Electronic Supplementary Material (ESI) for Soft Matter.

Supplementary materials: Measuring mechanical cues for modeling stromal matrix in 3D cell culture

Linda Srbova[1], Ossi Arasalo[1], Arttu J. Lehtonen[1], and Juho Pokki[1,*]

1 Department of Electrical Engineering and Automation, Aalto University, Espoo, Finland

* Corresponding author, E-mail: juho.pokki@aalto.fi

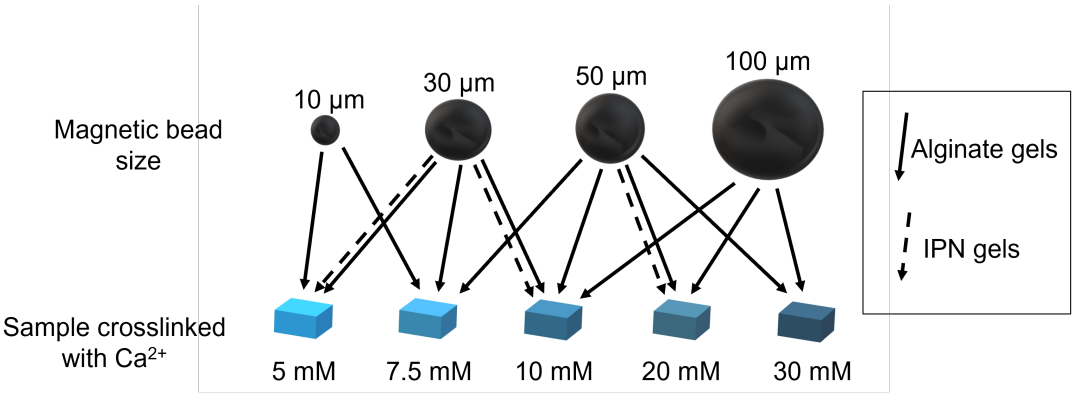

Figure S1: Diagram outlining the nominal diameters of the magnetic probes used to measure the hydrogels at the different crosslinker concentrations using the magnetic microrheometry.

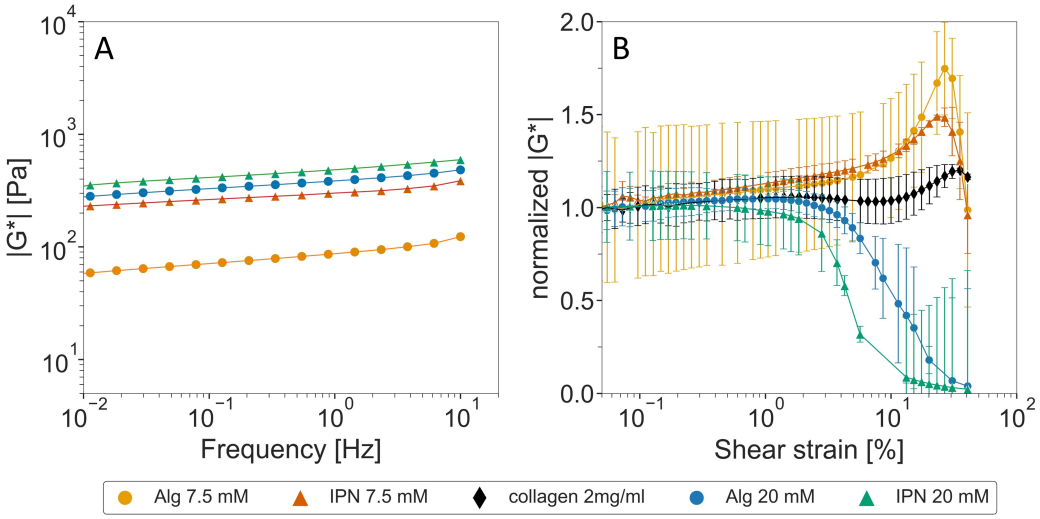

Figure S2: Oscillatory frequency and strain sweep tests in determination of the linear viscoelastic region (LVR) for the alginate and IPN hydrogels.
(A) Absolute shear modulus ($|G^*|$) as a function of frequency. Frequency sweeps of the alginate and IPN hydrogels measured from 0.01 to 10 Hz, at a 1 % strain and at a temperature of 20°C after 40 min of gelation. One measurement was performed for each condition.
(B) Values of $|G^*|$ normalized by the LVR value at 1 %. The mean values of the normalized $|G^*|$ are shown as a function of an oscillatory strain, with the normalized standard deviations in error bars (i.e. coefficient of variation). The values were obtained using strain sweeps measured from 0.05 % strain until a sample rupture. Two repetitions were performed for each condition.

Section S1: Bayesian modeling methods

A Bayesian model was constructed based on our previous work [4] to quantify the relationship between the hydrogel mechanical properties, and the degree of crosslinking, elevating hydrogel stiffness. The model enables comparisons of different experimental conditions, which are challenging based on the raw data alone, because the sample sizes vary, and larger magnetic probes are needed in stiffer hydrogels. Here, we have used a (piecewise) linear function with a Gaussian process (Eqs. 1–10) to model the relationship between the hydrogel viscoelasticity ($|G^*|$ and Φ) and the degree of crosslinking. We have taken into account the systematic biases, induced by the choice of the magnetic probes (Eqs. 11–16). Specifically, for the purpose, we have obtained posterior distributions that have been used to study the underlying mechanical behaviour of the hydrogel material, while we have minimized the contribution of the biases and measurement uncertainty.

Mean behavior

$$\mu^\gamma, z_i^\gamma, k_i^1, k_i^2, b_i^1 \sim \text{Normal}(0,1) \tag{1}$$
$$\sigma^\gamma \sim \text{Half-Normal}(1) \tag{2}$$
$$\gamma = \mu^\gamma + \sigma^\gamma z_i^\gamma \tag{3}$$
$$b_i^2 = (k_i^2\gamma_i + b_i^1) - k_i^1\gamma_i \tag{4}$$
$$\mu_i = k_i^2 + \text{S}(\gamma - \text{crosslinker}_i)(k_i^1 - k_i^2) \tag{5}$$
$$b_i = b_i^1 + \text{S}(\gamma - \text{crosslinker}_i)(b_i^2 - b_i^1) \tag{6}$$

SD-like heterogeneity

$$\alpha_i^\sigma \sim \text{Half-Normal}(1) \tag{7}$$
$$\rho_i^\sigma \sim \text{InverseGaussian}(1,3) \tag{8}$$
$$\eta_{ij}^\sigma, \sigma_i \sim \text{Normal}(0,1) \tag{9}$$
$$\mathbf{L}_i^\sigma = \text{Cholesky}(k(\text{crosslinker}_i|\alpha_i^\sigma, \rho_i^\sigma)) \tag{10}$$

Probe diameter effect

$$\sigma^{\text{probe}}, \sigma^{\text{probe }\sigma} \sim \text{Half-Normal}(1) \tag{11}$$
$$z_l^{\text{probe}}, z_l^{\text{probe }\sigma} \sim \text{Normal}(0,1) \tag{12}$$
$$\mu_l^{\text{probe}}, \mu_l^{\text{probe }\sigma} = \sigma^{\text{probe}} z_l^{\text{probe}}, \sigma^{\text{probe }\sigma} z_l^{\text{probe }\sigma} \tag{13}$$

Probe coating effect

$$\sigma^{\text{coating}} \sim \text{Half-Normal}(1) \tag{14}$$
$$z_k^{\text{coating}} \sim \text{Normal}(0,1) \tag{15}$$
$$\mu_k^{\text{coating}} = \sigma^{\text{coating}} z_k^{\text{coating}} \tag{16}$$

Experimental design

$$\sigma^{\text{sample}}, \sigma^{\text{holder}} \sim \text{Half-Normal}(1) \tag{17}$$
$$z_s^{\text{sample}}, z_h^{\text{holder}} \sim \text{Normal}(0,1) \tag{18}$$
$$\mu_s^{\text{sample}}, \mu_h^{\text{holder}} = \sigma^{\text{sample}} z_s^{\text{sample}}, \sigma^{\text{holder}} z_h^{\text{holder}} \tag{19}$$

Collagen conc. and temp.

$$\sigma^{\text{conc}}, \sigma^{\text{temp}} \sim \text{Half-Normal}(1) \tag{20}$$
$$z_c^{\text{conc}}, z_t^{\text{temp}} \sim \text{Normal}(0,1) \tag{21}$$
$$\mu_c^{\text{conc }\sigma}, \mu_t^{\text{temp }\sigma} = \sigma^{\text{conc}} z_c^{\text{conc}}, \sigma^{\text{temp}} z_t^{\text{temp}} \tag{22}$$
$$k^{\text{conc}}, b^{\text{conc}}, k^{\text{temp}}, b^{\text{temp}} \sim \text{Normal}(0,1) \tag{23}$$
$$\mu^{\text{conc}}, \mu^{\text{temp}} = k^{\text{conc}}\text{conc} + b^{\text{conc}}, k^{\text{temp}}\text{temp} + b^{\text{temp}} \tag{24}$$

Likelihood

$$|G^*| \sim \text{Normal}(\text{loc} = \mu_i\text{crosslinker}_i + b_i + \mu_{k[j]}^{coating} + \mu_{l[j]}^{\text{probe}} + \mu_{s[j]}^{\text{sample}} + \mu_{h[j]}^{\text{holder}} + \mu_{i[j]}^{\text{conc}} + \mu_{i[j]}^{\text{temp}}, \text{scale} = \text{Softplus}(\mathbf{L}_i^\sigma \eta_i^\sigma + \mu_{l[j]}^{\text{probe }\sigma} + \sigma_i + \mu_{c[j]}^{\text{conc }\sigma} + \mu_{t[j]}^{\text{temp }\sigma})) \tag{25}$$

In the equations, the subscript i denotes for the two measured materials: alginate and IPN hydrogels. The subscript j indicates the observed datapoints. The number of these

datapoints for alginate and IPN hydrogels are different, but the same index j is used for clarity. The further subscripts k, l, s and h denote different probe coatings and sizes, as well as sample and holder assignments used in the measurements, respectively. The brackets in the subscripts indicate the corresponding index assingment for the jth measurement. For instance, $h[j]$ indicates the jth datapoint's holder index.

A logarithmic transformation is taken for both viscoelastic properties ($|G^*|$ and Φ) before modeling the data. Logarithmic transformation is useful because the observed values are all positive and the relationships looks linear in the log-scale, thus simplifying the analysis.

The relationship between the amount of the crosslinker and the observed $|G^*|$ is modeled with a smoothed piecewise linear functions (Eqs. 1-6). Two linear functions cross at a switchpoint (crosslinking saturation point), noted as γ (Eq. 3, which is partially pooled), which is assumed to be similar across differnt hydrogel types (i.e. the alginate concentration is fixed in all IPN hydrogels at 5 mg/mL but due to sample preparation error there can be a certain variation). NB: The transition over this point is smoothed with a sigmoid function, denoted with (S). Further, the variable k_i^1 is the slope of the linear function until the switchpoint, and the variable k_i^2 the slope after the switchpoint. Correspondingly, the variable b_i^1 is the intercept of the first linear function and the letter b_i^2 is the intercept for the second linear function, which is determined so that the lines intersect at the switchpoint.

The heterogeneity in viscoelastic properties at the different crosslinker concentrations has been captured with a Gaussian process (Eqs. 7-10). The covariance function (k) has been chosen to be an exponentiated quadratic function [5]. This function's amplitude has a prior with a significant mass close to zero allowing constant heterogeneity to be a as function of the crosslinker concentration. The length scale parameter (ρ_i^σ) has a (zero) boundary avoiding prior to prevent an unrealistically varying signal.

The probe-related effects on viscoelasticity are covered in Eqs. 11-13. For example, the effects of the probe size on stiffness are visible in Fig. S5A. Thus, the probe-related effects are included in the model and assumed to be additive (Eq. 25) to the mean and heterogeneity values of the data at the logarithmic scale. The effects are assumed to be at the same magnitude for all measured conditions. In other words, for example, the heterogeneity effects of a probe with a 100 μm nominal diameter is the same for both the alginate and IPN hydrogels. Partial pooling (Eq. 13) was used to calculate the effects on both the mean viscoelasticity and the heterogeneity in viscoelasticity. Further, the accounted probe effects include errors caused by the intrinsic properties of the probes (sphericity and magnetization) and their effects on the magnetic force.

Further, the magnetic probe coating is only assumed to affect the mean value of viscoelasticity (Eq. 16), not the heterogeneity in viscoelasticity. This choice has been made by inspecting the raw data as we find no effects of the coating on viscoelasticity in a simple additive manner, as a function of the crosslinker concentration.

Effects of the experimental design have been incorporated in the model similarly as in [4]. These effects have been partially pooled at the holder and sample levels (Eq. 19). We have assumed the effects to be additive to the mean value of the observed measurements. With this choice of the parameters, the heterogeneity is assumed to be the variation within holders, as a function of the crosslinker concentration.

Collagen concentration and the effects of the temperature are assumed to affect the mean value with a linear trend as in Eqs. 23-24. On the other hand, their effects are assumed to affect the heterogeneity similarly as coating and probe effects, Eqs. 20-22. Different temperature and collagen concentration were experimented only with IPN samples, so these parameters only affect only IPN observations. The indices c and t "pick" the correct value

for each IPN measurement similar to all other effects in the model.

This defined model formulates how the crosslinker concentration has been assumed to affect the $|G^*|$, both its magnitude and heterogeneity within each sample. This same model has also been used for modeling Φ, however, for this modeling the magnitude has been assumed to be a single linear function, without a switchpoint. In this case, Eqs. 4-6 and the parameters γ and k_i^2 are unnecessary, and the mean of the likelihood (Eq. 25) simplifies to: $k_i^2 \text{crosslinker}_i + b_i^1 + \mu_{k[j]}^{coating} + \mu_{l[j]}^{\text{probe}} + \mu_{s[j]}^{\text{sample}} + \mu_{h[j]}^{\text{holder}} + \mu_{i[j]}^{\text{conc}} + \mu_{i[j]}^{\text{temp}}$.

The rheometry data has been also modeled with this same model but without the probe effects, because they have not been used in the measurements. The same switchpoint parameter has been used for both microrheometry and rheometry. The rheometry data has been normalized with the same constants as microrheometry. NB: It must be noted that the heterogeneity measured from the rheometry data is the variation between repeated measurements, therefore, these properties are not compared as the meaning would be ambiguous.

Section S2: Results on mechanics and mechanical heterogeneity

Mechanics of the hydrogels

Table S1: Composition of the alginate hydrogel samples at an alginate concentration of 5 mg/mL. Also, the magnetic and reference probe information are noted.

Crosslinker [mM]	5.0	7.5	10.0	20.0	30.0	syringe
magnetic probes [µL]	75	75	75	75	75	1
reference probes [µL]	75	75	75	75	75	2
vol. from alginate stock (7 mg/mL) [µL]	750	750	750	750	750	1
DMEM [µL]	143	139.5	136	122	108	2
0.75 M crosslinker [µL]	7	10.5	14	28	42	2
mixing times	15x	20x	18x	12x	10x	NA

Table S2: Summary of viscoelastic properties of the alginate samples, defined by rheometry (macro) and microrheometry (micro), for each crosslinker concentration.

		$\lvert G^* \rvert$ [Pa]		Φ [rad]	
Method	Crosslinker [mM]	Mean (SD)	Median	Mean (SD)	Median
macro	5.0	16 (6)	14	0.1 (0.00)	0.10
macro	7.5	134 (48)	128	0.12 (0.04)	0.10
macro	10.0	192 (71)	163	0.09 (0.00)	0.09
macro	20.0	576 (112)	578	0.13 (0.06)	0.10
macro	30.0	924 (533)	1024	0.19 (0.09)	0.14
micro	5.0	47 (86)	12	0.13 (0.06)	0.12
micro	7.5	111 (236)	34	0.18 (0.11)	0.16
micro	10.0	203 (196)	136	0.16 (0.06)	0.15
micro	20.0	695 (663)	494	0.17 (0.08)	0.14
micro	30.0	267 (503)	90	0.19 (0.09)	0.16

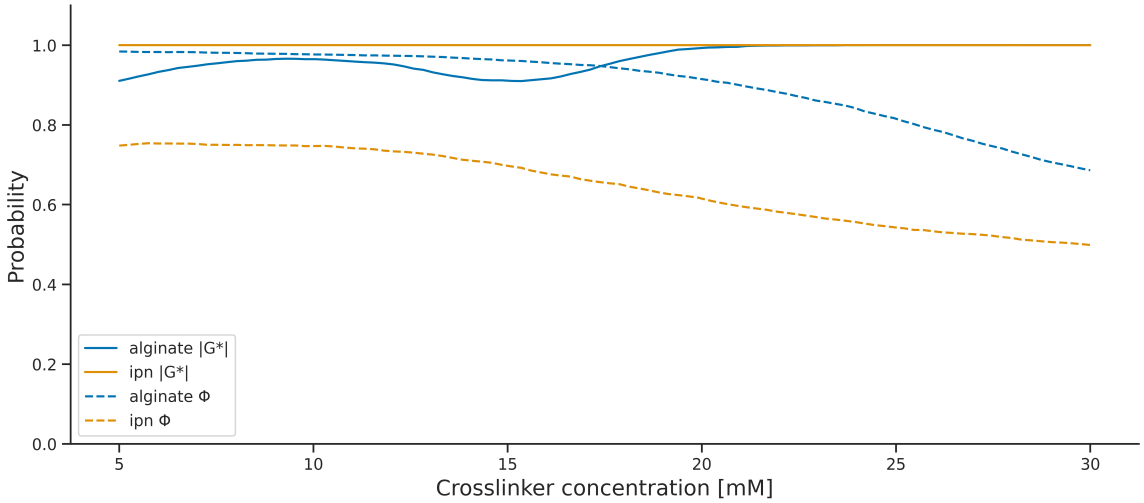

Figure S3: Comparisons of the mean stiffness values for the rheometry and microrheometry results in respect to posterior probability. Lines indicate the posterior probability in the direction of the difference between rheometry and microrheometry. For absolute shear moduli ($|G^*|$) (solid line), the high values in the direction of difference denote that the rheometry gives larger values than microrheometry. For phase-shift angles (Φ) (dashed line), the direction of the difference is the opposite: the high values denote that the microrheometry gives larger values than rheometry. The differences between the mean values that are over 0.1 are considered to be practically relevant.

Table S3: Posterior probabilities comparing the direction of difference between the rheometry and microrheometry. For absolute shear moduli ($|G^*|$), the high values in the direction of difference denote that the rheometry gives larger values than microrheometry. For phase-shift angles (Φ), the direction of the difference is the opposite: the high values denote that the microrheometry gives larger values than rheometry.

Crosslinker [mM]	$\|G^*\|$		Φ	
	alginate	IPN	alginate	IPN
5.0	91.0	99.9	97.0	62.9
7.5	95.4	NA	96.7	NA
10	96.5	99.9	96.3	60.0
20	99.3	99.9	85.5	46.5
30	99.9	NA	58.3	NA

Table S4: Posterior probabilities for the trend being positive, and increasing as a function of the crosslinker until the switchpoint (saturation point), and after that. NB: For Φ, this is just the slope parameter, because no switchpoint has been assumed (both probabilities are the same). The values above 0.1*std are assumed to have a practical effect (i.e the values are calculated as $P(k_i > 0.1 * std)$).

Condition	Probability before switchpoint		Probability after switchpoint	
	$\|G^*\|$	Φ	$\|G^*\|$	Φ
Microrheometry: alginate	99.9	59.6	0.2	59.6
Microrheometry: IPN	98.5	24.4	37.9	24.4
Rheometry: alginate	98.2	89.3	84.0	89.3
Rheometry: IPN	81.8	47.5	97.7	47.5

Table S5: Composition of the IPN hydrogels with 5 mg/mL of alginate, for the varied collagen and Ca^{2+} alginate-crosslinker concentrations.

Crosslinker [mM]	5.0	10.0	20.0	collagen [mg/ml]	syringe
Magnetic probes [µL]	75	75	75	1,2,3	1
Reference probes [µL]	75	75	75	1,2,3	2
Alginate (13.1 mg/mL) [µL]	400	400	400	1,2,3	1
Ca^{2+} crosslinker 0.75 M [µL]	7	14	28	1,2,3	2
DMEM/F12 [µL]	382.3	375.3	361,3	1	2
	271.5	264.5	250.5	2	2
	160.8	153.8	139.8	3	2
Collagen (9.48 mg/mL) [µL]	110.8	110.8	110.8	1	2
	221.5	221.5	221.5	2	2
	332.3	332.3	332.3	3	2

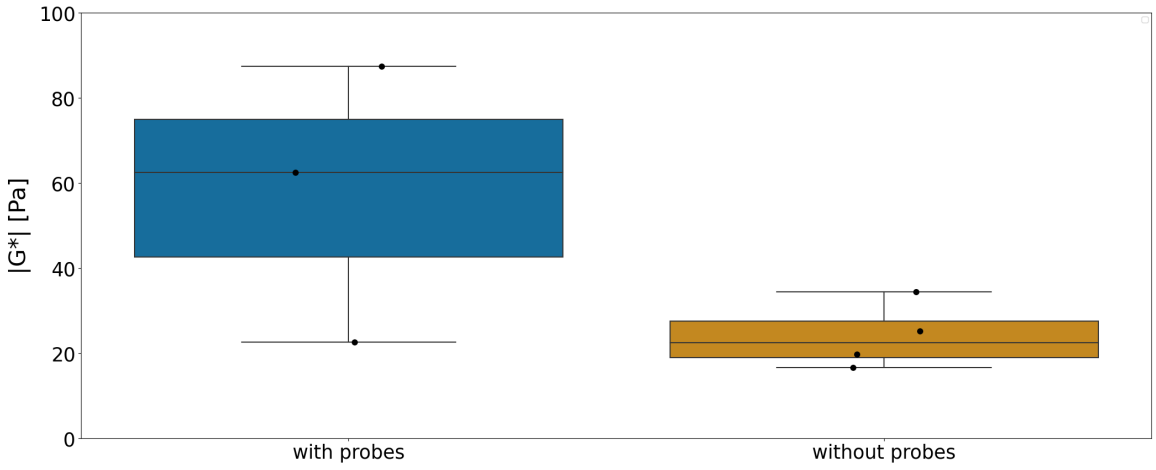

Figure S4: Comparison of absolute shear moduli ($|G^*|$) of the alginate hydrogels with a 5 mM concentration of the crosslinker, with and without the magnetic probes.

Table S6: Summary of viscoelastic properties of the IPN samples, defined by rheometry (macro) and microrheometry (micro), for each crosslinker concentration.

		$\|G^*\|$ [Pa]		Φ [rad]	
Method	Crosslinker [mM]	Mean (SD)	Median	Mean (SD)	Median
macro	5.0	256 (291)	133	0.14 (0.03)	0.12
macro	10.0	643 (93)	673	0.12 (0.01)	0.12
macro	20.0	1403 (213)	1293	0.14 (0.03)	0.14
micro	5.0	19 (22)	11	0.12 (0.06)	0.12
micro	10.0	86 (124)	27	0.18 (0.12)	0.16
micro	20.0	464 (430)	344	0.15 (0.07)	0.14

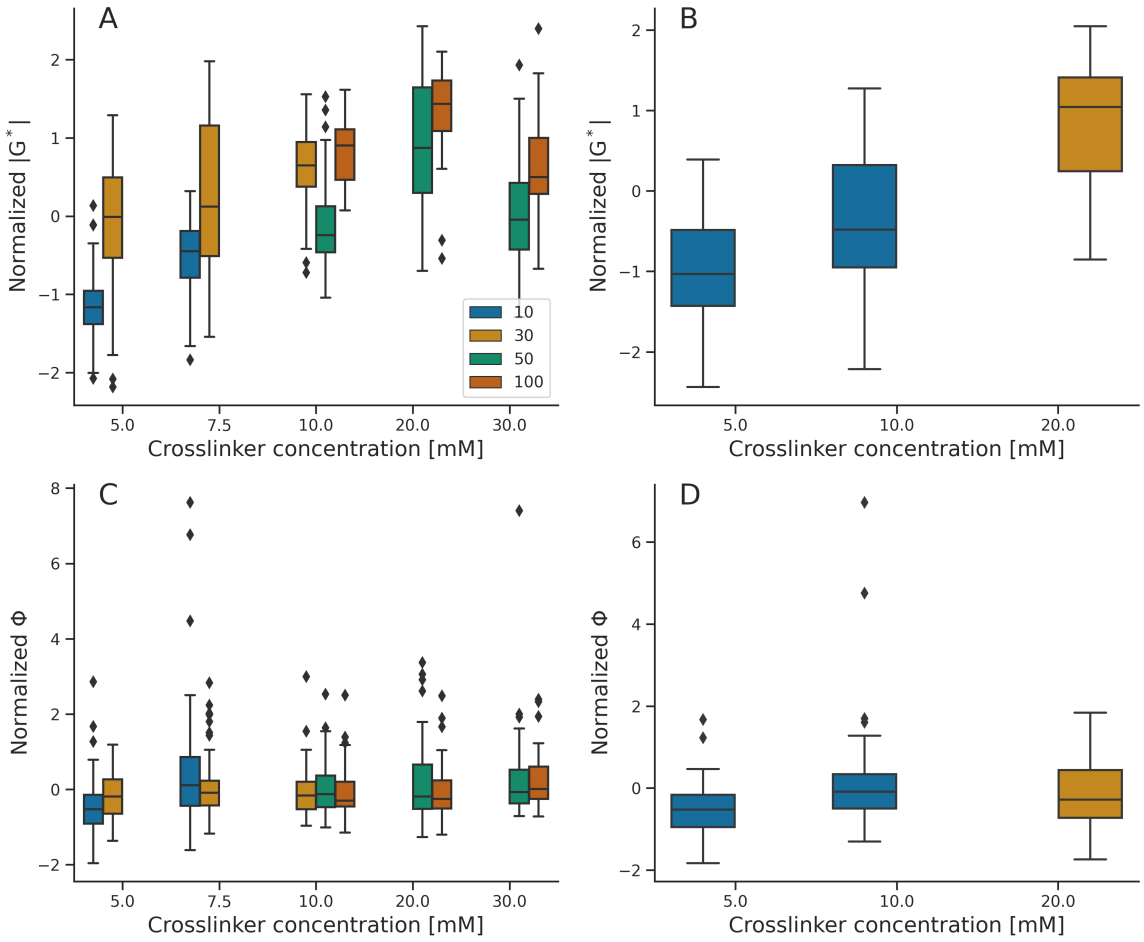

Figure S5: Magnetic microrheometer measurements grouped by the magnetic-probe size. (A-B) show the normalized values of $|G^*|$, and (C-D) show the normalized values of Φ. Alginate hydrogels are in (A, C) and the IPN hydrogels are in (B, D), as a function of the crosslinker.

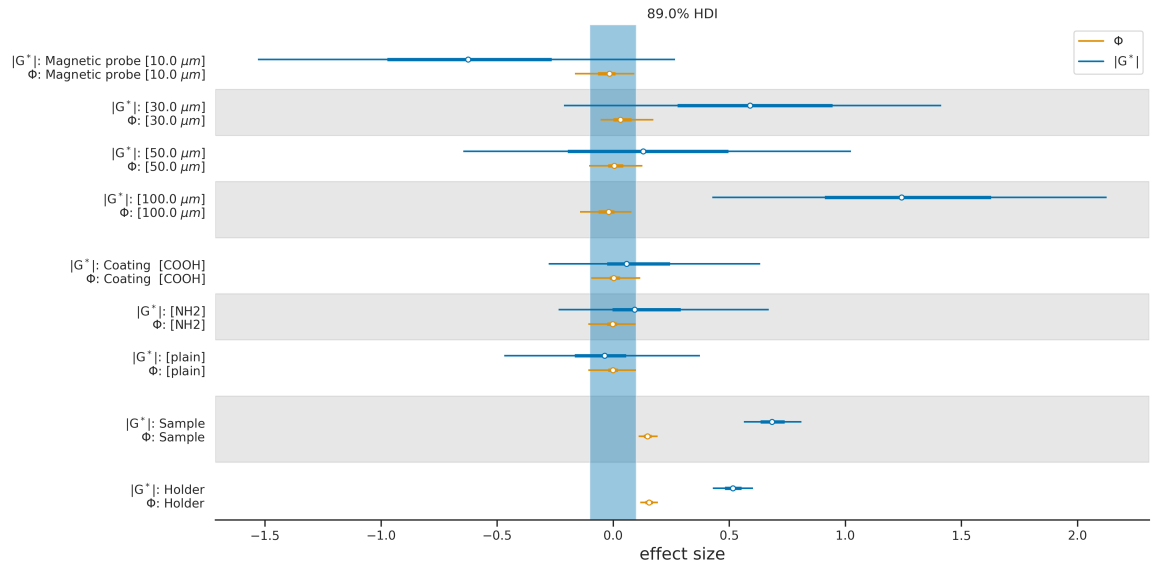

Figure S6: Estimated probe effects for the $|G^*|$ and Φ values. The probe size has a notable effect to the mean $|G^*|$ values. On the other hand, the probe coating effects on the mean $|G^*|$ and Φ values are non-existent. Sample and holder effects,especially effects to the $|G^*|$ are large indicating high contribution from experimental errors. Blue area is the region of practical equivalence (ROPE), which is defined to be $[-0.1, 0.1]$ of the standard deviation of the data [2].

Heterogeneity in viscoelasticity

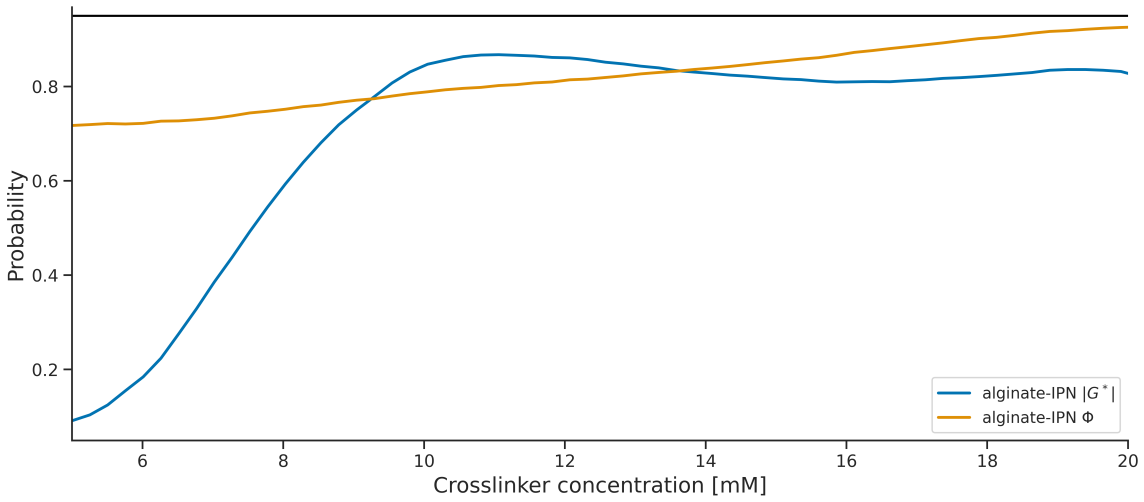

Figure S7: Probability of the alginate hydrogels having a lower heterogeneity in viscoelasticity than the IPN hydrogels, specifically, in $|G^*|$ and in Φ across the different crosslinker concentrations. The black line indicates a 95% significance threshold. The differences in the heterogeneity values are assumed to be pratically relevant if they are more than 0.1 of the standard deviation.

Table S7: Probability values comparing the increase in the heterogeneity for different pairs (m1 and m2).

Material	Crosslinker concentration		Pr(m2-m1)	
	m1	m2	$\|G^*\|$	Φ
alginate	10.0	20.0	49.19	3.42
alginate	10.0	30.0	88.78	1.38
alginate	20.0	30.0	61.26	0.1
alginate	5.0	10.0	2.27	0.18
alginate	5.0	20.0	42.29	5.54
alginate	5.0	30.0	82.05	2.48
alginate	5.0	7.5	0.04	0.0
alginate	7.5	10.0	2.81	0.15
alginate	7.5	20.0	50.93	5.72
alginate	7.5	30.0	87.94	2.35
IPN	10.0	20.0	51.59	85.74
IPN	5.0	10.0	99.84	5.26
IPN	5.0	20.0	99.9	93.38

Non-linear mechanical responses of the hydrogels

Table S8: Composition of collagen type 1 sample at a concentration of 5 mg/mL was used as a reference material for comparing the non-linear response behavior of the hydrogels.

	volume [µL]	syringe
DMEM/F12	678.5	1
Magnetic probes 10 µm	75	2
Reference particles	75	1
Collagen (9.48 mg/ml)	221.5	2

Probe displacements

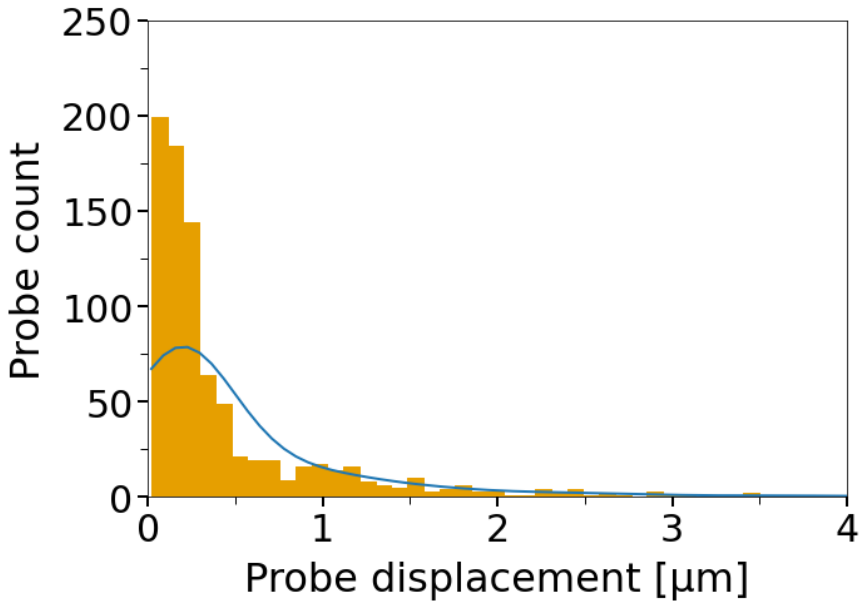

Figure S8: The histogram (orange) of displacements of all magnetic probes from data shown in Figs. 2 and 3. The displacement values are the maximum travelled distance in one direction from a probe's initial location.

Gelation of the hydrogels

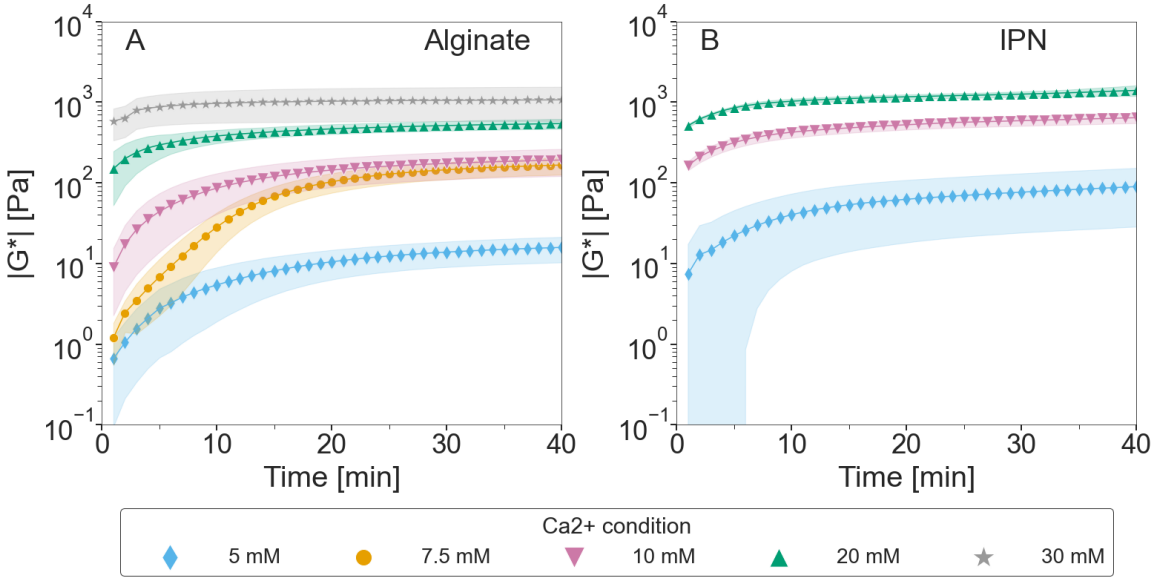

Figure S9: Oscillatory time sweep tests capture the kinetics of the gelation process ($|G^*|$ as a function of time) of alginate and IPN hydrogels at different crosslinking conditions, measured at 1 % strain and 5 Hz frequency using plate-plate rheometer. The plateau region of $|G^*|$ indicates the completion of network formation. The gelation curves for alginate (A) were collected at 20 °C and the gelation curves for IPN (B) were collected at 37 °C. Each hydrogel condition shows in average 2-6 replicates.

Section S3: The Bayesian model posterior distributions and model checking

Posterior distributions

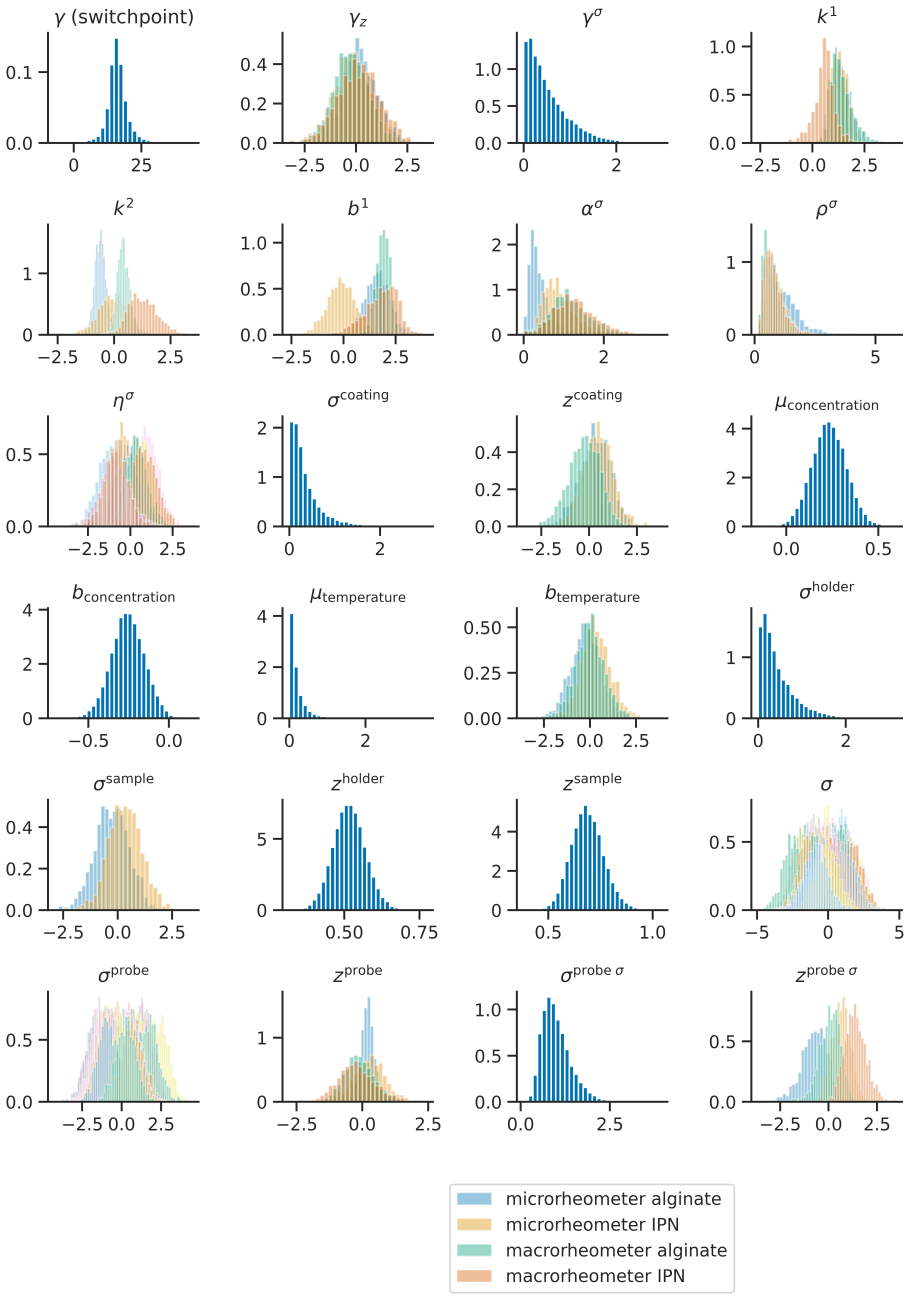

Figure S10: Posterior distributions for $|G^*|$.

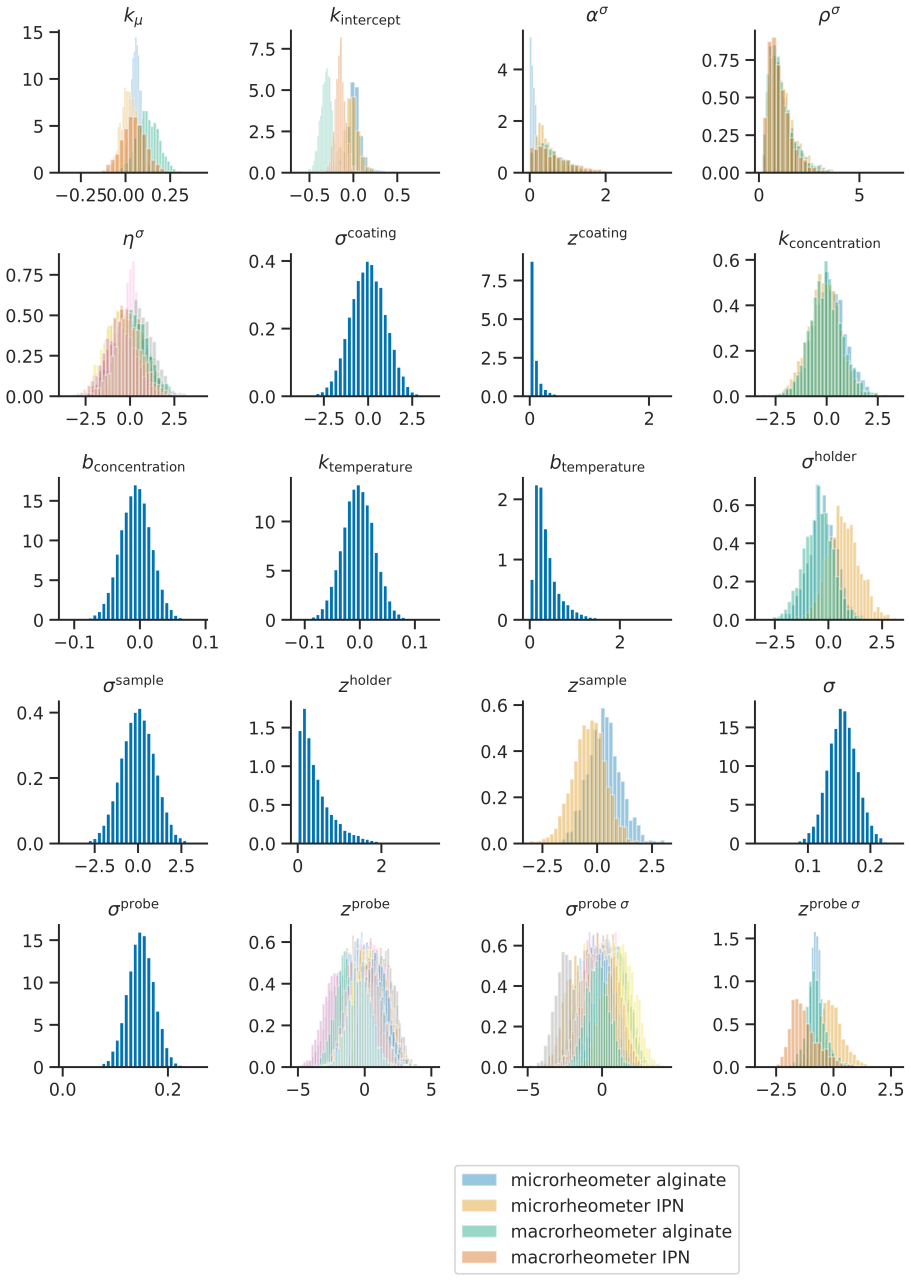

Figure S11: Posterior distributions for Φ.

Model checking

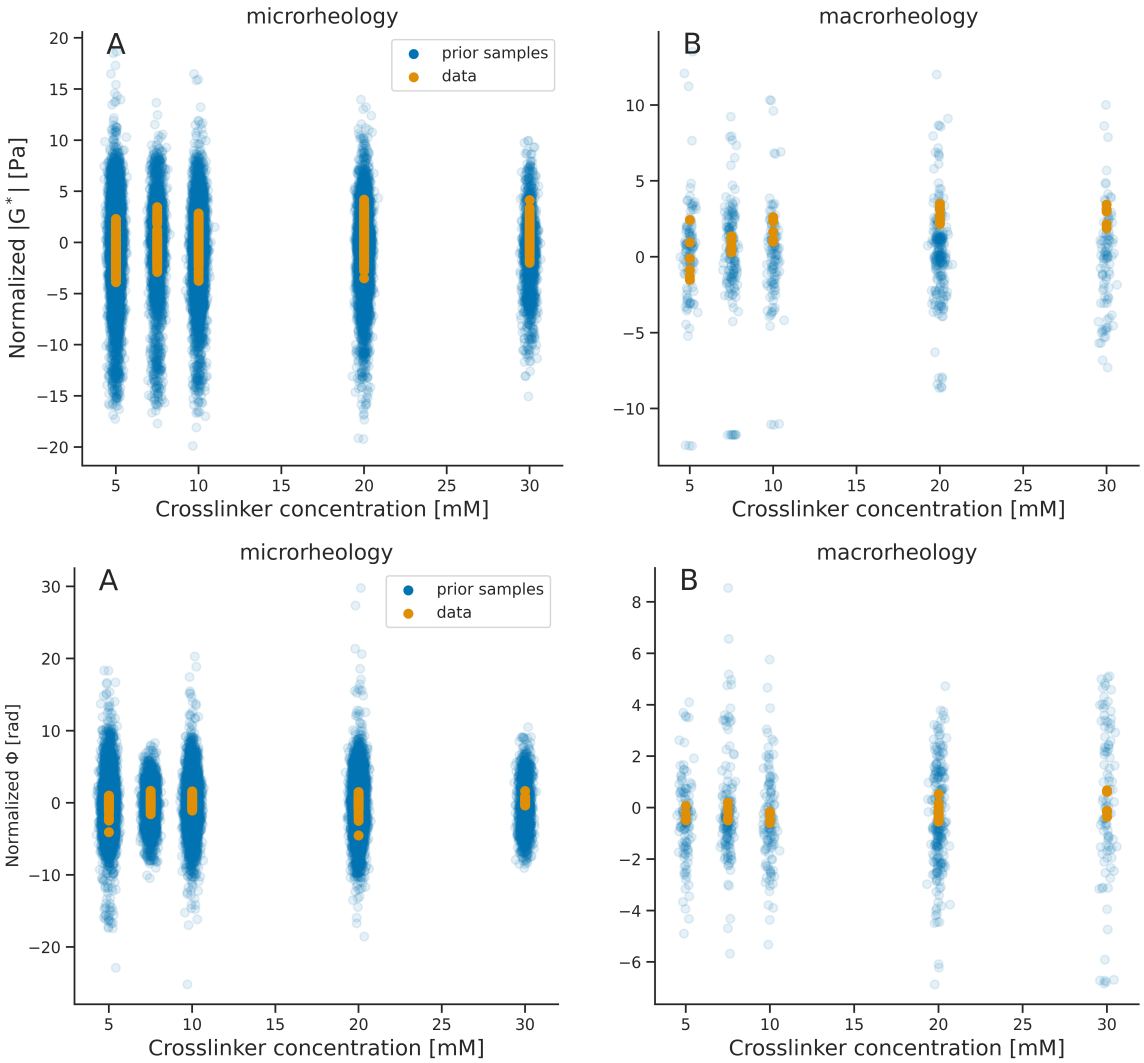

Figure S12: Prior draws from the model estimating $|G^*|$ (A, B) and Φ (C, D) for both microrheometry and rheometry.

The prior predictive samples can be seen in Fig. S12. The datapoints cover the range of the observed data. The model was implemented in the Tensorflow probability and the NUTS sampler was used for MCMC [1]. The MCMC diagnostics are as follows, all $\hat{R}$ (potential scale reduction factor) values are all below 1.1, effective sample sizes (ESS) are above 400 (4 parallel chains) and the Bayesian fraction of missing information (BFMI) is above 0.3. The posterior samples overlapped with the data, and the related Fig. The Arviz visualization library was used to check the posterior traces [3]. The code is also available in `https://github.com/arasalo1/switchpoint`.

References

[1] Joshua V Dillon, Ian Langmore, Dustin Tran, Eugene Brevdo, Srinivas Vasudevan, Dave Moore, Brian Patton, Alex Alemi, Matt Hoffman, and Rif A Saurous. Tensorflow distributions. arXiv preprint arXiv:1711.10604, 2017.

[2] John K Kruschke and Torrin M Liddell. The bayesian new statistics: Hypothesis testing, estimation, meta-analysis, and power analysis from a bayesian perspective. Psychonomic bulletin & review, 25:178–206, 2018.

[3] Ravin Kumar, Colin Carroll, Ari Hartikainen, and Osvaldo Martin. Arviz a unified library for exploratory analysis of bayesian models in python. Journal of Open Source Software, 4(33):1143, 2019.

[4] Arttu J. Lehtonen, Ossi Arasalo, Linda Srbova, Maria Heilala, and Juho Pokki. Magnetic microrheometry of tumor-relevant stiffness levels and probabilistic quantification of viscoelasticity differences inside 3d cell culture matrices. PLOS ONE, 18(3):1–23, 03 2023.

[5] Carl Edward Rasmussen and Christopher K. I. Williams. Gaussian processes for machine learning. Adaptive computation and machine learning. MIT Press, 2006.