INTRODUCTION

Fibrils composed of proteins that are connected via cross-β-sheet secondary structure are abundantly present in nature. These so-called amyloid fibrils are a versatile material and used to store and release peptide hormones, enable attachment to surfaces, and provide protection against environmental stresses. Their nanoscale organization, combined with their ability to organize into bundles and networks at larger length scales, makes amyloid fibrils also attractive materials for a variety of applications. Amyloid fibrils have been used to remove heavy metal ions from water, improve DNA and RNA transduction, and to build scaffolds for tissue regeneration. This last application relies not only on single fibril characteristics but also on the rheological properties of networks of fibrils. Amyloid fibrils of the food proteins lysozyme and β-lactoglobulin and the Parkinson’s disease related protein α-synuclein have been reported to organize into hydrogels. The viscoelastic response of these gels depends to a large extent on physical interfibril interactions which can be tuned using physicochemical parameters. Typically, the strength of the interaction, and therefore the fraction of entanglements in the network that become physically cross-linked, is the result of a long-range electrostatic repulsion and a short-range attraction. This means that with increasing ionic strength the charges on the fibrils become screened and the electrostatic barrier that counteracts the formation of cross-links is reduced. The elastic modulus $G^\prime$ of networks of β-lactoglobulin, lysozyme, and α-synuclein amyloid fibrils therefore increases with increasing ionic strength. For networks of α-synuclein fibrils, it has been shown that the short-range attraction is hydrophobic in nature, and as a consequence, the fraction of cross-linked entanglement points increases with increasing temperature.

The above-mentioned investigations were performed in simple buffer solutions. It is unknown how well the obtained insights into interfibril interactions translate to the more complex solution conditions typically present in applications. Here we investigate if the physical interactions that dominate the network response in simple buffer solutions still govern the response in more complex environments. We limit our investigations to networks of lysozyme amyloid fibrils and focus on cell culture conditions, as lysozyme amyloid fibrils have been shown to support cell attachment and spreading. Induce no cytotoxicity, and preserve chondrocyte phenotype and increase extracellular matrix deposition. We show enhanced energy dissipation in hydrogels of lysozyme amyloid fibrils in compositionally complex biological fluids compared to

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their viscoelastic response in simple buffers. We attribute this energy dissipation to the presence of interacting molecules in these solutions and especially to the adsorption of the abundantly present protein serum albumin to the fibril surface. Although the adsorption of molecules that are present in the medium and secreted by cells complicates gaining control over interactions, we argue that the observed energy dissipation may be beneficial. Matrix remodeling through stress relaxation is increasingly recognized as critical in controlling cell fate.17,18

### MATERIALS AND METHODS

**Amyloid Gels.** Gels of lysozyme amyloid fibrils were prepared from a 1 mM hen egg white lysozyme (Sigma-Aldrich) in 50 mM NaH₂PO₄ (Merck) of which the pH was subsequently set to 2 using HCl (Merck). To initiate amyloid fibril formation, the solution was seeded with 5% (equivalent monomer concentration) preformed lysozyme fibrils (see below). Capped glass vials containing 2 mL of this lysozyme solution were left to equilibrate for 3 weeks under quiescent conditions at 60 °C after which a gel had formed. The pH of the gel was returned to pH 7.3 by dialyzing them against 10 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol buffer (TRIS, Merck) buffer, pH 7.3. For the dialysis, ~8 mL buffer was added to the gel and exchanged every 2 days for 3 weeks. During this time the vials were kept in an incubator at 37 °C under 5% CO₂. Gels were stored with 2 mL of TRIS buffer on top to prevent drying out and kept at 4 °C until use. Following the same protocol, amyloid gels were prepared at lysozyme concentrations of 0.2, 0.5, and 2.0 mM lysozyme. To test the effect of solution conditions, lysozyme amyloid gels, prepared at 1 mM lysozyme, were dialyzed against phosphate-buffered saline (PBS, Gibco), PBS with 0.4 mM bovine serum albumin (BSA, Sigma-Aldrich), PBS with 3.5 mg/mL low molecular weight sodium hyaluronic acid (25 kDa), 50 %w % glycerol (Merck) in Tris buffer pH 7.3, PBS with 3.5 mg/mL high molecular weight sodium hyaluronic acid (1.5−2.2 MDa), high glucose Dulbecco's Modified Eagle’s Medium (DMEM), and pooled synovial fluid from Shetland ponies. Lysozyme amyloid fibril gels were chemically cross-linked by incubating them in a tissue fixation solution consisting of 3.7% formaldehyde (Sigma-Aldrich) in Tris buffer for 24 h. To reduce intramolecular S-bridges that possibly formed within or between the amyloid fibrils, gels were incubated in 3.7% β-mercaptoethanol (Sigma-Aldrich) in PBS for 24 h. Gels were measured directly after incubation. All solutions where sterile filtered before use, and the vials and caps were sterilized in 70% ethanol (Merck).

**Lysozyme Fibril Seeds.** Fibril seeds were prepared by fragmenting preformed lysozyme fibrils in five cycles of freezing in liquid nitrogen and subsequently thawing this solution in a water bath at 80 °C.

**Rheometry.** Lysozyme amyloid gels for rheometry experiments were die cut (8 mm diameter) and loaded onto a water-cooled Physica MCR 301 rheometer (Anton Paar). The experiments were performed in a plate−plate geometry with an upper plate diameter of 8 mm (PP08, Anton Paar). The parallel plate was lowered until a normal force was detected, indicating contact. The parallel plate was lowered a final 0.1 mm after contact to ensure full contact, and an oil trap was used to prevent evaporation of solution from the gel.

After transfer, the storage modulus G' was followed for 1 h at a strain of 0.5% and a frequency of 0.5 Hz. During this time period, typically a small increase in the elastic modulus was observed after which it attained a constant value. To ensure that measurements were performed in the linear response regime of the amyloid gel, stress−strain curves were obtained at a fixed strain rate. Based on the stress−strain curves, frequency spectra were recorded at a strain of 0.5%. Stress relaxation tests were performed on the same samples by subjecting them to a strain of 1%.

**Atomic Force Microscopy.** To prepare samples for atomic force microscopy (AFM), a suspension of lysozyme amyloid fibrils was diluted 1000 times in ultrapure water and deposited on freshly cleaved mica (Muscovite, V-1 quality, EMS). Samples were left to dry in air and imaged with an AFM (Multimode 8, Bruker) in tapping mode (air) using a NCS36/CR-AU tip B (1.75 N/m, 155 kHz, MicroMasch). Height images were collected at 1024 × 1024 pixels and 0.1 Hz.

To determine the persistence length, the AFM images of the fibrils were analyzed using the MATLAB (R2019b, MathWorks)-based Env Visualization package.19 Because the persistence length was of the same order of magnitude as the fibril contour length the method based on mean-square end-to-end distance was selected for analysis. The persistence length of the lysozyme fibrils was determined by random resampling using bootstrapping with replacement. For each bootstrap, the mean-square end-to-end distance was binned at equal length intervals. The persistence length was subsequently estimated by fitting the experimental data using the mean square end-to-end expression for a 2D wormlike chain model.

**Fluorescent Labeling of BSA.** A 200 μM BSA solution was prepared in 10 mM Tris pH 7.4, 10 mM NaCl (Merck), and 0.1 mM EDTA (Merck). To reduce cysteines, TCEP was added to the BSA solution to a final concentration of 10 mM and the mixture was incubated for 30 min at room temperature. Subsequently, the BSA solution was incubated with 2.3 mM maleimide-functionalized AlexaFluor488 (AF488, Thermo Fisher) for 1 h at room temperature in the dark. Excess dye was removed using a 2 mL Zeba Spin desalting column (ThermoFisher). The final BSA-AF488m concentration (C_	ext{BSA-AF488m}) was calculated using the following equation:

\[
C_	ext{BSA-AF488m} = C_	ext{BSA} \times \frac{\text{C}_{\text{AF488}}}{\text{C}_{\text{BSA}}} \times \frac{1}{\text{CF}}
\]

where C_{BSA} and C_{AF488} are the absorbance at 280 and 493 nm, respectively, ε_{BSA} is the molar extinction coefficient of BSA at 280 nm (ε_{BSA} = 43824 M⁻¹·cm⁻¹), and CF is the correction factor for the contribution to the absorbance at 280 nm (A_{280} = 0.11) of the AF488m dye (CF = 0.11).

**Total Internal Reflection Fluorescence (TIRF) Microscopy.** To visualize the fibrils in fluorescence microscopy experiments, either labeled or unlabeled BSA was added to 1 mM lysozyme fibrils (equivalent monomer concentration) in Tris buffer, pH 7.4, to a final BSA concentration of 10 μM. Fibrils were stained with 5 μM thioflavin T (ThT, Sigma-Aldrich). Images of the fibrils were obtained using a Nikon Ti-E microscope with a 100X CFI APO TIRF objective (Nikon) and an iXon 3 DU-870 EMCCD camera (Andor). ThT fluorescence was excited with an argon laser using the 457 nm line. We made use of a 455 nm excitation filter with a 10 nm bandpass and a 485 nm emission filter with a 30 nm bandpass.

**Microscale Thermophoresis.** The MicroScale Thermophoresis (MST) technique (Monolith NT.115, NanoTemper Technologies GmbH, Munich, Germany) was used to quantify the interaction between labeled BSA and lysozyme fibril seeds. BSA-AF488 (0.2 μM) was mixed with a series of 16 different lysozyme seed concentrations (from 0 to 50 μM equivalent monomer) in a 10 mM Tris-HCl, 10 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer. Samples were transferred to standard capillaries (Nonatemp), and measurements were performed three times at 37 °C with a constant blue LED power of 20% and with three different infrared laser powers (20−40−80%). The measurement procedure was composed of (1) a 5 s baseline for signal normalization, and then (2) a 30 s period with the infrared laser turned on, followed by (3) another 5 s after turning off the infrared laser. The data was analyzed using the MO. Affinity Analysis v2.1.2030 software using the “Thermophoresis with T Jump” strategy.

**RESULTS AND DISCUSSION**

The self-assembly of lysozyme into amyloid fibrils is usually induced at low pH and high temperature. The viscoelastic properties of networks of these fibrils has been characterized in detail at low pH.11 However, in the context of biological applications, including tissue regeneration, the viscoelastic response of the network near physiological conditions is more relevant. Although lysozyme is expected to be positively charged at both pH 2 and pH 7, the charge density will be considerably lower at pH 7. This lower charge density is expected to affect interfibril interactions and thereby network...
properties. To obtain insight into interfibril interactions and the resulting viscoelastic response of lysozyme amyloid fibril networks at physiologically relevant conditions, lysozyme amyloid fibril networks produced at low pH were dialyzed to obtain networks under solution conditions that are directly relevant for biological applications.

Lysozyme amyloid fibril networks produced at pH 2 at different protein concentrations were dialyzed against Tris buffer, pH 7.4. This approach did not change the overall appearance of the networks, and the networks remained self-supporting for the entire lysozyme concentration range tested. Samples of these networks were transferred to a rheometer, and their viscoelastic properties were quantified using oscillatory rheometry. Frequency sweeps show that the networks of lysozyme amyloid fibrils behave as viscoelastic materials (Figure 1a). In the tested concentration range the spectra are relatively featureless, both the storage modulus $G'(f)$ and the loss modulus $G''(f)$ only weakly depend on frequency. We did not observe crossovers between $G'(f)$ and $G''(f)$ in the probed frequency range. $G'$ is about 1 order of magnitude larger than $G''$ over the frequency range probed. With the above-mentioned features, the viscoelastic response of the lysozyme amyloid fibril networks as a function of frequency is reminiscent of that of chemically and some physically cross-linked networks rather than entangled fibril solutions.

The observed frequency behavior agrees with the response of lysozyme networks at low pH and observations for other amyloid fibril networks. The absolute values observed for $G'$ are however more than 1 order of magnitude larger than what has been reported previously for lysozyme amyloid networks at comparable, millimolar, equivalent monomer concentrations at low pH. The persistence length $l_p$ obtained from AFM images is of the order of a few micrometers, comparable to values for $l_p$ reported in the literature (Figure 1b,c). In the absence of changes in $l_p$, stronger attractive interfibril interactions, in Tris buffer of near neutral pH, may be responsible for the higher values of $G'$ observed here. However, differences in the setup of the rheometry measurements possibly also contributed to the observed difference.

To support the idea that strong interfibril interactions cause the lysozyme amyloid networks to behave as if they were cross-linked, we plot $G'$ at 0.1 Hz ($G_0$) and observe a strong increase of $G_0$ with the lysozyme concentration. In the literature, several models are available that describe the scaling relation between $G_0$ and the concentration of semiflexible polymers. To select the model that physically describes our system adequately, the most likely fibril deformation modes have to be considered. The mesh size of the amyloid fibril network was estimated using the approach previously verified for F-actin networks.

With this approach, we estimated the mesh size to be $\sim 0.5 \mu m$ for a 1 mM lysozyme solution, assuming that all the monomeric protein has been completely converted into double stranded fibrils. Because the persistence length of lysozyme amyloid fibrils is large compared to the mesh size in the tested concentration range there is no excess fibril contour length available between neighboring interfibrillar contact points. Models that rely on entropic stretching deformations are therefore not taken into account. Instead, we selected a scaling based on the non-affine floppy modes model that has previously been used to describe the response of networks of physically cross-linked actin bundles and $\alpha$-synuclein amyloid gels. In this model, network elasticity is attributed to bending modes between cross-links and the elastic modulus scales as.

$$G_0 \sim \frac{k \delta^2}{\xi \xi_m^2 l_c}$$

where $k$ is the bending modulus, $\xi$ the average mesh size, $l_c$ the distance between cross-links, and $\delta$ the strain. To account for the concentration dependence, this scaling relation simplifies to $G_0 \sim \frac{\xi_m^2}{\xi} l_c^{-4}$. The mesh size is a function of the concentration and scales as $\xi \sim (c_{\text{lysozyme}})^{-1/2}$. We assume that cross-links can only appear at entanglement points. The entanglement length scales with the concentration as $l_c \sim l_p (c_{\text{lysozyme}})^{1/5}$. Hence we expect $G_0 \sim (c_{\text{lysozyme}})^{13/5}$. This scaling relation is in good agreement with our observations (Figure 1d). Additionally, chemically cross-linking the protein fibrils using formaldehyde does not further increase the elastic modulus (Figure 1d). We therefore conclude that interactions between lysozyme fibrils in Tris buffer are so strong that $G_0$ of the network behaves comparable to the chemically cross-linked network.

Native lysozyme is stabilized by disulfide bridges. These disulfide bridges are broken to allow for aggregation into

![Figure 1.](https://doi.org/10.1021/acs.langmuir.1c00657)
amyloid fibrils, but can potentially reform after the dialysis step. To test if the formation of disulfide bonds between surface exposed thiol groups in the fibrils is responsible for the formation of chemical interfibril cross-links the disulfide bonds were reduced using β-mercaptoethanol. This reduction had however no effect on \( G_0 \) (Figure 1d). Because other covalent bonds are highly unlikely to form spontaneously, we conclude that the strong interfibril interactions are not the result of the formation of chemical bonds between the protein fibrils. Instead, physical interactions result in a strong attraction between fibrils.

For like-charged fibrils, the formation of physical cross-links is usually the result of a short-range attraction and a long-ranged electrostatic repulsion.\(^{11,13,25}\) To test if additional screening of the surface charge further decreases the repulsive component of the interfibril interactions, the ionic strength of the buffer solution was brought to physiological values by dialyzing the amyloid network against PBS. The effect of this increase in salt concentration was visible but moderate (Figure 2a).

For α-synuclein amyloid fibrils, hydrophobic interactions are an important source of interfibril attraction.\(^{13,25}\) As a result, the network response depends on the temperature: in networks of α-synuclein, amyloid fibrils contacts are lost at lower temperatures while the contacts effectively become stronger with increasing temperature.\(^{26}\) The networks of lysozyme amyloid fibrils are not sensitive to changes in temperature between 5 °C and 60 °C (Figure 2b). Neither the absolute values of \( G_0 \) nor the value of the loss tangent \( G'/G'' \) was affected by change in temperature. Over the whole temperature range, \( G' \) dominates the network response and the loss tangent remains low. Interfibril attraction is therefore most likely dominated by van der Waals interactions as reported.\(^{11}\)

Because the strong interactions between lysozyme fibrils in the network are physical in nature, they are sensitive to changes in the physicochemical environment.\(^{11,12}\) In biological applications, the networks of amyloid fibrils will be exposed to more complex environments than simple buffer solutions. In the development of amyloid fibril networks for tissue regeneration applications this means that the networks are exposed to cell culture media.\(^{14}\) Once implanted, the material will have to function in the presence of local biological fluids, e.g., the synovial fluid in joints when used as scaffolds for cartilage regeneration. To mimic solution conditions close to those in biological fluids, we dialyzed lysozyme amyloid networks against cell culture medium and synovial fluid. In both cases the measured \( G_0 \) is slightly higher but of the same order of magnitude as observed for the networks in PBS (Figure 3A,B). Again, both the elastic and viscous response as a function of frequency does not contain any crossovers between \( G' \) and \( G'' \) in the probed frequency range (Figure 3B). \( G''(f) \) is however shifted to much higher values, and the loss tangent changes from ~0.1 in PBS to values >0.5 in these compositionally complex solutions (Figure 3C). This suggests that additional viscous dissipation mechanisms exist in the relevant biological fluids.

A mechanism of energy dissipation that is always present in polymer networks is friction due to viscous drag of the individual fibrils.\(^{24}\) As a result of the presence of high molecular weight compounds, e.g., hyaluronic acid in synovial fluid or proteins in culture media, these fluids may be more viscous, which translates into an increase in drag of individual amyloid fibrils. To test this idea, either low or high molecular weight hyaluronic acid was added to the lysozyme networks in PBS and left to equilibrate. Considering the radius of gyration of the hyaluronic acids, estimated assuming Gaussian chain behavior and their flexibility and the mesh size of the lysozyme networks estimated from the density, we anticipate that the hyaluronic acid diffuses into the network. The hyaluronic acid concentrations used in this experiment were comparable to those found in synovial fluid.\(^{22}\) The enrichment of the PBS solution with hyaluronic acid had no effect on the viscoelastic
response of the network; the frequency spectra overlapped (Figure 3D). Control experiments in which the viscosity was increased by replacing the buffer solution with 50 wt % glycerol showed a similar picture; the loss tangent (\(G''/G'(0.1 \text{ Hz})\)) remained low at \(\sim 0.1\). We therefore assume that other mechanisms are responsible for the observed energy dissipation in both growth medium and synovial fluid.

In the absence of chemical interfibril cross-links, the amyloid fibril network can potentially rearrange under stress. Such network rearrangements would be a source of energy dissipation. To test if network rearrangements are possible, the amyloid hydrogels were exposed to larger stresses and subsequently visually inspected. We observed that the appearance of the amyloid hydrogels changed. Whereas initially the gels were opaque, they turned blueish after exposure to larger stresses (Figure 4A). This change in color indicates that rearrangements, from an isotropic distribution to more parallel orientations, have occurred in the network structure.\(^{28}\) We therefore hypothesize that the increase in energy dissipation observed in cell culture medium and synovial fluid is the result of enhanced lysozyme amyloid network reorganization under stress.

Stress relaxation test, in which we follow the normalized stress, of lysozyme amyloid fibril networks in different solutions show that relaxation is faster in cell culture media and synovial fluid compared to Tris buffer and PBS (Figure 4b). To further verify that the increase in the viscous dissipation is a result of stress-induced changes in network organization, we chemically cross-linked interfibril connections using formaldehyde and determined the \(G'_c\), \(G''_c\), and loss tangent in oscillatory rheometry experiments. While the value of \(G_c\) remains of the same order of magnitude after cross-linking, the loss tangent decreases to \(\sim 0.1\) (Figure 3B,C). We therefore conclude that in cell growth medium and synovial fluid, network reorganizations are facilitated.

In addition to various small molecules, both cell culture medium and synovial fluid contain a considerable amount of serum albumin, \(\sim 4 \text{ g/L} (\sim 60 \mu\text{M})\) and \(\sim 12 \text{ g/L} (\sim 180 \mu\text{M})\), respectively.\(^{29}\) The serum albumins present in our experiments are net negatively charged around neutral pH while lysozyme is positively charged. Their opposite charge may cause serum albumin to bind to the lysozyme amyloid fibrils. Experiments with fluorescently labeled BSA confirm this: in fluorescence microscopy experiments we observe colocalization between lysozyme amyloid fibrils and BSA (Figure 4c). Microscale thermophoresis experiments indicate that the equilibrium dissociation constant is of the order of a few micromolar and that the binding of lysozyme fibrils (equivalent monomer) to serum albumine is cooperative (\(n = 2\)) (Figure 4d).

We hypothesize that the attractive interaction between lysozyme amyloid fibrils and serum albumin, and possibly other components found biological fluids, is responsible for the enhanced stress relaxation. Indeed, when we complement PBS buffer with BSA in the dialysis step, the viscoelastic response of the amyloid fibril network becomes comparable to that of networks in cell culture medium or synovial fluid. In the presence of BSA, \(G''/G'\) of the fibril networks increases to values \(>0.5\) while \(G_c\) remains approximately constant (Figure 3B,C). Measurements in which we follow the stress relaxation also confirm this picture. The addition of BSA speeds the decrease in stress compared to lysozyme networks in PBS (Figure 4b). In the rheology experiments both lysozyme and BSA are present in millimolar concentrations. With an affinity of BSA for lysozyme fibrils in the micromolar range, the fibril surface is therefore expected to be covered with BSA in the rheology experiments. The binding of (bovine) serum albumin to the lysozyme fibrils seems to be responsible for the increase in energy dissipation. Relaxation of stresses in the amyloid network is the result of the unbinding and reformation of interfibril connections. In the presence of BSA the reformation of interfibril connections between lysozyme fibrils will however be hindered by the presence of lysozyme fibril-bound BSA. Rebinding will therefore occur on longer time scales in the presence of BSA compared to when BSA is absent. These longer time scales lead to the observed faster rate of network relaxation.

### CONCLUSION

Interfibril interactions physically cross-link networks of lysozyme amyloid fibrils. The presence of physical cross-links causes the viscoelastic properties of networks of lysozyme amyloid fibrils in compositionally complex biological fluids to differ considerably from the viscoelastic response in simple buffer solutions. The presence of serum albumin and potentially other molecules in these biological solutions results in enhanced energy dissipation. We attribute the increase in energy dissipation to the competition between the reformation of physical interfibril connections and the binding of serum albumin and other molecules in the biological solution to the fibrils.
The observed increased energy dissipation due to adsorption of proteins, and possibly other components, present in biological fluids and secreted by cells may have benefits. The hydrogels that are currently used for tissue regeneration applications often consist of chemically cross-linked networks of polymers. Cells have been reported to respond to the stiffness of their substrate, and substrate stiffness is thought to be one of the physical factors that regulates stem cell fate. In the design of artificial matrices for tissue regeneration the stiffness of the material is therefore typically taken into account. Real tissues are, however, viscoelastic materials with a loss tangent of ~0.2. In these viscoelastic materials, matrix remodeling through stress relaxation is increasingly recognized as being critical in determining cell fate. Stress relation is now recognized as an important design parameter for cell culture.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

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**ABBREVIATIONS**

PM, cell growth medium; SV, synovial fluid; \( G_0 \) G’ at 0.1 Hz; CL, cross-linking; BSA, bovine serum albumin; AFM, atomic force microscopy; MST, microscale thermophoresis

**REFERENCES**

(1) Maji, S. K.; Perrin, M. H.; Sawaya, M. R.; Jessberger, S.; Vadodaria, K.; Rissman, R. A.; Singru, P. S.; Nilsson, K. P. R.; Simon, R.; Schubert, D.; Eisenberg, D.; Rivier, J.; Sawchenko, P.; Vale, W.; Riek, R. Functional Amyloids As Natural Storage of Peptide Hormones in Pituitary Secretory Granules. *Science* 2009, 325 (5938), 328–332.

(2) Barlow, D. E.; Dickinson, G. H.; Orihuela, B.; Kulp, J. L.; Rittschof, D.; Wahl, K. J. Characterization of the Adhesive Plaque of the Barnacle Balanus Amphitrite: Amyloid-Like Nanofibrils Are A Major Component. *Langmuir* 2010, 26 (9), 6549–6556.

(3) Mostaert, A. S.; Giordani, C.; Crockett, R.; Karsten, U.; Schumann, R.; Jarvis, S. P. Characterisation of Amyloid Nanostructures in the Natural Adhesive of Unicellular Subaerial Algae. *J. Adhes.* 2009, 85 (8), 465–483.

(4) Icomimidou, V. A.; Vriend, G.; Hamodrakas, S. J. Amyloids protect the silkmoth oocyte and embryo. *FEBS Lett.* 2000, 479 (3), 141–145.

(5) Podrabsky, J. E.; Carpenter, J. F.; Hand, S. C. Survival of water stress in annual fish embryos: dehydration avoidance and egg envelope amyloid fibers. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 2001, 280 (1), R123–R131.

(6) Bolisetty, S.; Mezzenga, R. Amyloid-carbon hybrid membranes for universal water purification. *Nat. Nanotechnol.* 2016, 11 (4), 365–371.

(7) Dai, B.; Li, D.; Xi, W.; Luo, F.; Zhang, X.; Zou, M.; Cao, M.; Hu, J.; Wang, W. Y.; Wei, G. H.; Zhang, Y.; Liu, C. Tunable assembly of amyloid-forming peptides into nanosheets as a retrovirus carrier. *Proc. Natl. Acad. Sci. U. S. A.* 2015, 112 (10), 3096–3101.

(8) Das, S.; Jacob, R. S.; Patel, K.; Singh, N.; Maji, S. K. Amyloid Fibrils: Versatile Biomaterials for Cell Adhesion and Tissue Engineering Applications. *Biomacromolecules* 2018, 19 (6), 1826–1839.

(9) Li, C. X.; Born, A. K.; Schweizer, T.; Zenobi-Wong, M.; Cerrutti, M.; Mezzenga, R. Amyloid-Hydroxyapatite Bone Biomimetic Composites. *Adv. Mater.* 2014, 26 (20), 3207–3212.

(10) Yan, H.; Saiani, A.; Gough, J. E.; Miller, A. F. Thermoreversible protein hydrogel as cell scaffold. *Biomacromolecules* 2006, 7 (10), 2776–2782.

(11) Cao, Y. P.; Bolisetty, S.; Adamcik, J.; Mezzenga, R. Elasticity in Physically Cross-Linked Amyloid Fibril Networks. *Phys. Rev. Lett.* 2018, 120 (15), 158103.

(12) Pogostin, B. H.; Linse, S.; Olsson, U. Fibril Charge Affects α-Synuclein Hydrogel Rheological Properties. *Langmuir* 2019, 35 (50), 16536–16544.

(13) Semerdzhiev, S. A.; Lindhou, S.; Stefanovic, A.; Subramaniam, V.; van der Schoot, P.; Claessens, M. Hydrophobic-Interaction-Induced Stiffening of α-Synuclein Fibril Networks. *Phys. Rev. Lett.* 2018, 120 (20), 6.

(14) Reynolds, N. P.; Charney, M.; Mezzenga, R.; Hartley, P. G. Engineered Lysozyme Amyloid Fibril Networks Support Cellular Growth and Spreading. *Biomacromolecules* 2014, 15 (2), 599–608.

(15) Yang, L. J.; Li, H. Y.; Yao, L. X.; Yu, Y.; Ma, G. Amyloid-Based Injectable Hydrogel Derived from Hydrolyzed Hen Egg White Lysozyme. *Acta Omega* 2019, 4 (5), 8071–8080.

(16) van Dalen, M.; Karperien, M.; Claessens, M.; Post, J. Amyloid microwebs for cartilage repair. *Osseointegr.* *Cartilage* 2017, 25, S158–S159.

(17) Charrier, E. E.; Pogoda, K.; Wells, R. G.; Janney, P. A. Control of cell morphology and differentiation by substrates with independently tunable elasticity and viscous dissipation. *Nat. Commun.* 2018, 9, 13.
(18) Chaudhuri, O.; Gu, L.; Klumper, D.; Darnell, M.; Bencherif, S. A.; Weaver, J. C.; Huebsch, N.; Lee, H. P.; Lippens, E.; Duda, G. N.; Mooney, D. J. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. Nat. Mater. 2016, 15 (3), 326.
(19) Lamour, G.; Kirkegaard, J. B.; Li, H.; Knowles, T. P. J.; Gsponer, J. Easyworm: an open-source software tool to determine the mechanical properties of worm-like chains. Source Code Biol. Med. 2014, 9, 16.
(20) Aulisa, L.; Dong, H.; Hartgerink, J. D. Self-Assembly of Multidomain Peptides: Sequence Variation Allows Control over Cross-Linking and Viscoelasticity. Biomacromolecules 2009, 10 (9), 2694−2698.
(21) Ozbas, B.; Kretsinger, J.; Rajagopal, K.; Schneider, J. P.; Pochan, D. J. Salt-triggered peptide folding and consequent self-assembly into hydrogels with tunable modulus. Macromolecules 2004, 37 (19), 7331−7337.
(22) Schmidt, C. F.; Barmann, M.; Isenberg, G.; Sackmann, E. Chain dynamics, mesh size, and diffusive transport in networks of polymerized actin - a quasielastic light-scattering and micro-fluorescence study. Macromolecules 1989, 22 (9), 3638−3649.
(23) Heussinger, C.; Frey, E. Stiff polymers, foams, and fiber networks. Phys. Rev. Lett. 2006, 96 (1), 4.
(24) Lieleg, O.; Claessens, M.; Luan, Y.; Bausch, A. R. Transient binding and dissipation in cross-linked actin networks. Phys. Rev. Lett. 2008, 101 (10), 108101.
(25) Semerdzhiev, S. A.; Dekker, D. R.; Subramaniam, V.; Claessens, M. Self-Assembly of Protein Fibrils into Suprafibrillar Aggregates: Bridging the Nano- and Mesoscale. ACS Nano 2014, 8 (6), 5543−5551.
(26) Bianco, V.; Franzese, G. Contribution of Water to Pressure and Cold Denaturation of Proteins. Phys. Rev. Lett. 2015, 115 (10), 5.
(27) Temple-Wong, M. M.; Ren, S. W.; Quach, P.; Hansen, B. C.; Chen, A. C.; Hasegawa, A.; D’Lima, D. D.; Koziol, J.; Masuda, K.; Lotz, M. K.; Sah, R. L. Hyaluronan concentration and size distribution in human knee synovial fluid: variations with age and cartilage degeneration. Arthritis Res. Ther. 2016, 18, 18.
(28) Deek, J.; Chung, P. J.; Kayser, J.; Bausch, A. R.; Safinya, C. R. Neurofilament sidearms modulate parallel and crossed-filament orientations inducing nematic to isotropic and re-entrant birefringent hydrogels. Nat. Commun. 2013, 4, 2224.
(29) Bennike, T.; Ayturk, U.; Haslauer, C. M.; Froehlich, J. W.; Proffen, B. L.; Barnaby, O.; Birkeland, S.; Murray, M. M.; Warman, M. L.; Stensballe, A.; Steen, H. A Normative Study of the Synovial Fluid Proteome from Healthy Porcine Knee Joints. J. Proteome Res. 2014, 13 (10), 4377−4387.
(30) Loebel, C.; Mauck, R. L.; Burdick, J. A. Local nascent protein deposition and remodelling guide mesenchymal stromal cell mechanosensing and fate in three-dimensional hydrogels. Nat. Mater. 2019, 18 (8), 883.
(31) Jin, R.; Hiemstra, C.; Zhong, Z. Y.; Feijen, J. Enzyme-mediated fast in situ formation of hydrogels from dextran-tyramine conjugates. Biomaterials 2007, 28 (18), 2791−2800.
(32) Jin, R.; Moreira Teixeira, L. S.; Dijkstra, P. J.; Karperien, M.; van Blitterswijk, C. A.; Zhong, Z. Y.; Feijen, J. Injectable chitosan-based hydrogels for cartilage tissue engineering. Biomaterials 2009, 30 (13), 2544−2551.
(33) Jin, R.; Moreira Teixeira, L. S.; Dijkstra, P. J.; van Blitterswijk, C. A.; Karperien, M.; Feijen, J. Chondrogenesis in injectable enzymatically crosslinked heparin/dextran hydrogels. J. Controlled Release 2011, 152 (1), 186−195.
(34) Discher, D. E.; Janmey, P.; Wang, Y. L. Tissue cells feel and respond to the stiffness of their substrate. Science 2005, 305 (5751), 1139−1143.
(35) Watt, F. M.; Huck, W. T. S. Role of the extracellular matrix in regulating stem cell fate. Nat. Rev. Mol. Cell Biol. 2013, 14 (8), 467−473.
(36) Boettcher, K.; Grumbein, S.; Winkler, U.; Nachtsheim, J.; Lielig, O. Adapting a commercial shear rheometer for applications in cartilage research. Rev. Sci. Instrum. 2014, 85 (9), 093903
(37) Cameron, A. R.; Frith, J. E.; Cooper-White, J. J. The influence of substrate creep on mesenchymal stem cell behaviour and phenotype. Biomaterials 2011, 32 (26), 5979−5993.