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Authors

Ondeck, Matthew G

Engler, Adam J

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Mechanical Characterization of a Dynamic and Tunable Methacrylated Hyaluronic Acid Hydrogel

Matthew G. Ondeck
Material Science Program,
UC San Diego,
La Jolla, CA 92093

Adam J. Engler[1]
Material Science Program,
UC San Diego,
La Jolla, CA 92093;
Department of Bioengineering,
UC San Diego,
La Jolla, CA 92093;
Sanford Consortium for Regenerative Medicine,
La Jolla, CA 92037
e-mail: aengler@ucsd.edu

Hyaluronic acid (HA) is a commonly used natural polymer for cell scaffolding. Modification by methacrylate allows it to be polymerized by free radicals via addition of an initiator, e.g., light-sensitive Irgacure, to form a methacrylated hyaluronic acid (MeHA) hydrogel. Light-activated crosslinking can be used to control the degree of polymerization, and sequential polymerization steps allow cells plated onto or in the hydrogel to initially feel a soft and then a stiff matrix. Here, the elastic modulus of MeHA hydrogels was systematically analyzed by atomic force microscopy (AFM) for a number of variables including duration of UV exposure, monomer concentration, and methacrylate functionalization. To determine how cells would respond to a specific two-step polymerization, NIH 3T3 fibroblasts were cultured on the stiffening MeHA hydrogels and found to reorganize their cytoskeleton and spread area upon hydrogel stiffening, consistent with cells originally cultured on substrates of the final elastic modulus. [DOI: 10.1115/1.4032429]

Keywords: stiffness, crosslinking, Irgacure, fibroblast

Introduction

An environment that mimics intrinsic properties of the extracellular matrix (ECM) is becoming increasingly recognized as an essential cell culture element, facilitating appropriate adhesion and proliferation in vitro. This is due in part to observations that ECM mechanics, specifically elastic modulus or "stiffness" (i.e., a term commonly used in biology, which is meant to be synonymous with modulus), can drive a variety of cell processes, including stem cell differentiation [1,2], cell migration [3,4], and cancer cell activation [5–7]. The ECM is very dynamic and constantly being remodeled by matrix metalloproteinases [8], which cleave structural ECM proteins and then rebuilt by new ECM proteins that are synthesized and assembled by cells [9]. This process normally occurs during development [10], but in adults when this equilibrium is disrupted, pathological changes can result. For example, tumor progression is characterized by fibrosis, which leads to a stiffer ECM and disrupts the acinar morphology of cell structure in breast cancer [5]. Metalloproteinases are also upregulated in certain cancer types to facilitate cancer metastasis through a more pliable ECM [11]. Similarly, after a myocardial infarction, significant ECM protein deposition causes fibrosis and the formation of a scar, which limits myocardial function [12,13].

Many scaffolds have been employed to mimic ECM properties and present a more biomimetic substrate to cultured cells; several classic scaffold types modulate stiffness to create materials that are always soft or stiff, e.g., polyacrylamide [14]. However, there is growing recognition that dynamic materials are needed to mirror how tissues can change modulus or "stiffen" over time as described above, e.g., development, disease, etc. In response, many groups have created dynamic systems over the past several years including hydrogels that stiffen by diffusion-based Michael-type addition reactions [15], heat-induced crosslinking [16], and *p*H-dependent iron complexation [17] or hydrogen bonding [18]. UV-based, free radical-mediated crosslinking [19] offers precise control of when and where stiffening occurs versus methods that require the entire hydrogel to stiffen [17,18] or that crosslink too gradually [15]. However, it is not clear what dynamic stiffness range these hydrogels have and how closely they would match developmental or pathological changes. Many of these materials use large nonsulfated glycosaminoglycans as the polymer backbone to fabricate the hydrogel. This is due in part to the presence of several functional groups, which enables easy chemical modifications, support of cell adhesion, and relatively stiff structure. For example, HA supports cell adhesion via the surface receptor CD44 [20,21], it can be easily thiolated [15] or methacrylated [19,22] and can generate polymer networks with biologically relevant mechanics [1,4,5].

The precise control of photopolymerization enables the most effective measurement of stiffness and may yield the most control. Guvendiren and Burdick have previously synthesized a MeHA hydrogel that can be partially polymerized using a short crosslinker, dithiothreitol (DTT), subsequently soaked in a photoinitiator, Irgacure 2959, and exposed to 350 nm UV to completely crosslink the material [19]. Rather than use this dual-step polymerization technique to achieve a step increase in matrix stiffness, here we employ photopolymerization for sequential polymerization as it only occurs in the presence of UV versus DTT reactions. Moreover, UV polymerization can be switched on and off and therefore tune stiffness to fit a multitude of applications. Thus, with our characterization here, we believe that this system can be useful for a variety of different cell culture applications, especially ones that require a time-dependent dynamic material system.

Materials and Methods

MeHA Synthesis. All materials were obtained from Sigma (St. Louis, MO) unless otherwise indicated. MeHA was synthesized using a 50 kDa sodium hyaluronate precursor obtained from LifeCore Biomedical. The methacrylation protocol used was previously described in Guvendiren and Burdick [19]. Briefly, sodium hyaluronate was dissolved in de-ionized water at 1 w/v% overnight and was reacted with 10 or 20 M methacrylate anhydride for 12 hrs. The monomer solution was dialyzed against de-ionized water at 4 °C for 3 days and then was lyophilized for an additional

[1]Corresponding author.

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3 days. The lyophilized MeHA was analyzed by ^{1}H nuclear magnetic resonance (NMR) to determine methacrylate substitution ratio by normalization of the peaks at a chemical shift of 6 ppm, representing the methacrylate group, by peaks between 3 and 4 ppm, which represent native HA. Substitution ratios were computed from triplicate NMR analyses.

MeHA Hydrogel Synthesis. MeHA was weighed and dissolved at 1% and 3% in a 0.2 M Triethanolamine in phosphate-buffered saline (PBS; pH = 10). Irgacure 2959 was initially dissolved at 10% in 100% ethanol and then diluted to 0.05% in the MeHA solution, then photopolymerized using a transilluminator (4 mW/cm^2; UVP, Upland, CA) emitting 350 nm wavelength UV light. Twelve microliter of MeHA hydrogel solution was photopolymerized between two 12 mm diameter coverslips where one was gluteraldehyde-activated to permit hydrogel binding and the other was nonadherent dichlorodimethylsilane-activated to achieve easy detachment and create a free surface. This created ~100 μm thick hydrogels that could stay submerged and hydrated. Only for cell experiments, protein for cell attachment was added by mixing 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 50 mM N-hydroxysuccinimide, and 150 μg/ml type I rat tail collagen dissolved in PBS (Corning). The collagen-crosslinker solution was added to the hydrogel and incubated overnight at 37 °C.

To stiffen MeHA hydrogels as previously described in Guvendiren and Burdick [19], Irgacure 2959 was added at 0.05% v/v to hydrogel solutions in PBS (without cells) or added at 0.05% v/v to 1 ml of cell media (with cells). Following 30 min incubation, hydrogels were exposed to 350 nm wavelength UV light for an indicated amount of time and then was washed three times with sterile PBS or cell culture media to remove unreacted Irgacure 2959. For initial exposures, hydrogel solutions in PBS between coverslips were exposed to UV for the indicated amount of time to polymerize them. For sequential exposure, 1 ml of the 0.05% Irgacure solution was added per coverslip, incubated at 37 °C for 30 min, and then exposed for the indicated amount of time. Thus "1 + 1" conditions indicate 1 min exposure for the initial and sequential steps. To ensure that UV exposure was not detrimental to cells plated after initial crosslinking, polymerization in subsequent steps will be limited in duration, which has previously shown that it did not cause DNA damage [19,23].

Atomic Force Microscopy. An MFP-3D-Bio atomic force microscope (Asylum Research; Santa Barbara, CA) was used to determine hydrogel mechanical properties. Chromium/gold-coated, silicone nitride cantilevers (NanoWorld) with unsharpened pyramid-shaped tips having a half-opening angle of 35 deg and tip radius of 20 nm were used. Nominal spring constants for cantilevers were at least 40 ± 20 pN nm^{-1} and were calibrated using a thermal noise method [24]. Samples were immersed in PBS prior to indentation, and were then indented at a velocity of 2 μm s^{-1} until a trigger force of 2 nN was detected. Tip deflections were converted to indentation force for all samples using their respective tip spring constants and Hooke's Law. Indentation depth was computed from the difference between tip deflection into the hydrogel and glass. All AFM data were analyzed using custom-written code in Igor Pro to determine Young's Modulus as previously described [25] based on a Hertz model [26]. Data were fit from the maximal indentation back toward the contact point, and all the data were found to have monophasic behavior up to the maximal indentation, which scales with modulus. Measurements were made at least at five random locations across each hydrogel for multiple hydrogels per formulation and made after MeHA hydrogels were washed three times with PBS.

Cell Culture. To prepare for cell culture, MeHA hydrogels were washed three times with sterile PBS and sterilized with UV light for 30 min. Once sterile, the MeHA hydrogels were washed three more times with sterile PBS. NIH 3T3 fibroblast cells were cultured on the surface of the MeHA hydrogel at a cell density of 2×10^3 cells/cm^2 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and antibiotics. The density was chosen that ensures that cells are predominantly isolated from their nearest neighbors.

Immunofluorescent Staining. NIH 3T3 cells attached to MeHA hydrogels were fixed with 3.7% formaldehyde prior to and after MeHA hydrogels stiffening at days 1 and 3, respectively, then permeabilized using 1% Triton X for 15 min. Samples were rinsed and stained with 1:1000 rhodamine-phalloidin in a 2% ovalbumin blocking buffer for 60 min at 37 °C and 1:5000 DAPI to stain cell nuclei for 10 min at room temperature. Samples were rinsed and mounted with Fluoromount-G (Southern Biotech). Samples were imaged on a Nikon TiU inverted optical microscope using a 60× water objective. Images were captured on a CoolSnap HQ camera controlled by METAMORPH 7.6 software. IMAGEJ software (NIH) was used to analyze images and quantify cell area.

Statistical Analyses. Data comparisons were subjected to one-way analysis of variance (ANOVA) with post hoc Tukey correction, nonparametric Student's t-test with unequal variance assumption, or as indicated in respective captions. Significance was assigned for $P < 0.05$ though data for $P < 0.1$ are noted. Pooled data are represented as means ± standard deviation unless otherwise indicated. All the experiments were performed in triplicate with pooled data points unless otherwise indicated.

Results

Modulating MeHA Stiffness Using Polymer Concentration, UV Exposure, and Degree of Substitution. MeHA was synthesized by reaction of HA salt with methacrylic anhydride and examined by NMR, which confirmed 65% methacrylate substitution (Fig. 1(*a*)). When 1% and 3% w/v MeHA hydrogels were polymerized with increasing UV exposure, we found that matrix stiffness increased with both bulk polymer concentration and UV exposure time (Fig. 1(*b*) and Supplemental Table 1 is available under the "Supplemental Data" tab for this paper on the ASME Digital Collection). However, 3% MeHA hydrogels exponentially increased in stiffness with respect to UV exposure time while the 1% hydrogels increased in stiffness more linearly, likely because the lack of methacrylate in 1% MeHA limited the polymerization rate for the amount of free radical generation, which we chose to hold constant. Polymer concentration differences, especially at higher concentrations, could create stiffer or softer domains, so spatial variation of modulus was assessed, while hydrogels were largely homogeneous for 1% MeHA hydrogels polymerized by 1 min UV exposure, we found elevated spatial differences in 3% MeHA hydrogels (Fig. 1(*c*)), which when assessed by surface root mean squared changes are fourfold higher than 1% MeHA hydrogels.

UV exposure allows one to switch polymerization on and off, but it is not clear if that polymerization is additive during sequential polymerizations; if it is independent of prior crosslinking state, intermittent versus continuous crosslinking, i.e., 1 min + 1 min versus 2 min, should yield equivalent matrix stiffness. Surprisingly, 1% MeHA hydrogels from continuous UV exposure compared to sequential bouts of UV exposure did not show additive behavior (Fig. 2(*a*) and Supplemental Table 2 is available under the "Supplemental Data" tab for this paper on the ASME Digital Collection); sequential polymerization, e.g., 1 + 1 min and 2 + 2 min, was significantly stiffer than hydrogels stiffened for 2 or 4 min, respectively. However, 3% MeHA hydrogels behaved in a more additive fashion with the stiffness of both the 1 + 1 and 2 + 2 min approximately equaling the stiffness of the 2 min and 4 min hydrogels, respectively (Fig. 2(*b*) and Supplemental Table 2 is available under the "Supplemental Data" tab for this paper on the ASME Digital Collection). Stiffness variation between hydrogels increased with additional UV exposure time but that could be due to variation in Irgacure amount but not

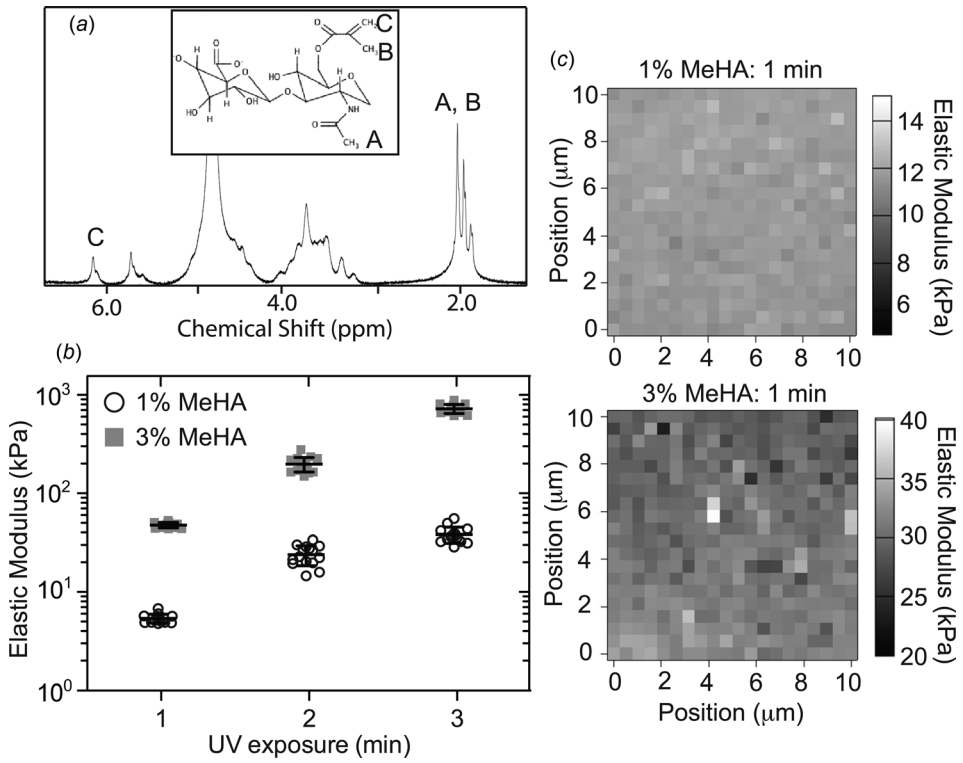

Fig. 1 Functionalization and characterization of MeHA hydrogels. (*a*) NMR spectrum of 50 kDa HA with methacrylate functionalization (~65% methacrylate functionalized). (*b*) Elastic modulus of 1% and 3% w/v of MeHA polymerized for 1, 2, and 3 min with 350 nm UV light. All samples are statistically different from one another based on one-way ANOVA with $p<10^{-4}$. (*c*) A 10 μm × 10 μm elastic modulus map for 1% and 3% MeHA gels UV polymerized for 1 min.

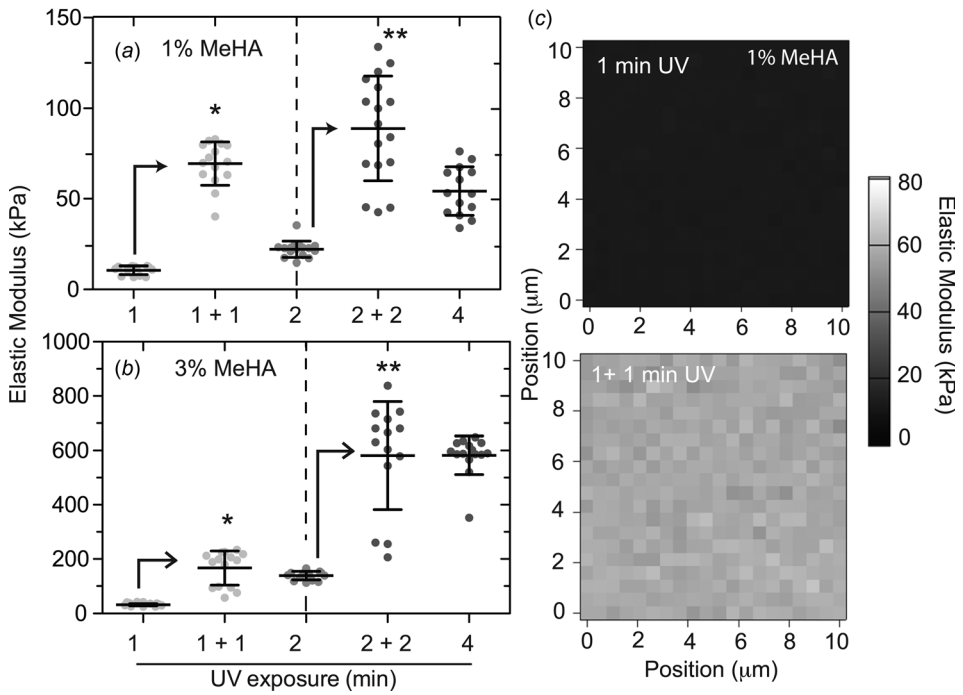

Fig. 2 Comparison of on-demand versus continuous stiffening. (*a*) Elastic modulus measured for 1% MeHA gels polymerized for 1, 2, and 4 min and gels polymerized for 1 and 2 min, stiffened additionally with 1 and 2 min of UV light exposure, respectively. Using nonparametric t-tests: *$p<10^{-12}$ and **$p<10^{-8}$. (*b*) Elastic modulus measured for 3% MeHA gels polymerized for 1, 2, and 4 min and gels polymerized for 1 and 2 min, stiffened additionally with 1 and 2 min of UV light exposure, respectively. Using nonparametric t-tests: *$p<10^{-7}$ and **$p<10^{-6}$. (*c*) A 10 μm × 10 μm elastic modulus map for 1% MeHA gel UV polymerized for 1 min then stiffened with an additional 1 min of UV light.

spatial variation; stiffness maps appeared spatially homogenously for both 1 min and 1 + 1 min hydrogels (Fig. 2(*c*)). These data suggest that dynamics created from UV is highly dependent on polymer concentration and are likely more coupled than hypothesized.

Changing bulk concentration changes the amount of methacrylate present for crosslinking. To independently change the degree of methacrylation without bulk HA changes, MeHA was synthesized with different amounts of methacrylate functionalization, i.e., ~38% and ~65%, and as confirmed by NMR (Fig. 3(*a*)). Stiffness for 3% MeHA hydrogels increased with 38% and 65% functionalization as well as UV exposure time (Fig. 3(*b*) and Supplemental Table 3 is available under the "Supplemental Data" tab for this paper on the ASME Digital Collection). The disparity between the moduli reveals the significance of the additional functionalization on the mechanics of the polymerized hydrogel.

Fibroblasts Respond to Dynamic MeHA Stiffness Via Spreading Changes. Dynamic MeHA hydrogel system can be used as an in vitro cell culture model that will allow cells to perceive both the soft and stiff matrix stiffness [19]. To demonstrate this over the specific ranges of parameters mentioned above, NIH 3T3 fibroblasts were cultured on the surface of 1% and 3% MeHA hydrogels that were polymerized for 1 and 2 min by UV exposure and that were covalently functionalized with collagen postpolymerization to support integrin-mediated adhesion. After cells were allowed to interact with the softer matrix for 1 day, MeHA hydrogels polymerized for 1 min initially were subsequently stiffened with an additional 1 min of UV exposure (1 + 1 min), resulting in a stiffness increase of 10–70 kPa and 30–170 kPa for 1% and 3% MeHA matrices, respectively (see Figs. 2(*a*) and 2(*b*)). At day 3 (2 days following matrix stiffening), fibroblasts cultured on the 1% 1 + 1 min gels exhibit disorganized actin fibers after matrix stiffening, indicative of cytoskeletal reorganization (Fig. 4(*a*)). Conversely, actin stress fibers were apparent in the 1 + 1 min 3% MeHA matrix condition as compared to the cells cultured on the 1% MeHA gels (Fig. 4(*a*), arrowheads). Cell area was analyzed as a metric to measure cytoskeletal remodeling. Fibroblasts cultured on the 1 + 1 min 1% MeHA gels exhibited an

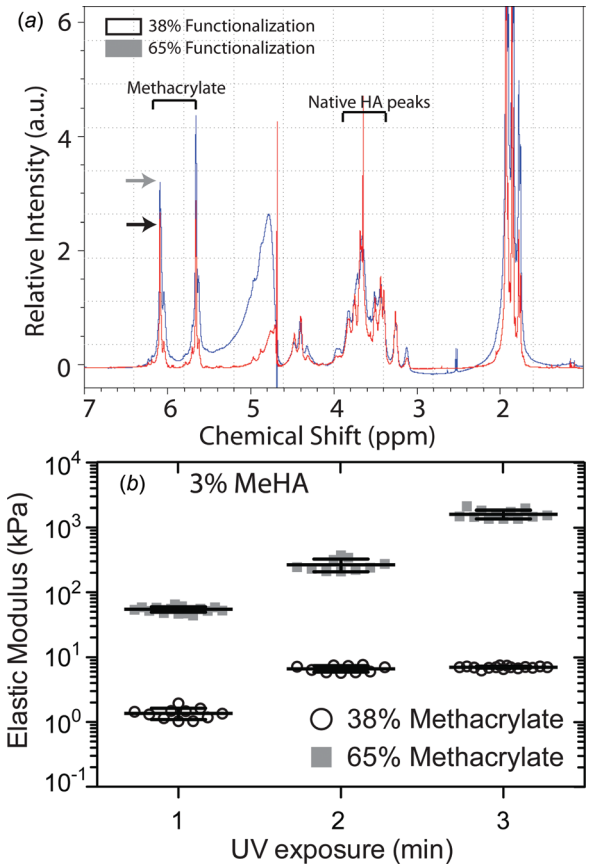

Fig. 3 Impact of degree of methacrylation on stiffness. (*a*) NMR spectrum of MeHA with 38% (black arrow) and 65% functionalization (gray arrow), with peaks representing the methacrylate group and HA indicated. (*b*) Elastic modulus of 38% and 65% methacrylate functionalized 3% MeHA polymerized for 1, 2, and 3 min with 350 nm UV light. One-way ANOVA indicated that conditions were statistically different with $p < 10^{-4}$ for UV exposure time within each methacrylation percentage, although post hoc Tukey analysis did not find a difference between 2 and 3 min exposure time for 1% MeHA.

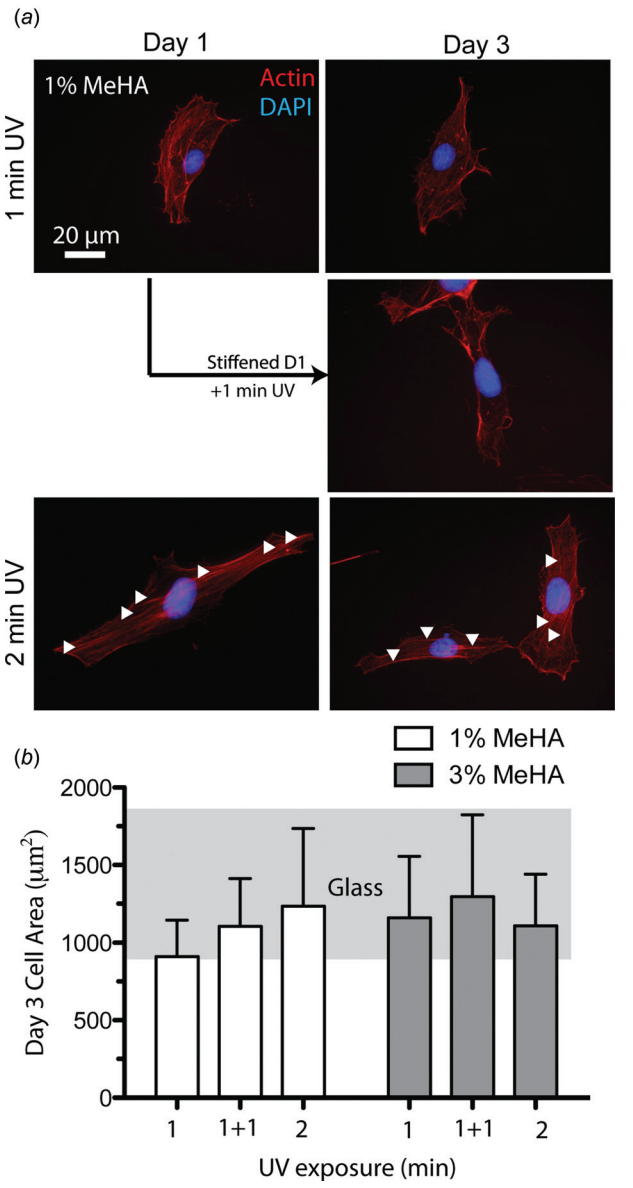

Fig. 4 Dynamic stiffening affects cell spreading. (*a*) NIH 3T3 fibroblasts were cultured separately on 1% MeHA gels UV polymerized for 1 and 2 min, and dynamic MeHA gels polymerized for 1 min and then stiffened on for 1 additional minute. Cultures were stiffened on day 1 and fixed on day 3. Arrowheads indicate stress fibers. (*b*) Fibroblast cell area was measured at day 3 for cells cultured on 1% and 3% MeHA gels polymerized for 1 and 2 min and 1 + 1 min stiffened gels. The gray background is the range of cell areas for fibroblasts cultured on tissue culture glass as a comparison. One-way ANOVA indicated that only the 1% MeHA conditions were statistically different with $p < 0.1$ for UV exposure time.

increase in cell area as compared to cells cultured on 1 min gels but were smaller than those cultured on the 2 min MeHA gels (Fig. 4(*b*)). Surprisingly, the cell area varied very little between fibroblasts cultured on the 1 min, 2 min, and 1 + 1 min 3% MeHA gels (Fig. 4(*b*)). This could be attributed to the very high stiffness of 3% MeHA hydrogels, which is above pathological significance where cells are sensitive to stiffness [27].

Discussion

A growing consensus among mechanobiology literature is that dynamic materials are required to more carefully direct cell behavior [14]. Despite the existence of numerous crosslinking methods that facilitate dynamic crosslinking [15–19] and likely yield changes in stiffness. While some haven been shown to polymerize gradually [15], the dynamic range of many of these systems is not uncertain. For example, Guvendiren and Burdick have used rheology to illustrate the dynamic changes for MeHA hydrogels [19], but how the system is affected by both internal and external factors is not clear. Since relatively small stiffness changes regulate many ubiquitous cell behaviors [14,23,27], e.g., proliferation, migration, and differentiation, knowing how polymerization properties affect stiffness is important, especially because they can be nonlinear. We will discuss these implications both for how MeHA parameters affected stiffness and how dynamic versus static stiffness can regulate cell behavior, especially in the context of fibroblasts used here.

MeHA Gel Mechanics. We found that polymerized MeHA hydrogel stiffness depends on a number of factors, including but not limited to UV exposure time, monomer concentration, degree of methacrylate functionalization, and the amount of initiator available for polymerization; each factor influenced polymerization to a different degree. For example, in Figs. 2(*a*) and 2(*b*) with an equal amount of UV exposure time, the moduli and rate of polymerization of a MeHA hydrogel from solution were not necessarily equal to that of a sequentially polymerized MeHA hydrogel; the 1% MeHA polymerization exemplifies this where the 1 + 1 and 2 + 2 min sequentially polymerized hydrogel stiffness was higher than that of the single polymerized 2 and 4 min hydrogels, respectively. In contrast, when monomer concentration is high, e.g., 3% MeHA, 1 + 1 and 2 + 2 min hydrogel stiffness is more additive since it is relatively equal to singly polymerized 2 and 4 min hydrogels, respectively. While the reason for this is unclear, the data imply that the rate of polymerization is dependent largely on the monomer concentration. When monomer concentration is low (e.g., 1% MeHA), it appears that the rate of methacrylate crosslinking cannot keep up with rate of initiator activation in the single polymerized hydrogels (2 min and 4 min gels) before reaching a point of crosslinking saturation. With less monomer, the hydrogel reaches the saturation point more quickly and leads to a softer hydrogel. The sequentially stiffened 1% MeHA hydrogel may bypass this initial saturation point because the secondary stiffening process, which uses additional Irgacure, will crosslink the leftover uncrosslinked methacrylate groups, generating a longer chain polymer with a higher modulus. This is evident by the 2 min and 4 min 1% MeHA gels being softer than the 1 + 1 and 2 + 2 min gels, respectively. However, when monomer concentration is high (e.g., 3% MeHA), it would take more crosslinking or a longer UV exposure to reach crosslinking saturation; therefore, we see equally stiff gels between the 1 + 1 min and 2 min gels and the 2 + 2 min and 4 min 3% MeHA gels. If the 3% MeHA hydrogels were polymerized longer, i.e., closer to crosslink saturation, we would expect to see behavior similar to 1% MeHA hydrogels. These behaviors demonstrate the importance of these factors in controlling polymer crosslinking and in enabling tunability of this system.

Cell Response to MeHA Matrix Stiffening. Cells perceive physical cues from their environment through integrins, which convert these signals that the cell can respond to by changes in the morphology/area or other perceivable properties including lineage [28–30]. Significant changes have been observed in stem cell differentiation [19] and cell maturation [15] in 2D when materials are stiffened, e.g., cells can transdifferentiate [19] or become increasingly mature [15], respectively. However in 3D, dynamic stiffening serves to enhance cell confinement [16], a property of matrices in 3D which has been observed in covalently but not ionically crosslinked systems [31]. For the range of materials tested here, we noted that fibroblast area did increase between the first and second polymerization steps for 1% MeHA and that cell area for sequentially polymerized hydrogels was nearly identical to continuously polymerized hydrogels of the same amount of time. We did not observe the same behavior for 3% MeHA hydrogels (Fig. 4(*b*)), but that is likely because the materials were well above physiological stiffness [27] and not due to other parameters including the collagen coating, as this has not been found to influence mechanics elsewhere [32]. Moreover, cells cultured on the 1 + 1 min matrix also appeared to exhibit a more disorganized actin cytoskeleton than cells on either the 1 min or 2 min gels at day 3, which is indicative of cytoskeletal reorganization following matrix stiffening. Similar results have been shown in other material systems with different cell types including mesenchymal stem cells [19] and cardiomyocytes [33], where cell area has shown to increase upon matrix stiffening. However it is important to note that in all of these systems, cell behavior was examined at a later time point, e.g., 2 days, allowing the cells to have equilibrated with their new environment. While smooth muscle cells do not appear to respond to dynamic changes in other cues, e.g., topography [34], a new line of inquiry based on how stem or other cells respond to stiffening immediately after it occurs could observe phenomenon that could be different from those seen at steady state here. Thus, our data and these new applications suggest that this MeHA hydrogel system could be used for a variety of application due to its tunable, dynamic mechanics and its large range of moduli.

While we and others have focused on responses to elastic properties, whether in 2D or 3D [1–7,19,27], it is important to note the growing body of literature on cell responses to viscoelastic properties [35–38]. A more complete description of how cells respond to their environment should involve such viscous influences. While materials that are widely used for these types of investigations are typically more elastic than viscous, e.g., polyacrylamide [39], natural matrices are exceedingly complex with strain-dependent properties [40]. Thus, one must note that even dynamic materials should require viscous characterization and/or be engineered to contain viscous properties.

Conclusion

In this study, we adapted a protocol for a dynamic MeHA hydrogel system and further analyzed the mechanics of the system. We have demonstrated the tunability of this dynamic MeHA matrix and identified several factors that effect polymerization and matrix mechanics, including the importance of degree of methacrylate functionalization and the amount of initiator available for polymerization, and UV exposure time. We also used AFM spatial stiffness maps to show the homogeneous nature of these hydrogels. Fibroblasts cultured on these hydrogels show that cells can change their spread area in response to a change in matrix stiffness within a single culture. These data implicate that dynamic rather than just static stiffness can be an important means of modulating cell responses and that MeHA hydrogels have a range of stiffness suitable to modulate these cell changes.

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