

Tuning the Range of Polyacrylamide Gel Stiffness for Mechanobiology Applications

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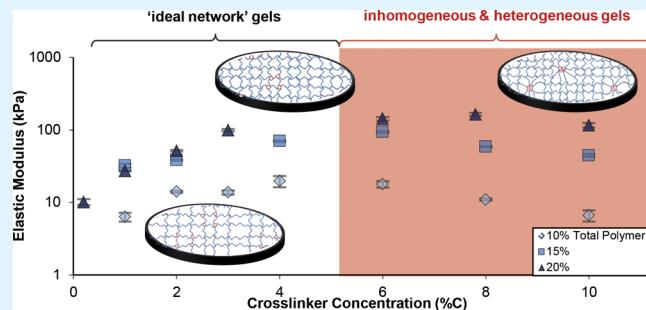
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Supporting Information

ABSTRACT: Adjusting the acrylamide monomer and cross-linker content in polyacrylamide gels controls the hydrogel stiffness, yet the reported elastic modulus for the same formulations varies widely and these discrepancies are frequently attributed to different measurement methods. Few studies exist that examine stiffness trends across monomer and cross-linker concentrations using the same characterization platform. In this work, we use Atomic Force Microscopy and analyze force–distance curves to derive the elastic modulus of polyacrylamide hydrogels. We find that gel elastic modulus increases with increasing cross-link concentration until an inflection point, after which gel stiffness decreases with increasing cross-linking. This behavior arises because of the formation of highly cross-linked clusters, which add inhomogeneity and heterogeneity to the network structure, causing the global network to soften even under high cross-linking conditions. We identify these inflection points for three different total polymer formulations. When we alter gelation kinetics by using a low polymerization temperature, we find that gels are stiffer when polymerized at 4 °C compared to room temperature, indicating a complex relationship between gel structure, elasticity, and network formation. We also investigate how gel stiffness changes during storage over 10 days and find that specific gel formulations undergo significant stiffening (1.55 ± 0.13), which may be explained by differences in gel swelling resulting from initial polymerization parameters. Taken together, our study emphasizes the importance of polyacrylamide formulation, polymerization temperature, gelation time, and storage duration in defining the structural and mechanical properties of the polyacrylamide hydrogels.

KEYWORDS: polyacrylamide, hydrogel, atomic force microscopy, elastic modulus, substrates for mechanobiology



1. INTRODUCTION

Polyacrylamide hydrogels are versatile materials with widespread use as cell culture substrates to study how cells sense and respond to the physical characteristics of their microenvironment.^{1,2} Changes in polyacrylamide stiffness impact cell spreading,³ shape,⁴ functional maturity,⁵ and differentiation status.⁶ Mechanically tunable polyacrylamide substrates can also be selectively functionalized with extracellular matrix (ECM) proteins to control cell adhesion.^{7,8} Protein functionalized gels can further be used to measure cell-generated forces by applying traction force microscopy (TFM) methods to the position of fiducial microbeads embedded within the material.^{9,10} TFM combined with ECM micropatterning on these substrates enables studying how cell shape influences cell force generation.¹¹ Recently, stem cell differentiation has been shown

to depend on polyacrylamide structure (gel porosity) and gel stiffness.^{12,13}

Polyacrylamide hydrogels are a highly swollen network of cross-linked acrylamide units and the stiffness of the material can be tuned by varying the amount of bis-acrylamide cross-linker and total acrylamide monomers in the gel precursor solution.² The total polymer content (T) and cross-linker concentration (C) are calculated and reported as follows

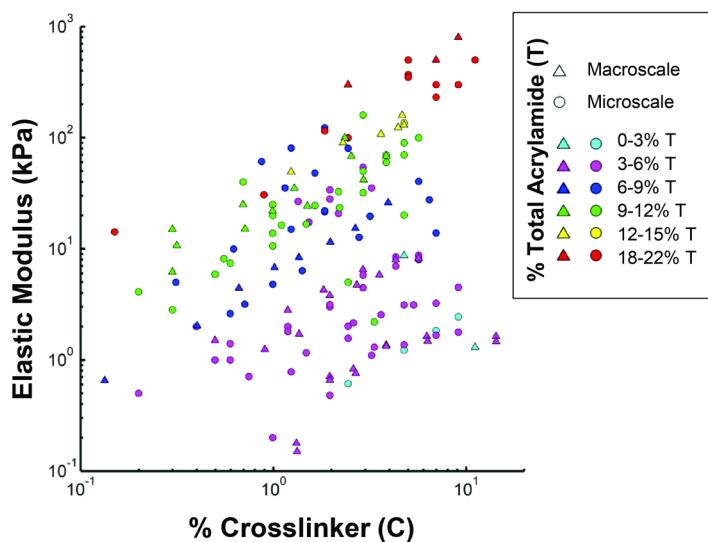
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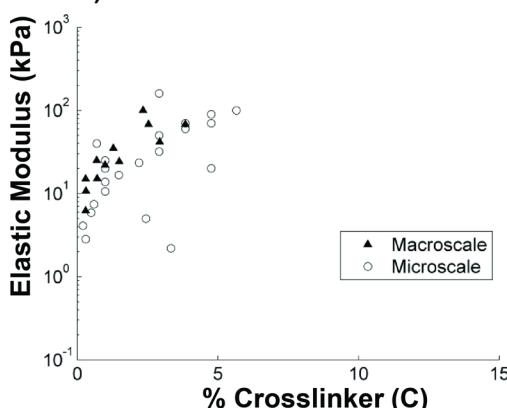
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A.) All studies



B.) 10%T



C.) 20%T

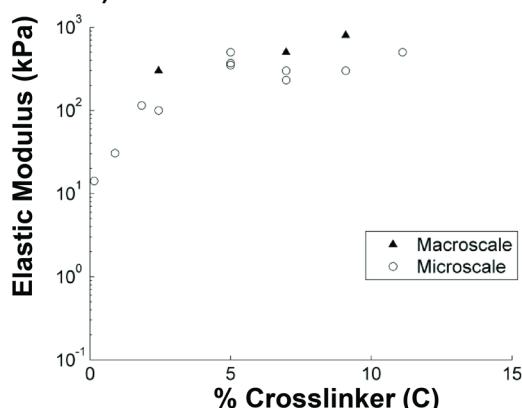


Figure 1. Mechanical characterization of polyacrylamide gels is highly variable because of measurement modes and gel synthesis conditions. (A) We conduct metadata analysis of 25 studies which report gel elastic modulus (E). The gel formulations are grouped by total acrylamide content (T) using different colors and the measurement type is represented by circles (●) and triangles (▲) for micro- and macroscale measurements, respectively. We analyze the relationship between cross-linker content (C) and E at approximately constant T in samples with (B) 10.02–10.6% T , and (C) 20.5–22% T .

$$T \text{ (w/v)} = \frac{\text{acrylamide (g)} + \text{bis(acrylamide) (g)}}{\text{total volume (mL)}} \times 100\% \quad (1)$$

$$C \text{ (w/w)} = \frac{\text{bis(acrylamide) (g)}}{\text{acrylamide (g)} + \text{bis(acrylamide)(g)}} \times 100\% \quad (2)$$

Polyacrylamide has been accepted as a linearly elastic material^{14–16} because it exhibits a constant storage modulus throughout a wide range of strains (0.01–1 dimensionless strain, see rheometry data in Storm et al.¹⁷). Thus, polyacrylamide gels can be mechanically characterized by time-independent recovery after mechanical loading.¹⁸ Linear elastic models assume materials undergo reversible deformation, not affected by the rate of loading. Yet polyacrylamide is a hydrogel and the viscous behavior of water can invalidate these assumptions in certain testing regimes. Thus, poroelastic models have been applied to polyacrylamide characterization

to include the contribution of viscous fluid flow to the elastic matrix of the polymer network during mechanical deformation.^{1,19–22}

Yet the reported elastic moduli values for the same polyacrylamide formulations span a large range and there is no single standard method for nano- and microscale characterization of these hydrogels.^{1,23} In this study, we examined 25 papers using polyacrylamide gels for cellular mechanobiology studies to find that in addition to high variability, stiffness measurements are sparse and disparate for gels with high cross-linker ($C > 5\%$) and high total polymer content ($T > 10\%$) (see Figure 1 and Table S1). The high variability in cross-study comparisons of gel properties can be due to the following factors: the indentation probe size (macro vs microscale probes¹), gel sample thickness,^{24–26} variability in the gelation time,²⁷ and nonlinear behavior at large strains.²⁶ From our metadata analysis and a report by Oyen,¹ total acrylamide content (T) is most significant factor in determining mechanical properties of polyacrylamide gels, yet the relation-

ship between gel elastic modulus and cross-link content (C) is unclear. For 10% T, it appears that increasing C increases the elastic modulus of the gel (see Figure 1B), a trend confirmed by Yeung and colleagues for 7.5% T at low cross-linker concentrations (0.01–1% C).⁴ Yet for formulations with 20% T, increasing C leads to a general plateau of stiffness (Figure 1C). The relationship between cross-linker concentration, structure of the polyacrylamide network, and resulting elastic modulus is not straightforward.^{1,28}

In addition to inherent differences in polyacrylamide characterization methods, the time hydrogels spend in storage can alter mechanical properties. Damljanovic and colleagues observed that polyacrylamide gels softened ~25% over time when stored in phosphate buffered saline (PBS) and a decrease in the elastic modulus when polymerized gels were heated to 37 °C in PBS and cell culture media.²⁹ This material softening was attributed to incomplete polymerization during cross-linking of the gel over 20 h as the material reached swelling equilibrium.²⁹ Other studies used light scattering experiments to quantify inhomogeneities in the gel network and noted that increasing storage time and temperature induced swelling and changed gel stiffness.^{21,30} Hydrolysis of amide groups into carboxylate anions, which proceeds time linearly during gel aging, may be responsible for the increased swelling,³¹ which results in decreases in network inhomogeneities after gel aging.³²

In this work, we aim to better understand the complex relationship between gel formulation (cross-linker and total polymer concentration) and the resulting stiffness. We measure the elastic modulus of a wide range of polyacrylamide gel formulations using one characterization approach: microscale indentation using an atomic force microscope (AFM). We ask whether polymerization temperature can change the stiffness trends we note across polyacrylamide formulations. We also evaluate how polyacrylamide gel stiffness changes during storage time because many mechanobiology studies require cells to be cultured long-term on polyacrylamide substrates (7 days to 4 weeks⁶) and these gels are often prepared in bulk to store for upcoming experiments. Finally, we provide design criteria for polyacrylamide gel formulations to yield well-defined gels whose mechanical characteristics follow polymer scaling laws.

2. MATERIALS AND METHODS

2.1. Materials. *Polyacrylamide Gels.* Stock solutions of 0.5 g/mL acrylamide (Sigma-Aldrich, 01696 Fluka) and 0.025 g/mL bis-acrylamide (Sigma-Aldrich, 146072) prepared in Milli-Q water were used to create polyacrylamide gel precursor solutions. The required amounts of acrylamide and bis-acrylamide stock solutions were diluted with Milli-Q water in a microcentrifuge tube to make the final concentrations needed to reach the desired formulation of total polymer and cross-linker.

Activated Coverslips. Glass coverslips were first cleaned of organic material for 30 min in piranha solution (3:1 ratio of sulfuric acid to hydrogen peroxide). Then the slides were rinsed with Milli-Q water and incubated for 10 min in 0.5% v/v (3-Aminopropyl)triethoxysilane (APTES, Sigma-Aldrich, 440140) in Milli-Q water. The silanized coverslips were baked at 55 °C until dry, allowed to cool, and incubated for 30 min in 0.5% v/v gluteraldehyde in Milli-Q water (based on previous methods³³). Coverslips were stored in a desiccator in the dark at 4 °C and used within a month of silanization.

2.2. Polyacrylamide Gelation. The gel precursor solution (acrylamide, bis-acrylamide, and water) was degassed in a vacuum desiccator for 1 h prior to gelation. To initiate gelation, 5 μL of 10% w/v ammonium persulfate (APS, Sigma-Aldrich, A9164) was added to ~995 μL of gel precursor solution followed by 0.5 μL of N,N,N',N'-

Tetramethylethylenediamine accelerator (TEMED, Sigma-Aldrich, 411019). The solutions were mixed by gentle pipetting before dispersing on the activated coverslips. Gels were polymerized at room temperature (23 °C) and at 4 °C to test the effect of polymerization temperature on the gel structure and stiffness. For all polymerization conditions, two 254 μm thick PDMS spacers (Stockwell Elastomerics, HT-6240) were placed on a square activated coverslip and a 12 mm diameter coverslip was placed on top of the spacers to provide an even gel surface. Twenty-five μL of gel precursor solution was pipetted into these coverslip sandwiches using capillary action. Most gels polymerized within 5 to 15 min. Gels were left undisturbed at room temperature for 30 min or overnight at 4 °C. Afterward, the gels were flooded with 1X PBS pH 7.4 (Life Technologies, 10010023) and the top coverslip was gently lifted from the gel surface using tweezers. Gels were stored at room temperature in PBS until indentation experiments were performed.

We evaluated the transmittance of the gels polymerized at 23 and 4 °C using a Shimadzu 1800 UV-vis spectrophotometer. After gelation, we used a scalpel to separate samples from activated coverglass and carefully loaded them into a 1 cm cuvette filled with PBS, ensuring full contact of the gel slab to the cuvette wall. We measured the transmittance (%) in the range 400–700 at 2 nm intervals.

2.3. Microscale Mechanical Characterization Experiments. *Atomic Force Microscopy Indentation (AFM).* We employed microscale mechanical testing to evaluate the properties of samples over a wide range of gel formulations and during gel storage. Excluding gel aging studies, we performed all AFM indentation experiments within 24 h of polymerizing the hydrogels. We used a WITec AFM (alpha300) with gold-coated tapping mode cantilevers (NanoWorld AG: Nanosensors, PPP-NCSTAuD-10). We characterized the cantilever stiffness to be between 8.45 and 8.5 N/m for probes used in this study using thermomechanical noise measurements.³⁴ After characterization, we attached one 50 μm diameter bead (Duke Scientific, 9050) to the tip of each probe using UV epoxy glue (Loctite 352).

For all AFM indentation experiments, we prepared sample holders from the lid of Falcon Easy-Grip Tissue Culture Dishes (60 × 15 mm) that were glued to a standard glass slide using 5 min epoxy. We applied a small amount of vacuum grease to the back of the gel coverslip to adhere it to the dish surface. We then flooded the dish with 1X PBS, loaded it into the AFM, focused the cantilever, and incubated the cantilever within the sample for at least 1.5 h to stabilize the system before obtaining measurements. We performed Optical Lever Sensitivity measurements³⁵ before analyzing each gel sample by indenting the glass surface using the following parameters: feedback control with 0.5 V set point, 1% p-gain, and 0.2% i-gain, force-distance using 0.2 μm pull and 0.6 μm push at 0.2 μm/s speed. We then approached gel surface with the following feedback threshold parameters: 0.5 V set point, 2% p-gain, 0.2% i-gain.

Force-Distance Measurements. We optimized the force-distance curve parameters to ensure that we obtained zero load data as well as ample indentation of our samples for analysis. We performed 5 indentations in 5 areas (25 indentations total per sample) at an approach and retraction rate of 3 μm/s. Approach and retraction distances were optimized for each gel formulation (see Table S2 for experimental details). We analyzed the force-distance curves by assuming the polyacrylamide is a linearly elastic substrate using the Hertz elastic contact model.³⁶ The majority of metadata we analyzed characterized polyacrylamide stiffness using the Hertz method.^{2,37–41}

2.4. Scanning Electron Microscopy for Gel Structure. We performed both environmental variable-pressure (VP) and field-emission (FE) scanning electron microscopy (SEM) to visualize the structure of polyacrylamide gels. For VPSEM, we prepared gels as described above and hydrated samples in ultrapure water overnight before imaging. Samples were blotted gently with a Kimwipe before loading onto the cool-stage of a Hitachi S-3400N SEM (Hitachi High-Technologies, Pleasanton, CA, USA). Imaging was done at 50 Pa, using cool-stage control at –20 °C (Deben UK Ltd., Suffolk, UK) and Backscattered Electron (BSE) detection at 15 kV accelerating voltage. For FESEM visualization samples were prepared as previously described and hydrated in PBS overnight. The samples were then

gradually dehydrated using a series of ethanol dehydration steps (50, 70, 90, 100% ethanol) and dried at their critical point with liquid CO₂ using the Tousimis Autosamdry Critical Point Dryer (815B, Rockville, MD) with 20 min of purge time. CPD has been used for SEM preparation of polyacrylamide hydrogels⁴² to avoid the damaging effects of air–liquid interfaces. The samples were dried with CPD for 1 h to achieve gradual exchange of 100% ethanol to liquid CO₂, followed by an increase in temperature to 31 °C and pressure to 1350 psi (the critical point of CO₂), and gradual bleeding of the resultant CO₂ gas. This enabled drying of fragile samples under negligible surface tension. Samples were subsequently mounted onto standard Aluminum pin stubs, and sputter coated with gold–palladium (5 nm) using a Denton DeskII sputter-coater (Denton Vacuum, LLC, Moorestown, NJ, USA). FESEM imaging was done using a Zeiss SIGMA (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) at 2 kV accelerating voltage and InLens Secondary Electron (SE) detection.

2.5. Analysis. Custom Matlab scripts were used to analyze the data using the Hertz model on the approach trace of the force–distance curve. We assumed a rigid indenter to convert between the reduced elastic modulus to material elastic modulus using the Poisson's ratio and approximated the Poisson's ratio as 0.48 based on micropipette aspiration measurements by Boudou.⁴³

We followed the approach by MacKay and Kumar⁴⁴ to employ Hertz fitting and first baselined our data to start with a flat region of zero deflection. We then identified the contact point (cp) on our approach curve using the following formula where, d_1 , d_2 , z_2 , and z_1 refer to the d vertical deflection and z position of the first data point and second data point to be fit⁴⁴

$$cp = \frac{(z_2 - d_2) - (z_1 - d_1) \left(\frac{d_2}{d_1} \right)^{2/3}}{1 - \left(\frac{d_2}{d_1} \right)^{2/3}} \quad (3)$$

As suggested by MacKay and Kumar,⁴⁴ the first data point is taken at 15 nm deflection, and the second data point is at 100 nm deflection or the maximum deflection if the sample does not reach 100 nm. Then, we fit the following Hertz relation for 2 μm beyond the contact point, where R is the radius of our probe, δ is the indentation depth ($z - d$), and ν is the Poisson's ratio of our material^{44–46}

$$F_{\text{sphere}} = \frac{4E\sqrt{R\delta^3}}{3(1 - \nu^2)} \quad (4)$$

When we evaluated the aging of gels, we compared the elastic modulus as calculated from force–distance data by the method described above on days 1, 3, 5, 7, and 10 after polymerization. We performed a Student's *t* test (unpaired, two-tailed) with a 95% confidence interval to determine if changes in mechanical moduli during gel storage were statistically significant. We performed Q–Q plot analysis of all the time points to verify that the data followed normal distributions and assumed that each indentation of the substrate was an independent measurement.

We analyzed the swelling ratio (Q_m = swollen gel weight/dried gel weight) of gels to determine how swelling altered the mechanical properties of gels during aging. We polymerized 200 μL of polyacrylamide in 0.65 mL microcentrifuge tubes (for 30 min and overnight at room temperature). We then took gels out of the tubes and immersed them in PBS for 10 days to allow for swelling. Gels were then gently blotted dry with a Kimwipe and weighed on a microbalance (range 0.16–0.26 g). Gels were completely dried for 24 h in a lyophilizer and weighed again (range 0.007–0.036 g) to calculate the swelling ratio.

3. RESULTS

3.1. Trends in Polyacrylamide Gel Elastic Modulus.

From the general trends observed in our metadata analysis (see Figure 1), we characterized polyacrylamide gels of different formulations to investigate the relationships between cross-

linker concentration, total acrylamide content, and mechanical properties. We measured the elastic modulus (Figure 2) of gels fabricated with a range of cross-linker concentration (0.25–11% C) and constant total acrylamide content (10, 15, and 20% T). For formulations with constant T, the elastic modulus increased with increasing cross-link concentration up to an inflection point after which the elastic modulus decreased (i.e., gels became softer) with increasing cross-linker content. This trend has been demonstrated previously^{27,28,47–50} and we identified unique inflection points for 3 different gel formulations. The inflection point shifts to higher cross-linker content as the total acrylamide content of the gels increases: 5% C for 10% T, 7% C for 15% T, and 8% C for 20% T (Figure 2A–C). For C lower than the inflection point, we observed a power law relationship of cross-linker concentration with the elastic modulus, as predicted from scaling theory of polymer networks^{49,51–53} (see power line fits in Figure 2).

We visualized the hydrogel structure of various polyacrylamide formulations to evaluate if submicron heterogeneities could be responsible for these stiffness trends. Across the same polymer content (T), we used field-emission scanning electron microscopy to compare gels with 2% C that follow the power law relationship (top row, Figure 3), 4–8% C gels at the inflection point where stiffness deviates from the power law (middle row, Figure 3), and 10% C gels that are well past the inflection point (bottom row, Figure 3). Gels at or above the inflection point (15% T 6% C, 20% T 8% C and all three of the 10% C gels) share large void features in their structure. Although we believe the gross features are due to sample preparation artifacts (see Discussion), there are obvious structural differences between samples below the inflection point and those at or above it. The surfaces of the 2% C gels appear smooth with ~25 nm holes scattered on the 10% T 2% C sample, which are likely also drying features. Although the features (voids and holes) we observed are likely drying artifacts, evaluating changes in these defects still provides insights of relative differences in the structure of gels following the power law relationship and those which are mechanically softer than predicted (compare top and bottom rows of Figure 3). We were not able to resolve the gel structure under environmental conditions using the variable-pressure SEM because the gel structures were smaller than the 50 μm resolution of the instrument (Figure S1). Figure S2 shows views of the gel sample edges, which provide oblique cross-sectional views of the gel structure at different thicknesses.

3.2. Altered Gelation Temperature. On the basis of previously reported models for altered polyacrylamide gel structure as a function of gelation kinetics, we polymerized gels at lower temperature (4 °C). The trend in elastic modulus with cross-linker concentration changed to a plateau region instead of a clear inflection point as compared to gels polymerized at room temperature (Figure 4). Gels polymerized at 4 °C overnight showed an overall larger maximum stiffness. 10% T, 8% C gels polymerized at 4 °C exhibited an average elastic modulus of 27 kPa, whereas the same formulation polymerized at room temperature was 10.9 kPa.

3.3. Aging Effects. We examined the stability of gels over time and examined the elastic modulus during storage for up to 10 days postpolymerization (Figure 5). The formulation of the gel altered its variation in stiffness during storage. Over 10 days, 10% T, 2% C gels demonstrated the largest increase in stiffness by a factor of 1.56 ± 0.13 as compared to 1 day after polymerization (significant at the 95% confidence interval by

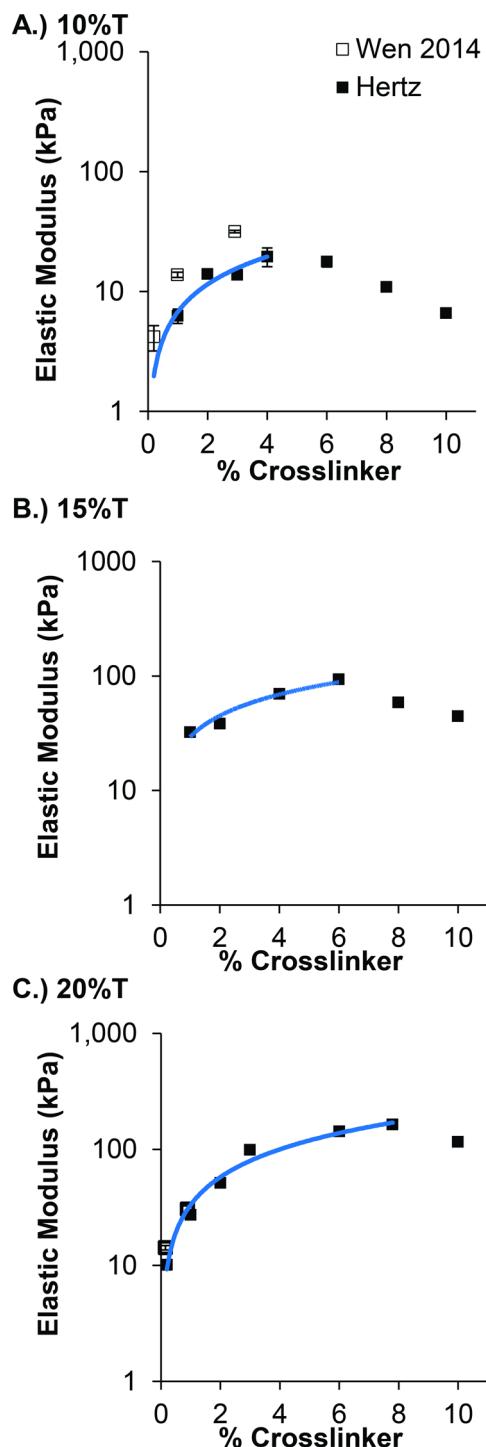


Figure 2. Relationship between cross-linker concentration and elastic modulus varies for total acrylamide content (T). Gels containing (A) 10, (B) 15, and (C) 20% total acrylamide exhibit an increase in elastic modulus with increasing cross-linker concentration following a power law up to inflection points at 4, 6, and 8% C, respectively. After the inflection point, the gel modulus decreases with increasing cross-linker. As verification of our characterization method, we also plot gel stiffness reported recently by Wen and colleagues in panel A. Elastic moduli for all samples were calculated using the Hertz (black rectangles, ■) fitting approach to microscale force-indentation experiments. The power fit (solid blue line: $R^2 = 0.91$ for 10% T, 0.95 for 15% T, and 0.98 for 20% T) expected from polymer theory is shown for data points before inflection. Error bars show standard deviation with $n = 25$ for each point.

the unpaired, two-tailed Student's t test). 15% T and 20% T gels, both with 2% C, also increased in stiffness by day 10 by a factor of 1.09 ± 0.05 and 1.24 ± 0.22 , respectively (both values significant at the 95% confidence interval by the unpaired, 2 tailed Student's t test). We compared the statistical significance of the elastic modulus at each time point as compared to day 1 after polymerization and summarize the results in Table S3. Overall, changes in gel stiffness become significant by day 3 after polymerization.

4. DISCUSSION

For each gel formulation with set total acrylamide content (T), we observed that the elastic modulus increases with increasing cross-linker content until reaching an inflection point after which the elastic modulus decreases. Several groups have reported similar trends in polyacrylamide stiffness as a function of cross-linker.^{27,28,47–50} In particular, ~10% T gels have been shown to decrease in their stiffness for cross-linker content greater than 5%.^{47,50} In our study, we identify the inflection points corresponding to altered gel material properties for 3 total polymer formulations: 5% C for 10% T, 7% C for 15% T, and 8% C for 20% T.

Decreasing stiffness at high cross-linker concentrations can be explained by a transition in the structure of the polyacrylamide gel network from an ideal to a clustered gel.^{54,55} Ideal hydrogels exhibit maximal elasticity because each tetrafunctional bis-acrylamide molecule is connected to four of the nearest neighboring acrylamide groups and spaced following a Poisson distribution to ensure maximal elastically effective chains.^{27,55} When the amount of cross-linker to monomer is in excess, there is insufficient acrylamide to connect to the available bis-acrylamide molecules. In addition, bis-acrylamide has low solubility in water; thus, at high concentrations, bis-acrylamide is more likely to aggregate in the gel precursor solution.^{54–56} When bis-acrylamide aggregates and the distribution of bis-acrylamide molecules becomes clustered, the bis-acrylamide units connect to only available acrylamide chains to avoid extending the growing acrylamide chains beyond their maximum end-to-end distance.⁵⁴

The clustering of cross-links in the gel leads to both structural inhomogeneity (spatial variation in cross-link density) and heterogeneity (phase-separated regions of the gel).⁵⁷ Both inhomogeneities and heterogeneities decrease the number of elastically effective chains and leads to softer materials compared to ideal networks.⁵⁸ These highly cross-linked aggregates (microgels) within the larger macrogel structure have been named “frozen blob clusters”^{1,27,28,30,58} as they form quickly during gelation.

Experimentally, the variation in gel density as a result of frozen blob clusters has been mostly measured by photon transmission intensity,^{30,58,59} small-angle (X-ray or neutron) scattering,^{60,61} and dynamic light experiments.^{49,62,63,63} Recently, Bush and colleagues used load relaxation curves at different indentation depths to demonstrate that mechanical characterization can also reveal heterogeneities in poly(ethylene glycol) hydrogels and found that the same sample yielded a range of indentation moduli as the material behavior varied with different indentation depths due to contributions of the gel structure.²²

We visualized the surface of the gels with FESEM and noted large void features that were likely due to sample processing methods. However, the range of heterogeneity in these post-CPD images suggests underlying differences in the gel network.

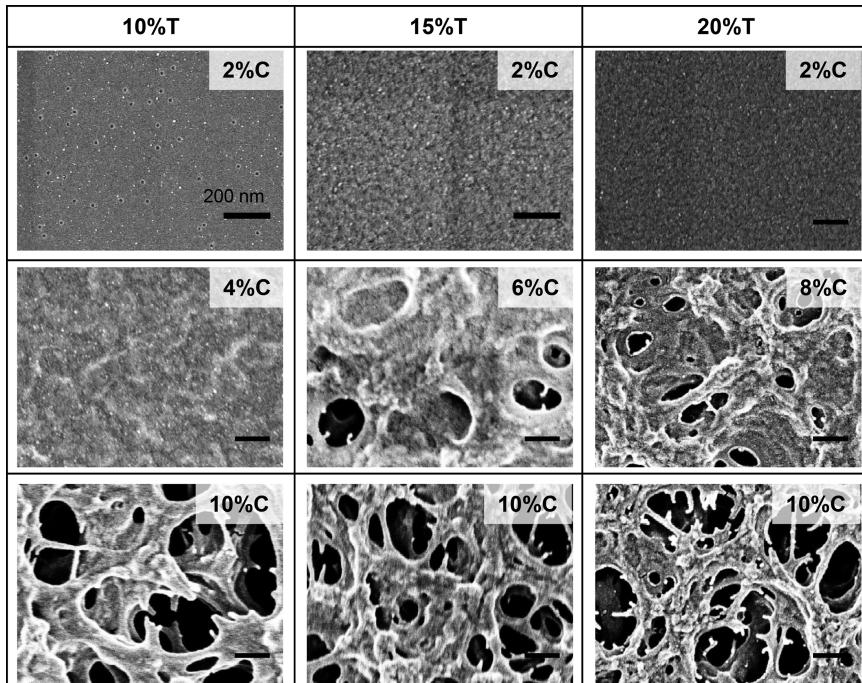


Figure 3. Surface FESEM images of various formulations of polyacrylamide gels. The large voids arise from the CPD processing of the substrates, while not indicative of gel pore size, they reveal different drying behaviors which suggest different nanostructure existed before the drying. Scale bars are 200 nm.

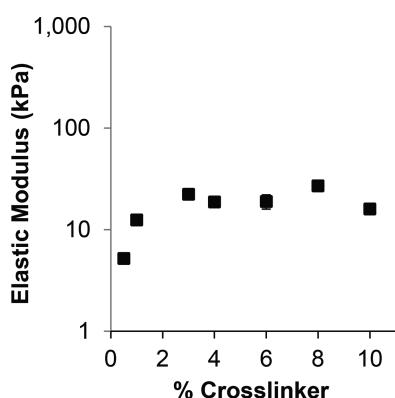


Figure 4. Lowering polymerization temperature to 4 °C increases gel stiffness for gels with 10% T and various cross-linker concentrations and changes the inflection point (4% C for gelation at 23 °C, Figure 2A) to a plateau for cross-linker concentration above 3%.

Replacing PBS in the gels by ethanol (dehydration) can lead to distortion of the hydrogel network by swelling and CPD can result in overall shrinkage of the gel,⁶⁴ which in combination with pump down procedures during critical point drying and sputter coating may have further opened up these defects especially in our thin gel samples ($\sim 250 \mu\text{m}$). Thus, we are not able to directly evaluate gel structure of various polyacrylamide formulations, yet we can compare postdrying structural differences between batch-processed gels and infer these are duly influenced by the original gel structure. The void defects are more severe and the gel network was disrupted to a greater extent for gels beyond the inflection point (10% C, last row on Figure 3) than for the other formulations, suggesting that perhaps more solvent and larger macroporous areas were initially present in these gels. Gels with the same total polymer content yet less cross-linker (15% T 6% C, 20% T 8% C)

exhibited fewer and smaller voids in their structure but were still different from the smooth 2% C gels. We further attempted to visualize gel structures for frozen blob clusters using environmental VP SEM to preserve the native hydrated state of the gels⁶⁵ but VP SEM lacked the resolution to visualize the gel ultrastructure.

Finally, we studied how reaction conditions during the free radical polymerization process can lead to structural heterogeneity.⁵⁷ Chain propagation is much more rapid than radical initiation,⁶⁶ especially at high cross-linker concentrations such that a small cluster quickly grows into a frozen blob during gelation due to chain cyclization and multiple cross-linking reactions happening to the same growing polymer (mechanisms reviewed recently by Lorenzo and Seiffert⁶⁷). The temperature at polymerization affects the dissociation of the initiator into free radicals, polymerization propagation, and acrylamide coupling reaction kinetics. In our study, we tested if decreasing the polymerization temperature (from 23 to 4 °C) could alter the rate of polyacrylamide chain propagation in an effort to decrease the formation of frozen blob clusters and therefore remove heterogeneity in the gel.

Decreasing the polymerization temperature in 10% T gels with various levels of cross-linker resulted in a higher elastic modulus. Instead of an inflection point, we observed a plateau in gel stiffness as cross-linker increased beyond 3% C. Structural contrast between frozen blob clusters and holes in the gel induces scattering which decreases light transmittance through the sample,⁵⁸ so we attempted to measure the transmittance of our gel samples to evaluate gel heterogeneity. However, we did not detect a difference in gel heterogeneity between samples polymerized at 23 and 4 °C relative to the inherent measurement variability.

The effects of polymerization temperature on gel structure and elasticity are complex and not well understood, as evidenced by contradictory findings in prior studies. Gelfi and

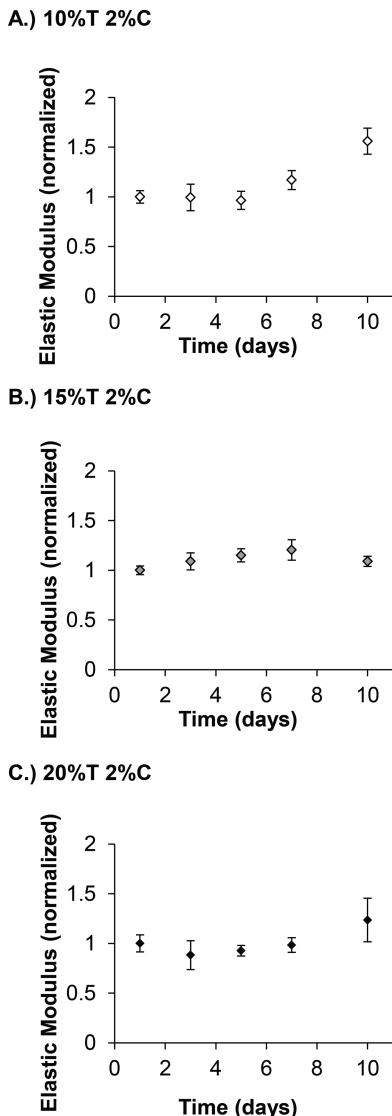


Figure 5. Gel formulation alters how mechanical properties change during gel aging over 10 days. Storage in PBS leads to an increase in the elastic modulus up to (A) 1.55 times for 10% T 2% C gels, (B) 1.09 times for 15% T 2% C, and (C) 1.24 times for 20% T 2% C gels. Measurements are normalized to 1 day after polymerization. Error bars show standard deviation with $n = 25$ for each point.

colleagues demonstrated that higher polymerization temperatures ($25\text{--}50\text{ }^{\circ}\text{C}$) led to more transparent gels with higher homogeneity but the substrate elastic modulus decreased.⁶⁸ Yet a study by Calvet and colleagues noted that 2% T required lower temperatures with decreasing cross-linker content to reach the maximum elastic modulus.³⁷ Thus, gel structure and elasticity, although linked, do not always respond in the same direction to temperature changes due to competing effects of altered kinetics, degradation, and molecular distribution of the reaction components. In our study, we note only that the 10% T gel formulations increased in heterogeneity and elasticity when we decreased the polymerization temperature.

Finally, we evaluated how storage of polyacrylamide gels altered their stiffness. We studied aging of “ideal” gel formulations (2% C with various total polymer T) below the inflection point for up to 10 days (Figure 5). All three samples (10, 15, and 20% T, all at 2% C) varied in their stiffness day to day. The changes were statistically significant as compared to

day 1 for most of the time points (see Table S3 for the *t* test analysis). The 10% T, 2% C sample exhibited the most aging, stiffening up to $1.55\times$ times over 10 days of storage in buffer.

We hypothesize that the 10% T, 2% C formulation gel polymerized incompletely in 30 min which led to deswelling and contributed to the stiffening during aging. We polymerized gels for 30 min as this is a commonly used polymerization time reported in protocols to produce polyacrylamide materials for mechanobiology experiments.² To evaluate if under-polymerization of our 10% T, 2% C gel sample caused its aging profile, we polymerized samples for both 30 min and overnight and calculated the swelling ratio (Q_m) (see Figure S3). We determined that the Q_m range is largest for the 10% T, 2% C samples polymerized for 30 min compared to the other formulations polymerized at 30 min and overnight. Thus, for formulations low in total acrylamide and cross-linker, 30 min may not be long enough gelation time for the reaction to go to completion. In chemically initiated radical polymerization, 90% of acrylamide molecules become polymerized in 1 h and eventually the gel reaches 99% conversion,⁵⁵ but this rate also depends on the cross-linker concentration as bis-acrylamide can cluster in solution. Thus, the time allotted for polymerization combined with gel formulation affect gel swelling behavior, which influences gel structure and mechanical properties as the material ages.

5. CONCLUSIONS

Taken together, these results demonstrating the complex relationship between T and C are important to consider when using polyacrylamide substrates to study cell response to substrate stiffness. These relationships may also change due to gel aging in a way that is dependent on gel formulation. Gels with frozen blob clusters increase their structural heterogeneity during swelling^{30,58,69,70} and formulations beyond the inflection points may exhibit even greater changes in mechanical properties during aging. Thus, full characterization of these substrates is important to create experimental controls and enable comparisons between studies.

In our experience, to achieve specific target stiffness values, one must maintain all of the following parameters constant: formulation of the gel (acrylamide, bis-acrylamide, initiator, and accelerator ratios), gelation temperature, gelation time, and storage time of the gel before use in experiments. In Table S4, we summarize the formulations and parameters that yielded “ideal” gels in our studies following a power-law response of increasing elastic modulus with increasing cross-linker content. These data will be useful to researchers wishing to achieve specific gel stiffness in their experiments, although we caution that changes in gel stiffness during gel storage and the degree of change depends on gel formulation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b09344.

Details of the metadata analysis, parameters used during mechanical characterization, and further details on hydrogel formulations and polymerization conditions (PDF)

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Author Contributions

Experiments were designed by A.K.D. and B.L.P. and conducted by A.K.D. The manuscript was written by A.K.D. and B.L.P. A.K.D. and B.L.P. approved the final manuscript.

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Notes

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ABBREVIATIONS

- AFM, atomic force microscopy
ECM, extracellular matrix
TFM, traction force microscopy
T, total polymer content
C, cross-linker concentration
E, elastic modulus
PBS, phosphate buffered saline
SEM, scanning electron microscopy
VP, variable pressure
FE, field emission
CPD, critical point dryer

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