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Manipulating the mechanics of extracellular matrix to study effects on the nucleus and its structure

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Abstract

Tissues such as brain, muscle, and bone differ greatly not only in their biological functions but also in their mechanical properties. Stiffness of the extracellular microenvironment affects processes such as cell polarization and DNA replication, thereby regulating nuclear size, shape, and levels of nuclear proteins such as the lamins that can modulate gene expression. Reductionist approaches have helped dissect the effects of matrix mechanics away from confounding biochemical signals. Here, we summarize materials and methods for synthesizing and characterizing soft and stiff synthetic hydrogels widely used for mechanobiological studies. Such gels are easily made to mimic the mechanical heterogeneity of fibrotic tissues. We also describe a nano-thin collagen film system, which enables control of fiber isotropy in addition to bulk material stiffness. With the different systems, we illustrate the effects of matrix stiffness on nuclear size, shape, and proteins including the lamins.

1. Introduction

In the structure-function paradigm that often guides modern studies in molecular and cell biology, structure generally gives rise to physical properties which in turn dictate function. However, some cell and tissue structures are dynamic, with feedback of properties into structures. The nucleus is among these malleable structures, and increasing evidence underscores its response to structures and properties outside of the cell.

In animals, the most abundant proteins are those in the high-order structural assemblies of the extracellular matrix (ECM), which not only define tissue microenvironments for the specialized cells within, but also present different physical properties to these resident cells (Leight et al., 2017; Wells, 2013). Mechanical ‘stiffness’ or microelasticity (in units of kPa) is often useful in characterizing the physical properties of tissues, and generally reflects the

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contributions of fibrillar collagens (Shoulders and Raines, 2009). Due to the varying concentrations of collagens among normal adult tissues, stiffness ranges over two orders of magnitude, from soft brain and fat (Flanagan et al., 2002; Georges et al., 2006; Patel et al., 2005) to stiff muscle (Ferrari et al., 1998), cartilage (Stolz et al., 2004), and rigid pre-calcified bone (Andrades et al., 2001; Engler et al., 2006; Holmbeck et al., 1999; Morinobu et al., 2003). Cells within these diverse tissues constantly probe the local micro-mechanical properties of their surroundings by adhesion and active pulling, sensing the stiffness as a resistance to imposed deformations. Extensive *in vitro* studies with many types of cells reveal a mechanical feedback in which cells reorganize their cytoskeleton and adjust their contractility, which are important to a broad range of cellular mechanosensing mechanisms (Wolfenson et al., 2016). On soft and malleable substrates, cell types that include adhesion-dependent primary cells (e.g. bone marrow-derived mesenchymal stem cells, MSCs) and many immortalized cell lines (e.g. lung adenocarcinoma A549s) downregulate actomyosin contractility, turn over focal adhesions, and ultimately generate much lower tension on ECM compared to cells on stiff or rigid substrates (Buxboim et al., 2014). This causes the cells on soft ECM to become less-spread and more rounded in terms of both cell and nuclear morphology.

The nucleus, as the largest organelle in a cell, is increasingly understood to be mechanosensitive (Alam et al., 2014; Cho et al., 2017; Guilluy and Burridge, 2015; Swift et al., 2013). Mechanical forces imposed on or by the cell must first propagate to the nuclear envelope before transduction into downstream signals in the nuclear interior (Irianto et al., 2016b). The nuclear envelope can in this respect be regarded as a multiplexer for transducing external mechano-signals in addition to being a physical barrier for material transport in and out of the nucleus. The nuclear envelope harbors thousands of regulatory proteins and complexes, and the inner nuclear membrane is lined with an organized meshwork of intermediate filament A- and B-type lamins. As with many other helix-rich structural proteins in the cell or the ECM (e.g. collagen-I), lamins are coiled-coils that assemble into higher-order filaments (Herrmann et al., 2009; Turgay et al., 2017). A-type lamins interact at least indirectly with genomic and telomeric chromatin, based on co-immunoprecipitation and on increased mobility of telomere ends upon depletion of lamin-A (Burla et al., 2016; Gonzalo and Eissenberg, 2016). B-type lamins (lamin-B1 and B2), unlike lamin-A, are permanently modified by a farnesyl group that anchors them to the inner nuclear membrane with higher affinity (Jung et al., 2013). Proteomic profiling of diverse tissues have shown high-collagen, stiff, and mechanically stressed tissues such as muscle and bone possess 10-fold or greater levels of lamin-A compared to low collagen, soft tissues such as brain or fat (Swift et al., 2013), suggesting a mechano-protective role for these nuclear proteins. Indeed, recent studies of constricted cell migration have demonstrated low lamin-A cells are especially susceptible to stress-induced disruption of the lamin-B meshwork (Harada et al., 2014) and nuclear envelope rupture (Denais et al., 2016; Irianto et al., 2017; Raab et al., 2016).

To study nuclear morphology and the lamins in relation to matrix stiffness, cells are often cultured on polyacrylamide (PA) hydrogels with tunable stiffness and controllable adhesion ligands (Swift et al., 2013). PA is a synthetic polymer in long-time use for protein separations and, more recently, for various cell culture applications (Pelham and Wang,

1998). The elasticity of PA gels can be easily adjusted by varying the concentration of monomers and crosslinkers, and the requirement for cell adhesion can be fulfilled by attachment of a thin layer of cell-binding ECM proteins such as collagen-I, fibronectin, or Matrigel. Mechanical properties of PA hydrogels have been extensively characterized by various macroscopic techniques including bulk tensile loading and rheology, but atomic force microscopy (AFM) indentation is best for assessing the microscale elasticity (Engler et al., 2007). PA has other important physicochemical properties: the hydrogels are >90% water, optically transparent, chemically inert, neutral in charge, and non-toxic to cells after polymerization. The simple composition allows for additional functional modifications to better mimic specific biological microenvironments so that more complex cellular responses can be studied (Fischer et al., 2012; Moeller et al., 2018). However, polymerization conditions and buffer parameters are critical, and accuracy in formulation and measurement are required to reproducibly obtain results for hydrogels that provide prescribed mechanical characteristics.

Living tissues are of course far more complex than polymeric gels, but their bulk mechanical properties are similarly determined by the density of collagenous ECM and by the degree of ECM crosslinking. Collagenase digestion can soften tissues in minutes, and the stiffening and fiber bundling effects of crosslinking are perhaps most evident in rigid fibrotic ‘scar tissue’ as well as a broad range of pathological conditions such as late stage liver cirrhosis and cancer (Levental et al., 2009; Zhao et al., 2018). Model systems that can recapitulate such diverse physiological changes are thus crucial to understand how cells and their nuclei respond to microenvironment cues. Here, we review methods to synthesize PA hydrogels of tunable, homogeneous stiffness (Engler et al., 2007; Swift et al., 2013), followed by more recent PA gels that allow for control of mechanical heterogeneity (‘scar-mimetic’ substrates) (Dingal et al., 2015), and finally thin collagen nanofilms that can be used to assess the effects of fiber crosslinking and alignment (Ivanovska et al., 2017). The effects on nuclear factors including lamins will be described for each system as an example of broad nuclear changes that can occur from manipulation of cell matrix.

2. Method for making polyacrylamide gels

2.1. Glass cleaning

Cleaning glass coverslips is the first step to ensure successful synthesis of PA gels as clean glass surface is crucial for gel attachment. Although PA gel on 18mm round coverslip is used here as an example, the gel size is indeed scalable when different size of coverslips are used.

1. Prepare RCA solution (DI water, hydrogen peroxide, and ammonium hydroxide, 2:1:1 ratio by volume, ~400 ml) in a beaker under a chemical safety hood.
2. Place the coverslips (both 18mm & 25mm in Table S1) in the RCA solution.
3. Heat RCA solution up to 70–80 °C for 10 minutes. During heating, place a thermometer in the beaker to monitor the temperature. Cover the beaker with aluminum foil to prevent evaporation.
4. Transfer all coverslips to a new beaker containing ~400 ml DI water.

5. Gently swirl the beaker containing DI water 2–3 times.
6. Transfer the 25 mm coverslips to a desiccator.
7. Allow these 25 mm coverslips to dry overnight. (18 mm coverslips will be used for glass functionalization in the next section.)

RCA is a solution first developed to clean silicon wafers and widely used in the semiconductor industry since. It is preferred in this initial step to 1) oxidatively breakdown organic contaminants, 2) dissolve oxide layers and 3) form a dissolvable metal-ammonia complex to remove metal ions (Kern, 1990). After RCA cleaning, a thin layer of water will form on cleaned coverslips, as the whole glass surface will have become hydrophilic without contaminants. The formation of this thin film of water can be used as a standard to check the quality of RCA cleaning.

Piranha solution (3:1 ratio of sulfuric acid to hydrogen peroxide) is another commonly used solution to clean coverslips (Denisin and Pruitt, 2016; Zayats et al., 2014), which can be used interchangeably with RCA solution. In this case, 30 minutes incubation of coverslips in piranha solution is recommended (no heating is necessary).

2.2. Chemical functionalization on coverslips for covalent gel binding

Perturbations such as pipetting during experiments, shear forces in some setups, or even cell contractility on thin gels may create wrinkles or partial detachment of hydrogels from coverslips, ultimately compromising the quality of the final gel product (Buxboim et al., 2010). Therefore, it is recommended to covalently connect the PA gel to the coverslips to prevent such issues. Although direct binding of PA gel to glass coverslip is difficult, a mediator which covalently binds to the glass coverslip on one side and the PA gel on the other is indeed available. Allyltrimethylchlorosilane (ATCS) has chloro-groups that can react and bind to glass covalently as well as a vinyl group which can integrate with PA gels (Fig. 1A-i).

Thus:

1. RCA-cleaned 18 mm coverslips (*not* 25 mm) are rinsed sequentially in water, ethanol, and chloroform (each ~400 ml) by gently swirling each beaker 2–3 times.
2. Place the rinsed coverslips in silanization solution – chloroform with 0.1 v/v% ATCS and 0.1 v/v% trimethylamine (TEA) – for 1 hr for functionalization (Fig. 1A-i).
3. Rinse the ATCS-silanized coverslips in chloroform, ethanol, and DI water (each ~400 ml) sequentially as in step 1.
4. Transfer the coverslips to a desiccator and dry overnight with the 25 mm coverslips to remove any excess water droplets.

Upon ATCS functionalization, the surface of the coverslips becomes hydrophobic and water droplets on coverslips now form a large contact angle between the solid and liquid surface,

which is the opposite of the hydrophilic properties prior to treatment. This phenomena can be used as a first visual check for the quality of ATCS functionalization.

Alternatively, a combination of 0.5% v/v (3-Aminopropyl)triethoxysilane (APTES) and 0.5% v/v glutaraldehyde (GA) in water can also be used for glass functionalization (Denisin and Pruitt, 2016). APTES binds to the glass surface first and then the remaining amine group (-NH₂) reacts with one of the aldehyde groups (-CHO) in GA. During acrylamide polymerization, the remaining free aldehyde group (-CHO) reacts with amine group (-NH₂) on polyacrylamide so that the PA gel covalently attaches to the surface (Fig. 1-ii).

ATCS is highly reactive with air, so old bottles may have white powder-like solid near the cap opening. Fresh ATCS is recommended if the silanized coverslips do not appear sufficiently hydrophobic. Indeed, after successful silanization, dehydration of PA gels in ethanol (~minutes) will not detach the gels from the coverslips and such gels can be rehydrated after being immersed in water (Buxboim et al., 2010).

2.3. Gelation

1. Prepare fresh precursor solution for soft and stiff gels based on the compositions listed in Table 1 (1 ml precursor solution per stiffness is usually sufficient for one experiment).
2. Prepare 10 w/v % ammonium persulphate (APS) solution (i.e. dissolve 1 g APS in 10 ml DI water).
3. Add the catalytic initiators 0.1v/v% N,N,N',N'-tetramethylethylenediamine (TEMED) and 1v/v% APS solution to each precursor solution, then vortex for 5 s to ensure complete mixing and to initiate the polymerization.
4. Quickly place ~7 μ l gel precursor per 1 cm² of glass surface (add a single droplet in the center of the coverslip). For 18 mm ATCS-treated coverslips, the droplet should be 20 μ l.
5. Quickly and gently cover with hydrophilic cover glass (RCA-cleaned 25 mm round coverslip) without applying pressure on top. This will result in a 'sandwich' of glass-gel solution-glass.
6. Allow polymerization to occur for 30min.

The polymerization reaction scheme is identical to homemade gels used in electrophoresis for the separation of proteins or nucleic acids. Bis-acrylamide is the crosslinker. By varying concentrations of acrylamide and bis-acrylamide, gels with varying stiffness can be achieved. The suggested recipes in Table 1 can be used to synthesize gels that match the stiffness range of a variety of tissues. For example, normal brain has a stiffness ~ 0.3 kPa; healthy liver or lung has a stiffness ~ 1–3 kPa; striated muscle is usually ~10 kPa and matrix on precalcified bones can be as stiff as 40kPa (Swift et al., 2013).

In step 5, the interaction between polymer and ATCS-silanized coverslips (Fig. 1A-i) allows no excess solution to disperse outside of the coverslip region when a hydrophilic coverslip is gently added on top of the droplet. Polymerization requires <30 minutes at room

temperature. Stiffer gels polymerize faster as more reagents are present in the precursor (~5 minutes for 40kPa and ~20 minutes for 0.3kPa). However, allowing reaction to occur for 30 minutes as in step 6 ensures complete reaction and limits dehydration or damage to the gel. Any excess precursor solution remaining in the Eppendorf tubes will also polymerize, which can be used to check the quality or extend of polymerization (Fig.1-iii). In step 4, 7 μ l gel precursor per 1 cm² of substrate surface has been determined empirically (trial and error), and reproducibly generates high quality PA gels.

7. After polymerization for 30 minutes, place the gel 'sandwich' into DI water and place on a shaker for 1 hour.
8. Gently peel off the large coverslip. The PA gel should be visible as a thin transparent layer on the 18 mm coverslip (for troubleshooting, see 2.6.4).
9. Rinse gels in DI water on the shaker for 5 minutes.

To coat PA gels with collagen-I:

10. Add crosslinker sulfo-SANPAH (50 μ g/ml in 50mM HEPES) over the whole gel surface and photo-activate under 365 nm UV light for 7 minutes. If PA gels (on 18 mm coverslips) are placed in a 12-well plate, 400–500 μ l of sulfo-SANPAH per well is recommended.
11. Wash away excess sulfo-SANPAH using DI water (5 minutes on shaker) after UV activation.
12. Submerge the coverslips in collagen-I solution (100 μ g/ml in 50 mM HEPES) overnight at room temperature with gentle shaking (for troubleshooting, see section 2.5–5).

Sulfo-SANPAH is a crosslinker that contains an amine-reactive N-hydroxysuccinimide (NHS) ester and a photoactivatable nitrophenyl azide. Other published protocols may add NHS directly into the reaction mixture during gel polymerization.

If fibronectin is preferred, 5 μ g/ml in PBS is recommended using the same protocol. The concentration can be varied depending on the purpose of the experiments.

2.4. Sterilization

Multiple methods have been developed to sterilize hydrogels including heat treatment, gamma or UV exposure, gas plasma, ethylene oxide, and supercritical carbon dioxide treatment. However, the selection of the method depends on the sensitivity of the modifications to PA gels (Dai et al., 2016). For example, although the PA gel described here is inert and can be sterilized by UV, modification of PA gel with a photosensitive cross-linker would render the system susceptible to UV exposure, so in such cases alternative sterilization methods would be preferred (Ifkovits and Burdick, 2007; Jariashvili et al., 2012; Sanford et al., 1998). For conventional PA gels here, we recommend 2 hr UV light exposure in the biosafety hood. Sufficient PBS is needed to immerse the hydrogels and prevent excessive drying during sterilization. If necessary, the safety hood blower should be turned off to minimize PBS evaporation.

2.5. Troubleshooting

1. The hydrophilicity/hydrophobicity of treated glass coverslips should be confirmed before proceeding to the next step.
2. TEMED and APS should be mixed thoroughly with precursor to ensure homogeneous polymerization.
3. After mixing TEMED and APS, the mixture should be quickly transferred on silanized coverslips and covered by a hydrophilic coverslip. Acrylamide polymerization occurs rapidly (~minutes at high concentrations). Significant delays during this step could result in premature polymerization of the droplet on silanized coverslips, preventing proper flattening of the gel surface.
4. After gel polymerization and prior to removing the hydrophilic coverslip, a 45 min – 1 hr wash in DI water or PBS is highly recommended. Otherwise, the hydrophilic coverslip will be difficult to be peeled off without damaging the gel surface. Extra caution is recommended when peeling of the hydrophilic coverslip in order to prevent defects on gels or breaking of hydrophobic coverslips.
5. Collagen-I solution for gel ligand coating must be prepared fresh, otherwise collagen can self-assemble in solution and affect coating efficiency and/or homogeneity.

2.6. Characterization of PA hydrogel by AFM

Different methods are available for characterizing the elasticity of PA hydrogels such as tensile loading, rheology, and AFM. We focus on AFM here as it offers many useful functions in addition to mere measurements of material properties. For example, there is a growing appreciation that AFM can be utilized in various research contexts, including characterization of protein-protein interaction strength, cell adhesion forces, as well as submicrometer scale imaging (Ivanov et al., 2011). The “force mode” has been widely used in biophysical studies (Ikai, 2010; Pecorari et al., 2017), and here we briefly illustrate how this mode can be used to characterize the elasticity of PA hydrogels.

AFM measurements should be taken under the same settings and conditions where the PA hydrogels would be applied. For most applications the PA gel is completely submerged in cell culture media on the AFM stage. Once the cantilever is lowered close to the surface of the gel, indentations are made at a tip velocity of 2 $\mu\text{m/s}$ over a total vertical distance of 4 μm (for both indentation and retraction). Multiple force-indentation curves should be obtained across the whole surface of the gel for an average of 50–60 force curves per matrix condition. The contact point for each indentation curve (or force-distance curve) is the point where the AFM cantilever deflection can be detected. The force-indentation curve between the contact point and the end of the indentation is subsequently analyzed and fit to the Hertz sphere or the Sneddon cone model depending on the shape of AFM tips, to obtain Young's modulus of the substrate. A Poisson ratio ν of 0.4–0.45 is recommended to calculate the elasticity of unknown matrices as this range is determined by macroscopic tension tests. Indeed, the computed elasticity is not dramatically affected by the Poisson ratio as variation between 0.3 and 0.5 affects the modulus by only 10% (Engler et al., 2004; Li et al., 1993).

Some characterization under recommended conditions has been done (Fig. 1B-E) as a guideline for those who intend to synthesize PA gels with desired stiffness. Thin ligand coating (e.g. with collagen-I) does not affect the elasticity of thick PA gels (Fig. 1B). Classic theory of rubber elasticity would predict that the elastic modulus of a polymer gel scales linearly with concentration of crosslinkers, which is overall true under log-scale for at least 3%, 5% and 10% acrylamide mixture confirmed by both pyramid and spherical tips under AFM measurement (Fig. 1C). The plot also shows excellent agreement with published measurements made by others using both AFM (4%) and bulk rheology methods (Mahaffy et al., 2000). However, high deviations can be seen at 3% acrylamide with 0.03 w/v% crosslinkers, which indicates PA gels in this elasticity range should be avoided in biological research (Fig. 1C). One possibility for this deviation might be percolation of bonds or material heterogeneity during synthesis. Although the thickness of gels can vary depending on the purpose of experiments (Fig. 1D), thinner gels tend to be mechanically stiffer than the theoretical value shown by both AFM measurements (Fig. 1E) and cell responses (Buxboim et al., 2010). Therefore, gel thickness should as well be taken into account to ensure correct elasticity.

2.7. Characterization of PA hydrogel by rheometry

Rheological measurement using rheometers is another commonly used approach to characterize the mechanical properties of hydrogels, but it measures shear modulus instead of Young's modulus (E) described in previous sections. Typically, samples are placed in a cup-and-bob rheometer, where the bob can have different geometries (e.g. plate or cone-shaped). In the rheometer, the plate or cone is resisted by a thin piece of metal called torsion bar during rotation. The shear stress can be calculated based on the known response of the torsion bar and the degree of actual twist during sample measurement. The shear rate is determined by the rotational speed and cone dimensions.

When a sinusoidal oscillation is applied, the response wave of the sample can be time-delayed with a phase shift δ . In case of purely elastic materials, δ is zero and for purely viscous materials, δ will be 90 degrees out of phase. This parameter gives a measure of the viscous versus elastic ratio of the material at a given frequency, from which the elastic and viscous components of the stress wave can be separated and the respective in-phase shear storage modulus G' and out-of-phase loss modulus G'' can be obtained. Rheology measurements of biological soft materials is a rapidly developing field (Chen et al., 2010) and detailed protocols can be found here (Callies et al., 2017).

2.8. Sample studies of nucleus utilizing soft/stiff PA gels

Several mechano-regulation mechanisms have been discovered in recent years using soft and stiff PA hydrogels, some of which have focused on nuclear envelope proteins such as lamins. On soft PA hydrogels (0.3 kPa), cells exhibit a low-tension phenotype in terms of rounded cellular and nuclear morphology, smaller spreading area, disassembly of stress fibers, as well as low levels of α smooth muscle actin (SMA, or ACTA2) expression within 1–2 days of culture. However, on stiff hydrogels (40 kPa), the opposite effect is seen (Swift et al., 2013). While culturing cells on soft substrates often results in nuclear membrane 'wrinkles' indicative of low tension, culturing cells on stiff substrates is sufficient to drive nuclear

spreading and tautness. Besides differences in nuclear morphology, immunofluorescence and western blots indicate lamin-A protein is more abundant in cells cultured on stiff substrates than those on soft gels, consistent with trends seen in diverse tissues. One interpretation is that cells somehow upregulate lamin-A levels on stiff surfaces to protect the nucleus against correspondingly higher tensile forces (Isermann and Lammerding, 2013; Swift et al., 2013). Indeed, ‘laminopathies’ resulting from lamin-A mutations (e.g. dilated cardiomyopathies and Hutchinson-Gilford Progeria Syndrome) typically cause defects that are most pronounced in stiff tissues such as muscle or bone (Cho et al., 2018; Swahari and Nakamura, 2016). Furthermore, frequent nuclear membrane rupture is seen in mutant cells or lamin-A depleted cells when cultured on stiff substrate where the tension is high (Tamiello et al., 2013; Xia et al., 2018). Therefore, in response to high tension from stiff substrate, a robust nuclear lamina seems required to maintain nuclear integrity. This notion is further supported by evidence that lamin-A determines nuclear stiffness and resistance to deformation (Irianto et al., 2016a; Pajeroski et al., 2007).

Studies using PA gels have further suggested lamin-A’s mechanosensitivity arises in part from its phosphorylation and turnover during interphase. Mass spectrometry-based measurements revealed multiple phosphorylation sites on lamin-A’s head (Ser22) and tail domains (Ser390, Ser404 and Thr424) that are all suppressed when cells are cultured on stiff hydrogels (Swift et al., 2013), suggesting high nuclear tension inhibits phosphorylation and degradation of lamin-A. This stiffness-mediated regulation via phosphorylation is further validated by phosphomimetic mutant studies, in which cells with S22A and S22E mutations were cultured on soft and stiff substrates. The ‘non-phosphorable’ S22A mutation on LMNA caused the nucleus to exhibit high-tension phenotypes on soft gels, whereas the phosphomimetic S22E mutant exhibited low-tension phenotypes even on stiff substrates (Buxboim et al., 2014), indicating that lamin-A mechanosensing might regulate key downstream cell responses to stiffness. The time scale for lamin-A regulation through phosphorylation is surprisingly rapid, as relaxation of tension via cell rounding (40-minute treatments of low-dose trypsin to partially detach cells) or 1–2 hr treatment with blebbistatin (myosin II inhibitor) on cells cultured on stiff substrates can result in rapid accumulation of phosphorylated lamin-A fragments. However, lamin-B remains roughly constant when cells are cultured on soft and stiff gels and no lamin-B phosphorylation can be detected (Lammerding et al., 2006). A possible mechanism bridging tension and lamin-A phosphorylation is that low tension in the cell and nucleus destabilizes the lamin-A coiled-coil dimers, favoring phosphorylation by constitutive kinases and promoting subsequent degradation. This mechanism of tension-inhibited phosphorylation provides the biophysical basis for a “use it or lose it” model, whereby unstressed lamin-A is degraded under low-stress conditions but stabilized under high-stress conditions (Buxboim et al., 2014; Cho et al., 2017).

3. Modification of PA gels for studies of nuclear responses to fibrotic environments

One variable of potential relevance to health and disease is mechanical heterogeneity, such as that which arises in fibrotic scar tissue upon acute (e.g. myocardial infarction) or chronic

(e.g. late stage liver cirrhosis) injury (Mann et al., 2011; Turgeman et al., 2008). Given that fibrosis is a major contributor to many diseases including cancer as well as normal and premature aging of adult tissues, a model system that can better mimic fibrotic tissues could help our understanding of pathological mechanisms and potentially lead to new therapeutic targets. To mimic the mechanically heterogeneous nature of fibrotic scar tissue as a minimal matrix model of scars (MMMS), co-polymerization of PA with collagen-I produced highly branched fiber bundles segregated heterogeneously at the subsurface of PA hydrogels (Dingal et al., 2015). By this method the hydrogel creates a soft and stable bulk system, whereas collagen-I self-assembles into stiff, randomly-distributed heterogeneous fractals similar to a thin section from fibrotic liver (Fig. 2A-i). Since collagen self-assembly can be viewed as a diffusion-limited cluster aggregation process (DLCA), the fractal sizes can be controlled conveniently by adjusting the concentration of collagen-I.

Fibrosis could occur in virtually all healthy tissues whose stiffness can range from 0.3 kPa (brain) to 40 kPa (bone) and beyond (Cox and Erler, 2011), and MMMS can potentially be used to model such processes for a wide variety of cell types. The mechanics of heterogeneously rigid scar tissue can be recapitulated by manipulating the concentration of acrylamide and collagen-I during co-polymerization. For instance, by mixing 0.4 mg/ml of collagen-I into the PA gel precursor, collagen fiber bundle surface coverages of ~30% can be achieved, which matches that of a muscular dystrophy for a mouse model as well as that of Duchenne muscular dystrophy skeletal muscle tissue cross-sections (~20–30%) (Dingal et al., 2015). Variations of such MMMS gels could thus prove useful for researchers particularly interested in studying the role of fibrosis in cardiovascular or musculoskeletal disease, and perhaps also for those studying the behavior of cancer cells in fibrotic tissues (Chin et al., 2016).

3.1. Synthesis of MMMS

The protocol for making MMMS is similar to synthesizing a conventional PA hydrogel, except the DI water in Table 1 is replaced by the collagen mixtures listed in Table 2. MMMS usually takes a longer time to polymerize than PA gels, so sufficient time is needed to complete the reaction. For MMMS, 60 minutes is recommended instead of 30 minutes (for troubleshooting, see 3.2–2). HEPES buffer is used to dissolve collagen because it is more compatible with collagen than PBS is (Sung et al., 2009), but requires a pH greater than 8.3 since a basic condition is required to allow collagen to self-assemble into bundles (Li et al., 2009). Upon co-polymerization with PA, the collagen bundles self-assemble into fractal structures similar to that seen in scars, but the bundles form underneath the surface. Therefore, ligand coating (e.g. with collagen or another ECM protein) is still needed to allow adhesion. The resulting MMMS gel is mechanically heterogeneous – stiff directly above the fractal collagen bundles, but soft elsewhere. As cells can ‘feel’ the stiffness of their substrates up to ~5 μm deep (Buxboim et al., 2010), different responses are seen from cells on MMMS and on standard homogeneous gels.

3.2. Troubleshooting

1. The pH of HEPES buffer is crucial as low pH can hinder gel polymerization.

2. Although 20 minutes is usually sufficient to polymerize conventional 0.3kPa PA gel, polymerization of MMMS takes longer due to the presence of embedded collagen bundles. A total of 60 minutes for MMMS polymerization is generally sufficient and is recommended (for all stiffness ranges). Again, any excess precursor solution can be used for quality control purposes.

3.3. Mechanical characterization of MMMS

Similar to elasticity measurements on conventional PA gels, AFM remains a powerful tool to characterize the material properties of MMMS. Due to the mechanical heterogeneity of MMMS, AFM scanning via 'contact mode' is applied to obtain elasticity topology, rather than probing random locations as in conventional PA gels. The spatial AFM resolution used in Fig. 2-ii is 1 μm , which emulates cellular length scales in matrix sensing. A silicon nitride cantilever probe with a pyramidal tip and spring constant of 30–100 pN/nm is used to indent the gels in this case. The elasticity map is then calculated from indentation curves using the Hertz model. (Dingal et al., 2015). Compared to conventional PA gels which are homogeneously $\sim 0.3\text{kPa}$ across the entire surface, MMMS have micron-scale fractal 'islands' that are stiffer ($> 1\text{kPa}$).

Just as adherent cells pull on the matrix laterally during mechanosensing, deformation induced by lateral pulling by a glass probe can be assessed on both conventional PA gels and on MMMS to qualitatively illustrate the mechanical heterogeneity of MMMS. In lateral pulling experiments (Fig. 2-iii), glass micropipettes are formed from glass capillaries (1-mm inner diameter, World Precision Instruments, Sarasota, FL) using a Flaming-Brown Micropipette Puller (Sutter Instrument, Novato, CA). Micropipette tips are first placed on the surface and then used to probe the gels embedded with microbeads (1- μm diameter FluoSpheres, Invitrogen) by constant displacement 20 μm parallel to the gel surface (white arrows, Fig. 2A-iii). Nearby fluorescent bead displacements are traced simultaneously (pink & blue on images and plots, Fig. 2A-iii) (Dingal et al., 2015). In general, stiff gels have higher resistance to lateral pulling-induced deformations, resulting in smaller bead displacements. For standard homogeneous PA gels, similar displacement curves are obtained when pulling from different directions (Fig. 2A-iii plots). However, different displacement curves are observed for MMMS when pulling towards versus away from the collagen bundles, indicating heterogeneity in stiffness on such gels.

3.4. Sample studies of nucleus utilizing MMMS

Early studies using MMMS offer unique insight into how cells perceive a mechanically heterogeneous environment differently from homogenous substrates. For example, early studies have shown that while crawling on the scars, well-spread cells polarize more with greater persistence (several hours longer) along the scar's long axis than less spread cells, suggesting that responses for spreading and non-spreading cells could be divergent. Mouse liver cancer cells (Dox-inhibitable MYC-expressed EC4 cells (Anderton et al., 2017; Nair et al., 2018)) cultured on stiff substrate exhibit more nuclear spreading and higher proliferation (4N population) than that on soft matrices (Fig. 2B). However, turning off MYC expression by Doxycycline results in decreased nuclear spreading and proliferation even on stiff gels (Fig. 2B), indicating stiffness-mediated proliferation and nuclear spreading is perhaps

regulated by MYC. Indeed, MMMS with 20% scar area in soft bulk PA gels showed a 100% increase in proliferating population (4N) and significant increases in nuclear spreading compared to those cultured on homogeneously soft PA gels (Fig. 2C), indicating that cell proliferation as well as morphology may be profoundly altered on fibrotic tissues. Moreover, lamin-A tends to be equally high in cells cultured on homogeneously stiff substrates and those on MMMS, but relatively low on soft perhaps due to phosphorylation (Fig. 2D). Therefore, counterintuitively, the heterogeneously stiff scars seem to somehow homogenize and dominate the cell response. Indeed, MSCs cultured on MMMS with soft bulk PA gels exhibit homogeneously high expression of SMA compared to cells cultured on soft gels which express low SMA (Dingal et al., 2015). Although further studies are needed to resolve molecular mechanisms, MMMS adds complexity to the homogeneous PA gel system, making the hydrogel a more suitable model for fibrosis (Green and Elisseff, 2016).

4. Fibrillar and crosslinked collagen films

Tissue stiffness correlates with increased deposition of fibrous ECM and also with increased covalent crosslinking (Lampi and Reinhart-King, 2018). The simplest polymer physics models show that gel stiffness is linearly dependent on crosslink density. Transcriptomic analyses for normal tissues of different stiffness reveal *COL1A1* and *COL1A2* are highly correlated not only with each other as expected of a stoichiometric complex (at the protein level), but also highly correlated with lysyl oxidases (*LOX*) crosslinking enzymes (Ivanovska et al., 2017). Transcriptome data for human cancers in TCGA (The Cancer Genome Atlas) include well-known fibrotic cancers such as hepatocellular carcinoma (HCC) and likewise reveal *LOXL1* and *LOXL2* scale with *COL1A1* either strongly (exponent for *LOXL1* is nearly 1) or weakly (exponent for *LOXL1* is <1) (Fig. 3A); expression is also increased in tumors versus adjacent normal tissue (Goldman et al., 2018). Although many genes such as *CD47* are stiffness-independent (Fig. 3A), some nuclear factors such as *GLI2* also correlate strongly with *COL1A1* (Fig. 3B). *GLI2* is upstream in a pathway that drives cancer cell proliferation and that correlates with low survival (Fig. 3B,C) (Omenetti et al., 2011; Shi et al., 2016). One can infer correlations with crosslinking, but reductionist experimental approaches are important.

To study the effects of collagen crosslinking on nuclear mechanosensitivity, a simple method was developed to generate a thin monolayer of fibrillar collagen that is either pristine or enzymatically crosslinked (Fig. 4A) (Cisneros et al., 2007; Ivanovska et al., 2017). The collagen nanofilms are fundamentally distinct from synthetic PA gels in terms of structure and thickness (nm in contrast to ~70 μ m PA gels). Nonetheless, some cell and nuclear responses in culture are very similar between soft gels and pristine films when compared to stiff gels and crosslinked films.

4.1. Synthesis of collagen nano-films

1. Prepare glycine buffer with 50 mM of glycine and 200 mM of KCl. Adjust the pH to 9.2 to ensure correct collagen D-spacing (for troubleshooting, see section 4.2–1).

2. Filter the buffer through a 0.2 μm filter to remove contaminants before addition of rat tail collagen.
3. Place 25mm mica in 6 well plates.
4. Add rat tail collagen-I to the filtered buffer solution, such that the final collagen concentration is 1 $\mu\text{g}/\text{ml}$. Once collagen is mixed, add 200 μl of this mixture to mica. Add the mixture drop by drop to ensure the liquid is dispersed on the entire mica surface.
5. Allow 30 min for collagen assembly on mica at room temperature under the biosafety hood.
6. Gentle aspirate away the liquid.
7. Wash with PBS (add PBS – swirl 6 well plate 2–3 times – aspirate away PBS).
8. UV sterilization (1hr) is optional as previous filtration is usually sufficient to remove contaminants.
9. If crosslinking is needed, prepare transglutaminase (TGM2) solution (30 $\mu\text{g}/\text{ml}$ in 50 mM Tris buffer, 5 mM CaCl_2 , and 2 mM dithiothreitol at pH 7.4). After step 7, add 200 μl TGM2 solution to the collagen film and agitate on a shaker for 4 hr at 37°C, followed by washing with PBS twice.

4.2. Troubleshooting

1. Collagen fibril self-assembly on mica is very sensitive to pH and electrolytes in the solution. The spacing among the collagen fibrils is pH-dependent, and clear separation can be achieved at pH ~ 9.2 as fibrils repel each other at pH's close to and above the isoelectric point of collagen molecules (pH = 9.3 under these specific buffer conditions). The formation of characteristic D-periodic structure is shown to depend on the ion content as well, which was observed when varying levels of potassium ions were present in the solution (Jiang et al., 2004).
2. Always prepare fresh solutions and filter the solution with a 200 microns filter to prevent bacterial growth.
3. Avoid cracks on the mica surface as they can affect the orientation of the fibrils.
4. After deposition of collagen fibrils, be sure to aspirate the liquid gently during all washing steps. Fibrils are not strongly attached to the surface
5. Rinse away TGM2 thoroughly (2–3 times) before seeding cells, as some cells cannot tolerate high levels of TGM2.

4.3. Film characterization by AFM

According to AFM imaging, fibrillar collagen film consists of self-assembled but highly aligned collagen fibers with a D-spacing of ~ 67 nm regardless of crosslinking, which is consistent with the literature (Fang and Holl, 2013). The average film height is ~ 2 nm, suggesting only one layer of collagen fibrils is formed (Fig. 4B) (Ivanovska et al., 2017).

The mechanical properties of the film can be altered by modulating the degree of collagen cross-linking. Pristine fibrillar films are anisotropic, with higher tensile strength in the long axis making them difficult to deform in this direction (Friedrichs et al., 2007). However, there is no interaction between adjacent parallel fibrils and even these fibrils are only loosely attached to the bottom mica substrate by weak electrostatic interactions, so displacements and deformations occur readily when forces are applied in the lateral direction. A cell can generate nano-Newton forces on the substrate (Sagvolden et al., 1999) and a similar amount of force applied laterally by the AFM tip is sufficient to displace the fibrils. However, such displacement is largely resisted after crosslinking (Fig. 4B).

4.4. Sample studies of nucleus utilizing collagen films

A cell perceives the stiffness of its environment by sensing the resistance to an active pulling or pushing, and with time this mechanosensing often affects the shape of the cell and its nucleus. On pristine films of collagen fibrils, the long axis is difficult to pull on, whereas deformation in the lateral direction is much easier, and so cells seem to sense a stiffer substrate along the fibers but a softer substrate in the perpendicular direction; consistent with stiffness-driven spreading, cells elongate along the fiber direction (Fig. 4C). However, on crosslinked films, the resistance is high in all directions, and so cells spread with no clear orientation despite the underlying alignment of fibers (Fig. 4C). Quantitation shows both the nucleus and the cell exhibit greater 2D projected areas on crosslinked films than on pristine fibrillar films, mapping very well into morphologies on PA gels of 50 kPa and 10 kPa, respectively (Fig. 4D). Cell and nuclear aspect ratios exhibit peaks of elongation that likewise suggest pristine films are sensed as softer than crosslinked films (Fig. 4D). Further study showed that higher lamin-A levels are expressed by MSCs on cross-linked collagen films and the nuclei tend to be correspondingly stiffer (Ivanovska et al., 2017), consistent with results for stiff PA gels (Fig. 2D). Furthermore, after a week in culture, the transcription factor RUNX2 accumulates more so in the nuclei of MSCs on cross-linked collagen films, which is consistent with osteogenesis induced by matrix stiffness.

5. Discussion

Much of our understanding of cell biology is based on culturing cells on rigid glass or rigid plastic (which is a synthetic polymer) while rigorously controlling the temperature and media at physiologically relevant conditions (i.e. 37 °C, neutral pH, serum supplements). Ignoring the diversity of mechanical cues presented to cells in normal and diseased tissues surrenders control of additional factors that affect cell and nuclear phenotype (Blau et al., 2015; Shulman and Nahmias, 2013). PA hydrogels have served as foundational materials for elucidating the likely effects of tissue stiffness on cells and their nuclei, and the practicality, simplicity, and reproducibility of PA gels will likely prove useful in further reductionist studies beyond mechanobiology. However, continued development of new materials methods that address additional factors such as stiffness heterogeneity, fiber crosslinking, or even dimensionality (Brown et al., 2018; Mealy et al., 2018), could be key to understanding how cells and nuclei interact with their microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

Several recent methods that we applied to nuclear-matrix studies are illustrated in detail including a polyacrylamide hydrogel, a minimal model of scars and a thin collagen nanofilm. Hints for troubleshooting and characterizations in addition to published measurements are also provided to ensure reproducibility. The matrix effects on nuclear lamina are used as examples to demonstrate the application.

Figure 1.

Synthesis of PA gels and AFM Characterization. (A) (i-ii) Overall reaction scheme of PA gel synthesis. (iii) Quality check using excess precursor solution remaining in Eppendorf tube. (B) Elasticity measured by macroscopic tension test and micro-indentation with AFM are consistent. Surface functionalization such as collagen coating exerts no effect on apparent elasticity of thick gels. The inset demonstrates the linearity of elasticity which is the slope. (C) AFM-determined elasticity for various compositions. Open circles represent data for 5.5% and 3% PA gels as determined by others by microrheology; the open star represents a data point from others for a cross-linked 4% PA gel analyzed by both AFM and rheology. (D, E) Soft PA gels, when cast as thin films, prove to be thickness-dependent in their apparent elasticity as measured by AFM indentation.

Figure 2.

'Scar-on-a-dish' MMMS is mechanically heterogenous. (A) (i) Scar-like islands of collagen-I bundles are heterogeneously entrapped in the subsurface of a soft PA hydrogel, demonstrated by both cartoons and imaging. A fibrotic liver thin section is provided as comparison. (ii) AFM elasticity characterization on homogeneous 0.3kPa PA gel and corresponding MMMS. (iii) Glass pulling experiment indicates MMMS is heterogeneous in stiffness. (B) Proliferation (or 4N population) and nuclear spreading of EC4 cells are shown on soft and stiff PA gels with/without MYC-inhibition. * $p < 0.05$ comparing to results on soft. The average and standard deviation is indicated by the bar and box, respectively, using software OriginLab 2018. (C) Proliferation (or 4N population) and nuclear spreading of EC4 cells are shown on soft, stiff and MMMS gels without MYC-inhibition. * $p < 0.05$ comparing to results on soft. (D) Nuclear spreading is increased with increased stiffness but MMMS with 0.3kPa bulk PA show nuclear spreading comparable to that on 10–20 kPa gels. Cells on

MMMS has lamin-A level similar to cells on stiff substrates. Lamin-A phosphorylation might be a mediator as it regulates stiffness-mediated lamin-A level.

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Figure 3.

Collagen-1 expression correlate with mRNA of crosslinking proteins and proteins that drives cancer cell proliferation. (A) RNA-seq data from TCGA shows that mRNA of crosslinking proteins LOXL1 and LOXL2 are highly correlated with expression of COL1A1 in hepatocellular carcinoma, whereas stiffness-independent CD47 shows no correlation (The link for the databased is provided in the supplement.). (B) GLI2 which correlates with collagen-I expression regulates proteins that drives proliferation in HCC. (C) RNA-Seq analysis implies crosslinking of matrix stiffens the microenvironment, leading to elevated expression of proliferation-related proteins in the nucleus and drive proliferation.

Figure 4.

Analysis of cell morphology and protein organization in MSCs cultured on pristine fibrillar and cross-linked collagen-I nanofilms. (A) Effects on MSC morphology after culture on (i) fibrillar (pristine) and (iii) cross-linked collagen-I nanofilms; (ii) nuclear height on the two types of films. (B) AFM amplitude-mode topographical images of molecular films of highly ordered collagen-I fibrils, showing D-periodic structure, self-assembled on rigid substrates in the absence (i, ii) and presence (iii, iv) of cross-linking. Collagen fibrils were deformed using the AFM stylus in lithography mode to apply a low dragging force across the film (ii, iv), thus showing the flexibility of the fibrils and the deconstruction of the ribbons to monomers in the absence of cross-linking (ii). (C) Immunofluorescence images of MSCs cultured for 24 h on (i) fibrillar and (iii) cross-linked collagen-I nanofilms with staining against F-actin (red) and DNA (Hoechst, blue). (ii, iv) Staining against collagen shows crosslinked films are not as severely deformed as the pristine ones. The random orientation of the dense regions of collagen protein suggests that the films are mechanically anisotropic, despite the topographically isotropic orientation of the fibrils evident from AFM images. (D) Comparison of matrix E -dependent cell morphologies of MSCs cultured on thick, isotropic polyacrylamide (PA) gels or thin collagen-I nanofilms. Plots of (i) cellular and (ii) nuclear spread area were fit to hyperbolic functions, with free parameters obtained from cells on PA

gels of controlled stiffness (blue) and cells on thin films (red squares), with the unknown effective stiffness felt by the cells on thin films superimposed to obtain the effective stiffness (10 kPa for pristine and 50 kPa for cross-linked collagen-I films). (E) Cellular aspect ratio (i, left axis), order parameter (i, right axis), and nuclear aspect ratio (ii) as functions of the gel matrix elasticity also showed consistency between morphology of pristine fibrillar and cross-linked films with 10- and 40-kPa PA gels, respectively. The figure is adapted from elsewhere (Ivanovska et al., 2017) with permission of the authors.

Table 1.

Composition in soft and stiff PA hydrogels.

	40% Acrylamide^a	1.5% Bis-AA^b	DI water
0.3kPa	7.5 v/v%	4.7 v/v%	87.8 v/v%
1kPa	9.0% v/v%	5.6 v/v%	85.4 v/v%
3kPa	11.2% v/v%	7.0 v/v%	81.8 v/v%
10kPa	15.0 v/v%	9.3 v/v%	75.7 v/v%
40kPa	25.0 v/v%	20.0 v/v%	55.0 v/v%

^{a)}Table 1. notes: The concentration of stock solution purchased from Sigma for acrylamide is 40%.

^{b)}The annotated solution of bis-AA need to make fresh from powder (e.g. 0.15 g Bis-AA dissolved in 10 ml DI water) every time before gel synthesis. This table is based on measurements in Fig. 1B-E and confirmed in various studies (Buxboim et al., 2014; Dingal et al., 2015; Swift et al., 2013).

Table 2.

Composition for MMMS. All concentration has been tested and characterized to ensure working (Dingal et al., 2015).

Final collagen conc.* (mg/ml)	Rat Tail Collagen-I stock solution (e.g. 4mg/ml)	Acetic acid stock solution (0.1M)	Vol. of HEPES (50mM, pH8.3) in 1mL mixture
0.4	100 µl	200 µl	700 µl
0.1	25 µl	50 µl	925 µl
0.025	6.3 µl	12.5 µl	981.2 µl

* Note: We use mg/ml here instead of v/v% because the stock concentration of purchased rat tail collagen from Corning varies from 3.5 to 4.2 mg/ml (depending on batch).