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A Novel Method to Make Polyacrylamide Gels with Mechanical Properties Resembling those of Biological Tissues

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[Abstract] Studies characterizing how cells respond to the mechanical properties of their environment have been enabled by the use of soft elastomers and hydrogels as substrates for cell culture. A limitation of most such substrates is that, although their elastic properties can be accurately controlled, their viscous properties cannot, and cells respond to both elasticity and viscosity in the extracellular material to which they bind. Some approaches to endow soft substrates with viscosity as well as elasticity are based on coupling static and dynamic crosslinks in series within polymer networks or forming gels with a combination of sparse chemical crosslinks and steric entanglements. These materials form viscoelastic fluids that have revealed significant effects of viscous dissipation on cell function; however, they do not completely capture the mechanical features of soft solid tissues. In this report, we describe a method to make viscoelastic solids that more closely mimic some soft tissues using a combination of crosslinked networks and entrapped linear polymers. Both the elastic and viscous moduli of these substrates can be altered separately, and methods to attach cells to either the elastic or the viscous part of the network are described.

Graphic abstract:

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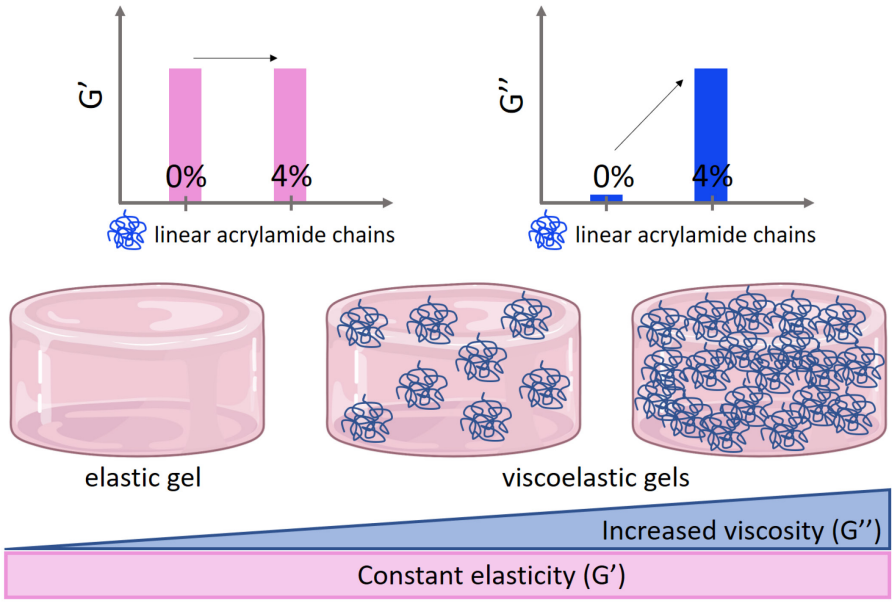

Polyacrylamide gels with independently controlled elasticity and viscosity.

Keywords: Viscoelasticity, Polyacrylamide, Viscosity sensing, Mechanosensing, Extracellular matrix

[Background] Biological tissues are viscoelastic materials that combine features of elastic solids and viscous fluids. Different tissues contain different amounts of the components that contribute to both viscosity (*e.g.*, hyaluronic acid) and elasticity (*e.g.*, collagen fibers), and their structure and proportion change during pathological processes. The widespread use of elastomers or hydrogels as substrates for cell culture (Pelham and Wang, 1997) has revealed the strong impact of substrate mechanics on cell structure and function and focused attention on the limited view of cell biology derived from studies of cells on traditional glass or plastic substrates (Janmey *et al.*, 2020). Until recently, studies of cellular response to substrate or matrix stiffness only examined how cells respond to elastic modulus alterations, with the viscous contribution being either neglected or uncontrolled. Theories of cell mechanosensing have also generally focused on the elastic resistance of the substrate. These limitations were largely due to the lack of suitable materials in which viscosity can be systematically varied within a viscoelastic substrate. There now exist three general approaches for introducing viscous dissipation in a viscoelastic substrate. In one, a polymerizing and crosslinking system, like a polyacrylamide gel, is designed to produce a network near the threshold of the sol-gel transition, such that there are significant elastic properties but also significant viscosity (Cameron *et al.*, 2011). A second method forms crosslinked polymer networks with two classes of crosslinkers: one is covalent and static and the other is non-covalent and dynamic. These first two classes of material are viscoelastic fluids, or viscoplastic material, meaning that if subjected to constant stress, they can deform without limits, as the crosslinks or entanglements rearrange to minimize energy in the deformed state (Chaudhuri *et al.*, 2016). The third class of material, which is the topic of this report, is viscoelastic solids. These materials dissipate substantial energy when they are deformed and slowly continue to deform in response to constant stress;

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however, eventually, they reach a steady state of deformation (strain) that depends on the magnitude of stress but not on the duration that it is applied. A detailed description of the differences between viscoelastic liquids and solids and a summary of how these three classes of material are constructed are provided in Chaudhuri *et al*. (2020).

Synthetic polyacrylamide (PAAm) hydrogels are widely used as a model system to study the effect of tissue elasticity on its behavior in health and disease (Beningo *et al*., 2002). PAAm can be easily tuned to the range of stiffness that reflects the physiological environment of cells, *i.e.,* from hundreds of Pascals up to tens of kilo Pascals. While the role of tissue elasticity in cell biology has been powerfully illustrated, little is known about the viscous aspect of tissue mechanics and how it determines cell structure and fate.

Upon polymerization, polyacrylamide hydrogels create a nearly purely elastic network, with a viscosity several orders of magnitude smaller than the elasticity (Basu *et al*., 2011). While many reports focus on controlling the elastic properties of hydrogels, our aim was to develop a strategy to introduce and control the viscosity in a viscoelastic network. Our protocol describes the preparation of high molecular weight linear polyacrylamide chains, which can be subsequently sterically entrapped in the crosslinked polyacrylamide network to serve as a viscous component of the gel that dissipates energy. This strategy allows the independent tuning of both the elastic and viscous properties of the hydrogel using the same chemical components as for the preparation of purely elastic polyacrylamide hydrogels. Moreover, it is possible to attach adhesive ligands using classical crosslinkers that can be bound to only the viscous part of the hydrogels (linear polyacrylamide chains), the elastic part of the hydrogels (polyacrylamide network), or both the elastic and viscous elements (as presented in Figure 1).

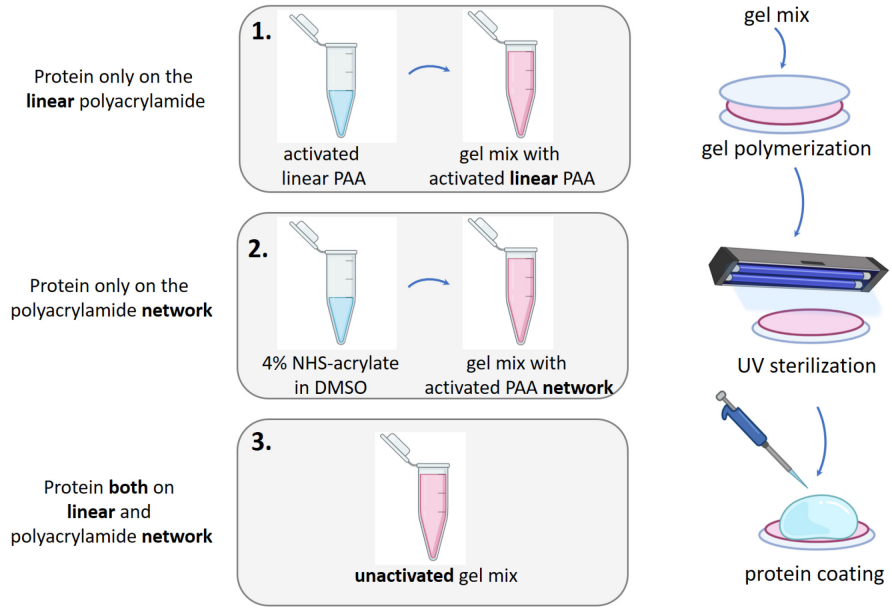

Figure 1. Illustration of the three methods to make viscoelastic gels presenting adhesion proteins. (1) Linear PAAm, where activated linear PAAm is used for the gel mix preparation; (2) the elastic network of crosslinked PAAm, where unactivated linear PAAm is mixed with an appropriate

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acrylamide/bis-acrylamide solution and NHS-acrylate monomers; or (3) both types of PAAm, where an unactivated gel mix is prepared and sulfo-SANPAH is used post-polymerization.

Materials and Reagents

1. 18-mm diameter glass coverslips (Menzel-Glaser, VWR, catalog number: MENZCB00190RA020)
2. 6-well plates (Corning, Fischer Scientific, catalog number: 07-200-83)
3. Pipette tips
4. 50-ml glass bottle
5. 1.5-ml and 0.5-ml conical tubes (Eppendorf, catalog numbers: 00030 120 086; 00030 121 023)
6. Aluminum foil
7. 0.1 M NaOH (Sigma-Aldrich, catalog number: 211465, store at room temperature)
8. (3-Aminopropyl)trimethoxysilane (3-APTMS) (Sigma, catalog number: 281778, store in a hood at room temperature)
9. Glutaraldehyde (Sigma, catalog number: 340855; store at 4°C)
10. SurfaSil Siliconized Fluid (Thermofisher Scientific, catalog number: TS 42800; store at room temperature)
11. Acetone (Sigma, catalog number: 179124; store at room temperature)
12. Methanol (Sigma, catalog number: 322415; store at room temperature)
13. 40% acrylamide solution (Bio-Rad, catalog number: 1610141; store in a hood at 4°C)
14. 2% bis-acrylamide solution (Bio-Rad, catalog number: 1610142; store in a hood at 4°C)
15. TEMED (Bio-Rad, catalog number: 1610801; store in a hood at room temperature)
16. Ammonium persulfate (APS) powder (Bio-Rad, catalog number: 1610700; store desiccated at room temperature)
17. Acrylic acid N-hydroxysuccinimide ester (NHS-acrylate) (Sigma, catalog number: A8060; store at -20°C)
18. Sulfo-SANPAH (sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate) (Sigma, catalog number: 803332-50MG; store at -20°C)
19. Dimethyl sulfoxide (DMSO) (Sigma, catalog number: 472301; store at room temperature)
20. HEPES (50 mM pH 8.2) (Sigma, catalog number: 83264; store at room temperature)
21. Phosphate-buffered saline (PBS) (Sigma, catalog number: 806552; store at room temperature)
22. MiliQ water
23. Protein of interest, *e.g.*, collagen I (Corning, catalog number: 354236) or fibronectin (MP Biomedicals, catalog number: MP215112601)

Equipment

1. 250-ml glass beaker

2. Set of micropipettes (from P1 to P1000)
3. Coverslip mini-rack
4. Shear rheometer (*e.g.*, Malvern Instruments, Kinexus stress-controlled rheometer)
5. Tweezers
6. Chemical hood
7. 4°C refrigerator
8. -20°C freezer
9. Incubator set at 37°C
10. UV light source at 320-365 nm
11. Centrifuge at 10,000 × *g*
12. Desiccator

Procedure

A. Glass coverslip functionalization
   1. Adhesive coverslips
      a. Pour 100 ml 0.1 M NaOH into a 250-ml glass beaker and preclean each coverslip by immersing in NaOH; allow them to air-dry.
      b. Under the chemical hood, pour 100 ml 3-APTMS solution into a 250-ml glass beaker and immerse the coverslips for 3 min.
      c. Wash the coverslips 3-5 times with 100 ml MilliQ water until no foam is observed in the water.

         CRITICAL STEP: Residual 3-APTMS will react with glutaraldehyde in the next step and produce an orange precipitate. If this happens, discard the coverslips and start from step a).
      d. Prepare 200 ml 0.5% glutaraldehyde in MilliQ water in a 250-ml glass beaker. Place the coverslips in a mini-rack and immerse in glutaraldehyde solution for 1 h.
      e. Air-dry the coverslips and store under vacuum at room temperature.
   2. Non-adhesive coverslips
      a. Under a chemical hood, prepare 100 ml 5% SurfaSil Siliconized Fluid in acetone.
      b. Immerse the coverslips in SurfaSil solution for 10 s and agitate to ensure a uniform coat.
      c. Rinse the coverslips with clean acetone.
      d. Rinse the coverslips with methanol.
      e. Air-dry the coverslips and store at room temperature.

B. Preparation of the linear polyacrylamide solution
   1. Prepare a 10% (w/v) solution of APS in H_2O before use. For long-term storage, aliquot the solution into 500-µl Eppendorf tubes and store in a -20°C freezer. Avoid freeze-thaw cycles. Solutions stored at room temperature are not stable. For the most effective polymerization, we recommend preparing a fresh 10% (w/v) solution of APS immediately prior to each gel

preparation.

2. In a 50-ml glass bottle, mix acrylamide, H_2O, TEMED, and 10% APS according to the recipe in Table 1.

   *CAUTION: The linear polyacrylamide solution can be made as **inert** or **activated** with the capability of binding adhesive proteins. If you aim to prepare **activated** linear polyacrylamide, substitute 1 ml H_2O with 1 ml 4% NHS-acrylate in DMSO, according to Table 1.*

   CRITICAL STEP: It is important to use very low amounts of polymerization initiator (APS) to ensure the formation of very long linear polyacrylamide chains with radii of gyration much larger than the mesh size of the crosslinked network of polyacrylamide. This prevents the linear chains from diffusing out of the polyacrylamide network during the time of the experiment.

Table 1. Composition of linear polyacrylamide solution in ml for a total volume of 10 ml

	40% acrylamide solution	H_2O	TEMED	10% APS	4% NHS in DMSO
Inert	1.25	8.72	0.005	0.025	-
Activated	1.25	7.72	0.005	0.025	1

3. Polymerize for 1 h at 37°C.
4. Cover the bottle with aluminum foil to protect it from light. Store at 4°C. If linear polyacrylamide is properly polymerized, it can be stored for months.

   CAUTION: The linear polyacrylamide solution is highly viscous; therefore, it is difficult to pipette it accurately. We suggest cutting the pipette tip before transferring and transferring the solution slowly.

C. Preparation of viscoelastic polyacrylamide gels

1. In an Eppendorf tube, mix acrylamide, bis-acrylamide, linear polyacrylamide, H_2O, and 4% NHS-acrylate in DMSO (if applicable) according to the recipe presented in Table 2.

   CRITICAL STEP: Mix the solution *very* gently by pipetting it up and down. It is important not to introduce air bubbles into the mixture. If you fail, you might need to degas the solution in a vacuum desiccator. The presence of air bubbles in the mixture will disrupt polymerization and change the mechanical properties of the resulting gels.

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Table 2. Composition of viscoelastic polyacrylamide gels in µl for a total volume of 500 µl

G' (kPa), G'' (Pa)	Acrylamide 40% stock	Bis-acrylamide 2% stock	H_2O	TEMED	10% APS	Linear polyacrylamide	4% NHS-acrylate in DMSO
1 kPa, 1 Pa	56	25	364	1.25	3.75	-	50
1 kPa, 200 Pa	69	25	30	1.25	3.75	321	50
5 kPa, 10 Pa	100	25	320	1.25	3.75	-	50
5 kPa, 500 Pa	100	37.5	21.5	1.25	3.75	286	50

2. Add TEMED and 10% APS according to Table 2, gently mix and pipet 100 µl gel mix onto the adhesive coverslip, and place the non-adhesive coverslip on top of the droplet.
3. After 15 min, add MilliQ water around the gel to avoid drying on the sides.
4. After an additional 15 min, remove the non-adhesive coverslip from the top of the gel, place the gel in a 6-well plate, and immerse in water.

   CAUTION: The mechanical properties of the gels depend strongly on the quality of the reagents used for the gel formulation, accuracy in pipetting, and mixing of the mixture. Most of the failures in gel polymerization come from mistakes in APS and TEMED pipetting and mixing or inappropriate storage of these solutions. Briefly, TEMED should be kept in the dark, and APS, which is not stable in water, should be freshly prepared for the most effective polymerization. We strongly recommend directly measuring the viscoelasticity of the gels using a rheometer after every separate preparation. In our experience, mixing and pipetting is not always identical, and G' tends to decrease as reagents get old, apparently due to oxidation. Thus, discrepancies in the measured G' and G" values can be as large as 50%.

D. Attachment of the adhesion proteins

Viscoelastic polyacrylamide gels consist of an acrylamide and bis-acrylamide network polymerized in the presence of linear polyacrylamide chains that are incorporated into the network, which determine its viscous properties. Our method allows for the covalent binding of proteins to the polyacrylamide network, linear polyacrylamide, or both.

1. Attachment of adhesion proteins to only the linear polyacrylamide
   a. To prepare activated linear polyacrylamide that can be crosslinked to the protein of interest, follow section "B. Preparation of the linear polyacrylamide solution" and the recipe from Table 1 for **activated** linear polyacrylamide. The resulting linear polyacrylamide chains will covalently bind proteins upon incubation with the protein of interest diluted to 0.1 mg/ml protein in 50 mM HEPES pH 8.2 for 2 h at room temperature or overnight at 4°C (both incubations result in uniform coating). For protein incubation, prepare a minimum of 80 µl 0.1 mg/ml protein solution per 18-mm diameter gel. The larger the gel, the more protein solution is needed.

CAUTION: Incubation with the proteins is performed following polymerization of viscoelastic gels using activated linear polyacrylamide chains immediately after their incorporation within the acrylamide/bis-acrylamide network. Linear polyacrylamide chains of the viscoelastic gel cannot be selectively activated after polymerization of the crosslinked network.

b. After incubation with the protein, rinse the gels 3 times with 100 µl PBS and store in PBS at 4°C until cell seeding, but not longer than a couple of days.

2. Attachment of the proteins to only the crosslinked polyacrylamide network
   a. 4% NHS in DMSO method
      i. Prepare fresh 4% NHS solution in DMSO.
      ii. Add 50 µl 4% NHS in DMSO per 500 µl gel mix volume according to Table 2.
      CAUTION: 4% NHS solution in DMSO is added to the gel mix prior to polymerization. The crosslinked polyacrylamide network of the viscoelastic gel cannot be selectively activated post-polymerization.
      iii. When APS and TEMED are mixed in, cast the gels between adhesive and non-adhesive coverslips, as previously described (Section C: Preparation of viscoelastic polyacrylamide gels).
      iv. Once the gel is rinsed and soaked in 50 mM HEPES pH 8.2, illuminate with UV light under the hood for 15 min. From this step forward, gels that will be used for cell seeding should be maintained under aseptic conditions.
      v. Immerse the gels in the protein of interest diluted to 0.1 mg/ml protein in 50 mM HEPES pH 8.2 and incubate for 2 h at room temperature or overnight at 4°C (both incubations result in uniform coating).
      vi. Rinse 3 times with PBS and store at 4°C until cell seeding, but not longer than a couple of days.
   b. NHS-acrylate in toluene method
      i. Under the chemical hood, prepare 0.5 ml 2% NHS-acrylate in toluene solution.
      ii. Prepare the gel mix without APS and TEMED following the recipe from Table 2, with one modification, *i.e.*, replace 50 µl 4% NHS in DMSO with 50 µl MilliQ water.
      iii. Add 100 µl 2% NHS-acrylate in toluene solution per 500 µl gel mix and agitate until the solution becomes uniformly turbid.
      iv. Leave for 5 min at room temperature and then centrifuge at 10,000 × *g* for 5 min to separate the toluene from the gel mix.
      v. Remove the toluene (upper) layer using a 200-µl pipette and transfer the gel mix to a new tube.
      vi. Add APS and TEMED to initiate polymerization and cast the gels between adhesive and non-adhesive coverslips, as previously described (Section C: Preparation of viscoelastic polyacrylamide gels).
      vii. Once the gel is rinsed and soaked in 50 mM HEPES pH 8.2, illuminate with UV light under the hood for 15 min. From this step forward, gels that will be used for cell seeding

should be maintained under aseptic conditions.

viii. Immerse the gels in the protein of interest diluted to 0.1 mg/ml protein in 50 mM HEPES pH 8.2 and incubate for 2 h at room temperature or overnight at 4°C (both incubations result in uniform coating). For protein incubation, prepare a minimum of 80 µl 0.1 mg/ml protein solution per 18-mm diameter gel. The larger the gel, the more protein solution is needed.

ix. Rinse with 100 µl PBS 3 times and store in PBS at 4°C until cell seeding, but not longer than a couple of days.

3. Attachment of proteins to both the linear polyacrylamide and crosslinked polyacrylamide networks

a. Soak the gels in 50 mM HEPES pH 8.2 for 15 min.

b. Prepare 5 mM sulfo-SANPAH in 25% DMSO and 75% 50 mM HEPES pH 8.2 (first dissolve sulfo-SANPAH in DMSO, then add HEPES in MilliQ water). A minimum of 80 µl 5 mM sulfo-SANPAH solution per 18-mm diameter gel is needed.

CAUTION: We strongly recommend preparing fresh sulfo-SANPAH solution prior to each gel preparation and not storing it longer than 1 day.

c. Cover the surface of each gel with 80 µl sulfo-SANPAH solution (for an 18-mm diameter gel) and illuminate with UV light (320-365 nm) for 10-15 min under the hood. From this step forward, gels that will be used for cell seeding should be maintained under aseptic conditions. Properly activated sulfo-SANPAH should change color from bright orange to a darker burnt orange or brown.

CAUTION: Do not overexpose the gels to UV light and do not allow the gels to dry during irradiation.

d. Rinse the gels 3-5 times with 100 µl 50 mM HEPES pH 8.2 until all the sulfo-SANPAH has been removed.

e. Immerse the gels in the protein of interest diluted to 0.1 mg/ml protein in 50 mM HEPES pH 8.2 and incubate for 2 h at room temperature or overnight at 4°C (both incubations result in uniform coating). For protein incubation, prepare a minimum of 80 µl 0.1 mg/ml solution per 18-mm diameter gel. The larger the gel, the more protein solution is needed.

f. Rinse 3 times with 100 µl PBS and store in PBS at 4°C until cell seeding.

CAUTION: We recommend using the gels within a couple of days of preparation and not placing them in long-term storage. On long time scales (day to weeks), linear polyacrylamide chains can diffuse out of the gel surface and alter their mechanical properties.

E. Cell seeding

1. Thirty minutes before seeding the cells, soak the gels in media and keep at 37°C.
2. Trypsinize the cells and prepare a cell suspension at the concentration of interest (to study cell-substrate interactions, single cells are typically needed).

3. Remove the media used for soaking the gels and replace with the cell suspension. Keep the gels in the incubator at 37°C. Cells will start to spread upon contact with the gel surface.

Data analysis

The primary data that characterize the gels are their shear storage or loss modulus as functions of time and shear strain, as introduced in Charrier *et al.* (2018). The magnitude of the viscous dissipation will be highly time dependent and also depends on the length of the linear polyacrylamide chains. The polymer length is highly dependent on the amount and activity of the APS initiator and is impossible to control precisely. Determination of the hydrodynamic radius from dynamic light scattering of very dilute solutions will provide a measure of this length, as described in Charrier *et al.* (2018).

For cells cultured on viscoelastic substrates, the most commonly measured quantities are cell area, shape, size of focal adhesions, motility, traction force, and proliferation. Examples of how these features change with substrates and vary among different cell types are provided elsewhere (Gong *et al*., 2018; Charrier *et al.*, 2020 and 2021; Mandal *et al*., 2020). Additionally, in Figure 2, we show the morphology and spreading area of the glioma cell line LBC3 grown for 24 h on 1 kPa and 5 kPa elastic and viscoelastic substrates. As can be seen, glioma cells are sensitive to substrate viscosity in an elastic modulus-dependent manner. For example, on G' = 1 kPa purely elastic gels, glioma cells were smaller than those on 1 kPa viscoelastic gels; on higher stiffness (G' = 5 kPa), the introduction of viscosity to the substrate did not cause a statistically significant increase in cell spreading area.

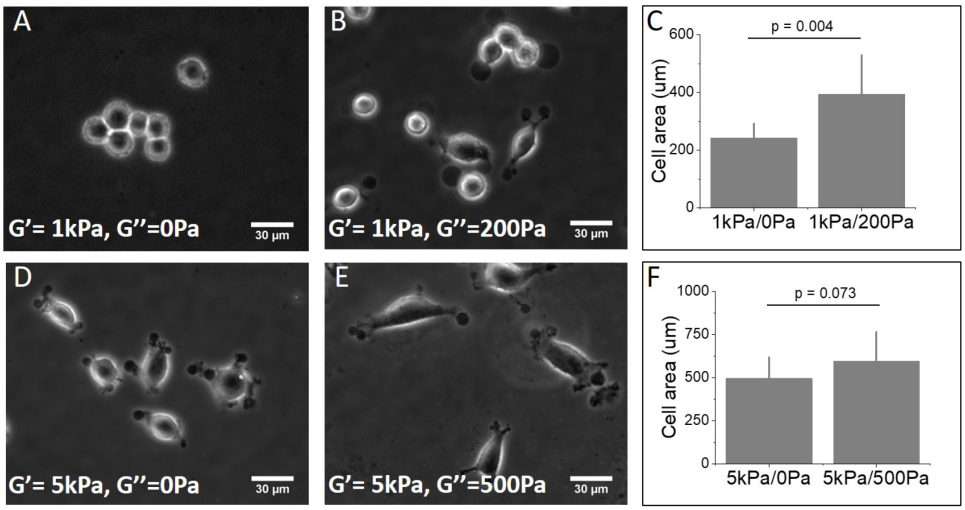

Figure 2. The effect of substrate viscosity on cell morphology and spreading area. LBC3 glioma cells grown for 24 h on 1 kPa elastic (A), viscoelastic (B), 5 kPa elastic (D), and viscoelastic (E) hydrogels, with 0.1 mg/ml fibronectin presented only on the crosslinked network of PAAm. Panels C and F show quantitation of the LBC3 cell spreading area for 1 kPa and 5 kPa elastic and viscoelastic gels.

Acknowledgments

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Competing interests

None of the authors have any competing interests to declare.

Ethics

No human or animal subjects were used in this study.

References

1. Basu, A., Wen, Q., Mao, X. M., Lubensky, T. C., Janmey, P. A. and Yodh, A. G. (2011). Nonaffine displacements in flexible polymer networks. *Macromolecules* 44(6) 1671-9.
2. Beningo, K. A., Lo, C. M. and Wang, Y. L. (2002). Flexible polyacrylamide substrata for the analysis of mechanical interactions at cell-substratum adhesions. *Methods Cell Biol* 69: 325-339.
3. Cameron, A. R., Frith, J. E. and Cooper-White, J. J. (2011). The influence of substrate creep on mesenchymal stem cell behaviour and phenotype. *Biomaterials* 32(26): 5979-5993.
4. Charrier, E. E., Pogoda, K., Li, R., Park, C. Y., Fredberg, J. J. and Janmey, P. A. (2020). A novel method to make viscoelastic polyacrylamide gels for cell culture and traction force microscopy. *APL Bioeng* 4(3): 036104.
5. Charrier, E. E., Pogoda, K., Li, R., Wells, R. G. and Janmey, P. A. (2021). Elasticity-dependent response of malignant cells to viscous dissipation. *Biomech Model Mechanobiol*. 20(1) 145-54.
6. Charrier, E. E., Pogoda, K., Wells, R. G. and Janmey, P. A. (2018). Control of cell morphology and differentiation by substrates with independently tunable elasticity and viscous dissipation. *Nat Commun* 9(1): 449.
7. Chaudhuri, O., Cooper-White, J., Janmey, P. A., Mooney, D. J. and Shenoy, V. B. (2020). Effects of extracellular matrix viscoelasticity on cellular behaviour. *Nature* 584(7822): 535-546.
8. Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S. A., Weaver, J. C., Huebsch, N., Lee, H. P., Lippens, E., Duda, G. N. and Mooney, D. J. (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater* 15(3): 326-334.
9. Gong, Z., Szczesny, S. E., Caliari, S. R., Charrier, E. E., Chaudhuri, O., Cao, X., Lin, Y., Mauck, R. L., Janmey, P. A., Burdick, J. A. and Shenoy, V. B. (2018). Matching material and cellular

timescales maximizes cell spreading on viscoelastic substrates. *Proc Natl Acad Sci U S A* 115(12): E2686-E2695.

10. Janmey, P. A., Fletcher, D. A. and Reinhart-King, C. A. (2020). Stiffness sensing by cells. *Physiol Rev* 100(2) 695-724.
11. Mandal, K., Gong, Z., Rylander, A., Shenoy, V. B. and Janmey, P. A. (2020). Opposite responses of normal hepatocytes and hepatocellular carcinoma cells to substrate viscoelasticity. *Biomater Sci* 8(5): 1316-1328.
12. Pelham, R. J., Jr. and Wang, Y. (1997). Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* 94(25): 13661-13665.