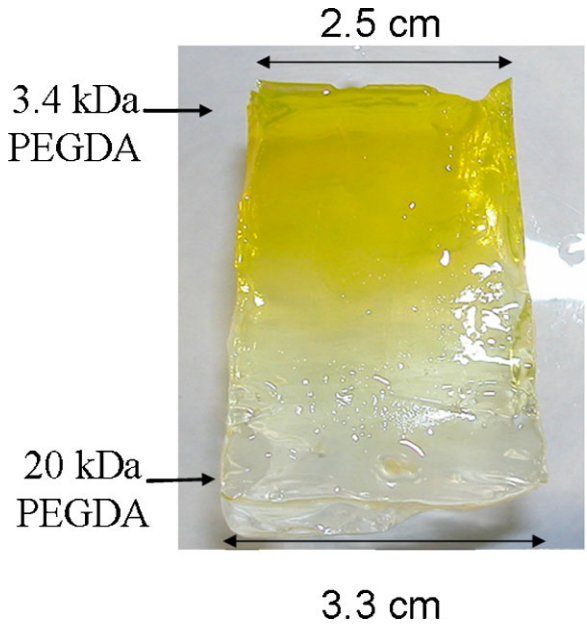

Figure 8. Gradient hydrogel exhibits patterned swelling. Fluorescein used to visualize gradient.

hydrogels with RAW 264.7 macrophages. After 48 h, macrophages are preferentially located on stiffer areas of the hydrogel (Fig. 9).

Discussion

Using PEGDA hydrogels of varying polymer chain length with controlled mixing and photolithographic patterning techniques, we are able to produce substrates with highly tunable spatial patterning of mechanical properties. Due to their excellent biocompatibility, these substrates can be used to study cell response to substrate rigidity in both two and three dimensions, offering a distinct advantage over mechanically patterned substrates developed to date. Hydrogels with gradient elasticity allow the rapid screening of the effect of an entire range of moduli on cellular behavior and may reveal changes in cellular behavior that might be missed when substrates with discrete elastic moduli are used. Hydrogels with distinct patterns of elasticity may allow the spatial patterning of multiple cell types or of cell behavior within a single cell type. We have also shown the spatial patterning of strain during mechanical loading within a single hydrogel, offering a unique opportunity to study cellular response to differential strain during static or cyclic loading. These hydrogels also offer an opportunity to view cell response to two or more rigidities or strains within a single microscopic field-of-view, minimizing sample-to-sample variation that may occur when comparing cells on separate substrates. As a simple demonstration of the utility of these substrates for the study of cellular behavior in

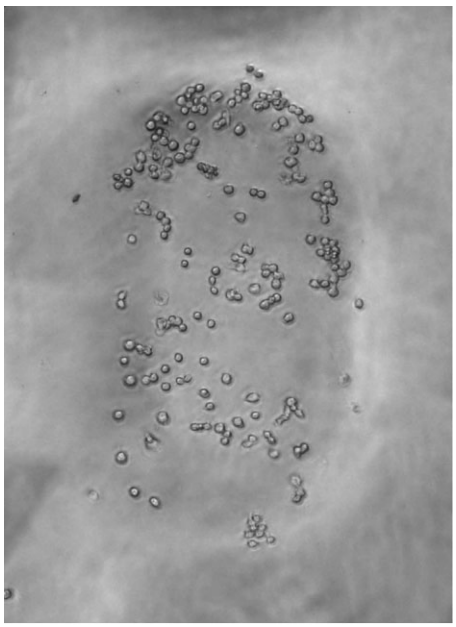

Figure 9. Hydrogels with patterned rigidity can influence cellular behavior. After 48 h, RAW 264.7 macrophages are preferentially attached to stiffer areas of the hydrogel.

response to substrate mechanical properties, we showed macrophage clustering on stiffer gel areas after 48 h of culture. In the future, we plan to more thoroughly characterize this behavior as well as the behavior of other cell types cultured on these substrates.

In addition to their applications in the study of cell response to substrate rigidity, these hydrogels also offer the potential for use as a tissue-engineering scaffold, where spatial patterning of stiffness might be desired for compliance matching with native tissues. Gradient hydrogels, for example, might be used in applications such as tendons, where stiffness changes dramatically between the muscular and bony insertions. Hydrogels with rigidity patterned in discrete areas may be used as a tool in the engineering of organs with complex organization of multiple cell types. Patterned hydrogels that exhibit anisotropy may be used in applications such as heart valves, where native tissues are anisotropic (Stella and Sacks, 2007).

Conclusion

The study of cell response to substrate rigidity is a young field, with almost unlimited potential for growth. Substrates with controlled rigidity, such as those described here, may serve as powerful tools for the study of substrate rigidity in development, health, and disease. They also form attractive substrates for tissue-engineering applications. The possibility of guiding differentiation using substrate rigidity is particularly exciting, as it offers a new strategy for the engineering of complex, three-dimensional tissues.

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