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Hydrogels with Reversible Mechanics to Probe Dynamic Cell Microenvironments

Adrianne M. Rosales,*[a] Sebastián L. Vega,[b] Frank W. DelRio,[c] Jason A. Burdick,*[b] and Kristi S. Anseth*[a],[d]

Abstract: The relationship between ECM mechanics and cell behavior is dynamic, as cells remodel and respond to changes in their local environment. Most *in vitro* substrates are static and supraphysiologically stiff; thus, platforms with dynamic and reversible mechanical changes are needed. Here, we developed hyaluronic acid-based substrates capable of sequential photodegradation and photoinitiated crosslinking reactions to "soften" and then "stiffen" the hydrogels over a physiologically-relevant range of moduli. Reversible mechanical signaling to adhered cells was demonstrated with human mesenchymal stem cells. *In situ* hydrogel softening (from ~14 to 3.5 kPa) led to a decrease in cell area and nuclear localization of YAP/TAZ, and subsequent stiffening (from ~3.5 to 28 kPa) increased cell area and nuclear localization of YAP/TAZ. Each photoreaction was cytocompatible and tunable, rendering this platform amenable to studies of dynamic mechanics on cell behavior across many cell types and contexts.

The native extracellular matrix (ECM) is dynamic, especially during instances of remodeling during development, disease, or acute injury. During these processes, many changing factors impact cell behavior, including bulk matrix mechanics. For example, the cardiac ECM stiffens as soon as one week after myocardial infarction due to fibrotic scar formation, thereby impacting cellular differentiation potential.[1] As another example, mesenchymal stem cells (MSCs) retain information about past substrate mechanics[2]; prolonged culture on pathologically-stiff substrates predisposes cells to a pro-fibrotic phenotype even after the mechanical stimulus is removed.[2-3] Thus, understanding the cellular response to changing mechanical environments is important; however, *in vitro* cell culture substrates with *in situ*, bidirectional changes in mechanics remain elusive.

Although several strategies to reversibly control bulk matrix mechanics in the presence of adhered cells have been developed, there remain shortcomings. External triggers such as pH[4] and temperature[5] may not be biocompatible for certain cell types, whereas stimuli such as calcium ion concentration,[6] competitive

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binding partners,[7] or magnetic particle incorporation[8] contain components that may alter cell signaling or require a sustained stimulus. Another challenge is capturing a tunable and relevant range of elastic moduli over which to study cell behavior. As a complementary method to the aforementioned strategies and to address these issues, this work establishes a route to reversibly alter hydrogel mechanics with on-demand photochemical reactions (Figure 1).

Light is a desirable trigger due to exquisite spatiotemporal control, noninvasiveness to cells, and photokinetic tunability. Several recent studies have expanded photochemical tools to control properties of dynamic cellular microenvironments, including mechanics.[9] Although many of the previously reported photoresponsive substrates are unidirectional,[10] here, orthogonal wavelengths of light trigger substrate softening and subsequent stiffening. Importantly, the dynamic elastic modulus range of the presented hydrogels spans ~3-30 kPa, allowing for biomimetic conditions of multiple tissues and ECM processes. This range was accomplished via modular modification of hyaluronic acid (HA) polymers with crosslinking functionalities.

HA was modified with three moieties (Figures 1A, S2, and S3): *o*-nitrobenzyl acrylates (x=13 mol%), methacrylates (y=40 mol%), and a cell-adhesive peptide containing the fibronectin-derived RGD domain (z=1 mol%). Acrylates are more susceptible to nucleophilic attack than methacrylates under base-catalyzed Michael addition conditions due to increased electrophilicity of the β-carbon;[11] thus, the addition of a di-thiol (dithiothreitol DTT) led to an initial hydrogel that preferentially contained crosslinks with the photodegradable *o*-nitrobenzyl group (Figure 1B). These initial crosslinks can be degraded at a user-defined timepoint via controlled exposure of 365 nm light (Figure 1C). Compared to other softening methods such as hydrolytic degradation,[12] the rate of degradation and thus the timescale of softening can be decoupled from the chemistry of the crosslinker – light dose, intensity, or wavelength can be used to tune the *o*-nitrobenzyl photocleavage.[10a, 13] Substrate softening does not disturb the methacrylates, which can be subsequently polymerized at a user-defined timepoint upon introduction of a photoinitiator and 400-500 nm light to stiffen the substrate (Figure 1D). Importantly, this step does not require any additional crosslinkers because the methacrylates are already present on the polymeric backbones, which avoids inhomogeneous crosslinking that may occur with the diffusion of large molecules. Furthermore, this step is amenable to many photoinitiators, but it is particularly well-suited to those that absorb visible light, as the macromers exhibit significant absorption below 400 nm before and after photodegradation (Figure 1E).

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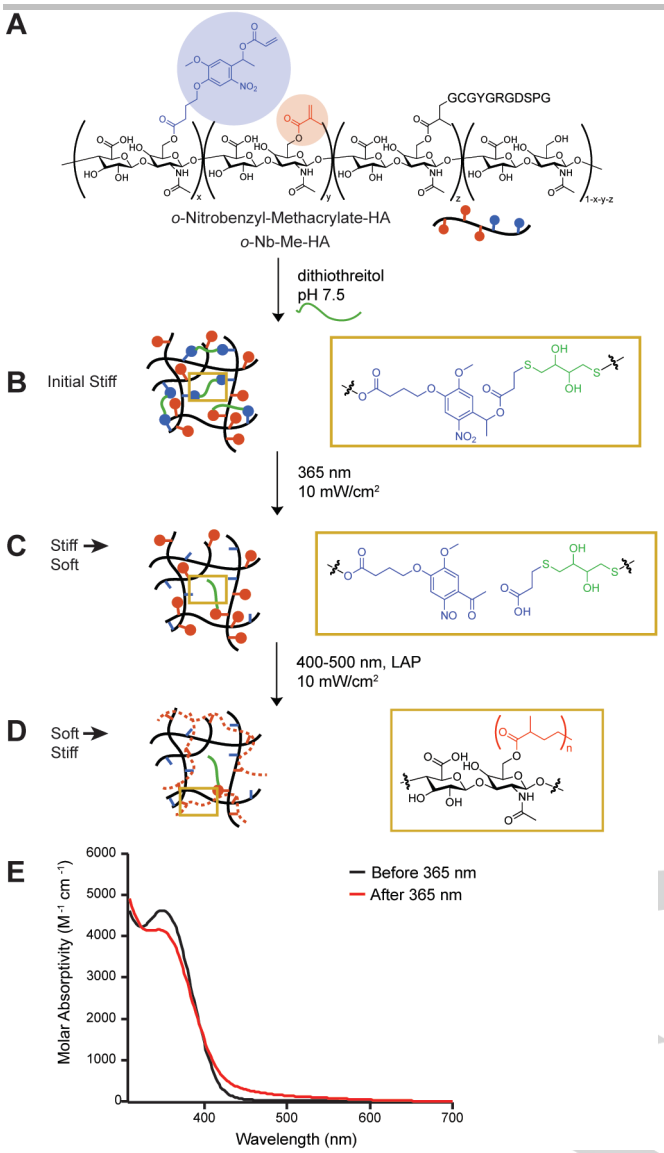

Figure 1. Dynamic hydrogel crosslinking with photochemical reactions. A) HA polymers were modified with *o*-nitrobenzyl-acrylates (13 mol%, blue) and methacrylates (40 mol%, red). B) Initial hydrogel formation proceeded via a Michael-type addition reaction at pH 7.5 using dithiothreitol (DTT, green) in a 1:1 ratio to the acrylates. C) Subsequent light exposure (365 nm) leads to *o*-nitrobenzyl photocleavage to soften the hydrogel. D) Hydrogel re-stiffening occurs via radical polymerization of the methacrylates using 400-500 nm light and a photoinitiator (LAP, 3.3 mM). E) Absorbance spectra of *o*-NB_{13}-Me_{40}HA indicates a maximum at 350 nm, which decreases with photodegradation.

Both shear rheology and atomic force microscopy (AFM) were performed to characterize the mechanical properties of the hydrogels. Shear rheology demonstrated a crossover of the storage modulus (G') and loss modulus (G") within 3 minutes (Figure 2A, top); by 50 minutes, G' was ~2 orders of magnitude larger than G". At approximately 3 hours, 90% of the final storage modulus was achieved, which for this formulation was 4 kPa, or a Young's modulus, *E*, of 12 kPa assuming a Poisson's ratio of 0.5.[14] Surface force measurements corroborated

this value, yielding an *E* of 14.8 ± 0.8 kPa (Figure 2B, top). By design, the cross-links are stable covalent thiol-ene linkages, so the hydrogels exhibit elastic, time-independent properties.

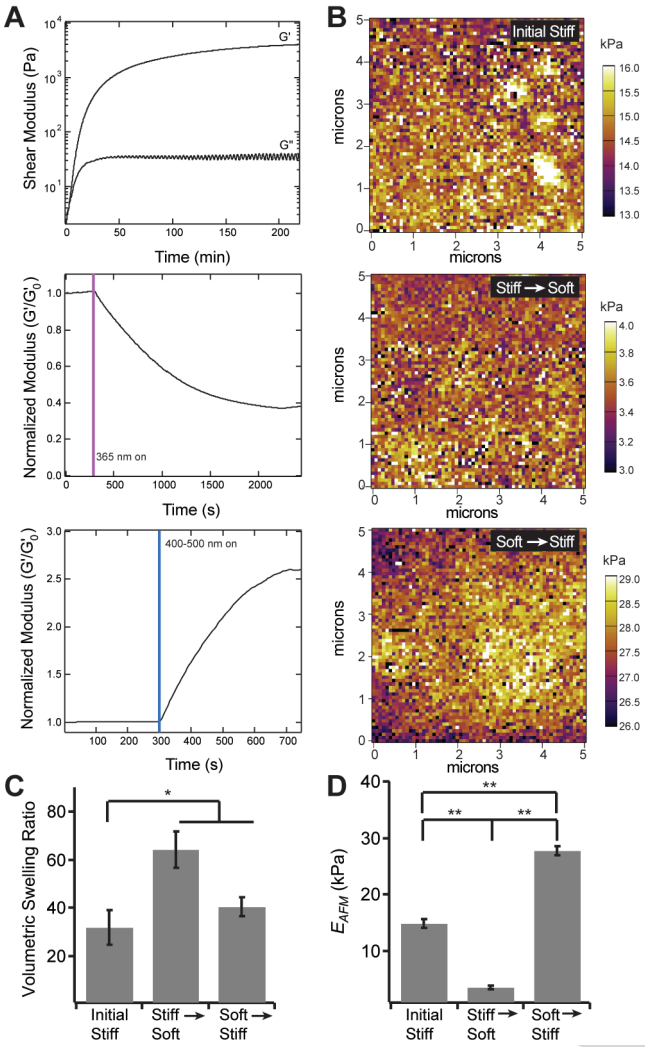

Figure 2. Characterization of reversible changes in mechanics. A) Shear rheology shows hydrogel formation proceeds over 3 h, with the crossover of G' and G" occurring in 3 min (top). Exposure to 365 nm light leads to a decrease in G' (middle); new crosslinks form for a LAP-laden hydrogel upon exposure to 400-500 nm light (bottom). B) Representative AFM images of *o*-NB_{13}-Me_{40}HA before exposure to light (top, ~14.8 kPa), after 365 nm exposure (middle, ~3.5 kPa), and after additional crosslinking (bottom, ~27.7 kPa). C) Bulk hydrogel swelling ratio increases upon hydrogel softening from 32% to 60% and decreases after hydrogel stiffening to 40% (mean ± s.d.) D) AFM measurements of elastic modulus (mean ± s.d.) show switching from 14.8 kPa (Initial Stiff) to 3.5 kPa (Stiff→Soft) to 27.7 kPa (Soft→Stiff). **: $P < 0.01$, *: $P < 0.05$

In situ, on-demand hydrogel softening was confirmed via shear rheology on thick hydrogel samples exposed to 365 nm light at an intensity of 10 mW/cm^2 (Figure 2A, middle), a previously identified cytocompatible dose.[10a, 15] Prolonged light exposure led to a plateau in the photodegradation at 40% of the initial modulus, which may indicate that a small portion of the methacrylates reacted during initial hydrogel formation. It is important to note

that the degradation kinetics in Figure 2A are slow compared to previously published systems[13] due to the necessity of thick samples for handling and coincident light attenuation (~50% attenuation at 60 μm depth for this formulation). At the surface, where there is minimal light attenuation, AFM measurements showed a softened *E* of 3.5 ± 0.3 kPa after 600 s exposure (Figure 2B, middle). These data indicate a photodegradation rate constant similar to previously published *o*-nitrobenzyl hydrogel systems (Figure S4).

Subsequent hydrogel stiffening was conducted using photoinitiated chain polymerization of the remaining methacrylates, thereby imparting reversible mechanics to this system. Hydrogels were first incubated with 3.3 mM LAP photoinitiator, then exposed to visible light in the 400-500 nm range (Figure 2A, bottom). At 405 nm, the molar absorptivity of the hydrogel (1050 $M^{-1}cm^{-1}$) and photoinitiator (25 $M^{-1}cm^{-1}$)[16] are much less than that of the hydrogel at 365 nm (4200 $M^{-1}cm^{-1}$); the hydrogel had minimal light attenuation, and exhibited a plateau in the increased G' after 420 s. For this formulation, the complete chain polymerization corresponded to an *E* of 27.7 ± 0.8 kPa, as measured by AFM (Figure 2B, bottom). The softened and re-stiffened moduli reported here represent the limits for an HA modification of x=13% and y=40%, but a smaller range can easily be achieved by changing the time of light exposure to yield partial degradation or polymerization.

Each crosslinking state of the hydrogel corresponded to a distinct volumetric swelling ratio (Figure 2C): ~30% (Initial Stiff), 60% (Stiff→Soft), and 40% (Soft→Stiff). These swelling ratios reflected the changes in hydrogel crosslinking density and hydrophilicity due to the photodegraded *o*-nitrobenzyl groups.

For these types of elastic 2D substrates, previous studies have shown that an increased modulus promotes cell spreading and elongation by providing resistance to cellular traction forces.[17] Thus, to probe whether this system could be used to deliver dynamic mechanical cues to adhered cells *in situ*, human mesenchymal stem cells (hMSCs) were first seeded on non-exposed hydrogels (Initial Stiff, 14.8 kPa), then softened (Stiff→Soft, 3.5 kPa) at 24 hours and subsequently stiffened (Soft→Stiff, 27.7 kPa) at 72 hours (Figure 3A). Cell spread area and roundness were assessed on each stiffness condition. Representative f-actin images (Figure 3B) show spread cells for all conditions. As hydrogel stiffness was decreased, cell area decreased as well (Figure 3C). Upon hydrogel stiffening, cell area increased again, though the cells remained slightly smaller than the initial condition. Cell roundness followed a reverse trend and increased upon hydrogel softening, then decreased upon hydrogel stiffening (Figure 3D); roundness was recovered for the Soft→Stiff condition. These data indicate the substrate mechanics significantly change between the three conditions, and hMSCs sense these changes *in situ*.

As another marker of mechanosensing, the nuclear localization of Yes-associated protein/transcriptional coactivator with PDZ binding motif (YAP/TAZ) was assessed (Figure 3E). The nuclear localization of YAP/TAZ has widely been shown to increase for cells on stiff 2D substrates, further regulating behaviors such as stem cell

differentiation.[18] Here, YAP/TAZ nuc/cyt ratio decreased on the Stiff→Soft hydrogels, although all ratios remained above 1 (Figure 3F). This result indicates some YAP remained in the nucleus, which agrees with the observation that cells exhibited some spreading on the softened substrates. YAP/TAZ nuc/cyt ratio subsequently increased on the Soft→Stiff hydrogels (Figure 3F) to levels similar to the Initial Stiff condition. Finally, nuclear YAP/TAZ ratio positively correlated with cell area, with more spread cells exhibiting higher YAP/TAZ ratios (Figure 3G).

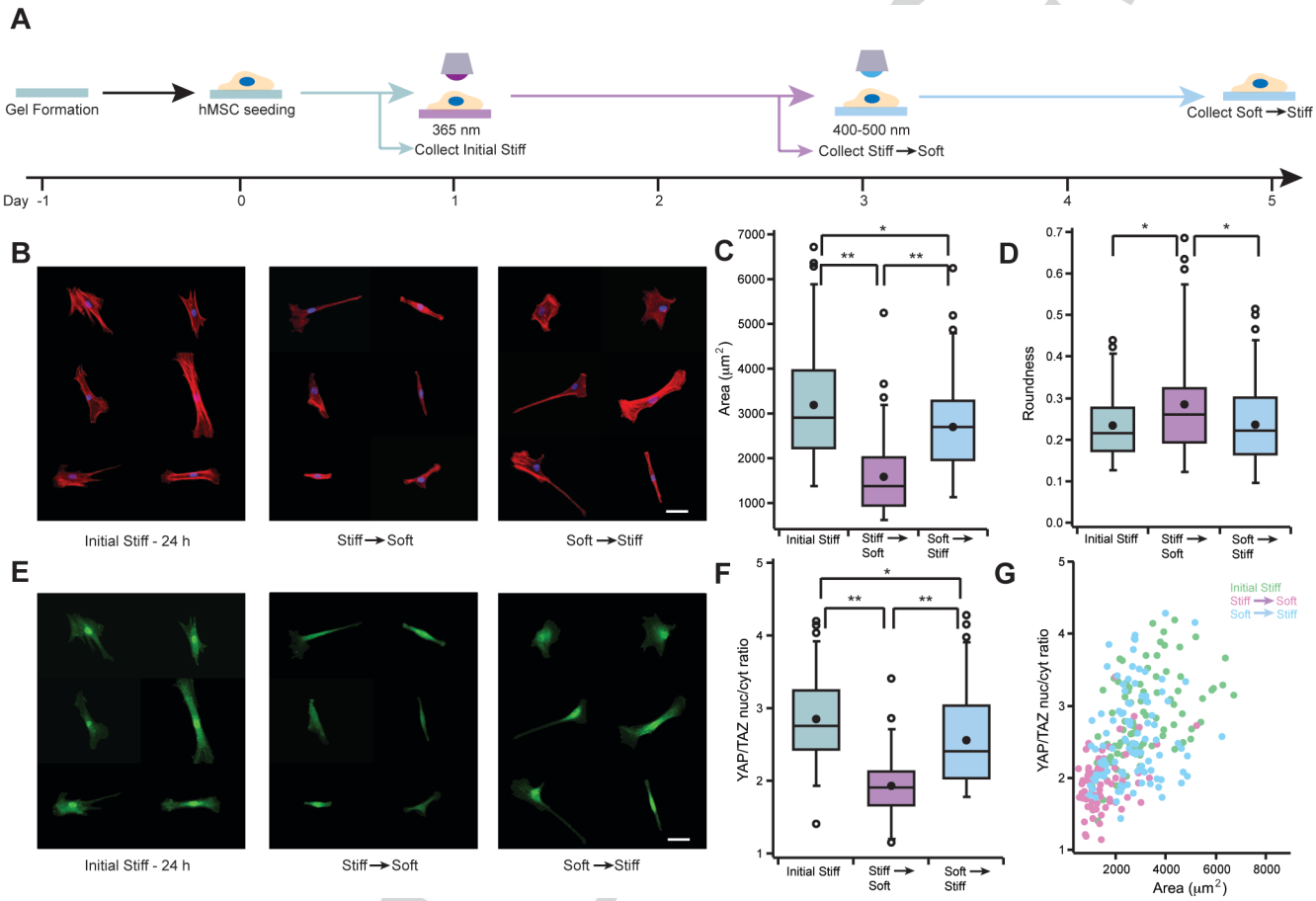

Figure 3. Cell response on *o*-NB$_{13}$-Me$_{40}$HA gels exposed *in situ*. A) Cells were seeded on Initial Stiff (14.8 kPa) gels on day 0, and exposed to 365 nm light on day 1 (3.5 kPa), followed by 400-500 nm light with LAP on day 3 (27.7 kPa). Samples were analyzed on day 1 (Initial Stiff), day 3 (Stiff→Soft), and day 5 (Soft→Stiff). (B) Representative maximum projections of single cells stained for f-actin (red) and nuclei (blue) and quantification of (C) cell area and (D) roundness on gels exposed *in situ*. (E) Representative maximum projections of the same single cells stained for YAP/TAZ (green), quantification of (F) YAP/TAZ nuc/cyt ratio, and (G) a scatter plot of YAP/TAZ nuc/cyt ratio versus cell area on gels exposed *in situ*. Scale bars = 50 μm; **: $P < 0.01$, *: $P < 0.05$, n > 70 cells.

Altogether, these data indicate that dynamic mechanical cues can be delivered to adhered cells *in situ* with the developed substrates. As a control, hydrogels were exposed to the aforementioned light conditions *before* cell seeding to assess cell behavior on the substrates without exposure to light (Figure S5) or photodegradation products (Figure S9). Cell area, roundness, and YAP/TAZ nuc/cyt ratios exhibited similar trends on the static hydrogels. To ensure that light exposure does not interfere with cell attachment, cell density was measured across the different conditions and no differences were observed (Figures S6 and S7). Furthermore, cell area

and nuclear YAP/TAZ ratio were measured on non-photoresponsive hydrogels to ensure that light alone does not affect cell behavior (Figure S8). Finally, no changes in nuclear YAP/TAZ ratio were observed over the length of the culture period on static hydrogels (Figure S10). These data indicate that the change in substrate mechanics dictates the change in cell phenotype observed here.

In conclusion, this work has demonstrated a unique strategy for on-demand, dynamic control of hydrogel mechanics with light. The theoretical moduli limits are tunable with acrylate and methacrylate modifications of the HA polymers, and further tunability could be achieved via partial degradation or photopolymerization. To demonstrate reversible cellular mechanosensing, hMSCs were shown to respond to dynamic substrate mechanics via changes in cell area and nuclear YAP/TAZ ratio. It is anticipated that the developed hydrogels will have wide applicability in mechanobiology and "mechanical memory"[2b, 3] studies relevant to disease and regenerative medicine.

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Keywords: gels • matrix mechanics • stem cells • photochemistry

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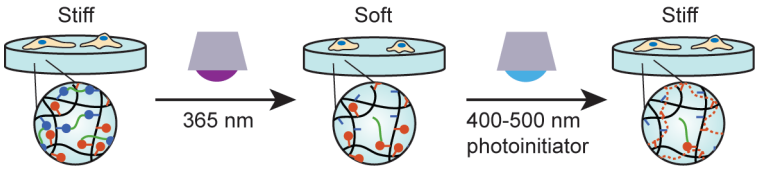

Hydrogels with reversible mechanics have been developed from hyaluronic acid polymers capable of photodegradation and photopolymerization, capturing dynamic aspects of native microenvironments. Cellular mechanosensing was demonstrated with human mesenchymal stem cells as the substrate mechanics changed *in situ*.

Adrianne M. Rosales, Sebastián L. Vega, Frank W. DelRio, Jason A. Burdick, and Kristi S. Anseth**

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