Designer biomaterials for mechanobiology

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Biomaterials engineered with specific bioactive ligands, tunable mechanical properties and complex architecture have emerged as powerful tools to probe cell sensing and response to physical properties of their material surroundings, and ultimately provide designer approaches to control cell function.

A variety of physical forces are ever present throughout the human body, ranging from the pumping of blood by the heart and flow-induced shear stress in blood vessels to tensional and compressive stresses from skeletal muscle contraction, tendon ligament stretching, joint loading and the vibrations of vocal folds phonation. While such forces are vital for the physical movements that enable us to breathe, move or digest, mechanical forces are also critical regulators of biochemical signalling, cell behaviour and tissue function. At the tissue level, physical forces regulate dorsal closure, epithelial morphogenesis and skeletal development during embryogenesis, extracellular matrix (ECM) remodelling during tissue homeostasis, vascular inflammation and sprouting, and repair of injured tissue during wound healing[1–4]. At the cellular scale, cell-generated contractile forces play a fundamental role in assembling the cytoskeleton and organizing the cellular architecture, which affects activation of biochemical signalling pathways and downstream gene transcription, ultimately controlling cell adhesion, migration, proliferation, differentiation and apoptosis[5–7]. Given the profound impact of mechanical forces on most, if not all, cellular functions, understanding how physical forces are converted into biochemical signals, a process called mechanotransduction, is imperative in order to comprehend embryonic development, regeneration and disease[3,8,9].

Biomaterials have been instrumental in the endeavour to reveal mechanotransduction processes. The first class of materials employed to study the impact of matrix mechanics on cellular function were hydrogels based on isolated natural ECM components such as collagen I, fibrin or basement membrane constituents (for example, Matrigel). These materials bear structural resemblance and cell adhesive properties comparable to those in *in vivo* microenvironments[10,11], wherein cells are embedded in a 3D fibre-rich network composed of proteins, glycoproteins and proteoglycans[2]. However, in the reconstituted native ECMs, the nonlinear stress–strain mechanics, the fibrous structural features and the compositionally constrained biological ligands are typically intertwined, which makes it difficult to identify the contribution to cell behaviour of each individual material property, such as the binding affinity for cells, mechanical stiffness, porosity, fibrous organization and viscoelasticity (Fig. 1a). These challenges in manipulating natural ECM properties initially hampered researchers' ability to isolate which aspects of the ECM affected cell signalling and behaviour; however, the limitations ultimately inspired the development of novel synthetic designer biomaterials with tunable material properties to parse out how cells sense, probe and integrate physical forces.

In this Commentary, we will discuss various approaches to control specific properties of synthetic analogues of ECMs, and how these materials have revealed previously unappreciated mechanisms of mechanotransduction. We will describe the key parameters that have been placed under synthetic control and the biology that has been revealed by these capabilities (Fig. 1b). In particular, we will focus on materials that enabled investigations into the impact of stiffness, degradability and viscoelasticity on cell behaviour in settings where cells are cultured on the surface of such materials (2D) and embedded within them (3D). We will also comment on ongoing developments that may lead to new concepts and paradigms in the field of mechanobiology in the coming years.

Substrate stiffness

Historically, the role of physical forces in regulating bone tissue remodelling and embryonic development was already appreciated by Wolff, Roux and Thompson[7], yet it was not until the 1980s that the importance of mechanical forces in controlling cell behaviour was welcomed by the broader scientific community[9]. Instead of using rigid tissue culture dishes, Harris and Stopak seeded cells on soft silicone films, a material platform that enabled one to witness cells generating forces via wrinkling of the underlying substrates during cell spreading and migration[12]. Although often credited as the first study showing that non-muscle cells exerted traction forces, their work also demonstrated that mechanobiology, a nascent field that until then predominantly relied on animal models, could be studied *in vitro*; and materials could be employed as a tool to study mechanotransduction, a groundbreaking approach preluding the era of modern mechanobiology.

Since then, multiple covalently crosslinked polymer hydrogels were developed as cell-compatible substrates to study the mechanics of cell adhesion and migration, including polyacrylamide (PAAm) gels and polydimethylsiloxane (PDMS), arguably the two most extensively used materials in the field. The advent of a variety of surface chemistries enabled the synthetic coupling of ECM proteins to the surface of these substrates, allowing one to tune the density of conjugated biological ligands. In addition, the stiffness of the substrates could be tuned by directly changing the ratio of polymer and crosslinker solution, curing temperature or duration of curing. Unlike native fibrous matrices, these synthetic hydrogels exhibit linear elastic behaviour, such that the bulk modulus (stiffness) is not affected by matrix deformation or strain rate[13]. Such synthetic characteristics offered the opportunity to decouple biochemical signals from substrate

stiffness, a feature that was unattainable in native ECM-derived hydrogels.

Using this material toolbox, a remarkable degree of insight in how cells sense and respond to mechanical forces has been revealed. Perhaps the most important discovery was that matrix rigidity alone can regulate cell morphology, function and fate[14]. Cells adherent to stiff substrates display larger spreading area and proliferate more compared to cells adherent on softer substrates[15,16]. Although the mechanisms are not fully understood, there is increasing evidence suggesting that cells sense substrate stiffness via their adhesions and cytoskeletal machinery[17]. Briefly, when a cell makes contact with a fibrous ECM protein such as fibronectin, anchoring molecules known as integrins engage with the fibronectin and undergo morphological changes that enable integrins to cluster into nascent adhesions[18]. While these nascent anchoring structures stabilize the cell on the substrate, actin strands that are polymerized in the lamellipodia of the cell, migrate from the lamellipodia towards the centre of the cell, a phenomenon called retrograde flow. When in proximity to nascent adhesions, the flowing actin strand interacts with adhesions as a catch bond, a non-covalent bond whose lifetime increases with increasing tension. As such, upon engagement, a (small) inside-out traction force generated by the retrograde flow is transduced to the integrins bound to the fibronectin fibres. On soft substrates, this probing force will cause the ECM to move with the integrins and thus no resistance will be applied to the catch bond, eventually leading to dissociation of the bond. In contrast, on stiff substrates, the ECM fibres will resist against the probing force, therefore engaging the catch bond, which now generates traction on the nascent adhesion, resulting in stretching and unfolding of adaptor proteins including talin and vinculin located within the adhesion[2]. As such, cryptic binding sites within talin and vinculin are now exposed to bind other focal adhesion proteins triggering a series of phosphorylation events and activating enzymes including focal adhesion kinase (FAK), proto-oncogene tyrosine-protein kinase Src, and small GTPase Ras homologue gene family, member A (RhoA). In turn, RhoA phosphorylates myosin light chain (MLC) through its downstream kinase ROCK, which engages actomyosin contractility, thus generating more traction on the focal adhesion. This mechanism leads to growing and maturing focal adhesions and the formation of filamental actin (F-actin) stress fibres in cells spreading on stiff substrates, but not on soft matrices[5,7].

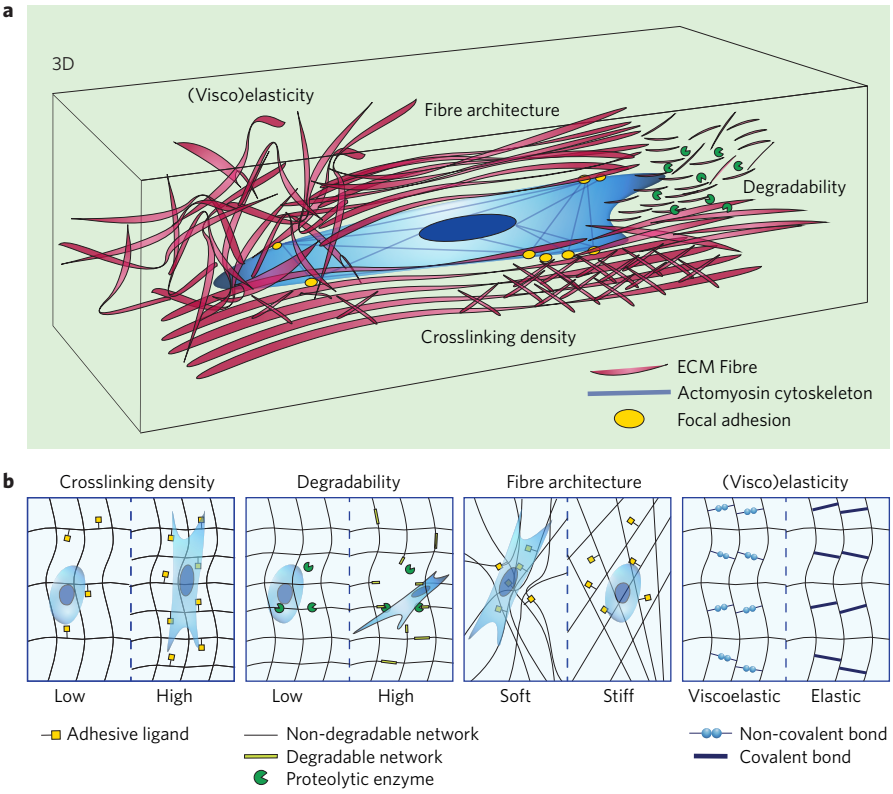

Figure 1 | Designer approaches to engineer biomaterials for mechanobiology. **a**, Schematic of a cell embedded in a fibrous-rich, mechanically anisotropic 3D microenvironment. **b**, Multiple designer parameters to orthogonally control specific properties of synthetic analogues of extracellular matrices, such as crosslinking density, matrix degradability, fibre architecture and viscoelastic mechanics.

The ability of cells to tune their cellular contractility in response to matrix stiffness has been postulated to underlie the phenomenon of durotaxis, a trait of cells to preferentially migrate from soft to stiffer substrates[19]. Durotaxis, coined by Wang and colleagues, can be investigated by seeding cells on substrates harbouring zones with softer and stiffer stiffnesses, which are created by either spatially controlling the crosslinker concentration or by tuning the amount of UV-exposure using gradient masks[20]. These approaches enable one to alter not only the baseline stiffness of the material, but also the steepness of the gradient. Unlike cell spreading, which is dependent on the absolute substrate stiffness, directional migration depends on the steepness of the stiffness gradient[21], and therefore suggests that mechanisms other than the actomyosin contractility, such as microtubule dynamics may underpin durotaxis[22].

Given that the nucleus of a cell is mechanically coupled to focal adhesions, substrate stiffness has also shown to regulate nuclear mechanics, chromatin remodelling and gene transcriptional expression that determines differentiation of stem cells into specialized lineages[23,24]. Indeed, seminal work from Discher and co-workers revealed that bone marrow stromal cells (BMSCs) can differentiate into different lineages as a function of substrate stiffness, a phenomenon mediated by stiffening of the nucleus by lamin-A proteins, which modulates nuclear entry of retinoic acid receptors[25]. BMSCs on very soft hydrogels preferentially adopt a neurogenic phenotype, while cells on intermediate stiff substrates switch on transcription of genes associated with myogenic differentiation, and cells on stiff substrates commit to the osteogenic lineage[26]. Thus, matching the substrate stiffness to the mechanics of specific tissues of interest is sufficient to prime BMSCs into tissue-relevant lineages. In addition to the physiologic role for stiffness, aberrantly high matrix stiffness was found to promote epithelial–mesenchymal transition (EMT) of epithelial cells and migration of cancer cells to metastasize[10,11,27]. Taken together, these studies demonstrate that matrix stiffness is a critical physical parameter that mediates normal and pathological states of cells.

The strain-independent, elastic properties of PAAm and PDMS not only supported

studies of stiffness, but also allowed for the measurement of traction forces generated by cells. In a method referred to as traction force microscopy (TFM), deformations in the material and corresponding forces were detected by tracking fiduciary beads embedded in PAAm substrates, micropatterned features on PDMS, or bending of micropillars[28]. Using TFM, it was discovered that adherent cells continuously probe their physical environment and change the degree of force generation depending on the stiffness of the substrate. That is, cells pull harder on stiff versus soft matrices, allowing cells to match their stiffness to the stiffness of the substrate[15].

Matrix degradability

Although the simplicity of 2D platforms provides a reductionist approach to study mechanotransduction, cells in many physiological settings are embedded within a 3D matrix. This limitation has spurred substantial efforts towards developing cytocompatible materials to support encapsulated (3D) culture[2,29]. In contrast to 2D substrates that are polymerized before cells are seeded, encapsulating cells in three dimensions requires the material components and crosslinking procedures to be biocompatible. Given the toxicity of the crosslinkers and acrylamide monomer, traditional PAAm gels are therefore unsuitable for encapsulating cells in three dimensions. Analogously, PDMS is a non-aqueous material and therefore cannot be used for this purpose. Advances in macromolecular chemistry and material science has enabled the design of new biocompatible materials to impart the hierarchical structure, biological complexity and dynamic mechanical properties of natural ECMs[30,31].

Pioneering work from Hubbell and co-workers adapted a Michael-type addition reaction as crosslinking chemistry to form poly(ethylene glycol) (PEG)-based 3D hydrogels. The mild and cytocompatible yet efficient nature of the chemical reaction permitted *in vitro* encapsulation of various cell types, demonstrating successful transition from a 2D substrate cell culture platform to a 3D cell-encapsulation hydrogel system[32]. The synthetic nature of these polymers preserves the well-defined relationship between modulus and crosslinking density and permits tunability of stiffness independent of the concentration of conjugated ligands. Tuning the crosslinking density yields a wide range of hydrogel mechanical properties. In addition, bioactive ligands derived from natural ECMs can be directly conjugated during cell encapsulation and hydrogel formation processes to support cell viability, spreading and matrix degradation, and remodelling in three dimensions, which explains why this design strategy quickly populated the biomaterials field. Building on similar strategies, polysaccharide (for example, hyaluronan, dextran, gelatin and alginate)-based materials have also been widely introduced for 3D cell culture, and advances such as oxime and click chemistry have been introduced as additional crosslinking chemistries[31,33]. Compared to PEG molecules, these polysaccharides provide many more sites on the backbone available for chemical modifications, offering greater flexibility for tuning ligands and stiffness.

Intriguingly, early attempts to encapsulate cells in 3D hydrogels revealed a completely opposite trend compared to traditional 2D culturing substrates. In contrast to planar surfaces, embedded cells in soft matrices spread well and display a polarized morphology, but remained round in stiff hydrogels, suggesting that, in addition to stiffness, matrix degradation — the breakage of crosslinks via passive hydrolysis or cell-mediated enzymatic cleavage — is a key component in regulating cell morphology in a 3D microenvironment[34]. It was further shown that the differentiation of human mesenchymal stem cells (hMSCs) was regulated by scaffold degradability[35]. However, when encapsulated in non-degradable, ionically crosslinked alginate hydrogels, lineage commitment of these MSCs was determined by matrix stiffness[36]. The physical crosslinking mechanism in this case allows significant cellular reorganization of the material and adhesion ligand presentation. Hence, the apparently divergent responses of cells in non-degradable versus degradable hydrogels underscores the importance of employing materials with distinct crosslinking mechanisms, molecular structure and dimensionality to elucidate how cells transduce physical cues from their 3D microenvironment.

Despite the promising success in recapitulating the tunability of material parameters in three dimensions, it remains a major challenge to precisely tune mechanical properties in fully synthetic matrices without interfering with other physiochemical factors. Increasing bulk hydrogel stiffness is generally associated with increasing crosslinking density, which concomitantly alters the matrix porosity, degradation, medium diffusion, mass transport, and thus the swelling properties of the gel. Swelling of hydrogels, typically more dramatic for soft matrices, has hindered the ability to generate structures with precise geometries and, important to mechanobiology, might introduce unwanted mechanical stresses in the system. To address this problem, we recently developed a synthetic dextran hydrogel with tunable hydrophobicity as a means to control non-swelling/swelling features. Using this system to tune hydrogel porosity independent of matrix degradability and stiffness, we showed that matrix degradability governs the mode of 3D endothelial cell invasion from single-cell to multicellular, strand-like invasion required for angiogenesis[37]. This example illustrates again how gaining control of confounding material properties provides a path to better understanding how specific material parameters are being transduced by cells.

Transient nonlinear mechanics

Most mechanobiology studies have relied on hydrogels that exhibit simple linear elastic mechanics. However, more complex mechanical properties exist in many native ECMs, including nonlinear mechanics due to their fibrous nature, as well as time-dependent effects such as plasticity and viscoelasticity[2].

One approach to introduce the fibrous character of native ECMs has been explored using electrospinning, a method used to fabricate fibrous matrices with tunable mechanics and user-defined fibre architecture with physiologically relevant dimensions and scales[38,39]. The nonlinear elastic characteristic of fibrillar networks promoted increased focal adhesion ligand density, enhanced adhesion signalling, cell spreading and proliferation signalling by recruiting local fibre assembly on soft fibrous substrates, revealing an inverted mechanosensing mechanism compared to traditional 2D hydrogel surfaces[38,39]. The synthetic fibre networks allow cells to assimilate their surroundings and transduce mechanical signals through architectural remodelling over multiple length scales, proving a useful platform to elucidate mechanobiology through integrin clustering and focal adhesion assembly at the nanoscale as well as through actomyosin activity between focal adhesions to probe stiffness at microscale.

To impart viscoelastic features, Mooney and colleagues modulated the nanoscale architecture of various molecular weights of alginate and employed an ionic-mediated non-covalent crosslinking strategy to form a library of alginate hydrogels with different stress relaxation kinetics. In contrast to covalent bonds, ionic bonds can break under physiological stresses, rendering plastic deformation of the material, which enabled the discovery of cells spreading and proliferation even on 2D soft viscoelastic substrates to comparable extents of those cultured on elastic stiff substrates. Further studies reveal that bone marrow cells

encapsulated in the 3D matrices with faster stress relaxation exhibited augmented spreading, proliferation and osteogenic differentiation through enhanced integrin recruitment and clustering, increased actomyosin contractility and nuclear translocation of yes-associated protein (YAP)[40]. Such unconventional findings imply that time-dependent, nonlinear mechanical characteristics can serve as an additional physical regulator in directing cell behaviour, ECM remodelling and stem cell fate.

Spatiotemporal control

The recent development of light-based conjugation strategies has allowed material properties to be changed non-invasively during an experiment. Pioneering work from Anseth and co-workers demonstrated that they could decorate 3D PEG hydrogels with photolabile nitrobenzyl ether-derived acrylate moieties, allowing for on-demand scaffold degradation via external photoirradiation stimuli. This chemistry approach allowed them to also demonstrate the capabilities to trigger encapsulated stem cells to spread into the gel and differentiate at will[41]. In a follow-up study, the team incorporated a thiol-ene photoreactive chemistry (reactive to visible light) to conjugate biological ligands and the previously established photocleavable reaction (reactive to UV light) to degrade the polymer backbone in PEG hydrogels that were crosslinked via a strain-promoted azide-alkyne cycloaddition (SPAAC) method[42]. Using these orthogonal crosslinking strategies together demonstrated the capability of spatiotemporal regulation of multiple materials properties such as integrin-binding ligand presentation and network erosion, in real time. The photocleavable chemistry also permitted *in vitro* modulation of hydrogel mechanical properties, such as reducing the modulus of an initially stiff substrate to a softer one while cells remained adhered to the substrate. This combination revealed a role for microRNA-21 and YAP/TAZ transcriptional co-activators as intracellular mechanical rheostats in transducing mechanical properties, and imparting some mechanical memory, in influencing stem cell plasticity. While briefly retaining past mechanical memory from stiff substrates yielded reversible activation of YAP, persistent mechanical dosing triggered constitutive nuclear localization of runt-related transcription factor 2 (RUNX2)-dependent YAP signalling even after the mechanical dose was removed[43]. This work illustrates how new synthetic capabilities have uncovered previously unappreciated insights into how cells respond to their local environment, as well as a practical perspective of how culturing and expanding stem cells outside of *in vivo* conditions affect their function and differentiation. In this case, the use of switchable mechanics was essential to demonstrate that cells can remember, for a time, their past mechanical conditions[43,44]. Matrix stiffening using this strategy has recently revealed that it can trigger activation of hepatic stellate cells into a fibrotic phenotype, suggesting the importance of such stiffening in the development of liver fibrosis[45].

Outlook

The evolution of mechanobiology studies highlights the ongoing development and advancement in material approaches to further understand how cells transduce material cues in ever more complex environments[46]. Initial studies were on linear elastic 2D substrates, and the field has begun to move into 3D encapsulating materials, fibrous materials and even those with time-dependent mechanical properties. Such transitions emphasize the importance of recapitulating the complexities of native ECMs over multiple length scales as an essential step towards understanding how mechanical cues regulate cellular function and stem cell fate. Despite the exciting progress in biomaterial design, there are still very few successes that truly demonstrate the ability to simultaneously and independently regulate biochemical, structural and mechanical cues within one single synthetic platform. Such challenges can be leveraged by engineering novel biocomposites with integrated material properties to capture the heterogeneous and anisotropic characteristics of the ECM. Although emerging trends using photosensitive molecular switches to manipulate mechanical properties *in vitro* have successfully demonstrated dynamic modulation of mechanical stiffness of synthetic ECMs in both two and three dimensions, such exogenous stimuli are not yet readily applicable to *in vivo* studies due to absorption of light in deep tissues/organs. Therefore, novel chemistries such as non-covalent reversible reactions that can be additionally manipulated to respond to physiological stimuli (for example, temperature, electrical potential, pH and enzymatic activity) have begun to gain increasing attention as alternative hydrogel crosslinking strategies to offer dynamic mechanical tunability that can be applied in *in vivo* settings[30,31,33]. Furthermore, other promising classes of materials, such as self-assembling peptide- or polypeptide-based materials, are also being developed to build complex fibrous scaffolds with hierarchical structures and nonlinear viscoelastic mechanics, offering additional platforms to probe complex mechanical events such as molecular association and energy dissipation in more native ECM-like settings[47,48]. Finally, the limitation of long-term cell encapsulation due to degradation of these materials requires the design of novel strategies to maintain the mechanical integrity of the material by matching the kinetics of matrix degradation with the rate of ECM production of the embedded cells. Extending the experimental lifetime of 3D culture systems from days to weeks will be critical for connecting short-time mechanosensing and biochemical signalling to long-term effects of these signals on cell function and tissue development. The future of this field is bright, as innovations in materials continue to drive our understanding of mechanobiology ever forward. ❐

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