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The Plot Thickens: The Emerging Role of Matrix Viscosity in Cell Mechanotransduction

Marco Cantini, Hannah Donnelly, Matthew J. Dalby, and Manuel Salmeron-Sanchez**

Cell mechanotransduction is an area of intense research focus. Until now, very limited tools have existed to study how cells respond to changes in the extracellular matrix beyond, for example, mechanical deformation studies and twisting cytometry. However, emerging are a range of elastic, viscoelastic and even purely viscous materials that deform and dissipate on cellular length and timescales. This article reviews developments in these materials, typically translating from 2D model surfaces to 3D microenvironments and explores how cells interact with them. Specifically, it focuses on emerging concepts such as the molecular clutch model, how different extracellular matrix proteins engage the clutch under viscoelastic-stress relaxation conditions, and how mechanotransduction can drive transcriptional control through regulators such as YAP/TAZ.

1. Introduction

The tissues where cells reside in vivo are mechanically dynamic and dissipative environments, where rapid and adaptive processes drive the interactions between cells and their surroundings.[1] However, standard materials used in cell culture provide static environments to cells; similarly, most synthetic systems designed to mimic native tissues or to promote healing after an injury focus on elastic mechanical properties and neglect to consider their viscous nature. Only recently, an expanding body of work has been tackling the role of time-dependent (viscoelastic) mechanical properties in driving cell behavior.[2–4] Nevertheless, while the mechanisms behind cell response to elastic systems have been widely explored and are at the basis of currently accepted paradigms underpinning cells' ability to sense and respond to mechanical cues, the understanding of cellular response to viscous interactions remains in its infancy.

Indeed, the adhesion, migration, proliferation, and differentiation of a number of cell types have been modulated by varying the stiffness of elastic substrates, such as synthetic extracellular matrices (ECMs) based on polyacrylamide hydrogels.[5] These substrates behave as elastic solids: when a constant stress or strain is applied to them, they respond with a strain or a stress that is also constant with time, storing mechanical energy until the force or deformation is released and the material returns fully to its original configuration (**Figure 1**A-1). As such, these synthetic materials are mechanically "elastic," and, albeit they do not fully recapitulate the properties of native ECMs, they have been instrumental in understanding the process of mechanotransduction, i.e., how cells sense mechanical stimuli and convert them into a biochemical response. Natural ECMs exhibit some combination of properties of elastic solids and viscous liquids: the former grant them elastic resistance to applied forces conferring mechanical strength to the tissue, while the latter allow them to adapt and rearrange under stress. Indeed, a purely viscous system "flows": the strain increases linearly (and irreversibly) with time when a constant force is applied (Figure 1A-2)—this is called creep; alternatively, the stress diminishes linearly when a constant strain is applied—this is called stress relaxation. Materials that combine elastic and viscous features, i.e., viscoelastic materials, are mechanically "dynamic": they dissipate forces applied, e.g., by cells residing within them, and they show a time-dependent mechanical response (Figure 1A-3), usually quantified using the characteristic times associated to stress relaxation or creep (Figure 1B). In particular, relaxation times, which provide an empirical measure of the rate of stress decay in response to a deformation, are frequently used to characterize this type of materials, as they allow a rapid comparison to native tissues or ECMs.[3] Viscoelastic materials can also display permanent or "plastic" deformations: upon removal of the applied stress, the material does not recover its initial configuration, but rather retains a certain level of deformation due to microstructural rearrangements (Figure 1A-3). This can be measured via a creep-recovery test, where the ratio of the remaining strain to the maximum one can be used to quantify the degree of plasticity of the material.[6]

The viscoelastic nature of the network that forms the skeleton of natural ECMs and of the synthetic hydrogels that mimic them is not the only cause of their dissipative behavior. Indeed, their high water content contributes to it, as the long-range migration of water molecules through their porous matrix allows stress to relax upon application of a constant strain, a phenomenon known as "poroelasticity."[3,7] Nonetheless, most studies using hydrogels to address the role of time-dependent

Dr. M. Cantini, Dr. H. Donnelly, Prof. M. J. Dalby,
Prof. M. Salmeron-Sanchez
Centre for the Cellular Microenvironment
University of Glasgow
Glasgow G12 8QQ, UK
E-mail: marco.cantini@glasgow.ac.uk;
manuel.salmeron-sanchez@glasgow.ac.uk

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mechanical properties on cell response consider them as simple viscoelastic materials, while the poroelastic effects are not taken into account. This simplification is acceptable when the diffusion time of the solvent is much higher than the timescales of viscoelastic relaxation and of the (cellular) processes that are studied.[7]

In this review, we will first elucidate the current paradigms in cell mechanotransduction, with a focus on the molecular mechanisms involved in mechanosensing at the interface between cells and their environment. Then, we will discuss recent developments in the design of synthetic materials with controlled dissipative properties, focusing on the impact of viscous interactions on the cell mechanotransductive response.

2. Mechanotransduction at the Cell–Matrix Interface

In this section, we review the molecular mechanisms through which cells sense the mechanical properties of both natural or synthetic environments. Sensing of the mechanical environment is a critical first step toward cell decision making; as such, mechanobiology at cell adhesions is crucial for tissue homeostasis, development and the outcome of many diseases.[8]

The ability of cells to sense and respond to mechanical stimuli is termed mechanotransduction. This requires the sensing of external forces and the transduction of this information, triggering a specific intracellular signaling response. The cytoskeleton plays a critical role in mechanotransduction by linking cellular components, e.g. cytoskeletal components and the nucleus, to integrins, which together comprise the force sensing apparatus.[9] Sensing of the mechanical environment enables cells to rapidly respond to whole tissue parameters such as ECM stiffness, influencing decisions regarding the form, function and fate of the cells.[10,11]

2.1. Integrin-Mediated Adhesion

In order to understand how cells sense and respond to their external environment, cell adhesion must first be considered. Cellular distribution of different types of adhesions is dependent on both cell type and the composition and mechanical properties of the ECM.[12] Cells express various cell surface adhesion receptors (including integrins, syndecans, and other proteoglycans, cadherins and other cell–cell adhesion molecules); however, the integrin family of transmembrane heterodimeric receptors is the best studied and is the key player in mechanotransduction.[13,14] The extracellular domains of the integrins bind specific motifs present in ECM proteins, such as fibronectin, vitronectin, collagen, and other.[15] Integrin-ligand binding induces conformational changes that unmask their short cytoplasmic tails, promoting linkage to the actin cytoskeleton and recruitment and assembly of intracellular structural or regulatory proteins, forming integrin adhesion complexes (IACs) at the cell membrane.[14–17] Several of these intracellular molecules have been shown to exhibit tension-dependent conformational changes that alter their kinase activities, leading to cellular response.[18]

Marco Cantini is a Research Fellow at the Centre for the Cellular Microenvironment of the University of Glasgow. After his bachelor's and master's degrees at Politecnico di Milano, he earned a Ph.D. in biomedical and biomechanical engineering through a joint graduate program of the three Italian Technical Universities. He then held postdoctoral positions at the Universitat Politècnica de València and at the University of Glasgow, working on cell–protein–material interactions to control cell fate and on material-based strategies to engineer tissue repair. As UKRI fellow and proleptic lecturer, he seeks to engineer microenvironments to harvest stem cell response to viscosity.

Hannah Donnelly is a post-doctoral researcher in the Centre for the Cellular Microenvironment at the University of Glasgow. Hannah completed her bachelor's degree at the University of Glasgow, and went on to carry out her Ph.D. there, investigating biomaterial strategies to gain insight into dynamics of stem cell self-renewal. Her research interests are in engineering cellular microenvironments to model stem cell niches for differentiation and self-renewal, with a focus on the cellular metabolome.

Manuel Salmeron-Sanchez is professor of biomedical engineering and co-director with Matt Dalby of the Centre for the Cellular Microenvironment at the University of Glasgow. He did a Ph.D. in Valencia and postdoctoral research at the Charles University in Prague and KU Leuven. He was full professor in Valencia and visiting professor in Georgia Tech before relocating his group to Glasgow in 2013. He develops materials that promote high-efficiency presentation of growth factors and the role of viscosity in mechanotransduction. Manuel is interested in in vitro systems for drug screening and disease modeling, along with materials for cancer research, living (bacteria-based) biomaterials, and regenerative medicine.

Integrin function is subject to complex and tight regulation, from both biochemical (activation) and mechanical (mechanotransduction) perspectives.[13] Several recent reviews detail

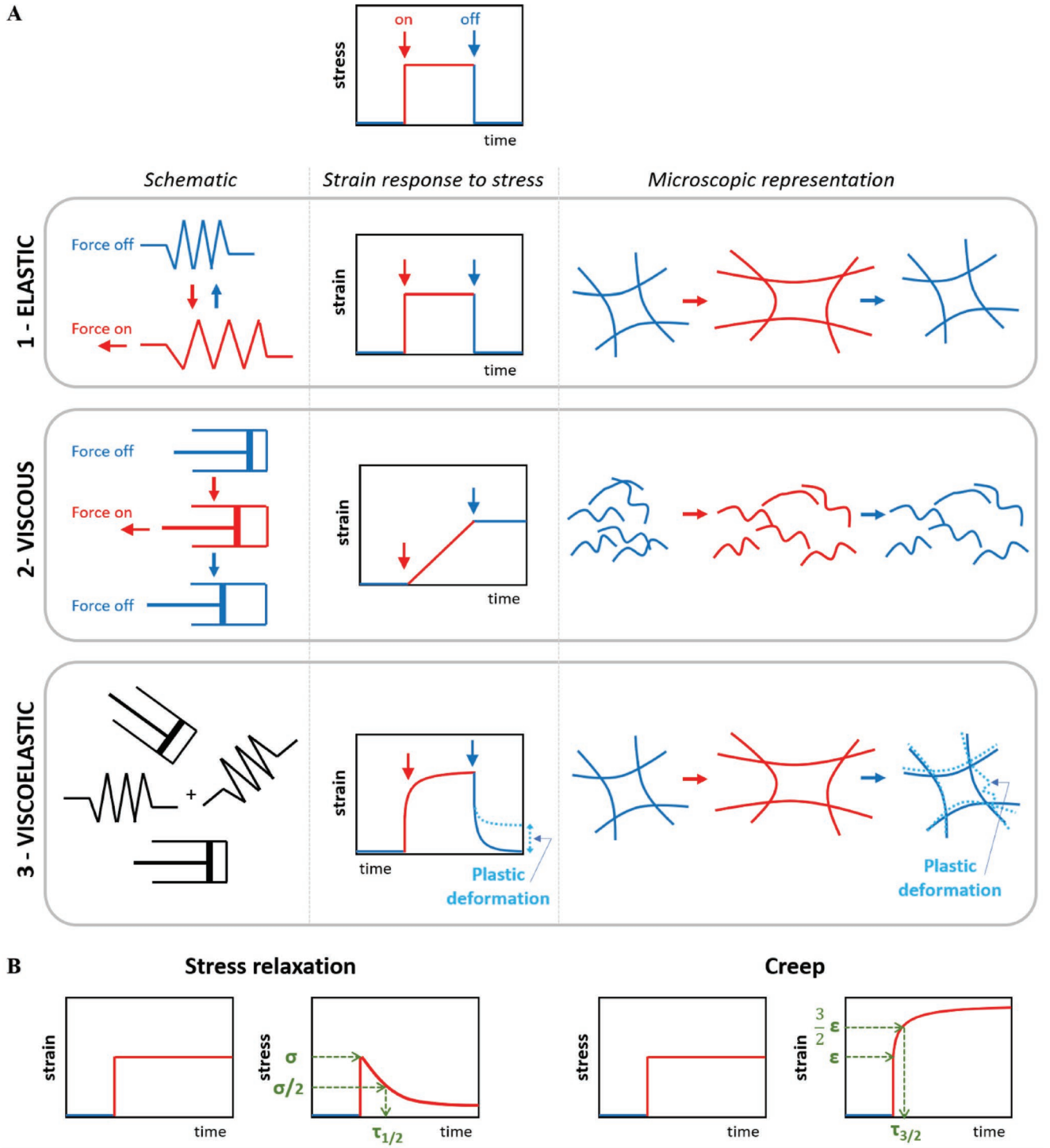

Figure 1. Sketch of elastic, viscous, and viscoelastic substrates. A) After application of a constant stress (force "on," red line), an elastic material (1), represented as a spring, deforms instantaneously, as elucidated by its sharp strain response to the applied stress. This deformation is maintained until the applied force is removed (force "off," blue line). In a viscous system, represented by a dashpot (2), the deformation instead increases linearly and irreversibly, and the original configuration of the material is not recovered after removal of the applied force, as elucidated in its microscopic representation. Natural and synthetic polymer networks are viscoelastic and behave as a combination of springs and dashpots (3), with time-dependent responses to an applied stress, as evidenced by the strain response curve. Moreover, when the stress is removed, the material can exhibit permanent or plastic deformation (represented by the dotted blue lines). Adapted under the terms of the Creative Commons Attribution License.[1] Copyright 2017, the Authors. Published by Elsevier. B) Standard tests to measure the viscoelastic properties of a material: stress relaxation, which measures the decrease of the stress after application of a constant strain, and creep, which instead measures the strain increase after application of a constant stress. Empiric measures of viscoelasticity include the time $\tau_{1/2}$ after which the stress is halved and the time $\tau_{3/2}$ it takes for a material to creep to 150% of its initial strain.

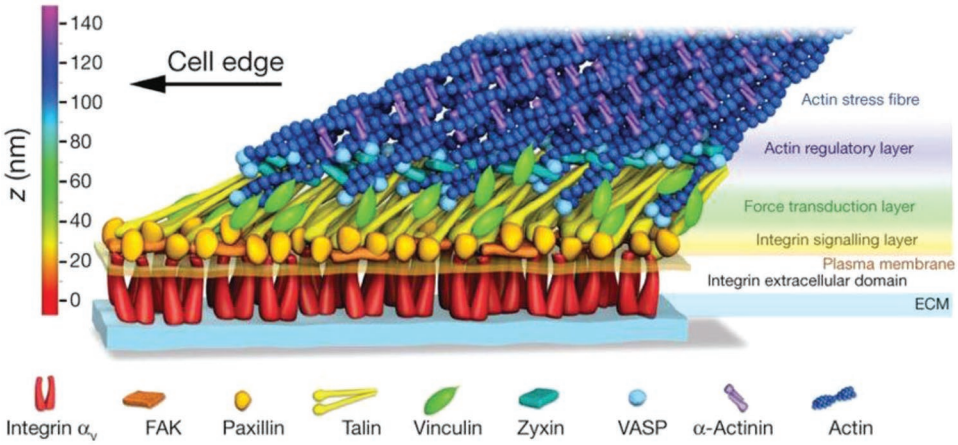

Figure 2. Model of focal adhesion molecular architecture. Schematic shows integrins bound to the ECM and the three overlapping layers of the focal adhesion core linking the ECM to the actin cytoskeleton: integrin signaling layer, force transduction layer and the actin regulatory layer. Reproduced with permission.[35] Copyright 2010, Springer Nature.

both this biochemical[13,19,20] and mechanical[12,21,22] integrin regulation.[13] Further to this, different integrin types exhibit different ECM binding rates,[23,24] and, as will be discussed below, this dictates how cells sense the rigidity of their environment.[23] As such, regulation of the integrin type at the cell membrane suggests that cells can tune how they sense and respond to environmental stiffness, for example, in healthy or pathological tissues.

2.1.1. Architecture of Focal Adhesions

The hierarchal structure of the linkage of integrin-actin is complex. The large number of proteins involved in this process has been intensely studied and is termed the "adhesome".[25] Recent proteomic analysis on the adhesome include over 2000 proteins as IAC components, with a consensus 60 defined as central to IAC formation,[26] encompassing 180 protein–protein interaction nodes.[27] The formation, disassembly and maturation of IACs is tightly regulated in time and space.[28] Cells form membrane protrusions—lamellipodia, filopodia, or both—that are driven by polymerization of actin filaments.[29] Protrusions are stabilized by adhesions that link the actin cytoskeleton to the ECM protein, and actomyosin contraction generates traction forces on the substrate.[30–32] Nascent IACs are small and transient, with relatively short lifetimes.[30,31] Nascent IACs that do not disassemble instead enlarge, growing initially at the rear of the lamellipodium;[28] IACs then cluster to form focal complexes. Focal complexes mature into larger, elongated supramolecular complexes called focal adhesions (FAs). FAs can themselves further mature into fibrillar adhesions, which support ECM synthesis and remodeling.[33,34] Focal complexes may assemble without actomyosin-mediated force, and rapidly disassemble if no external force is applied. However, as soon as they connect to the force machinery, focal complexes mature into FAs.[1,28]

Superresolution microscopy has provided insight into the molecular architecture of FA structures.[35] Integrins and actin are separated by a ≈40 nm core region that consists of partially overlapping nanoorganized molecular layers. The intracellular layer of FAs is composed by scaffolding, docking and signaling proteins that collectively serve as an interface between the transmembrane components directly linking the ECM to the actin cytoskeleton.[35,36] Such FAs are large, dynamic macromolecular assemblies; in total these plaques span <150 nm (**Figure 2**).[37] The signaling layer, closest to the plasma membrane, contains the highly phosphorylated signaling proteins focal adhesion kinase (FAK) and paxillin. Next, the force transduction layer, comprised of adaptor proteins talin and vinculin, links the integrin complex to the actomyosin machinery. From these adaptor proteins, talin tethers span the whole layer to regulate the ultrastructures, with talin heads binding integrins and its rod binding actin up to 30 nm away from the integrins, forming integrin–talin–actin complexes that work as mechanical linkages.[35,36] The most distal actin regulatory and actin stress fiber layers contain zyxin, VASP and α-actinin; these lie up to 60 nm away from the integrin layer.[35,36] FA architecture and layer organization are demonstrated in Figure 2.

2.1.2. Adhesion in Three Dimensions

Initially, the primary model for studying mechanosensing and FAs was largely carried out on fibroblasts cultured on 2D planar substrates of uniform stiffness (e.g., tissue culture plastic).[34] However, questions were raised as to the physiological relevance of these in vitro adhesions compared to their in vivo counterparts.[38] For example, culturing fibroblasts on flat substrates induces artificial polarity between the upper and lower surfaces of these normally nonpolar cells, and indeed, study of fibroblast behavior in 3D collagen gels leads to distinct differences in morphology and migration.[39] Early (2D) work has highlighted the remarkable molecular heterogeneity in adhesions formed by cells cultured on various substrates;[40] however such studies do not identify which adhesions are akin to those formed in vivo.

3D microenvironments composed of single or multiple ECM proteins can alter cellular responses and have revealed that this

response is multifaceted. Cukierman et al. originally found that dimensionality, molecular composition and mechanical properties all played important roles in mediating differences in adhesion formation and downstream signaling.[41] This study revealed that 3D adhesions are elongated and morphologically distinct from the FA and fibrillar adhesions typical of cultured cells, with low levels of FAK phosphorylation, contrasted with the highly phosphorylated FAK of 2D FAs.[40] 3D adhesions have higher abundance of the major fibronectin receptor $\alpha_5\beta_1$ compared to 2D FAs.[41] This could suggest higher tension in 3D adhesion, since the $\alpha_5\beta_1$ integrin binds directly to the synergy site of fibronectin, an interaction associated with stabilization of 2D adhesions that are under tension.[42,43] Increased stability could be responsible for the observed increase in longevity of 3D matrix adhesions compared to 2D FAs.[44]

Currently, the molecular composition of adhesions remains less well defined in 3D microenvironments than in 2D substrates, but, in general, the molecular composition of 3D adhesions appears to involve the same key players as those of 2D substrates. Differing combinations of proteins have been identified between different systems; for example, β_1 integrin, vinculin, and paxillin are found in 3D adhesions in fibrin,[45] along with zyxin, tensin-1, and talin in collagen gels.[46,47] Furthermore, changes in abundance, e.g. of $\alpha_5\beta_1$,[41] or in phosphorylation of proteins, e.g., the autophosphorylation site tyrosine 397 on FAK,[43] demonstrate that dimensionality is not enough to explain the differences, adding to the complexity of these adhesions. These differences appear to be dependent on many factors, such as dimensionality, matrix mechanics, and ligand availability, making it difficult to fully define the machinery involved. Nevertheless, investigation using simpler 2D systems is valuable in elucidating translation to 3D, and ultimately provides insight into more complex in vivo systems.

2.2. Mechanisms of Mechanotransduction—the Molecular Clutch

FAs are extremely dynamic, mediating the link of cells to their microenvironment by a series of bonds that dynamically engage and disengage.[48] Cell–ECM adhesions towards the leading edge of the cell drive cell movement as myosin-powered contractility and actin polymerization push against the membrane (**Figure 3**).[49,50] The force exerted by myosin-powered contractility and actin filament elongation drives the membrane

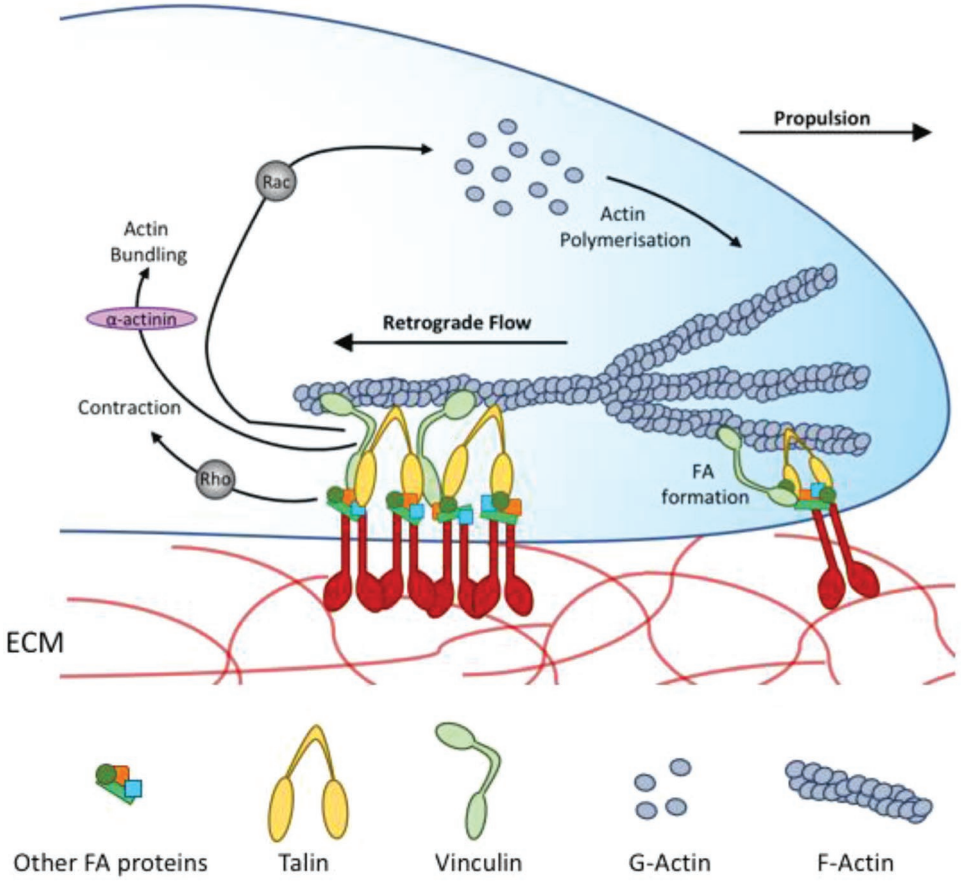

Figure 3. The molecular clutch. In the clutch model, actin polymerization and myosin contractility at the leading edge of the cell generate a retrograde flow of F-actin that generates force for integrins to pull on the ECM. As a consequence, talin unfolds and vinculin binds to stabilize the clutch. This generates tensional forces across these molecules and regulates their activation states. Talin and vinculin bind to many other signaling and scaffolding proteins, such as FAK and paxillin, which then produce downstream signaling events, regulating the activity of Rho-family GTPases. Talin and vinculin activation states regulate their dynamic turnover, and downstream signaling pathways that contribute to events such as greater contractility and FA growth through Rho, or enhanced actin polymerization at the leading edge through Rac.

forward, but only if the pushing force is in opposition with actin filaments that are ligated to the ECM. If the actin filaments are not ligated, the force of continuous polymerization leads to the backward motion of the filament network, known as retrograde flow.[51,52] Engagement of these filaments with IACs results in slowing down of retrograde flow, but due to the continuous polymerization of actin it fosters actin protrusion away from the cell center, thus pushing the cell membrane and generating cellular protrusions. As such, retrograde flow inversely correlates with cell migration speed.[50,52,53] The flow is partially transmitted to adaptor proteins and integrins, meaning molecules closer to the ECM experience progressively slower retrograde speeds.[54,55] Retrograde flow is observed for different types of actin structures, from lamellipodia to stress fibers, and similarly in cadherin-based cell–cell adhesions.[56] In short, IACs formed at the leading cell edge couple actin-ECM through FA adaptor proteins; this results in a traction force on the ECM and net forward movement of the cell (Figure 3).[57]

This link between actin and ECM appears to be central to adhesion-based mechanotransduction and has been termed the "molecular clutch" or, specifying its main components, "actin–talin–vinculin–ECM (e.g., fibronectin) clutch." The molecular clutch model has long been proposed to explain actin cytoskeleton and cell migration dynamics, but has now been used, somewhat counter-intuitively, to also explain force transduction in response to substrate stiffness.[58,59] Indeed, cell adhesion to a substrate involves engagement of the clutch: this entails unfolding of talin and its stabilization by vinculin. Talin unfolding is mediated through the force generated at the actin-integrin links of the protein, and it needs time to be completed: unfolding is faster the quicker the force is built up on pulling coming from both sides of the protein (actin filaments and integrins)—this is the force loading rate, and is the key concept to be considered. To engage the clutch, talin unfolding has to occur before integrins unbind from the ECM (this happens with a characteristic biochemical lifetime). On the other hand, if integrin unbinding (from the ECM) happens before talin unfolding, the clutch cannot engage and the adhesion is not stabilized. Based on this, the clutch model can explain cell response to substrate rigidity or stiffness. At low stiffness (<5 kPa), the time needed to build up force to unfold talin is high (i.e., the force loading rate is low); as a result, integrins unbind from the ECM before talin is unfolded and the clutch does not engage (i.e., the adhesion slips). On the contrary, on stiff surfaces (>5 kPa), force is built up quickly (i.e., the force loading rate is high); this leads to talin unfolding and stabilization by binding to vinculin (i.e., the adhesion grips). The clutch is engaged and stabilized and cell adhesion to the ECM is secured.[58,59] Further to this, differences in timescales of integrin binding will affect matrix stiffness sensing; for example, both binding and unbinding rates to fibronectin are higher for integrin $\alpha_v\beta_6$ than for $\alpha_5\beta_1$.[23] This means that the timescale in which the clutch can be engaged is affected based on what types of integrins are being expressed, suggesting that cells can tune their response to matrix rigidity by regulating expression of different types of integrin.[23,24]

At present, there is no evidence to support major differences in mechanisms of mechanosensing in 3D compared to 2D. A molecular clutch mechanism has been demonstrated in 3D collagen hydrogels, where local fibril stiffness and dimensionality led to alterations in adhesion protein dynamics.[47] Low stiffness in 3D resulted in increased turnover of adhesion proteins due to decreased ability to form stable adhesions. In stiffer 3D substrates, as for 2D, protein turnover is reduced due to stable adhesion formation,[47] i.e., in stiffer matrices adhesions grip, while in softer matrices adhesions slip.[43] Further to this, diffusion rates of both actin and paxillin in 3D adhesions demonstrate dynamics consistent with a clutch-like mechanism.[60]

The molecular clutch model was also recently used to understand the interplay between surface stiffness and ligand (e.g., RGD) density.[61] On rigid substrates such as tissue culture plastic or glass it has been long established that increased ligand density is required for adhesion formation.[62] A more complex behavior was instead observed on substrates with controlled substrate stiffness and ECM ligand (RGD) density. Oria et al. indeed showed that at low stiffness, close to that of softer tissues (but still >5 kPa), focal adhesions were longer when RGD spacing was increased from 50 to 100 nm. On the other hand, at higher stiffnesses increased ligand spacing led to focal adhesion collapse: at 100 nm RGD spacing the rigidity threshold above which focal adhesions collapsed was around 30 kPa, whereas at 50 nm spacing, the collapse occurred over 150 kPa. This behavior was explained considering the integrin–ligand linkages as molecular clutches that are recruited when the force load increases. The recruitment of more clutches redistributes the force load among them, thus reducing the total force each individual clutch is exposed to. However, when maximum recruitment is reached, no further distribution can occur and the adhesion collapses.[61]

2.3. Transcriptional Responses to Mechanotransduction

Adhesion dynamics thus lead to cell interpretation of mechanical inputs, requiring the generation of contractile forces in the cytoskeleton through which the cell actively interrogates its surroundings. These interactions induce mechanically activated signaling that regulates cell spreading, traction and RhoA activity to modulate cell behavior.[13,14] The YAP/TAZ complex (Yes-associated protein/Transcriptional coactivator with PDZ-binding motif) is an important factor in the transduction of mechanical signals. Both YAP and TAZ are mechanosensitive transcriptional activators with critical roles in development,[63] cancer,[64] and organ size control.[65] YAP/TAZ activity in a cell is controlled by two signaling branches; first, biochemically by the Hippo pathway, in which cadherin–cadherin interactions lead to sequestration of YAP in the cytosol, and second, mechanically whereby cytoskeletal tension induced by cell spreading promotes YAP nuclear translocation and downstream transcriptional activity. This mechanosensitive pathway has been demonstrated in 2D, where, on soft substrates, cell spreading is reduced and YAP remains cytosolic. On the other hand, on stiff substrates, where cytoskeletal tension is increased and nuclear pores are stretched, YAP nuclear localization increases.[66] This demonstrates that YAP/TAZ activity is governed directly by cell shape and polarity, which is in turn dictated by the cytoskeletal structure; physical cues such as these are converted by YAP/TAZ into gene expression signatures and coherent,

context-dependent biological responses.[67,68] For example, mesenchymal stem cells (MSCs) on stiff substrates (above 5 kPa) undergo osteogenic commitment via this mechanosensitive mechanism, where nuclear translocation of YAP subsequently activates the master regulator of osteogenesis runt-related transcription factor 2 (RUNX2).[69]

As such, YAP/TAZ is commonly referred to as a mechanical rheostat,[69,70] providing readouts of how cells are processing environmental mechanical input. Yet YAP/TAZ regulation within complex multifaceted (e.g., 3D) microenvironments is not yet well understood.[71] Until recently, much work on YAP/TAZ regulation has been performed in 2D culture scenarios, i.e., cells seeded on gels; yet, as discussed, translation of these systems to more in vivo-like 3D hydrogel models can result in altered or divergent behaviors.[71,72] For example, in purely elastic systems, increased stiffness in 3D does not always lead to increased cell spreading (as for 2D): this response is dependent on other material characteristics such as local degradability and stress relaxation times.[72,73] Caliari et al. addressed this using hyaluronic acid hydrogels with controlled stiffness and degradability, where they demonstrate that dimensionality alone cannot explain altered YAP/TAZ signaling in 3D. Here, YAP nuclear import is decreased when 3D gel stiffness is increased, yet when a degradable element is added YAP/TAZ nuclear translocation is significantly enhanced.[72] As such the interplay of these characteristics should be considered in tandem. Contrastingly, there appears to be no difference in YAP/TAZ regulation in response to dimensionality in viscoelastic materials, as elucidated in the following sections.

3. Mechanotransduction on Dynamic Interfaces

While it has been long established that cells respond to the elasticity of the culture substrate, recent material approaches have been developed to capture the dynamic nature of ECMs, in a bid to understand and exploit the dynamic (i.e., viscoelastic, including elastic and dissipative components) interactions between cells and their surroundings (**Figure 4**).[2–4] On the one hand, earlier approaches have tackled the role of dissipation at the interface, with a focus on the dynamic behavior of cell-adhesive ligands on the material surface.[2] These studies have eventually proved that controlling the surface mobility of ligands can recapitulate their in vivo viscous behavior, in turn tuning cell response via mechanotransductive pathways.[74,75] However, more recent approaches have been dedicated to the control of the bulk viscoelastic properties of hydrogels, revealing the role of substrate creep or stress relaxation in cell mechanotransduction, both when cells are cultured on these gels (2D culture) or encapsulated within them (3D culture).[3,4]

The prospect to mimic the dynamic aspects of cells interactions has driven studies showing that surface mobility can alter cell behavior (Figure 4). Kuhlman et al. first showed that increasing the tether length of the synthetic adhesive peptide RGD to the underlying substrate enhanced cell spreading.[76]

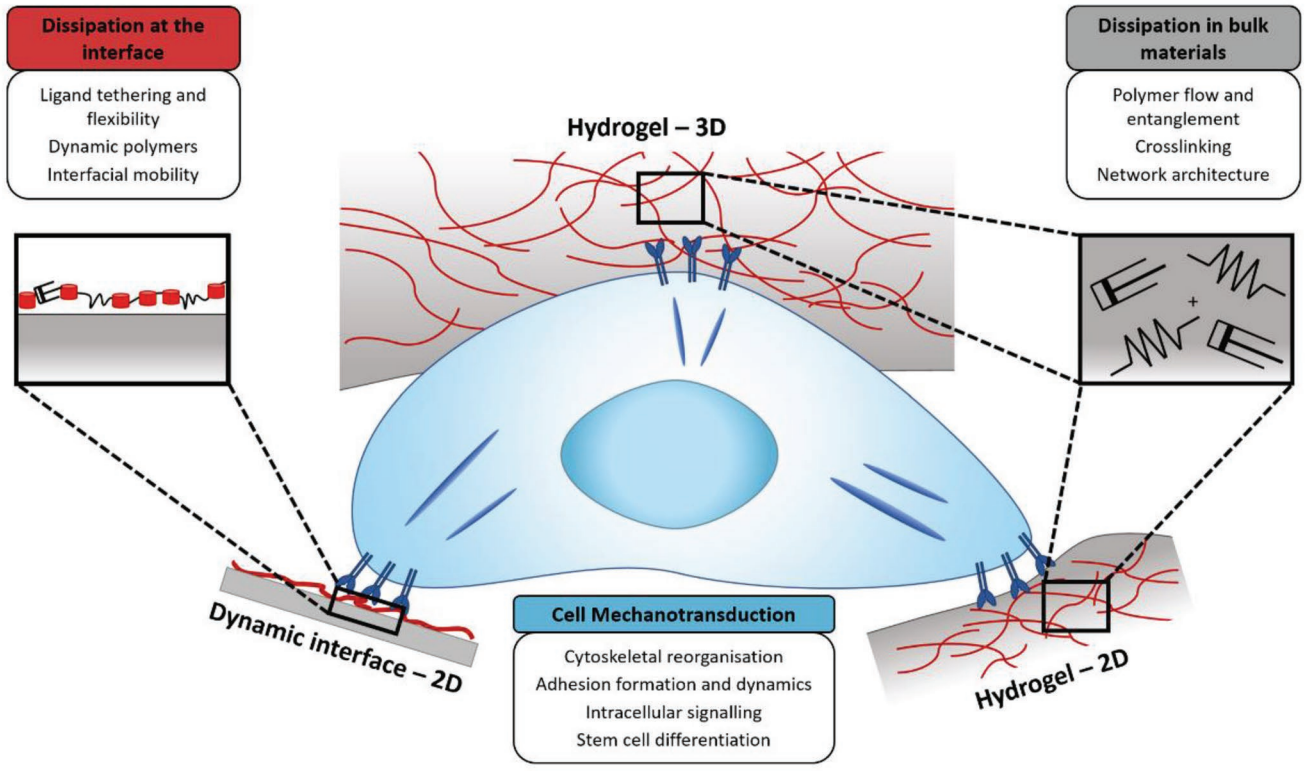

Figure 4. Overview of the material systems used to study the effect of dissipative interactions on cell mechanotransduction. Dissipation is controlled by the dynamics of the cell–material interface or by controlling the viscoelastic properties of a bulk hydrogel, with cells cultured on top of its surface (2D) or encapsulated within it (3D).

The authors found that the added mobility granted by longer tethers mediated an altered mechanotransductive response: the mobility of the tethered peptides allowed their cell-mediated reorganization on the nanoscale, allowing integrin clustering and promoting the formation of focal adhesions. Further to this, Curran et al. realized that dynamic surfaces with varying $-CH_3$ chain length were able not only to modulate cell adhesion, but also the expansion and phenotype maintenance of mesenchymal stem cells (MSCs), revealing the effect that dynamic interactions can have on cell fate.[77] Trappmann et al. further implied that ligand flexibility, determined by the number of covalent attachment points to the substrate per adhesion molecule, is crucial in eliciting a change in the adhesion and differentiation of stem cells independently of substrate stiffness.[78] This change was found to be mediated via clustering of the integrin complexes into focal adhesions and signaling through the ERK/MAPK pathway. This finding was later disputed by Wen et al., who found that substrate stiffness regulated cell differentiation independently of protein tethering.[79]

Other approaches to tune the dissipative properties at the material interface include the design of molecularly dynamic biomaterials based on polyrotaxane copolymers.[80] In particular, Yui and co-workers found that the hydrated molecular mobility granted by these copolymers and sensed by the cells via an adsorbed protein layer affected the attachment and morphology of adherent fibroblasts.[81,82] Moreover, these surfaces were able to control the adhesion and the switch between adipogenic and osteogenic differentiation of MSCs via the regulation of cytoskeletal tension through the Rho-ROCK signaling pathway.[83] Using induced pluripotent stem cells, surfaces with higher molecular mobility were further found to activate the Rac1/Cdc42 cytoskeletal signaling pathway and to promote N-cadherin expression, leading to cardiomyogenic differentiation.[84] Based on these findings, the modulation of surface mobility emerged as a powerful mechanotransductive modulator of cell fate via control of cytoskeletal signaling pathways. Moreover, by functionalizing these dynamic copolymers with RGD, the authors revealed that enhanced mobility is also able to mediate rapid cellular responses via faster ligand recognition and integrin binding.[85]

In other studies, dissipation at the interface has also been modulated via translation of the mobility or fluidity of the underlying substrate to the adsorbed interfacial protein layer which mediates cell adhesion. For example, we used polymeric substrates with increasing mobility (due to changes in the polymer side chain length) to control the interfacial flexibility of bound adhesive proteins: this not only affected cell adhesion and the ability of the cells to reorganize the underlying protein matrix, but it also eventually tuned differentiation in a cell contractility-dependent manner.[86,87] Similarly, crosslinking of a polymeric substrate has been used to regulate molecular mobility at the interface. For example, using thin polymer coatings with varying crosslinking degree, it was determined that surface mobility affects early cell response, including adhesion and reorganization of the adhesive proteins.[88] Using poly(dimethylsiloxane) with varying crosslink density, Mirzadeh et al. found an optimum molecular mobility of the substrate that prompted the best cell attachment and proliferation,[89] and Murrell et al. realized that tuning the viscosity of the substrate could regulate the collective movement of epithelial cells.[90] Liquid-like, noncrosslinked polymers were also found to reduce cell spreading and enhance the formation of spheroids due to the dissipation of cell-generated traction forces on a fluidic surface compared to an elastic one.[91] Some reports have found that cell adhesion can occur on the surface of low viscosity liquids, but this was enabled by the assembly of mechanically strong nanosheets of proteins at the interface.[92]

However, this evidence on the effect of dissipation on cell behavior arise from studies on interfaces which possess both viscous and elastic components, and the role of viscosity remains to be isolated. Within this respect, supported lipid bilayers (SLBs), membranes "supported" by an underlying rigid surface, provide a versatile and easy to manipulate viscous systems, without the contribution of elasticity (Figure 1A-2).[75] They can be employed as interfaces with easily tunable characteristics, including biospecificity, density of predesigned ligand molecules, and, most importantly, lateral mobility, which is driven by the viscosity of the bilayer.[93] Seminal studies have developed SLBs for the dynamic display of cell adhesion peptides, such as IKVAV (derived from laminin) or RGD (derived from fibronectin).[94,95] In particular, Garcia et al. found a cell type- and ligand-specific decrease in cell attachment on more fluid interfaces. They proposed that the differences in lateral mobility of the ligands would act in a manner that resembles the effect of varying substrate compliance, with the diminished cell spreading on the more mobile surfaces being analogous to cell behavior on a soft surface.

With the aim of dissecting the signaling mechanisms behind cell response to dynamic surfaces, Kourouklis et al. designed a similarly laterally mobile system based on the directed self-assembly of RGD-functionalized amphiphilic block copolymers, which share the amphiphilicity and mobility of lipids.[74] The authors found that these laterally mobile polymer films trigger a decrease in the size of focal adhesions at increasing mobility. Indeed, low interface mobility allows actomyosin-driven contractile forces to be sufficiently sustained, supporting the maturation of integrin clusters into focal adhesions and leading to cell spreading. Despite a monotonic decrease in focal adhesion size at increasing mobility, the authors observed a biphasic behavior in cell spreading, which was first reduced by an increase in ligand diffusion and then recovered at even higher values of mobility. This phenomenon was ascribed to a contractility-independent mechanism: the mobility of the ligands increases the probability of ligated integrins to find each other and form clusters; at high mobility, these clusters can eventually compensate for the lost contribution of the focal adhesions allowing cell spreading. However, if the mobility is high enough, other authors have observed that the loss of physical force acting on ligated integrins can cause a switch in downstream integrin signaling, resulting in their endocytosis.[96] Besides these viscosity-driven effects of mechanotransduction on cell attachment and adhesion, the mobility of SLBs has been further shown to be able to regulate the differentiation of MSCs.[93]

In our group, we investigated the mechanisms of cell response to surface viscosity within the framework of the molecular clutch model, which, as elucidated in the previous section,

has been proposed to explain mechanotransduction and, in particular, cell response to elastic substrates.[58] We used RGD-functionalized lipids of different diffusion coefficient (DOPC, 3.6 $\mu m^2\ s^{-1}$, and DPPC, 0.1 $\mu m^2\ s^{-1}$) to develop SLBs with varying surface viscosity (≈10^{-6}–10^{-4} Pa s m) (**Figure 5**A).[75] Cell response, in terms of size, cytoskeletal organization, focal adhesion formation and phosphorylation of focal adhesion kinases, was found to increase with the viscosity of the substrate. Actin retrograde flow, which is inversely proportional to the force exerted by a cell on a surface, was instead faster on less viscous, more mobile, surfaces. As a consequence, the translocation of the mechanosensitive YAP protein to the nucleus was enhanced on the more viscous surfaces, which eventually promoted myogenic differentiation of C2C12 cells. These results suggested that cells use the same mechanisms to sense both purely viscous surfaces and elastic materials: the molecular clutch, otherwise engaged by rigidity, here engages when the mobility is sufficiently low to cause the exposure of binding sites in mechanosensitive proteins, such as talin, reducing the actin flow and leading to the formation of focal adhesions, force transduction and cell differentiation (Figure 5B–D).

4. Mechanotransduction in Viscoelastic Materials

While tuning the dynamic properties of the interface between substrate and cells allows for a partial mimicking of the viscous interactions occurring in vivo, a more comprehensive approach involves adjusting the viscoelastic properties of biocompatible hydrogels, hydrated polymer networks which have been extensively used as artificial ECMs.[97,98] Traditionally, these materials have been designed or considered as elastic solids (Figure 1A-1). Recently, several strategies have been devised to control their dissipative behavior in order to better capture their ability to mimic the time-dependent mechanical properties of native ECMs (Figure 1A-3). In this section, we will review these recent advances in hydrogels' design, focusing on the mechanotransductive pathways elicited by exposing cells to changes in the viscous properties of the substrate, either by culturing them on top of the gels (2D culture) or within them (3D culture) (Figure 4). For an in-depth analysis of the approaches,

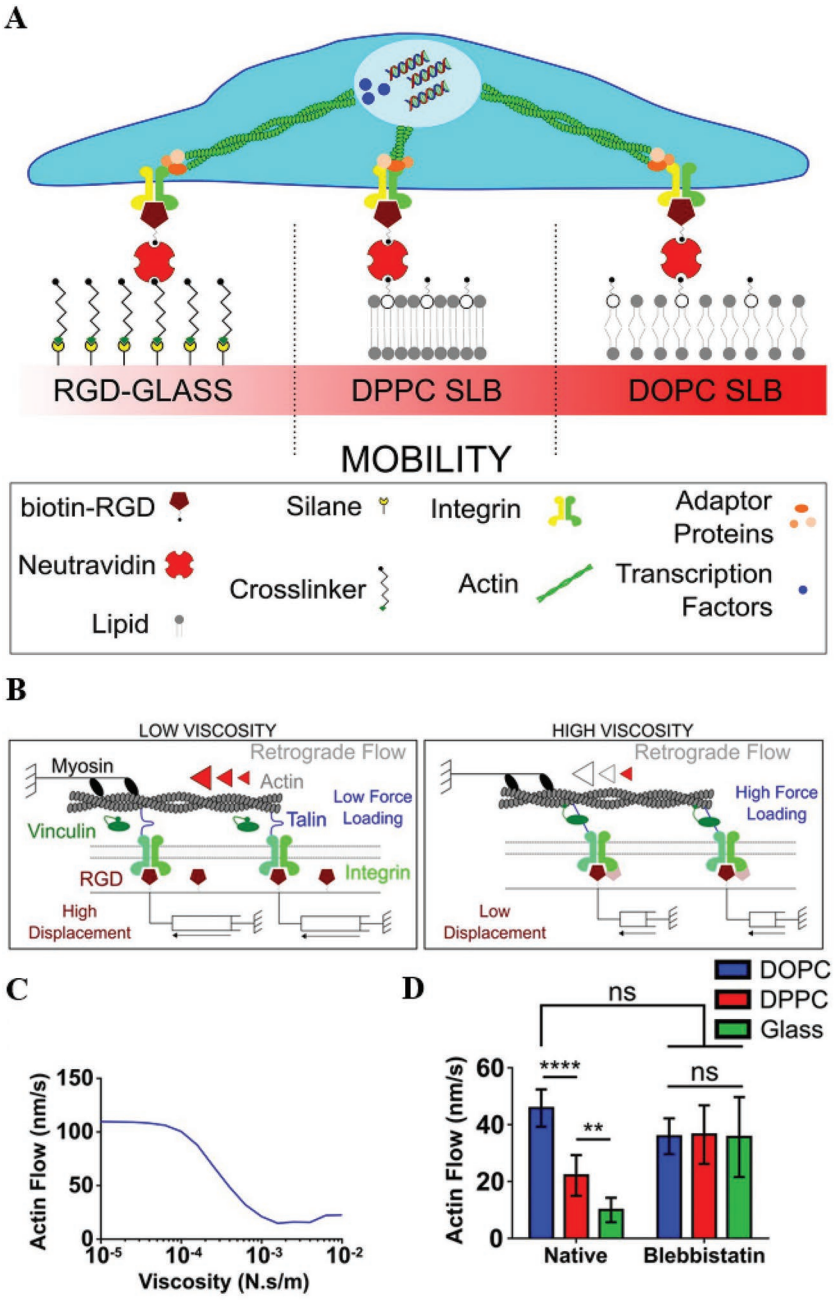

Figure 5. The molecular clutch explains cell mechanotransductive response to surface viscosity. A) Sketch of the system, based on supported lipid bilayers, used to control surface viscosity. A rigid surface based on salinized glass is used as control. The mobility of the ligand, RGD, is controlled by the surface viscosity of the bilayer, composed either of fluid-phase 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or gel-phase 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). B) Sketch of the effect of surface viscosity on the molecular clutch. On low viscosity surfaces (left, DOPC), high ligand mobility leads to slow force loading, which does not allow talin unfolding and does not slow actin retrograde flow. On high viscosity surfaces (right, DPPC), low ligand mobility leads to higher loading rate, talin unfolding, focal adhesion growth and reduction of the actin flow. C) Model prediction for the actin flow at increasing surface viscosity. D) Measured actin flow in C2C12 cells in normal conditions, showing a decrease at increasing viscosity, and in the presence of blebbistatin, a myosin inhibitor, which ablates cell mechanosensing ability. Reproduced under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0.[75] Copyright 2018, the Authors. Published by the US National Academy of Sciences.

based on physical or chemical cross-linking, that have been employed to modulate hydrogel viscoelasticity and of the experimental methods that can be used to characterize the time-dependent properties of these materials, the reader can refer to recent reviews.[3,4,97]

As anticipated in the introduction, most studies characterize the viscous behavior of the substrate by measuring the relaxation of the stress after application of a constant strain (Figure 1B, left): the relaxation time $\tau_{1/2}$, for example, indicates the time after which the stress is halved. Hence, short relaxation times indicate a fast stress relaxing material with a strong dissipative behavior. On the other hand, other authors use the creep response, which measures the increase of the strain after application of a constant stress (Figure 1B, right). In this case, $\tau_{3/2}$ indicates the time at which the strain is 150% of its initial value; again, high creep dissipative materials are characterized by shorter $\tau_{3/2}$. In the studies reviewed here, these relaxation times are usually within the timescale of cellular processes (seconds to hours) and hence much shorter than diffusion times of water molecules through the porous matrix of the gels (leading to poroelasticity). Under these conditions, cell response is ascribed to the viscoelastic nature of the polymer network, and poroelastic effects are not considered;[7,99] for an in-depth analysis of viscoelastic and poroelastic effects in hydrogels, the reader can refer to a recent review.[7] Further methods have been used to fully characterize the viscoelastic properties of gels;[3] of these, dynamic mechanical testing, in which a sinusoidal stress or strain is applied to the substrate, is the most common. By measuring the amplitude and the phase shift of the response, this method allows to determine the storage modulus (G' for shear, E' for tension or compression), which is indicative of the elastic behavior of the substrate, and the loss modulus (G'', E''), which is instead a measure of its viscous behavior. The loss tangent, defined as the ratio between loss and storage modulus, is often referred to as a measure of substrate viscosity. In most of the studies reviewed in this section, hydrogels are engineered so that they possess similar storage or elastic properties, while only their viscous component, measured as stress relaxation, creep or loss modulus (at a specified frequency), varies.

4.1. Cell Response on 2D Viscoelastic Substrates

A summary of the studies that have explored the effect of viscoelasticity on cell response in 2D scenarios is shown in **Table 1**. An early approach to modulate the viscoelastic properties of hydrogels was devised by Cameron et al., who managed to obtain a family of collagen I-functionalized polyacrylamide gels with similar elastic properties and varying viscous behavior by controlling the concentration of monomer and cross-linker.[100]

Table 1. Viscoelastic hydrogels and cell studies in 2D.

Material	Origin of viscoelasticity	Ligand type	E [kPa]	G' [kPa]	G'' [Pa]	tanδ	$\tau_{1/2}$ [s]	$\tau_{3/2}$ [s]	Cell type	Functional studies	Refs.
Polyacrylamide hydrogels	Physical entanglements	Collagen I	≈13.5	≈4.7 (at 0.005 rad s^{-1})	1–130 (at 0.005 rads^{-1})	–	–	–	Human MSCs	Morphology, adhesion, proliferation, differentiation, migration	Cameron et al.[100] Cameron et al.[101]
Alginate hydrogels	Ionic crosslinking	RGD	1.4–9	–	–	–	–	–	U2OS and 3T3 fibroblasts	Morphology, adhesion, YAP translocation	Chaudhuri et al.[102]
Alginate hydrogels	Ionic crosslinking	RGD	2.8–49.5	–	–	–	79 – 519	–	C2C12	Morphology, adhesion, proliferation	Bauer et al.[103]
Layer-by-layer poly(*N*-isopropylacrylamide)-*co*-(acrylic acid) microgel film	Internal crosslinking of the particles	Collagen I	≈107	–	–	0.8– 1.8	–	–	Human dermal fibroblasts	Morphology, adhesion, migration	Chester et al.[106]
Polyacrylamide hydrogels	Entrapment of linear polymer chains	Collagen I, fibronectin	–	≈5 (at 1 rad s^{-1})	10–490 (at 1 rad s^{-1})	–	–	–	3T3 fibroblasts, rat primary hepatic stellate cells	Morphology, adhesion, differentiation	Charrier et al.[107]
Poly(ethylene glycol) based hydrogels	Adaptable boronate ester crosslinking	RGD	–	≈13 (at 1 rad s^{-1})	≈0–1700 (at 1 rad s^{-1})	≈0–0.13 (at 1 rad s^{-1})	–	–	3T3 fibroblasts	Morphology, adhesion, YAP translocation	Marozas et al.[108]
Poly(ethylene glycol) based hydrogels	Photo-induced addition–fragmentation chain transfer reaction	RGD	–	≈2	≈20–300 (at 1 rad s^{-1})	≈0.01– 0.27 (at 1 rad s^{-1})	≈14– 214	≈47– 158	Human MSCs	Morphology	Marozas et al.[109]

By increasing the polymer content and reducing the crosslinking density, the authors were able to manufacture gels with the same storage modulus as highly cross-linked elastic gels ($G' \approx 4.7$ kPa, $E' \approx 13.5$ kPa at 0.005 rad s^{-1}), but with an enhanced creep response ($G'' \approx 130$ Pa vs 1 Pa), probably as a result of polymer physical entanglements that allow the material to flow: the density of crosslinks controls the elastic response while the ratio between chemical crosslinks and physical entanglements modulates the viscosity of the material. Culture of human MSCs on these gels revealed an increase of cell spreading on high creep dissipative gels, which was, however, accompanied by a decrease in the size and maturity of focal adhesions. Similarly to the observation made by Kourouklis et al., for substrates with high ligand mobility,[74] this behavior was found to be contractility independent. Here, the authors attributed this response to the cells attempting to maintain tensional homeostasis via an increase in cell spreading as the inherent high creep of the substrate induced a loss in tractional force and cytoskeletal tension (**Figure 6**A).[100] The authors further found that high loss modulus substrates allowed for increased differentiation potential in the presence of myogenic, osteogenic or adipogenic supplements. Moreover, the propensity of MSCs for spontaneous myogenic differentiation on the high creep gels led to the identification of the key mechanotransductive pathways involved in this response.[101] Indeed, the authors found that Rac1 was upregulated by the increased substrate creep, possibly via the guanine exchange factor Tiam1, which, at the same time, mediated the downregulation of Rho (Figure 6B). This was accompanied by higher expression levels of smooth muscle cells induction factors, including soluble factors (TGF-β_1), ECM proteins (collagen I, IV, and laminin) and, most importantly, the cell–cell contact adhesion molecule N-cadherin, in a response that resembles the one observed by Seo and co-workers for cells on molecularly dynamic interfaces.[84] Furthermore, Rac1 activation, through regulation of actin polymerization within lamellipodia, facilitated enhanced cell motility, confirming that viscosity has an effect on cell migration, as previously observed in the case of epithelial cell sheet movement on viscous surfaces.[90]

Another seminal work in cell response to bulk dissipation used instead physically crosslinked hydrogels as stress relaxing substrates compared to their elastic, covalently crosslinked counterpart.[102] Indeed, physical crosslinking allows the polymer network to flow, causing time-dependent deformations which can also be plastic. On the other hand, covalently crosslinked gels exhibit only low levels of stress relaxation at long timescales during bulk compression; this is related to water migration out of the gel (poroelastic effects).[3,103,104] In this work, cell culture on stress relaxing ionically crosslinked RGD-functionalized alginate gels with similar initial elastic modulus (in the range 1.4–9 kPa) as their covalently crosslinked elastic counterparts showed that stress relaxation induced an enhanced cell spreading only at low initial elastic modulus and high ligand density. This was mediated via β_1 integrins, actin polymerization, and actomyosin contractility, leading to increased nuclear translocation of mechanosensitive YAP, and was accompanied by local substrate remodeling associated with the plastic deformation of the substrate. Using a computational model, the authors concluded that this behavior is a consequence of molecular clutch-based adhesions (**Figure 7**). Using a similar system based on alginate, the same group was able to manufacture gels with a larger range of elastic moduli (2.8–49.5 kPa) and with either elastic or stress relaxing behavior (with $\tau_{1/2}$ in the range 79–519 s), in a bid to simulate the viscoelastic properties of healthy and diseased muscle tissue. Stress relaxing hydrogels enhanced cell spreading, as in their previous work, as well as the proliferation of myoblasts; this effect was reduced on stiffer gels.[103]

Other hydrogel systems with controllable viscoelastic properties are based on microgel films. These are produced by deposition, usually layer-by-layer, of discrete hydrogel particles (microgels) with sizes ranging from the nanometers to micrometers.[105,106] An earlier report showed that their viscoelastic behavior, beside granting them the ability to self-heal after stretching in the absence of covalent crosslinking of the film, affected cell behavior.[105] This was further addressed by Chester and co-workers, who designed a family of layer-by-layer microgel thin films with similar elastic modulus (≈107 kPa) but decreasing loss tangent (from 1.8 to 0.8) by tuning the internal crosslinking of the particles.[106] The loss tangent of the films was found to be the main regulator in fibroblast migration on these viscoelastic films: on more dissipative substrates, an increase in ROCK activity prompted amoeboid migration, with ellipsoid cells loosely attached to the substrate. On the other hand, a decrease in loss tangent below 1.4 was accompanied by a transition to mesenchymal migration, with elongated cells and increase in Rac signaling. While these results are at odds with the observations by Cameron et al., who instead observed Rac upregulation on high creep gels,[100,101] the discrepancy might be attributed to the different system used or to the difference in initial elastic modulus (107 vs 13.5 kPa).

Given the variability of the observed cellular responses and of the mechanisms believed to be responsible, Charrier et al. designed a system to decouple substrate viscosity from cell-mediated matrix reorganization by sterically entrapping high molecular weight linear polyacrylamide within covalently crosslinked networks of the same polymer.[107] This strategy allowed to manufacture a system where elastic (G') and loss moduli (G'') could be independently modulated, and adhesion ligands could be attached to the elastic network, to the viscous chains or both. The authors concluded that cell spreading is hindered by viscous dissipation if plastic remodeling is not permitted, impeding the local reorganization and clustering of adhesion molecules. Indeed, fibroblasts cultured on gels with a similar storage modulus ($G' \approx 5$ kPa, $E' \approx 15$ kPa at 1 rad s^{-1}) and varying loss modulus ($G'' \approx$ 0–500 Pa) showed a decrease in cell area at increasing stress relaxation when only the crosslinked elastic matrix was functionalized with adhesion proteins. On the other hand, functionalization of the full system allowed cells to rearrange the viscous component of the gel, and cell spreading was recovered. In any case, focal adhesions were always found to be smaller on dissipative substrates due to the inability to sustain larger traction forces, in accordance with the observations by Cameron and co-workers.[100] Interestingly, when only the viscous component of the gel was functionalized, cells were able to attach to the gels only if the ligand was fibronectin; no attachment was observed when collagen I was used. This, together with the

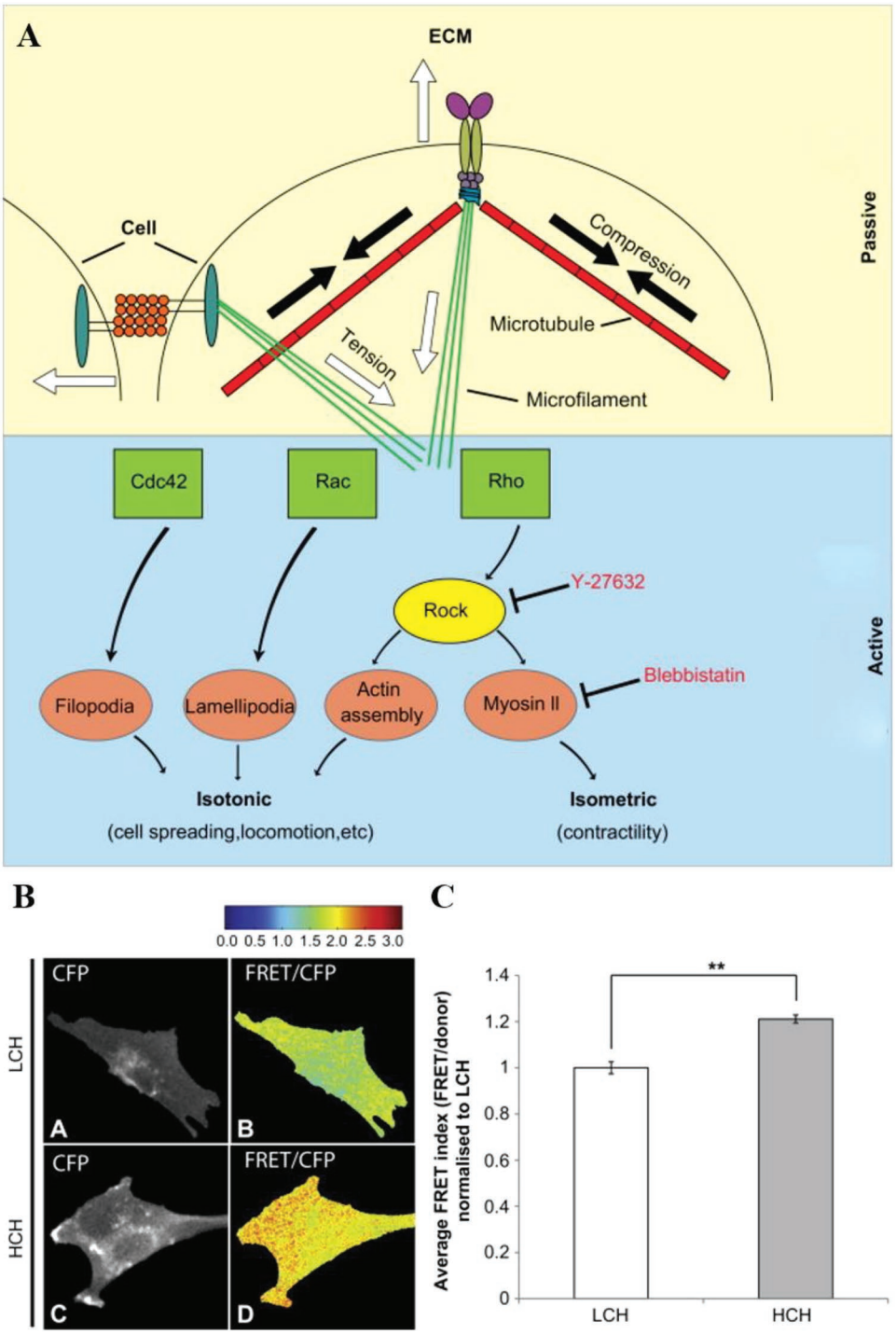

Figure 6. Tensional homeostasis and Rac1 activation explain cell response to high creep hydrogels. A) According to the cellular tensegrity model cells maintain their shape stability through a balance of tensional and compressive forces. Tensional forces acting on the microtubules are maintained passively through a tensed network of microfilaments connected to the ECM or to other cells and are generated actively by actomyosin contractility or cell distension. These active forces are regulated via the Rho protein family, including Rac. On high creep hydrogels (HCH), the substrate creep causes a loss of passive tension, which is balanced by cell spreading and leads to the upregulation of the activity of Rac. Reproduced with permission.[100] Copyright 2011, Elsevier. B) Confocal images and C) quantification of the FRET index of a Rac1 biosensor, indicating upregulation of Rac1 on high creep hydrogels compared to low creep elastic ones (LCH). Higher activation of Rac1 is evident at the periphery of the cells, where it sustains lamellipodial activity. Scale bar is 20 µm. Reproduced with permission.[101] Copyright 2014, Elsevier.

observation that cell spreading and focal adhesion formation in stress relaxing gels also showed a level of ligand-dependence, revealed that cell sensitivity to viscosity is ligand-specific, as anticipated by Garcia et al. using SLBs.[95] The authors hypothesized that the engagement of different integrins could change the timescale of cell mechanosensing, resulting in the

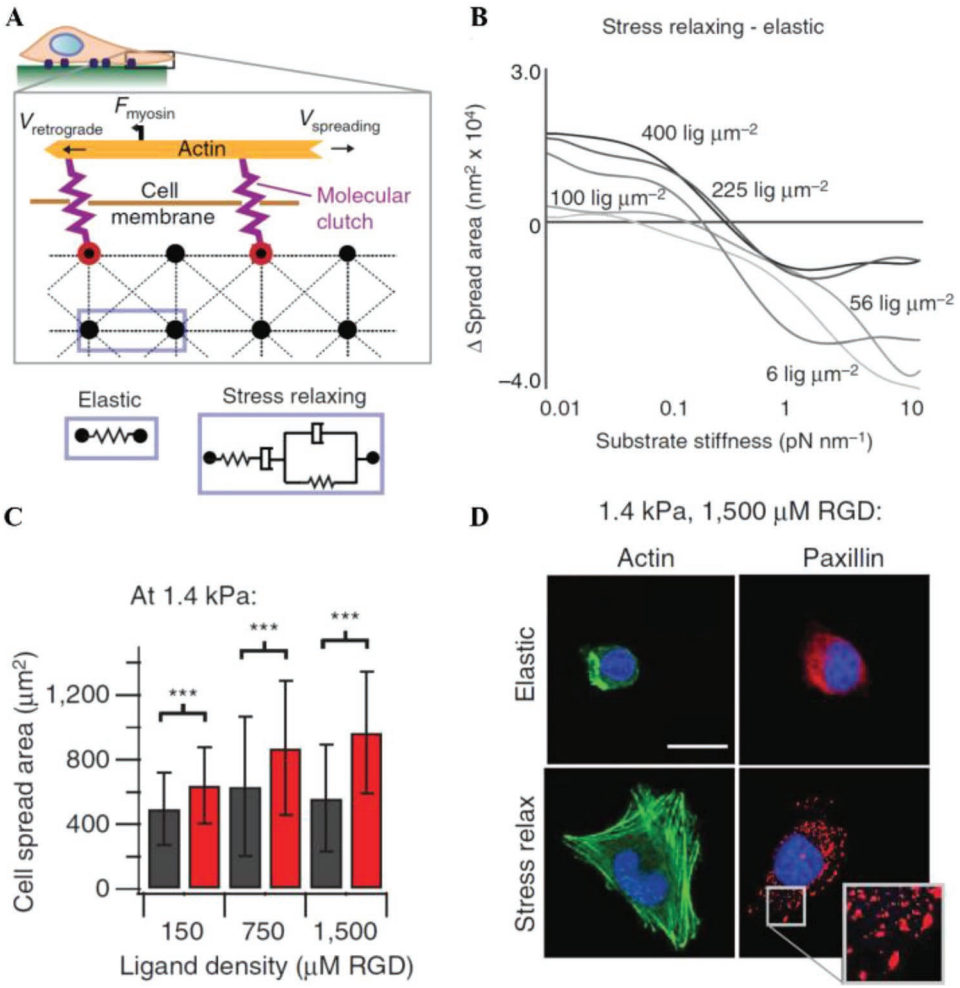

Figure 7. Computational model predicts increased cell spreading and plastic deformation on stress relaxing substrates at low initial stiffness and high ligand density. A) Sketch of the computational model, comprising actin retrograde flow coupled to the substrate through the molecular clutch. The substrate is modeled as either elastic or stress relaxing. B) Difference in cell spreading area between stress relaxing and elastic substrates predicted by the model; at high ligand density and low stiffness the model predicts the increase in cell area which is observed experimentally. C) Cell spreading as a function of ligand density on elastic (gray) or stress relaxing (red) soft gels. D) Focal adhesion formation on soft stress relaxing gels, indicated by paxillin (red) staining and mediated via plastic deformation of the substrate. Scale bar is 25 μm. Reproduced with permission.[102] Copyright 2019, Springer Nature.

cell effectively sensing different values of dissipation for the same gel.[107]

The role of time-dependent phenomena in cell mechanosensing of viscous interactions was then explored by Gong and co-workers, who used analytical and computational models to examine the dynamics of molecular clutches and substrate relaxation in cell response to viscoelasticity.[99] Using a canonical molecular clutch model, the authors concluded that the relationship between the timescale of the substrate relaxation and the timescales of clutch binding and of FA lifetime effectively explain the role of viscosity in cell spreading. As such, viscoelasticity alone, without the contribution of the plastic rearrangement of the substrate, can explain the results observed in previous studies where stress relaxation allows an increase in cell spreading at low values of elastic modulus.[102]

In order to explore experimentally the timescale dependence of mechanotransduction in response to the relaxation of viscoelastic substrates, Marozas et al. recently developed a family of gels with reversible boronate ester cross-links that allow to tune their viscoelastic spectra in the frequency range 0.1 to 10 rad s^{-1}, while maintaining similar elastic modulus ($G' \approx 13$ kPa, $E' \approx 39$ kPa). However, fibroblasts cultured on these substrates did not reveal a sensitivity to their frequency-dependent viscoelastic properties. Indeed, viscoelastic gels with varying loss tangent spectrum induced a similar decrease in cell spreading, FA formation and YAP translocation to the nucleus compared to their elastic counterpart.[108] While these results seem at odds with previous studies which revealed increased cell spreading and nuclear YAP translocation at increasing stress relaxation, crucial differences in the mechanical properties of the materials can explain this behavior: these boronate ester gels do not undergo plastic deformation, which has been proved to be fundamental to enhance cell response,[107] and are much stiffer. Indeed, stiff stress-relaxing gels ($E \approx 49.5$ kPa)

have been already observed to reduce cell size compared to their elastic counterpart.[103] Marozas et al. have also recently reported the development of a novel gel system that allows reversible photoinduced local changes in viscoelasticity, paving the way to its spatiotemporal control.[109] Human MSCs cultured on these soft gels ($G' \approx 2$ kPa) responded to a bulk change in viscoelasticity by contracting and deforming the substrate, while local induced viscoelasticity under a single cellular protrusion prompted the instantaneous retraction of all protrusions, with the effect dissipating at increasing distances from the viscoelastic region.

4.2. Cell Response within 3D Viscoelastic Matrices

Besides being used as substrates for 2D cell culture, hydrogels can also be employed to encapsulate cells, mimicking more closely the 3D meshwork environment that cells encounter in native ECMs. However, standard synthetic elastic hydrogels confine the cells within a restrictive environment inhibiting cellular processes; gels need to be modified, for example, by adding controlled degradation capability, to be more conducive to cell adhesion, proliferation and growth.[97,98] Similarly, tuning their viscous properties to exhibit the stress relaxation that is displayed by native or reconstituted ECMs has been revealed to facilitate these cell functions (**Table 2**).[3,4]

Through the combination of alginate chains of different molecular weights, varying crosslinking densities and PEG spacers covalently coupled to the polymer, Chaudhuri et al. managed to tune the relaxation time $\tau_{1/2}$ of ionically crosslinked RGD-functionalized alginate gels from ≈1 h (slow relaxing) to ≈1 min (fast relaxing), a range of timescales that is relevant to cell phenomena such as adhesion and spreading.[110] Encapsulation of MSCs within these substrates indeed showed that not

Table 2. Viscoelastic hydrogels and cell studies in 3D.

Material	Origin of viscoelasticity	Ligand type	E [kPa]	G' [kPa]	G'' [Pa[	tanδ	$\tau_{1/2}$ [s]	$\tau_{3/2}$ [s]	Cell type	Functional studies	Refs.
Alginate hydrogels	Ionic crosslinking, molecular weight of chains, crosslinking density, and steric hindrances (PEG spacers) between chains	RGD	≈9, ≈17	≈3 (at 1 Hz)	≈0.1–0.8 (at 1 Hz)	–	≈60 – 3300	–	3T3 fibroblasts and mouse MSCs	Morphology, adhesion, differentiation, YAP translocation	Chaudhuri et al.[110]
Alginate hydrogels	Ionic crosslinking, molecular weight of chains	RGD	≈17	–	–	–	≈50 – 800	–	Human MSCs	Differentiation, bone formation in vivo (rat calvarial defect)	Darnell et al.[111]
Alginate hydrogels	Ionic crosslinking, concentration and molecular weight of PEG spacers	RGD	≈3	–	–	0.023–0.084 (at 10^{-4}–1 Hz)	≈30 – 18 500		3T3 fibroblasts, mouse MSCs	Morphology, adhesion, proliferation, differentiation	Nam et al.[112]
Poly(ethylene glycol)-based hydrogels	Sliding hydrogel	RGD	≈10	–	–	–	–	–	Human MSCs	Morphology, differentiation	Tong and Yang[113]
IPN of hyaluronic acid and collagen I	Dynamic hydrazone crosslinking	Collagen I	–	0.009–0.510 (at 1 Hz)	–	–	≈55 – >18 000	–	Human MSCs	Morphology, adhesion	Lou et al.[114]
IPN of alginate and collagen I	Ionic crosslinking	Collagen I	–	0.25–2.5 (at 0.1– 10 Hz)	≈25–150 (at 0.1– 10 Hz)	2.3– 6.4 (at 0.1– 10 Hz)	≈100 – 3000	–	Human MSCs	Immunomodulation	Vining et al.[117]
Poly(ethylene glycol)-based hydrogels	Dynamic hydrazone crosslinking	RGD	1.8–27	–	–	–	≈10 – >1000	–	C2C12	Morphology	McKinnon et al.[118]
Poly(ethylene glycol)-based hydrogels	Thioester exchange	RGD	–	≈1.7 (at 1 Hz)	–	–	≈11 000		Human MSCs	Proliferation, morphology	Brown et al.[119]
Poly(ethylene glycol)-based hydrogels	Adaptable boronate crosslinking	RGD	≈14 –16	–	–		≈0.3 – 1		Human MSCs	Morphology, adhesion, YAP translocation	Tang et al.[120]
Alginate gels	Ionic crosslinking, molecular weight of chains, PEG spacers	None	≈3, 20	–	–	≈0.05–0.12	≈60 – 7000	≈40–5000	Bovine chondrocytes	Morphology, proliferation, chondrogenic phenotype	Lee et al.[121]
Poly(ethylene glycol)-based hydrogels	Dynamic hydrazone crosslinking	None	–	≈20 (at 1 rad s^{-1})	≈10–25 (at 1 rad s^{-1})	≈0.0005–0.0012 (at 1 rad s^{-1})	≈4×10^3– 3 $\times 10^6$	–	Porcine chondrocytes	Proliferation, chondrogenic phenotype	Richardson et al.[122]

only cell spreading, but also proliferation increased with faster stress relaxation, as previously observed in 2D.[102] Moreover, while an initial elastic modulus of 9 kPa promoted adipogenesis at levels decreasing with $\tau_{1/2}$, a higher elastic modulus of 17 kPa favored osteogenesis. When these stiffer osteogenic gels were also fast relaxing (with $\tau_{1/2}$ similar to fracture hematoma and early fracture callous), they prompted the formation of a more interconnected bone matrix compared to their slow relaxing counterpart. This was found to be mediated by local rearrangement of RGD ligands through β_1-based adhesions (without paxillin localization, indicating that focal adhesions were not formed), actomyosin contractility and nuclear translocation of YAP, while inhibition of Rho or Rac did not diminish osteogenesis (**Figure 8**A).[110] Fast relaxing alginate gels were further proved to promote remodeling and bone formation in vivo in rat calvarial defects.[111]

This effect of stress relaxation on cell adhesion and osteogenic differentiation in 3D has also been recently confirmed in another family of ionically crosslinked alginate gels;[112] in this case, their viscous properties were tuned, independently of the initial storage modulus, simply by varying the concentration and molecular weight of PEG spacers covalently grafted to the polymer chains. These improved stress relaxing gels were also able to support the formation of focal adhesions in 3D, with paxillin colocalization with the integrin clusters and increased FAK phosphorylation.

The role of ligand clustering in mediating cell mechanotransduction in response to dissipative interactions in 3D was also explored using other systems.[113,114] For example, Tong and Yang developed sliding hydrogels with mobile crosslinks based on polyrotaxanes that allowed for myosin contractility-driven rearrangement of the RGD ligands; this provoked changes in the morphology and eventually enhanced the differentiation potential of stem cells.[113] Lou et al. instead used an interpenetrating polymer network (IPN) hydrogel system based on slow relaxing dynamic covalent crosslinking of hyaluronic acid ($\tau_{1/2} >$ 4000 s) and fast relaxing collagen I ($\tau_{1/2} \approx$ 50s) to mimic both the viscoelasticity and the fibrillarity of native ECMs.[114] These two-stage stress relaxing IPN hydrogels showed that the viscous relaxation of the hyaluronic acid network controlled by its dynamic hydrazone crosslinking is fundamental for the reorganization of the collagen I fibers and the formation of focal adhesions, which were instead inhibited in controls where the hyaluronic acid crosslinks were static. The crucial role of matrix rearrangement and plastic deformation in cell response to 3D viscoelastic materials was further confirmed in other studies employing collagen gels or IPNs.[6,115,116] An alternative strategy to produce IPNs with controlled viscoelastic properties and fibrillarity for cell encapsulation was recently proposed by Vining et al., who were able to vary the viscoelastic properties of the matrix via secondary covalent crosslinking of a ionically crosslinked alginate network interpenetrated with collagen I, without impacting the architecture and the interpenetration of the IPN.[117]

The use of the dynamic covalent crosslinking strategy employed by Lou et al. to produce tunable viscoelastic 3D microenvironments for cell encapsulation was first demonstrated by McKinnon et al.[118] Indeed, reversible hydrazone crosslinking in RGD-functionalized PEG gels allowed them to synthetize covalently adaptable networks, where crosslinks could be broken in response to stress and rapidly reformed, with controllable stress relaxation ($\tau_{1/2}$ ranging from ten to thousands of seconds). C2C12 cells were able to spread within gels with stress relaxation in the range of the one observed in muscle tissue ($\tau_{1/2} \approx$ 100 s), eventually fusing into multinucleated myotubes, while more elastic gels constrained cell growth. The same group proposed an alternative strategy based on thioester exchange to produce an adaptable hydrogel with dynamic crosslinking and $\tau_{1/2}$ of ≈11 000 s.[119] Although this rate of relaxation is slower than the ones reported for native tissues, these thioester gels allowed spreading and proliferation of encapsulated MSCs compared to their static controls. Dynamic crosslinking has also recently allowed to expand the exploration of the effect of stress relaxation in 3D culture toward very short timescales (seconds or less), which have not been reached with other approaches and are relevant to many cellular processes.[120] Using dynamic boronate bonds, Tang et al. developed hydrogels with relaxation times $\tau_{1/2}$ in the range ≈0.3–1 s, showing that this fast relaxation allows an increase in cell volume and spreading, mediated by cell-driven remodeling of the surroundings, and in YAP translocation to the nucleus compared to elastic gels. Moreover, YAP/TAZ signaling was accompanied by changes in nuclear volume and shape, suggesting a possible role of the nucleus in the cell mechanotransductive response to viscoelasticity.[120]

Independently of the explored timescale of stress relaxation, all the previous studies revealed adhesion-mediated mechanisms of cell mechanotransduction, since all gels were either functionalized with adhesive ligands, typically RGD, or interpenetrated with an adhesive collagen network (Figure 8A, Table 2). Within this respect, stress relaxing materials triggered a similar response in both 2D and 3D culture: indeed, most studies concur in identifying the crucial role of the local remodeling of the matrix through integrin-based adhesions, with a subsequent translocation of YAP to the nucleus. However, research on this topic is still immature, and further work is needed to explore, e.g., the effect of initial elastic modulus, relaxation timescales and ligand types.

Finally, Lee and co-workers, encapsulating chondrocytes within stress relaxing alginate gels in the absence of adhesive ligands, revealed cell sensing of mechanical confinement as an adhesion-independent mechanotransduction mechanism for cells embedded within a 3D environment (Figure 8B).[121] Indeed, while a more elastic matrix ($\tau_{1/2} \approx$ 2 h) restricted cell volume, upregulating IL-1β secretion, cartilage degradation and cell death, faster relaxation ($\tau_{1/2} \approx$ 1 min) allowed cell volume expansion, chondrocyte proliferation and formation of an interconnected cartilage matrix. This suggests that cells modulate their volume to sense the viscoelastic properties of their surroundings. Moreover, fast relaxing gels allow the formation of an interconnected cartilage matrix by dissipating elastic stresses and undergoing plastic deformation.[121] This role of stress relaxation in non-adhesive hydrogels was recently confirmed by Richardson et al.[122] Using dynamic hydrazone crosslinking to modulate stress relaxation times in a range from hours to months, they found that an intermediate relaxation time of about 3 days prompted enhanced cellularity and cartilage matrix deposition within their gels.

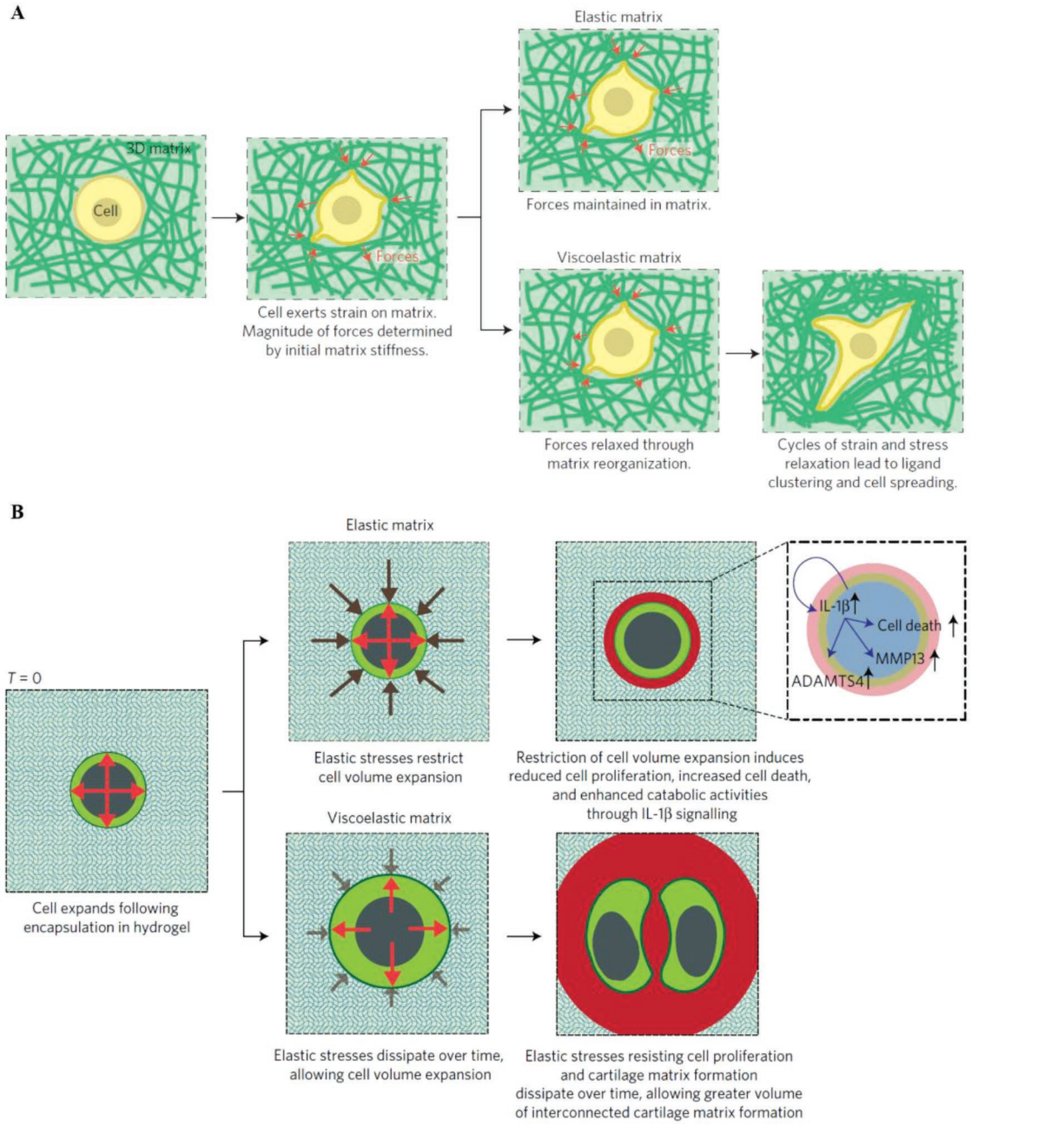

Figure 8. Adhesion-dependent and adhesion-independent mechanotransduction mechanisms within 3D stress relaxing hydrogels. A) In a ligand-functionalized hydrogel, cells bind to the polymer network via integrins and sense the matrix properties via actomyosin contractility; if the matrix is viscoelastic, force dissipation allows remodeling and ligand clustering, upregulating mechanosensitive pathways and promoting, e.g., osteogenic differentiation of MSCs. Reproduced with permission.[110] Copyright 2015, Springer Nature. B) In a nonadhesive hydrogel, cells sense stress relaxation via modulation of their volume; while an elastic matrix restricts cell volume expansion, a viscoelastic one allows it, facilitating cell proliferation and matrix secretion. Reproduced with permission.[121] Copyright 2017, Springer Nature.

5. Conclusions and Outlook

The ECM is a viscoelastic material in nature. When cells pull on the ECM—and this happens in a variety of processes including development, cancer, and regeneration—a dynamic, time-dependent, mechanical relationship is established between the cell and its environment. This relationship has important consequences, as cells are mechanosensitive entities and their behavior (e.g., signaling) will be triggered by this time-dependent, viscoelastic, interaction with the ECM. However, most of the understanding that we have about mechanotransduction has been through the use of pure elastic substrates, mainly polyacrylamide hydrogels functionalized either with fibronectin or collagen.[59] This has allowed the field to progress

in the basic understanding of cell response to stiffness, e.g., through the use of the molecular clutch model, and in the definition of design principles to engineer novel substrates for tissue regeneration, e.g., via the controlled differentiation of stem cells towards defined lineages.[79]

Over the last few years, researchers have started contemplating the emergence of viscoelasticity as an additional trigger of cell behavior, designing materials with controlled viscoelastic properties. Engineering the viscous component of synthetic materials is a complex task: while elastic properties can be simply modulated, e.g., just by tuning the crosslinking density, the control of the viscous component is significantly more subtle, and indeed more challenging. For example, strategies to control the viscosity of hydrogels have been based on i) using physical entanglements that can slide as stress/strain is applied on the system;[100] ii) using ionically crosslinked systems;[102] iii) altering the polymer architecture by, e.g., varying the molecular weight between crosslinking points and adding additional steric hindrances between chains;[110] and iv) using adaptable dynamic crosslinking strategies.[118]

A question underlying further designs of viscoelastic, dynamic materials, is whether cell response to purely viscous substrates can be understood following the same principles that govern cell response to surface elasticity. The use of model surfaces based on RGD-functionalized supported lipid bilayers demonstrated that a modified version of the molecular clutch model can unravel the role of viscosity in cell adhesion and differentiation. However, more developments using this type of well-defined model surfaces are needed to further improve our understanding of the molecular mechanisms involved in cell response to viscous interactions.[75] Indeed, recent breakthroughs have shown that the same principles can begin to explain cell behavior in complex viscoelastic systems.[99]

The field is now equipped with new tools to engineer 2D and 3D systems that provide more relevant viscoelastic environments to cells.[3] However, engineering materials to understand cell response to dissipative components comes with additional complexity: while elastic materials deform under the application of stress and come back instantly to the original mechanical state once the force is removed, viscoelasticity comes with memory. It is not only that the deformation of a viscoelastic material changes with time under the application of cellular forces (e.g., creep or stress relaxation), but also that, when additional forces are applied by the cells before the material has fully recovered from the initial pulling (as cells interact dynamically with the environment, e.g., in cell migration), the deformation will change depending on the past mechanical history of the system.[123] Yet we know that cells come with a mechanical memory as well[69]—we foresee exciting developments to understand this reciprocal dynamic interaction based on mechanical memory.

In addition, the mechanical properties of viscoelastic materials depend on the rate at which they are deformed, and precisely the force loading rate is the key parameter that determines cell mechanotransduction according to the clutch model.[58] In this respect, the timescale at which the cell probes and reacts to its environment becomes crucial, and this demands the development of new materials with tunable viscoelastic spectra. This means not only that new characterization of tissues has to be done to have reliable measurements of their dissipative component, but also that new technologies will have to be developed to engineer highly reproducible substrates with controlled elastic and viscous properties. There is no doubt that we have only scratched the surface: understanding and engineering viscoelasticity will bring additional challenges and opportunities to the broad community of biomaterials scientists in the future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

dissipative interactions, mechanotransduction, molecular clutches, viscoelasticity, viscosity

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