nature methods

SUPPLEMENTARY INFORMATION

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Water content, not stiffness, dominates Brillouin spectroscopy measurements in hydrated materials

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Supplementary Methods

Hydrogel preparation

Polyethylene oxide (PEO)

PEO hydrogels were made from powder with an average molecular weight of 1, 4, and 8 MDa (Sigma-Aldrich). Mixtures of 1.5%, 5.0%, and 8.0% (v/v) PEO (corresponding to ε = 0.985, 0.95 and 0.92, respectively) were prepared in distilled water with continuous magnetic stirring at 1000 rpm for at least 10 hours until the mixture was homogeneous. Data shown in the main text were from a single experiment with one hydrogel for each combination of water content and molecular weight. One sample volume from each hydrogel was taken for refractometry, one for Brillouin microscopy and 2-3 sample volumes for rheometry, as described below. PEO hydrogels were not allowed to swell and were mixed and stored in a sealed container to minimize evaporation. The density of PEO hydrogels was calculated according to:

$$\rho = \frac{m_w + m_p}{\frac{m_w}{\rho_w} + \frac{m_p}{\rho_p}} \tag{S1}$$

where m_w, ρ_w, m_p and ρ_p are the mass and density of water and polymer (ρ_w = 1.00 g/cm^3, ρ_p = 1.21 g/cm^3), respectively. A replicate experiment is described in Supplementary Note.

Polyacrylamide (PA)

PA hydrogels were prepared following a published protocol[1]. Hydrogel stiffness was adjusted by varying the ratio of acrylamide, bis-acrylamide and distilled water. For all samples, the initial concentration of acrylamide (Sigma-Aldrich) was 10% (w/v). N-methylene-bis-acrylamide (Sigma-Aldrich) was added at an initial concentration of 0.03, 0.06, 0.10, 0.15, 0.23, and 0.30% (w/v). The mixtures were centrifuged at 500g for 5 minutes, followed by the addition of 0.1% (w/v) ammonium persulfate (Sigma-Aldrich) and 0.1% tetramethylethylenediamine (Sigma-Aldrich) to initiate polymerization. The mixture was cast into disk-shaped molds (diameter 25 mm, depth 10 mm) and allowed to polymerize for 15 minutes. The hydrogels were then removed and immersed fully in a large container of distilled water to swell freely. Data shown in the main text were from a single experiment with 4 replicate hydrogels made from the same stock solution for each cross-linker concentration. Individual hydrogels were followed over time to measure mass, Young's modulus and longitudinal modulus by Brillouin microscopy. A second experiment using the same preparation is shown in Supplementary Note.

Hydrogel mass m was measured for each replicate immediately after polymerization and at 0, 12, 24, 36, and 71 hours using an electronic balance with a precision of 1 mg to track swelling (Supplementary Figure 1a). Brillouin microscopy, compression tests and refractometry were performed for each replicate at 12, 24, 36, and 71 hours after polymerization, as described below.

The water content ε of PA hydrogels was calculated as

$$\varepsilon = \frac{V_w}{V} \tag{S2}$$

where V_w is the volume of water within the hydrogel and V is the total hydrogel volume. V_w is determined by the initial water volume at the start of polymerization, $V_{w,0}$, and the volume of water imbibed during swelling, ΔV_w, such that $V_w = V_{w,0} + \Delta V_w$. Likewise, $V = V_0 + \Delta V_w$, where V_0 is the initial hydrogel volume determined by the volume of the mold. $V_{w,0}$ was calculated according to

$$V_{w,0} = V_0 - \frac{m_a}{\rho_a} - \frac{m_b}{\rho_b} \quad \text{(S3)}$$

where m_a, ρ_a and m_b, ρ_b are the mass and density of acrylamide (ρ_a = 1.13 g/cm^3) and bis-acrylamide (ρ_b = 1.235 g/cm^3), respectively. ΔV_w was calculated according to

$$\Delta V_w = \frac{m - m_0}{\rho_w} \quad \text{(S4)}$$

where ρ_w is the density of water and m_0 is the hydrogel mass measured immediately after polymerization. The density of the hydrogel was calculated according to

$$\rho = \frac{m}{V} \quad \text{(S5)}$$

The swelling ratio Q of the hydrogel was calculated according to:

$$Q = \frac{V}{V_0} = \frac{1 - \varepsilon_0}{1 - \varepsilon} \quad \text{(S6)}$$

where $\varepsilon_0 = V_{w,0}/V_0$ is the value of ε immediately after polymerization (Supplementary Figure 1b).

Brillouin microscopy

Experimental setup

Supplementary Figure 2a shows the optical setup for Brillouin microscopy, as described in a previous study[2]. An objective lens (20X, NA = 0.5, Olympus) focused the light of a single longitudinal mode DPSS laser (λ=561nm; 30 mW, Cobolt Jive) emerging from single-mode optical fiber (Kineflex, OptiQ) to illuminate a volume of approximately 7 μm^3. Backscattered light from this volume was collected by the same objective and coupled into a single-mode optical fiber delivering the light to a custom-built spectrometer, consisting of an interferometer[2], a virtually imaged phased array (VIPA) and a Neo sCMOS camera (Andor). The interferometer suppresses Rayleigh peaks before the signal passes through the VIPA (LightMachinery Inc.) etalon that spatially separates the frequency components. The spectral resolution of the VIPA spectrometer used to produce the data in Figure 1a and 1b of the main text was 0.7 GHz with a finesse of 56 and an FSR of 39 GHz. For the data shown in Figure 1c and 1d of the main text, the spectral resolution was 0.4 GHz with a finesse of 70 and an FSR of 30 GHz. For each PA or PEO hydrogel, three randomly-selected locations were measured in the hydrogel, with 50 spectra acquired at each location and averaged. The illumination and detection side single mode optical fibers together ensure that the optical system embodies a Type II confocal microscope[3]. This was important because measurements assume homogeneity within the sample volume.

Data analysis

The Brillouin modulus M was calculated following measurements of the Brillouin frequency shift ω_b, refractive index n, and density ρ, according to:

$$M = \rho \left(\frac{\lambda\, \omega_b}{2\, n \sin\frac{\theta}{2}} \right)^2 \quad \text{(S7)}$$

where λ and θ are the wavelength and angle between the incident and scattered wave vectors, taken to be 561 nm and π, respectively. The Brillouin frequency shift was measured based on the frequency difference between the Rayleigh and Stokes peaks, as described below. Density was calculated according to Equations S1 or S5. Refractive index was measured using an Abbe refractometer (Bellingham and Stanley Ltd., London). For PEO hydrogels, a small sample (~0.05 ml) was taken and placed in the refractometer for measurement. For PA hydrogels, thin films were prepared from the same stock solution for each cross-linker concentration, allowed to swell in distilled water, and samples were cut out for refractometry at each time point.

Raw images of spectra were calibrated to convert pixel location to frequency. To do this, we identified the line in the sCMOS image connecting the Rayleigh, Stokes, and anti-Stokes peaks across two orders. A peak finding algorithm was performed to locate the pixel location of the six peaks x_j along this line, where j ranges from 1 to 6 (Supplementary Figure 2b). A moving average was used to reduce noise. The position of each peak was then used to map pixel location to frequency f based on the known free spectral range (FSR) of the VIPA according to $f = ax^2 + bx + c$, where a, b and c were determined by minimizing the least squared error of the following relationships:

$$\begin{bmatrix} f(x_2) \\ f(x_5) \\ f(x_4) - f(x_1) \\ f(x_6) - f(x_3) \\ \big(f(x_3) - f(x_2)\big) - \big(f(x_2) - f(x_1)\big) \\ \big(f(x_6) - f(x_5)\big) - \big(f(x_5) - f(x_4)\big) \\ \big(f(x_6) - f(x_4)\big) - \big(f(x_3) - f(x_1)\big) \end{bmatrix} = \begin{bmatrix} 0 \\ FSR \\ FSR \\ FSR \\ 0 \\ 0 \\ 0 \end{bmatrix} = \begin{bmatrix} x_2^2 & x_2 & 1 \\ x_5^2 & x_5 & 1 \\ x_4^2 - x_1^2 & x_4 - x_1 & 0 \\ x_6^2 - x_3^2 & x_6 - x_3 & 0 \\ x_3^2 - 2x_2^2 + x_1^2 & x_3 - 2x_2 + x_1 & 0 \\ x_6^2 - 2x_5^2 + x_4^2 & x_6 - 2x_5 + x_4 & 0 \\ x_6^2 - x_4^2 - x_3^2 + x_1^2 & x_6 - x_4 - x_3 + x_1 & 0 \end{bmatrix} \begin{bmatrix} a \\ b \\ c \end{bmatrix} \quad \text{(S8)}$$

where x_1, x_2 and x_3 are the pixel locations of the Stokes, Rayleigh and anti-Stokes peaks of the first order, and x_4, x_5 and x_6 are the pixel locations of the Stokes, Rayleigh and anti-Stokes peaks of the second order. The pixel locations match those identified in Supplementary Figure 2b.

Lorentzian functions were then fitted to the Stokes peak of the second order and the anti-Stokes peak of the first order, and the frequency difference between these peaks, Δf, was calculated (Supplementary Figure 2b). The Brillouin frequency shift ω_b was then calculated according to:

$$\omega_b = \frac{1}{2}(FSR - \Delta f) \quad \text{(S9)}$$

Measurement of Young's modulus

PEO hydrogels

Measurements were performed using a controlled shear rate rheometer (AR 2000; TA Instruments) and Rheology Advantage software (TA Instruments). The rheometer used a cone plate configuration with a 40 mm diameter and 2° cone angle. Excess hydrogel was removed after lowering the cone. We first measured the viscoelastic storage modulus G' at 1 Hz over a range of oscillatory strain (Supplementary Figure 3a). These measurements confirmed that 1% strain lies within the linear elastic range. We then measured G' and the viscoelastic loss modulus, G'', over

a range of frequencies at 1% strain (Supplementary Figure 3b). Assuming incompressibility, the Young's modulus was calculated as $E = 3G'$, where G' was measured at 1 Hz and 1% strain. All measurements were done at 25°C.

PA hydrogels

The instantaneous elastic modulus of PA hydrogels was measured in unconfined uniaxial compression using an Instron Model 5866 fitted with a 50 N load cell. The samples were preloaded to 0.01-0.05 N for one minute, then compressed to 0.7 mm at a crosshead speed of 0.5 mm/min for 2 or 3 cycles. Digital calipers with a precision of 0.01 mm were used to measure the thickness of the hydrogel and its diameter prior to compression, from which the area was calculated. Stress and strain were then calculated, and the compression modulus was obtained from a linear regression applied to the second or third cycle of the stress-strain curve (Supplementary Figure 4). All measurements were done at room temperature.

Reporting summary

Further information on the experimental design is available in the Life Sciences Reporting Summary linked to this article.

Code availability

The custom Matlab code to analyze the data is available on request.

Data availability

The data sets generated and analyzed during the current study are available on request.

References

1. Tse, J. R. & Engler, A. J. Preparation of hydrogel substrates with tunable mechanical properties. *Curr. Protoc. Cell Biol.* **47**, 10.16.1-10.16.16 (2010).

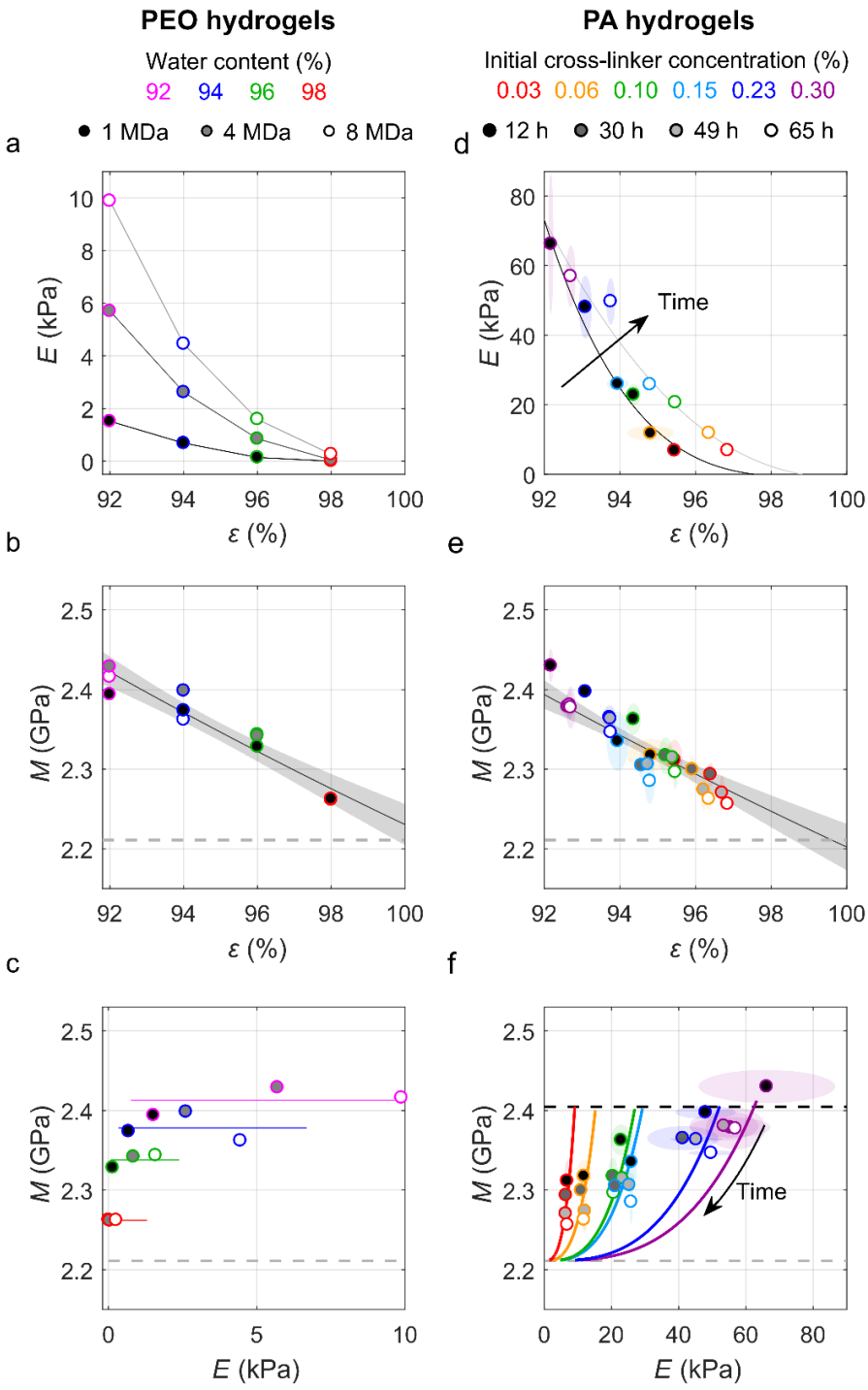
2. Lepert, G., Gouveia, R. M., Connon, C. J. & Paterson, C. Assessing corneal biomechanics with Brillouin spectro-microscopy. *Faraday Discussions* **187**, 415-428 (2016)

3. Wilson, T & Sheppard, C. J. R: *Theory and practice of scanning optical microscopy* (Academic Press, London, 1984).

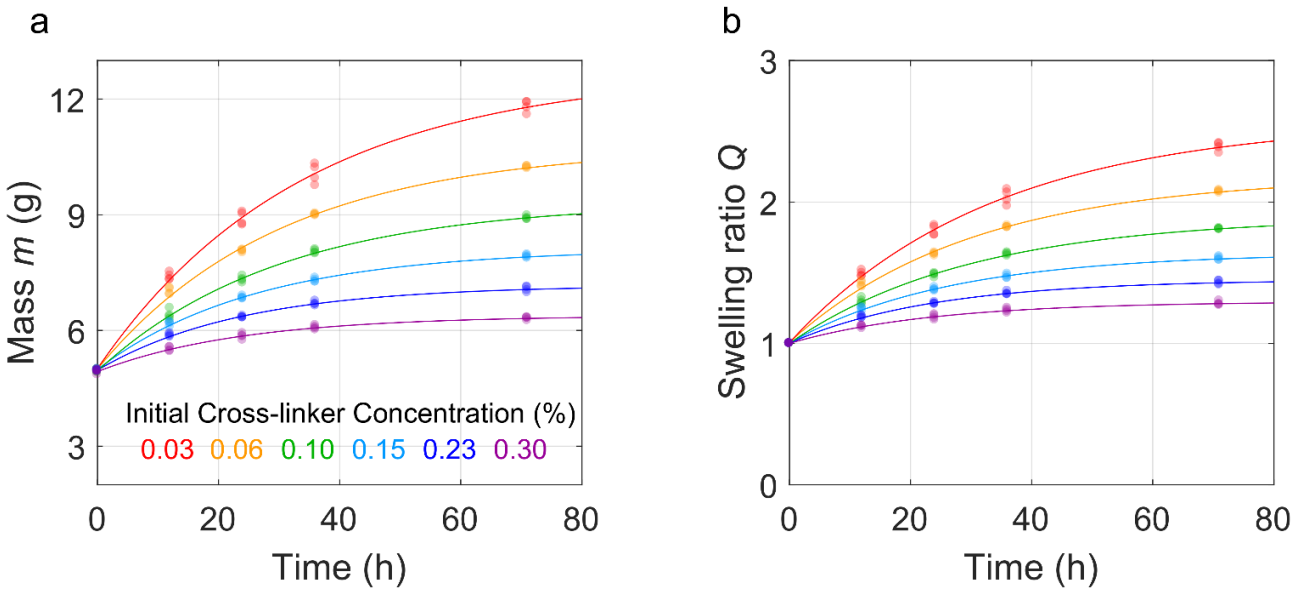
Supplementary Note

The data shown in the main text were replicated in a separate set of experiments. PEO and PA hydrogels were prepared and analyzed as described in Supplementary Methods. PEO hydrogels included 1, 4 and 8 MDa with ε = 0.92, 0.94, 0.96 and 0.98. Young's modulus E for PEO hydrogels was measured by rheometry. PA hydrogels included 0.03, 0.06, 0.10, 0.15, 0.23 or 0.30% (w/v) bis-acrylamide and 10% (w/v) N-methylene-bis-acrylamide initial concentration, measured at 12, 30, 49 and 65 hrs after polymerization. PA hydrogels were polymerized in disk-shaped molds of 20 mm diameter and 5 mm thickness. Young's modulus E for PA hydrogels was measured by unconfined uniaxial compression, with a maximum displacement of 0.25 mm. The longitudinal modulus M was measured by Brillouin microscopy. For the PEO studies, the VIPA spectrometer had a resolution of 0.4 GHz, a finesse of 70 and an FSR of 30 GHz. For the PA studies, the VIPA spectrometer had a resolution of 0.7 GHz, a finesse of 56 and an FSR of 39 GHz.

This second set of experiments exhibited the same behavior as shown the main text. Briefly, E for PEO hydrogels increased with increasing molecular weight for any given value of ε due to increased entanglement between polymer chains (Supplementary Figure SN1a). All values of M collapsed onto a single curve predicted by Equation 2 of the main text when plotted versus ε (Supplementary Figure SN1b), and there was no clear relationship between M and E (Supplementary Figure SN1c). For PA hydrogels, E decreased with increasing ε (Supplementary Figure SN1d). Despite different swelling times and cross-linker concentrations, all values of M collapsed onto a single relationship that depended on ε, as predicted by Equation 2 of the main text (Supplementary Figure SN1e). No single relationship could describe M in terms of E, but Equations 2 and 3 from the main text predicted the trajectory of how M and E changed during swelling (Supplementary Figure SN1f).



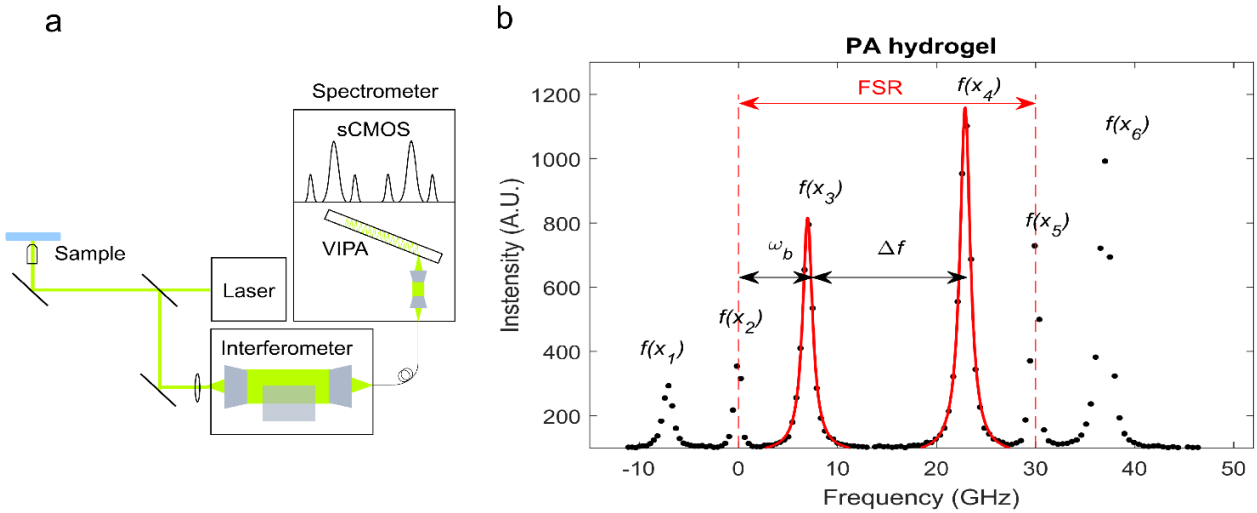
Supplementary Figure SN1: Replicate experiments for PEO (a-c) and PA (d-f) hydrogels give similar results as shown in the main text. For PEO hydrogels, each data point represents a single hydrogel, with colors and marker shading representing water content and molecular weight, respectively. For PA hydrogels, each data point represents the mean of 4 hydrogels, and ellipses represents the 95% confidence bounds on the data (±2 SD in each direction). Colors and marker shading represent the initial cross-linker concentrations and swelling times, respectively. These figures otherwise follow the format described in the main text.



Supplementary Figure 1

Polyacrylamide hydrogel swelling.

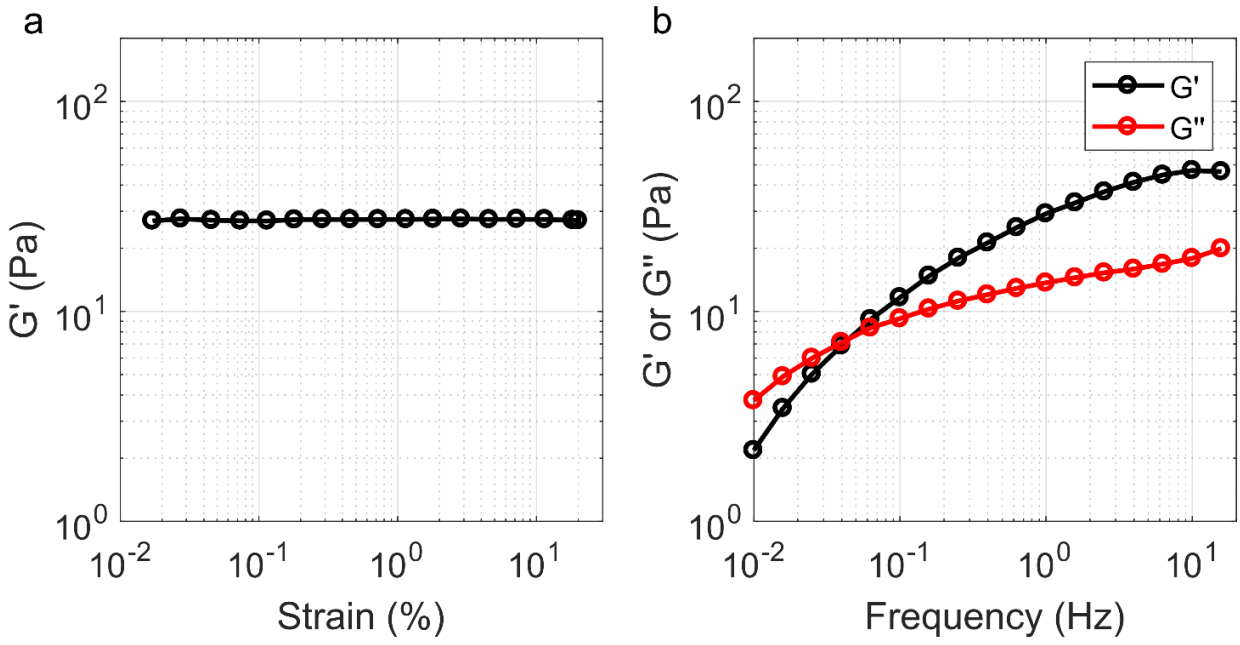
a, Hydrogel mass m increased during swelling, with larger swelling observed for lower cross-linker concentrations. **b**, The swelling ratio Q increased during swelling, as calculated by equation S6. Data are taken from the studies shown in Fig. 1c,d. Each data point represents an individual hydrogel made from the same stock solution. Data from four hydrogels are shown here. Curves show exponential fits.



Supplementary Figure 2

Setup of the Brillouin microscope and associated frequency analysis.

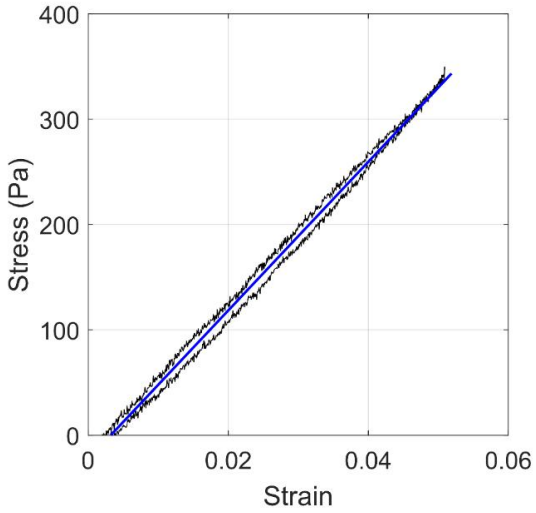
a, Schematic of the Brillouin microscope. Laser light is directed into an inverted confocal microscope. Backscattered light is collected and filtered by an interferometer to reduce the intensity of the Rayleigh peak by up to 40 dB. The filtered signal is passes through a VIPA to separate spectral components that are detected by an sCMOS camera. **b**, Pixel locations in the spectrum are converted into frequency (Supplementary Methods) to identify the Brillouin frequency shift ω_b after the peaks are fitted by a Lorentzian function, where $\omega_b = (\mathrm{FSR} - \Delta f)/2$. FSR, full spectral range. $f(x_i)$ represents the frequency at pixel location x_i, as needed for equation S8. Similar results were obtained for each individual Brillouin measurement, 50 of which were acquired at each location, averaging over three locations per hydrogel.



Supplementary Figure 3

Representative measurement of the Young's modulus of a PEO hydrogel by rheometry.

a, The viscoelastic storage modulus (G') as a function of oscillatory strain magnitude. **b**, The viscoelastic loss modulus (G'') as a function of frequency. Young's modulus E was calculated as $E = 3\,G'$. For this sample, the molecular weight was 8 MDa with ε = 1.5%. Similar results were obtained for each of the 2–3 hydrogel samples per condition.



Supplementary Figure 4

Representative measurement of the Young's modulus of a PA hydrogel by uniaxial unconfined compression.

The stress-strain data were used to calculate Young's modulus as the slope of the linear regression to a full cycle (blue line). The initial bis-acrylamide concentration of this sample was 0.06%, measured after 12 h of swelling. One similar compression measurement was done per hydrogel per time point.