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Viscoelastic hydrogels for 3D cell culture

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In tissues, many cells are surrounded by and interact with a three-dimensional soft extracellular matrix (ECM). Both the physical and biochemical properties of the ECM play a major role in regulating cell behaviours. To better understand the impact of ECM properties on cell behaviours, natural and synthetic hydrogels have been developed for use as synthetic ECMs for 3D cell culture. It has long been known that ECM and tissues are viscoelastic, or display a time-dependent response to deformation or mechanical loading, exhibiting stress relaxation and creep. However, only recently have there been efforts made to understand the role of the time-dependent aspects of the ECM mechanics on regulating cell behaviours using hydrogels for 3D culture. Here we review the characterization and molecular basis of hydrogel viscoelasticity and plasticity, and describe newly developed approaches to tuning viscoelasticity in hydrogels for 2D and 3D culture. Then we highlight several recent studies finding a potent impact of hydrogel stress relaxation or creep on cell behaviours such as cell spreading, proliferation, and differentiation of mesenchymal stem cells. The role of time-dependent mechanics on cell biology remains largely unclear, and ripe for further exploration. Further elucidation of this topic may substantially advance our understanding of cell–matrix interactions during development, homeostasis, wound healing, and disease, and guide the design of biomaterials for regenerative medicine.

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1. Introduction

The ECM is a heterogeneous soft scaffold made up of proteins and biopolymers, such as collagens, laminins, proteoglycans, and hyaluronic acid, that provides physical support and biochemical signalling to cells in tissues.[1,2] Hydrogels have been used extensively as synthetic ECMs for 3D cell culture to elucidate the impact of specific physical and biochemical cues on cell behaviors.[3–5] Hydrogels are cross-linked hydrophilic polymer networks that have high water content. The polymers can be fully synthetic, such as polyethylene glycol[6–8] (PEG), naturally derived, such as agarose[9] or alginate,[10,11] or from natural ECM components, such as hyaluronic acid,[12] fibrin,[13] reconstituted basement membrane matrix,[14] or type-1 collagen.[15] The natural ECM component derived hydrogels contain bioactive ligands that engage receptors on cells,[16] while many of the other polymers, such as PEG, agarose, or alginate, are considered to be inert. For inert hydrogels, bioactivity can be introduced by coupling bioactive ligands, such as the cell adhesion motif RGD,[10,17] to the hydrogel.

Using hydrogels as synthetic ECMs, the impact of the elastic modulus of ECM on various cell behaviours has now been established. The elastic modulus is a measure of stiffness, and is defined as the slope of the stress–strain curve over small strains. On collagen-coated polyacrylamide hydrogels used for 2D culture of cells, it has been found that an increased elastic modulus impacts cell migration,[18,19] neuro-

nal branching,[20] cell spreading,[21,22] malignancy in breast cells,[23] differentiation[24,25] and stemness[26] of stem cells, proliferation,[27] and various other important biological processes. However, many cells function in a 3D microenvironment where they are surrounded by ECM and other cells, and a 3D microenvironment has been found to be critical for capturing specific biological processes and behaviors.[14,28,29] In hydrogel based 3D culture, it has similarly been found that altered stiffness alone impacts biological processes, though some of the results depend on the particular choice of material systems.[30–34]

While these studies have powerfully illustrated the role of mechanics in cell biology, ECMs in tissues are not just elastic, they are viscoelastic, and the role of the viscous aspects of ECM mechanics on cell behaviours remains less clear. Materials that are viscoelastic exhibit some combination of properties of elastic solids and viscous liquids. Two key features of viscoelastic materials are that they dissipate energy due to deformation, while purely elastic materials will store energy, and that they display a time-dependent mechanical response. In particular, viscoelastic materials exhibit stress relaxation in response to a deformation, or creep in response to an applied mechanical stress.[35] Some viscoelastic materials can also be plastic, or exhibit irreversible or permanent deformations in response to an applied strain or stress. Many soft tissues and ECMs have been found to be viscoelastic.[35–37] Some examples of tissues and biological materials that have been demonstrated to exhibit substantial viscoelasticity include brain,[36] adipose tissue,[38] liver,[39] breast,[40] muscle,[41] skin,[42] embryonic tissue,[43] developing tissue,[44] fibrin clots,[45] coagulated bone marrow,[37] a fracture haematoma,[37] and an early fracture callus.[46]

A number of recent efforts have developed hydrogels with tunable viscoelasticity to present a microenvironment that more closely matches native ECM that is viscoelastic. These studies have established that the time dependent aspects of matrix mechanics, related to the viscosity but independent of the initial stiffness, can have a substantial and unexpected impact on various important cell behaviours. We discuss this new and emerging area of research in this mini-review. We review the viscoelasticity of hydrogels, describing both the characterization of viscoelasticity and the molecular mechanisms that give rise to viscoelasticity, discuss newly developed approaches to tuning the viscoelasticity of hydrogels, and highlight recent studies demonstrating an impact of viscoelasticity on cell behaviours. As this is a mini-review, the discussion is not meant to be comprehensive but rather a selective discussion, highlighting a few recent papers. The reader is referred to a number of recent reviews for a more comprehensive overview of hydrogels for 3D culture and cell–ECM mechanotransduction.[47–52]

2. Hydrogel viscoelasticity

2.1 Characterization of viscoelasticity and plasticity

Various approaches have been utilized to characterize viscoelasticity in hydrogels. In this section, these approaches are reviewed. It should be emphasized that all tests provide information about the time-dependent mechanics of the materials, therefore providing complementary information. Typically, measurements of viscoelasticity of hydrogels are made using a rheometer to exert and measure shear stresses and strains, or using a mechanical tester to apply and measure stress and strain in compression or tension. A number of microscale techniques have emerged and are being increasingly used to characterize viscoelasticity, so these are also described in this section. In all tests, an important consideration is nonlinearity, as many viscoelastic hydrogels can exhibit a mechanical response that is also dependent the magnitude of stress or strain, or are nonlinear elastic.

Using shear rheology or compression testing, one standard test used to measure the viscoelastic properties of hydrogels is the stress relaxation test. In this test, a constant strain, ε_0, is applied and the responding stress, $\sigma(t)$ is measured over time (Fig. 1A and B). An elastic material would maintain a constant stress, while viscoelastic materials exhibit stress relaxation. Either the stress, or the relaxation modulus, $G(t)$, defined as $G(t) = \frac{\sigma(t)}{\varepsilon_0}$, can be reported. In this modality of measurement the relaxation modulus, corresponding to the resistance to deformation, is linearly related to the stress. The stress relaxation response can be characterized empirically, for example using the time at which it takes for the stress to relax to half its original value or $\tau_{1/2}$. A covalently crosslinked polyacrylamide hydrogel exhibits minimal stress relaxation under compression, while various soft tissues exhibit substantial stress relaxation with $\tau_{1/2}$ ranging from ~10 s–200 s (Fig. 1B). Alternatively, the stress relaxation response can be fit to a linear viscoelastic model, and the best-fit parameters can be reported. Some common models used to fit stress relaxation responses include the Maxwell model, consisting of a spring in series with a dashpot, the standard linear solid model, consisting of a Maxwell model in parallel with a spring, and the Maxwell–Weichert model, consisting of multiple Maxwell models in parallel.[53] Importantly, the strain at which the stress relaxation test is conducted can be important, as some hydrogels, such as collagen or fibrin gels, exhibit strain-enhanced stress relaxation[54] (Fig. 1C). At higher strains, $\tau_{1/2}$ can be reduced by several orders of magnitude in collagen gels.[54]

A complement to the stress relaxation test is the creep test, in which a constant stress, σ_0, is applied, and the responding strain, $\varepsilon(t)$, is measured over time (Fig. 1D). Either the strain, or the creep compliance, $J(t)$, defined as $J(t) = \frac{\varepsilon(t)}{\sigma_0}$, can be reported. An elastic material will exhibit a constant strain over time, while a viscoelastic material will display an increase in strain or compliance, described as material creep, over time. As with the stress relaxation, creep responses can be described empirically, for example by the time at which it takes for a material to creep to a strain that is 150% of the initial strain. Alternatively, the creep response can be fit to a linear viscoelastic model. The creep response can be dependent on the magnitude of the stress applied, as has also been found in collagen gels[54] (Fig. 1E).

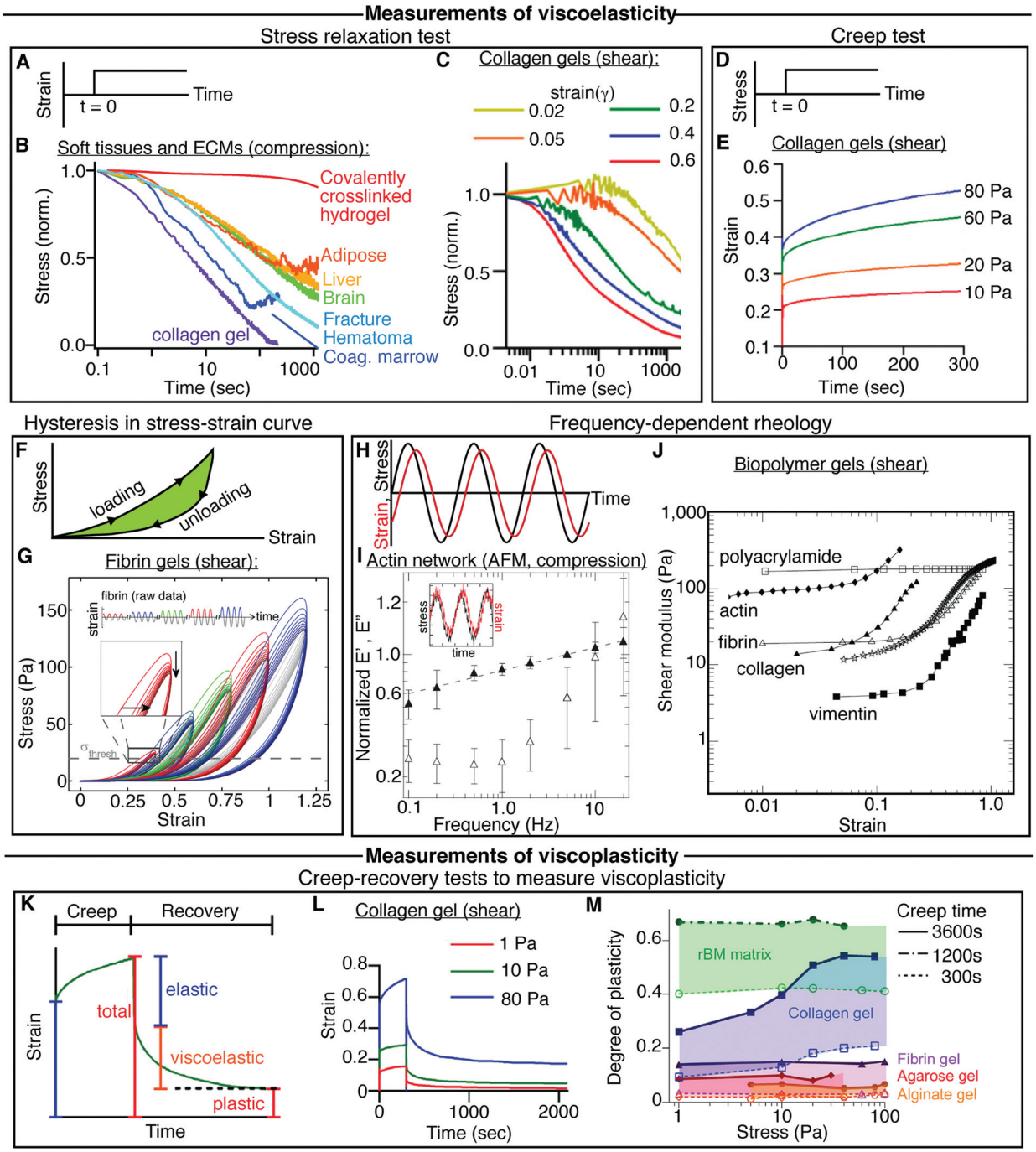

Fig. 1 (A) In a stress relaxation test, a constant strain is applied, and the resulting stress is measured over time. (B) Covalently crosslinked hydrogels exhibit minimal stress relaxation while various soft tissues and reconstituted ECMs exhibit substantial stress relaxation, indicating their viscosity. Panel from ref. 87. (C) Collagen gels exhibit strain-enhanced stress relaxation. Panel from ref. 54. (D) In a creep test, a constant stress is applied, and the resulting strain is measured over time. (E) Creep in collagen gels depends upon the applied stress. Panel from ref. 59. (F) Hysteresis in the stress strain curve during loading/unloading indicates energy dissipation and viscoelasticity. (G) Sequential stress–strain tests on fibrin gels indicates non-linearity of the stress–strain tests, and history dependent hysteresis in the stress–strain curve. Panel from ref. 55. (H) In frequency dependent rheology tests, a sinusoidally varying stress or strain is applied to the material, and the responding strain or stress is measured. Quantification of the in-phase response reveals the storage, or elastic, modulus, while quantification of the out-of-phase response reveals the loss, or viscous, modulus. (I) Quantification of the storage (filled triangles) and loss modulus (empty triangles) in actin networks reveals both parameters to be frequency dependent. Panel adapted from ref. 56 (J) The shear storage modulus of various biopolymer gels is strain dependent. Panel adapted from ref. 57, with permission from Nature Publishing Group. (K) Creep-recovery tests can be used to measure viscoplasticity, with the degree of plasticity indicated by the ratio of irreversible strains at long times in the recovery phase to the total strain during the creep test. (L) Creep-recovery tests of collagen gels show that plasticity, and stress dependence of the plasticity. (M) Creep-recovery measurements of various biopolymer gels demonstrate the plasticity to be dependent on the creep time in all the gels, and the creep stress in collagen gels. Panels K–M adapted from ref. 59, with permission from Elsevier.

In stress–strain tests, or tests where the strain, or stress, in the material is increased at a defined rate to a certain level (loading) and then decreased back to 0 (unloading), viscoelasticity manifests itself as hysteresis in the stress strain curve (Fig. 1F). The area between the curves represents the energy dissipated in the material during the loading and unloading cycle. As viscous liquids dissipate energy while elastic solids store energy, the degree of hysteresis corresponds to viscosity of the viscoelastic material. Hysteresis can depend on the magnitude of the stress or strain amplitude and rate used in the experiment. Fibrin and collagen gels both exhibit a nonlinear stress–strain response that exhibits substantial hysteresis and is history dependent[55] (Fig. 1G).

A fourth common approach to measuring viscoelasticity in hydrogels is dynamic mechanical testing. This method involves application of a sinusoidal stress or strain to the material, and measurement of the responding strain or stress (Fig. 1H). By comparing the amplitude and phase shift of the response, the storage, or elastic, modulus (G' or E' for shear or compression/tension respectively) and the loss, or viscous, modulus (G'' or E'' for shear or compression/tension respectively) can be determined as a function of frequency. Specifically, for a given frequency of oscillation, f, if strain, ε, is described as $\varepsilon = \varepsilon_0 \sin(2\pi ft)$, and the stress can be described as $\sigma = \sigma_0 \sin(2\pi ft + \delta)$, then the shear storage modulus can be calculated as $G' = \frac{\sigma_0}{\varepsilon_0}\cos(\delta)$ and the shear loss modulus can be calculated as $G'' = \frac{\sigma_0}{\varepsilon_0}\sin(\delta)$. For noisy data, G' and G'' can be calculated as $G'(f) + iG''(f) = \tilde{\sigma}(f)/\tilde{\varepsilon}(f)$, where $\tilde{\sigma}$ and $\tilde{\varepsilon}$ are the Fourier transforms of σ and ε, respectively. Under compression or tension, the storage modulus, E', and the loss modulus, E'', can be similarly calculated. The loss tangent, defined as $\tan(\delta) = \frac{G''}{G'}$, is often used to indicate how viscous the material response is. As the loss tangent may be frequency dependent, frequency must be specified. For example, both the storage and elastic modulus of dendritic actin networks are dependent upon the applied frequency, with the storage modulus displaying a weak power-law relation with frequency[56] (Fig. 1H). As with the other tests, the measured moduli can be nonlinear and dependent on the oscillation amplitude of the input stress or strain. For example, many biopolymer gels, including actin networks, fibrin gels, collagen gels, vimentin networks, and neurofilament networks, exhibit nonlinear elasticity in the form of strain or stress stiffening, or an increase in the shear storage modulus at greater strains or stresses[57,58] (Fig. 1J).

Many viscoelastic materials exhibit plasticity, or display permanent deformations in response to an applied strain or stress. While it has long been known that some hydrogels are plastic, few studies have focused on characterizing plasticity in hydrogel materials. In a recent study, we introduced a robust approach to characterizing plasticity in hydrogels[59] (Fig. 1K). Creep and recovery tests were performed, in which the stress was released following a creep test, and the strain was measured over time. Plasticity was characterized by quantifying the degree of plasticity, defined as ratio of strain remaining at long times, to the maximum strain induced during the creep test. Interestingly, the degree of plasticity was dependent both on the timescale of the creep test, and in some cases, the magnitude of the stress applied for various hydrogels (Fig. 1L and M). When the degree of plasticity depends upon the timescale of the creep test, the material can be referred to as viscoplastic. Notably, while many traditional engineering materials exhibit plasticity only when a sufficient stress, defined as the yield stress, is applied, plasticity was observed even at very small stresses in many of the hydrogel materials tested.[59]

While viscoelasticity and plasticity of hydrogels are most often measured using bulk techniques, a number of techniques are available to probe these properties at the microscale, a length-scale more relevant to cell–matrix interactions. One class of these techniques are indenter based techniques, involving probing the surface of a material with a probe, and include microindentation and Atomic Force Microscopy (AFM). Micro-indentation involves indentation into a sample with a rigid micron-scale probe, and measuring the resulting force with a micro-newton scale force gauge.[60] This tool has been used to measure the elastic modulus, stress relaxation, and plasticity of tissues and reconstituted collagen gels.[60,61]

AFM involves probing of a sample with a micron-scale cantilever that behaves like a Hookean spring for small deflections, and which can have a nanometer scale tip.[62] With a known or measured cantilever spring constant, detection of cantilever deflection with sub-nm resolution using an optical lever can lead to force resolution on the order of pico-newtons. The elastic modulus of hydrogels or cells can be determined from measured force-indentation curves, by fitting the curves to a model that takes into account the geometry of the cantilever tip.[63,64] AFM has been used extensively to characterize the elastic modulus of hydrogel substrates.[21,65] The storage and loss modulus can also be determined by performing AFM-based microrheology experiments, where sinusoidal oscillations are applied to the surface or tip.[66,67] AFM-based microrheology of cells demonstrated hysteresis in stress–strain loops and that the storage modulus increased with frequency following a weak power law.[67] AFM based microrheology can be used to perform nonlinear measurements to measure the stress or strain dependence of the viscoelasticity. Such measurements on reconstituted dendritic actin networks, which are found at the leading edge of crawling cells, revealed a regime of stress stiffening followed by a regime of reversible stress softening.[56] While capable, AFM based microrheology is not typically used to measure the viscoelastic properties of hydrogels.

Particle-based microrheology represents another approach to measuring mechanical properties at the microscale, and can be either passive or active. In passive particle-based microrheology, micron-size probe particles are embedded within a material, and the fluctuations of the particles due to Brownian motion are monitored, typically through dynamic light scattering[68] or direct imaging of the motions of individual beads.[69,70] If the particle size is larger than the mesh size of the gel, fluc-

tuations of the beads are limited by the viscoelastic properties of the gel. G' and G'' can be calculated from the equation $\tilde{G}(s) = \frac{k_B T}{\pi a s \langle \Delta \tilde{r}^2(s) \rangle}$, where k_B is Boltzmann's constant, T is temperature, a is radius of the particle, s is frequency in the Laplace domain, $\tilde{G}$ is the Laplace transformation of the complex shear modulus $G^*(\omega) = G'(\omega) + iG''(\omega)$, and $\langle \Delta \tilde{r}^2(s) \rangle$ is the Laplace transformation of the mean square displacement of the bead, $\langle \Delta r^2(s) \rangle$ (ref. 68). Alternatively the creep compliance, $J(t)$, can be calculated simply as $J(t) = \frac{\pi a \langle \Delta r^2(s) \rangle}{k_B T}$ (ref. 71). An advantage of particle-based microrheology relative to indentation-based techniques is that mechanical properties in the interior of the gel can be probed. However, a limitation of this technique is that it cannot be used to measure viscoelastic properties in hydrogels with a storage modulus greater than ~50 Pa, as fluctuations of beads in stiffer hydrogels are below the resolution of current measurement techniques.[72] With stiff hydrogels, particle-based microrheology can still be useful in identifying fluid-gel transitions and has been used to monitor the gelation of PEG-based hydrogels,[73] as well as the spatio-temporal dynamics of degradation of protease-degradable PEG hydrogels by cells.[74]

To better measure the properties of stiffer hydrogels and tissues, active particle-based microrheology can be used. In this approach, forces are applied to probe particles, either with an optical trap[75] or magnetic tweezers,[76] and the responding movement of the particle is measured. Even in stiffer gels, a sufficient force can induce measurable movements in the beads. As the responding bead displacement is a function of the viscoelastic properties of the surrounding medium, this technique can be used to determine viscoelastic properties of hydrogels. Such techniques have been applied to measure the viscoelastic properties of reconstituted ECMs and tissues.[77,78]

2.2 Molecular origins of viscoelastic behaviours in hydrogels

There are various molecular mechanisms that can give rise to viscoelasticity in hydrogels. As viscosity corresponds to energy dissipation, any molecular phenomenon that dissipates energy will lead to viscoelasticity. One source of viscoelasticity arises in physically, or non-covalently, crosslinked hydrogels. In these hydrogels, when a stress or strain is applied, crosslinkers in strained or stressed regions can unbind, allowing subsequent polymer matrix flow. In some cases, cross-linkers can then rebind following flow. This behavior is observed in weakly crosslinked collagen gels,[54] ionically crosslinked alginate gels,[79] and reversibly crosslinked PEG hydrogels.[41] Importantly, deformations of these materials can be plastic, as rebinding of crosslinkers and/or the lack of an elastic recovery force, secures the matrix in its configuration following flow.

In addition to crosslinker unbinding and polymer flow, various other mechanisms can give rise to viscoelasticity in hydrogels. The movement of entangled polymers, or "loose ends" of crosslinked polymers can also lead to viscoelasticity. Depending on the pore size and the nature of entanglements, polymers may be able to reptate out of entanglements when the hydrogel is stressed or strained.[80] If the entanglement carries a force or restricts deformation, release of the entanglement would then lead to stress relaxation or creep. Another mechanism that gives rise to viscoelasticity is protein unfolding, as the process of protein unfolding takes work, and thereby dissipates energy.[81,82] As proteins can refold, this represents a viscoelastic process that can be reversible and elastic, instead of plastic. Finally, hydrogels contain a high fluid content, and the movement of fluid in general dissipates energy. Therefore, even a covalently crosslinked hydrogel that is perfectly crosslinked, or containing no "loose" ends, will exhibit a finite loss modulus due to the water content. Such a covalently crosslinked hydrogel would not exhibit plasticity.

Time dependent mechanical responses of hydrogels can also occur due to poroelasticity. Poroelastic effects involve the movement of water through a porous matrix, and occur when a mechanical test results in a change of volume or geometry of the sample. For example, stress relaxation of covalently crosslinked hydrogels can occur under compression due to water leaving the periphery of the gel over time.[79] This movement is not instantaneous due to the small pore size of the hydrogels, which restricts the flow of water. Importantly, poroelastic effects can be geometry dependent, as the timescale for poroelastic stress relaxation under compression will depend upon the geometry of the sample, with larger samples exhibiting a longer timescale of stress relaxation.[79] In contrast, viscoelastic effects should be independent of the macroscopic geometry of the hydrogel.

2.3 Approaches to modulating viscoelasticity in hydrogels

Given the various molecular origins of viscoelasticity in hydrogels, numerous approaches have been devised to modulate the viscoelastic properties of hydrogels. In the area of hydrogels used for cell culture, an early approach to tuning the viscoelastic properties was presented by Cameron and colleagues using polyacrylamide hydrogels.[83] By varying both the concentration of acrylamide and bis-acrylamide crosslinker, this group developed a set of hydrogels with the same storage or elastic modulus, and a varying loss modulus and creep response. In their approach, a higher polymer concentration combined with a lower crosslinking density led to a hydrogel with a higher loss modulus but the same storage modulus. As the gels were covalently crosslinked, presumably the time dependent aspects of the mechanical response were mediated through the movement of loose ends of polymer chains, and it would be anticipated that these gels would be viscoelastic but not viscoplastic. While this approach has been demonstrated thus far only in polyacrylamide gels, presumably this could be extended to other covalently crosslinked hydrogels.

In physically crosslinked hydrogels, a number of different approaches to modulating viscoelasticity become possible. Physically-crosslinked hydrogels are typically viscoelastic, so one approach is to directly compare physically crosslinked hydrogels to covalently crosslinked hydrogels, by tuning the crosslinker concentrations so that the initial elastic response of both gels are similar. For example, ionically-crosslinked algi-

nate hydrogels exhibit substantial stress relaxation and a frequency dependent storage modulus while covalently crosslinked alginate hydrogels exhibit a frequency-independent storage modulus and stress relaxation under compression only due to poroelastic effects.[84] As stress relaxation in ionically crosslinked alginate hydrogels is thought to occur from crosslinker unbinding followed by polymer matrix flow, the ionically crosslinked alginate hydrogels are viscoplastic.[79] In a related approach, varying the concentration of both physical and covalent crosslinks can be used to tune viscoelastic responses, as was recently demonstrated in a peptide-based hydrogel material system.[85]

In another crosslinker-based approach, titrating the ratio of multiple weak crosslinkers can lead to different viscoelastic properties in hydrogels. This approach was demonstrated in PEG hydrogels, where McKinnon and colleagues utilized two hydrazine based bonds with different affinities alone or in combination to crosslink the hydrogels (Fig. 2A).[86] They

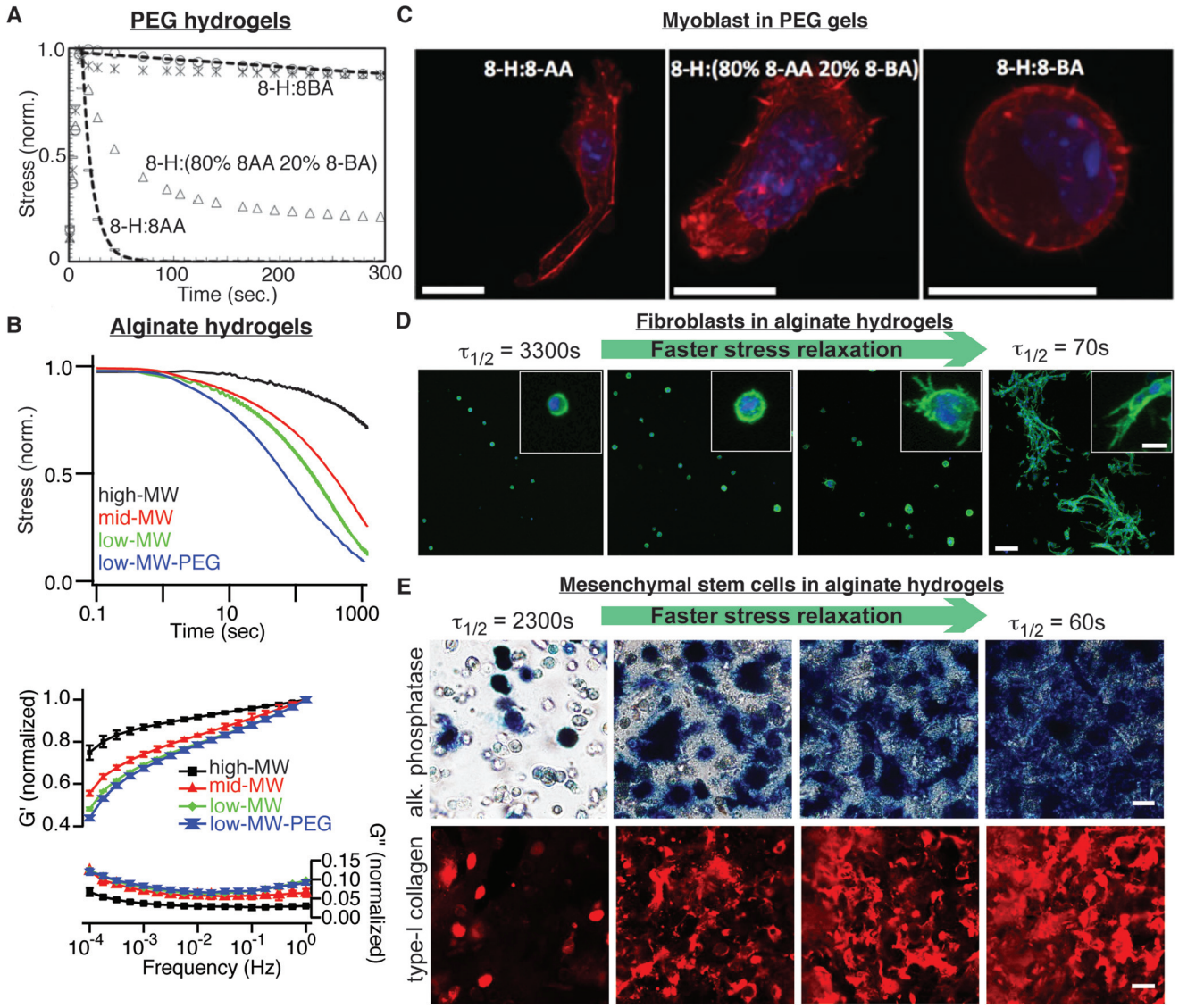

Fig. 2 (A) Stress relaxation tests of PEG hydrogels crosslinked with different functional groups. 8:H:8AA indicates crosslinking *via* aliphatic hydrazine end groups and aliphatic aldehyde groups; 8-H:8BA indicates crosslinking *via* aliphatic hydrazine groups and benzaldehyde groups; and 8-H:(80% A 20% 8-BA) indicates a combination of the two types of crosslinking. (B) Alginate hydrogels formed from alginate with different molecular weights (MW), or coupled with a short PEG spacer, exhibit different rates of stress relaxation. Faster stress relaxation corresponds to a higher loss modulus, and a greater rate of decrease in the storage modulus with frequency. (C) C2C12 myoblasts encapsulated in the different PEG hydrogel formations exhibit different morphologies. Actin indicated in red and nucleus in blue. Scale bars are 20 μm. (D) 3T3 fibroblasts encapsulated in RGD-coupled alginate hydrogels with different levels of stress relaxation exhibit different levels of cell spreading and proliferation. Actin shown in green and nucleus in blue. Scale bar is 100 μm for larger images and 20 μm for inset. (E) Alkaline phosphatase and type-1 collagen staining of MSCs encapsulated in RGD-coupled alginate hydrogels with an initial elastic modulus of 17 kPA but varying stress relaxation levels. ALP staining corresponds to osteogenic differentiation. Scale bars are 25 μm. Panels A and C are from ref. 41, and are reprinted with permission from John Wiley and Sons, and panels B, D, and E are from ref. 87.

demonstrated the development of hydrogels with the same initial elastic response, but varying stress relaxation rates, with $\tau_{1/2}$ ranging from ~10 s–6000 s. Some of the hydrogels were viscoplastic, while others were viscoelastic.

In addition, alteration of polymer architecture can also lead to tunable viscoelasticity in physically crosslinked hydrogels. In ionically crosslinked alginate hydrogels, reduction of the alginate polymer length, while increasing the concentration of ionic crosslinker and holding the overall concentration of alginate in the hydrogel constant, led to faster stress relaxation in the hydrogels while maintaining the same initial elastic response (Fig. 2B).[87] Further, covalent crosslinking of short PEG chains to the alginate, using carbodiimide chemistry, led to hydrogels with even faster stress relaxation and an increase in the loss modulus. It was hypothesized that the PEG molecules acted as spacers, which provided steric hindrance to crosslinking of the alginate chains, thereby promoting faster stress relaxation. Faster stress relaxation corresponded to a greater decrease in the storage modulus at lower frequencies, and a higher loss modulus, confirming that different viscoelastic tests provide complementary information. With these two approaches, $\tau_{1/2}$ was tuned from ~60 s–3600 s, while holding the initial elastic modulus constant. Importantly, this range covers the range of stress relaxation times measured in a number of soft tissues (Fig. 1B).

2.4. Impact of altered stress relaxation on cells

With the development of hydrogels with varying stress relaxation, creep, or viscosity, various studies have examined the impact of these properties on cell behaviours in both 2D culture and 3D culture. In 2D culture, several efforts have been made to understand the impact of time-dependent mechanics on cell spreading. On collagen-coated acrylamide hydrogels, increased stiffness will lead to greater cell spreading.[21,22] The current thinking about this mode of mechanotransduction is based on the idea of cells establishing a tensional homeostasis.[88–91] Cells are thought to exert contractile forces on substrates through integrin-based adhesions, and sense the elastic modulus by gauging resistance to these forces. Various molecules involved in the adhesion structures or contractile apparatus have been implicated as being force sensitive and mediating this response intracellularly, including talin,[92] vinculin,[93] and lamins.[94] Under this mechanism, it would be expected that increased stress relaxation, creep, or viscosity would reduce resistance to cell contraction over time, and therefore decrease cell spreading.

Several studies of cell spreading on viscoelastic substrates in 2D culture have presented findings counter to the simple expectation based on current models of mechanotransduction. On collagen-coated polyacrylamide gels, a greater loss modulus or increased creep promoted enhanced cell spreading.[83] Similarly, in alginate hydrogels, cells spread on soft, viscoelastic hydrogels to a greater extent than they did on elastic hydrogels with the same initial elastic modulus.[84] Further, when cultured on fibrillar dextran hydrogels, cell spreading on soft dextran substrates was diminished when welds, or rigid crosslinks, between the fibers, were added.[95] On the alginate and dextran hydrogels, increased cell spreading on the viscoelastic hydrogels was found to be associated with enhanced ligand clustering, presenting one possible explanation for the observed results, as ligand clustering is known to mediate various signaling pathways.[96,97] This enhanced clustering of hydrogel material could also lead to local stiffening. Alternatively, stress relaxation could potentially enhance the lifetime of integrin-ECM bonds that behave as slip bonds, as a decrease in force on individual bonds will lead to an increase in bond lifetime.[98] These highlight the rich complexity of cell–matrix mechanotransduction on viscoelastic 2D substrates.

Several studies have now examined the impact of hydrogel viscoelasticity in 3D culture on the morphological changes of cells. In 3D culture, cells are fully encapsulated in the hydrogels. As hydrogels used as synthetic ECMs are typically nanoporous, the hydrogel provides a physical barrier to processes involving cell shape change, cell movement, or cell expansion. Some covalent crosslinking approaches are cytotoxic and incompatible with 3D culture, limiting the number of approaches available for these studies. In PEG hydrogels, it was found that myoblast cells were able to spread in hydrogels with faster stress relaxation ($\tau_{1/2}$ ~10 s), but exhibited a purely rounded morphology in PEG hydrogels that were more elastic ($\tau_{1/2}$ ~6000 s)[41] (Fig. 2C). In the stress relaxing PEG hydrogels, embryonic stem cell derived motor neurons were able to extend neurites into the hydrogel.[99] Similarly, as the rate of stress relaxation was increased in RGD-coupled alginate hydrogels, with $\tau_{1/2}$ reduced from ~1 hour to ~1 min, fibroblast cells exhibited a greater degree of cell spreading and proliferation (Fig. 2D). In covalently crosslinked hydrogels, it has long been known that cells stay rounded and proliferation is inhibited. Previous work has bypassed this limitation by making the hydrogels degradable. The result of cells spreading in fast relaxing PEG and alginate hydrogels suggest that stress relaxation in these hydrogels may provide a complementary approach to facilitating cell behaviours that involve cell shape or volume change. Many of the natural ECM based material systems used for 3D cell culture, including collagen, fibrin, and reconstituted basement membrane matrix (*e.g.* matrigel) do exhibit substantial stress relaxation,[54,59] raising the possibility that fast relaxation may be a key aspect of these material systems that is underappreciated. As in 2D culture, enhanced clustering of ligands or local stiffening, resulting from matrix flow and densification, could contribute to the observed alteration of behaviors of cells in stress relaxing materials.

Hydrogel viscoelasticity also regulates the differentiation of mesenchymal stem cells (MSCs) in 3D culture. In alginate hydrogels, both the initial elastic modulus and the rate of stress relaxation were found to impact osteogenic differentiation of MSCs.[87] At an initial elastic modulus of 17 kPa, faster stress relaxation promoted osteogenic differentiation, by the MSCs (Fig. 2E). Faster stress relaxation not only promoted osteogenic differentiation, but also facilitated the formation of an interconnected, type-1 collagen rich, mineralized matrix by

the differentiated cells. The differentiation results complemented previous work finding that an elastic modulus of 20–30 kPa in slow-relaxing ionically crosslinked alginate hydrogels optimally promoted osteogenic differentiation of MSCs.[31] Contrastingly, in covalently crosslinked RGD-coupled hyaluronic acid (HA) based hydrogels, no osteogenic differentiation was observed at any stiffness, unless the hydrogels were engineered to be degradable.[100] One possible explanation that reconciles the contrasting results, is that mechanotransduction in MSCs may be sensitive to the difference in stress relaxation between slow-relaxing viscoelastic alginate hydrogels and more elastic covalently crosslinked HA hydrogels. Some minimal level of stress relaxation may be required for MSCs to undergo osteogenic differentiation in 3D culture. However, there are various other differences between the material systems and the studies that could alternatively contribute to the difference in results.

3. Conclusions

The use of viscoelastic hydrogels for 3D cell culture has emerged over the last several years. Here we sought to review different approaches to analysing hydrogel viscoelasticity, some of the underlying mechanisms that give rise to hydrogel viscoelasticity, and the established approaches to precisely modulating hydrogel viscoelasticity. While there are various methods used to characterize viscoelastic responses, stress relaxation tests have been most commonly used in recent studies of hydrogel viscoelasticity.

We also highlighted several studies revealing that time-dependent mechanics of viscoelastic hydrogels play a key role in several processes including cell spreading, proliferation, and stem cell differentiation. These early results suggest that time-dependent mechanics may be relevant broadly towards regulating cell biology, and are a distinct and critical parameter of the microenvironment that regulates cell function in tissues. As many ECMs and tissues are also nonlinear elastic,[57,101] an important question moving forward is how time-dependent and nonlinear mechanics jointly regulate cell behaviours. Further, various diseases, including breast cancer[102,103] and emphysema,[104] have been associated with alterations in tissue stiffness. Therefore, a pressing question is whether alterations in stress relaxation play a key role in the progression of these or other diseases.

From a mechanistic perspective, the mechanisms regulating the impact of stress relaxation, creep, or the loss modulus on cells remain unclear. Some of the results from culturing cells on viscoelastic hydrogels in 2D culture contrast expectations based on current theories of mechanotransduction. In 3D culture, reduced mechanical confinement and enhanced ligand clustering have both been identified for cells in hydrogels with fast stress relaxation. Are these mechanisms sufficient to explain the impact of stress relaxation on cell behaviours, or are there other complementary mechanisms occurring? Further, depending on the underlying mechanism of hydrogel viscoelasticity, some viscoelastic hydrogels can be viscoplastic. The importance of plasticity *versus* viscoelasticity in impacting cell behaviours remains yet to be elucidated.

We anticipate that the next few years will see an emergence of alternative approaches to modulating hydrogel viscoelasticity and plasticity, perhaps in combination with modulation of nonlinear elasticity, in new material systems, insights into new biological processes that are regulated by stress relaxation, and new mechanistic insights into how cells sense and respond to time-dependent mechanics.

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