

The Mechanical Environment of Cells in Collagen Gel Models

Global and Local Effects in Three-dimensional Biological Hydrogels

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Abstract It is becoming increasingly clear that cells behave differently in two-dimensional (2D) culture than in three-dimensional (3D) tissues, and that 3D culture models and new tools for probing them are needed for advancing our knowledge of mechanobiology. Cells physically interact with their surrounding extracellular matrix; they are able to sense the local stiffness, tension, and deformation within the matrix and, in turn, are able to remodel the matrix and generate forces with long-range effects. In tissues with sufficiently high cell density, the cells interact and generate coordinated forces which can be regulated by controlling the macroscopic mechanical boundary conditions. Understanding this dynamic reciprocity between the cells, matrix, and external environment is critical for determining how the cells sense, transduce, and respond to their mechanical surroundings. However, even in simplified models of 3D tissues, quantification of local (non-linear viscoelastic) mechanical properties is problematic, and the transfer of strain and stress to the cells is complicated by non-affine, non-uniform deformation of the cell/matrix composite. This review focuses on methods for characterizing and modulating the mechanical environment of cells cultured within reconstituted collagen gels, the most extensively utilized in vitro models of native 3D tissue.

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1 Introduction

The mechanical environment surrounding cells guides the development, remodeling and pathogenesis of tissues *in vivo*. For example, blood vessels remodel with hypertension, muscles atrophy when a limb is placed in a cast, and skin contracture is reduced with splinting following injury. The need for more quantitative study of the effects of mechanical cues on cell behavior, i.e., mechanobiology, has led to the development of a broad range of tools as highlighted in this volume. Although our knowledge of the mechanisms behind mechanotransduction have increased dramatically in recent years, the majority of the powerful new methods used to study cells are applicable only to two-dimensional (2D) cultures.

Cells have repeatedly been shown to behave differently in 2D culture than in three-dimensional (3D) models and tissues [100, 102]. For example, the “pancake” cell shape characteristic of 2D culture is not found in 3D gels, rather stellate, bipolar, or rounded cells are observed. Further, cell proliferation and protein biosynthesis are generally suppressed in cells entrapped in protein-based gels relative to culture on 2D tissue culture surfaces, and cell motility, an important means of reorganization of the surrounding matrix, is not limited to in-plane migration. In some cases, contradictory responses to mechanical stimuli are observed 2D and 3D systems. For example, in 2D motility is maximal on higher stiffness substrates in contrast to faster tumor cell movement in 3D matrices of lower protein concentration (and lower stiffness) [139]. In cyclically stretched 2D systems, adherent cells generally align along directions of minimal stretch [68], whereas cells cultured within uniaxially stretched 3D protein gels generally align in the direction of stretch along with the fibrous proteins [113]. As a final example, breast epithelial cells develop like tumor cells when cultured on 2D surfaces, but in 3D basal lamina protein gels (Matrigel) they revert to normal growth behavior [101].

Reasons for discrepancies in cell behavior between 2D and 3D environments are undoubtedly complex and include differences in stiffness (3D models are generally compliant compared to standard 2D culture) [100], the alignment [54] and microstructure [43] of fibrous structures in protein-based 3D systems, concentration of ligands available for binding, specialized cell–matrix adhesions [28], and more symmetric adhesions over surface of cell [44]. Differences may arise due to “dimensionality” itself as the cells are surrounded by fluid in 2D and simply adhered to the surface of a material in contrast to being entirely encased in the material in 3D. Imagine the difference of walking on a carpet compared to trying to walk through a massive tangle of yarn. To separate these effects which confound the study of mechanobiology, controlled manipulation of the physical environment of cells cultured within 3D tissue models is necessary while keeping the biochemical environment constant. This review focuses on methods for modulating the mechanical environment of cells cultured within reconstituted collagen gels, the most highly utilized *in vitro* model systems for the study of cell behavior in 3D.

Collagen is the major structural protein in connective tissues. Self-assembly of acid soluble collagen at 37°C to form a “stiff” gel was first observed approximately 40 years ago [53, 64]. Under these conditions collagen molecules and aggregates of molecules spontaneously assemble. Reconstituted collagen gel models populated with cells were developed by Bell et al. [10] in the late 1970s and utilized for the basis of tissue engineered skin and blood vessels. Although successful for wound healing treatments (Apligraf[®], Organogenesis Inc.), collagen gels have not been successfully utilized for substitution of blood vessels or other load bearing tissues as collagen gels neither obtain the stiffness and strength of mature tissue nor the native collagen hierarchy, even with long-term mechanical conditioning (months). Primarily, these biopolymer gels are utilized as model systems to study cell behavior such as cell contraction and migration in simulated “developmental” or “healing” environments (depending upon boundary conditions) [50].

Cells cultured within collagen gels are surrounded by “native” extracellular matrix (ECM) proteins and can remodel their surrounding fibrous matrix. This remodeling is modulated by local and global physical cues, and it results in changes to the mechanical properties. This “dynamic reciprocity” between the cells and matrix resembles that which occurs in vivo [50]. Remodeling of ECM (reviewed by Daley et al. [29]) is a key aspect of tissue development and fibrosis, is critical for “functional tissue engineering,” and is a major advantage of utilizing the collagen gel model system. Synthetic polymeric hydrogels fail to capture this fibrillar structure and remodeling, although synthetic hydrogels can be engineered to degrade with ECM production by the cells filling in the gaps [62]. Structural guidance and anisotropy can also be engineered into collagen gels by alignment of collagen fibrils via pre-processing (magnetically or mechanically) which provide additional physical cues that are difficult to incorporate into synthetic systems.

Despite many studies of the mechanics of collagen fibrillation [119] and the use of collagen gels for understanding cell–matrix behavior in 3D [108], issues remain in the use of this popular in vitro model for the controlled study of mechanobiology. This brief review is not meant to cover the “3D mechanobiology” literature exhaustively, rather we aim to clarify the terminology of the field and highlight how collagen gels can be used both in short- and long-term studies for different purposes, and how changes in global boundary conditions can be used to alter local cell behavior.

In the next section, we introduce the creation of collagen gel models with detailed discussion of the effects of collagen concentration, cell density, collagen sources, and extraction methods on the gel. Then in Sect. 3, the structure and physical properties of collagen gels are discussed. We describe methods for altering the mechanical environment using various static and dynamic boundary conditions in Sects. 4 and 5, respectively. The local environment of the cell within the fibrous matrix and calculation of cell-generated forces are discussed in Sect. 6. Finally, Sect. 7 provides a general discussion and look to the future use of 3D collagen gel model systems.

2 Composition and Creation of ECM Models

2.1 Creation of a Cell-populated Collagen Gel: The Basics

Cell-populated collagen gels are created by mixing together concentrated solutions of purified collagen, cells, and media. The solution is “cast” (poured into a mold), allowed to “gel” (fibrillate by self-assembly), and cultured under a variety of mechanical and chemical environments. Typically, they are cultured either attached to a dish/surface or gently released from the surface following gelation and allowed to culture free floating (see Sect. 4). *Compaction* of the gel by the resident cells is most striking in free floating gels where traction forces generated by migration of the cells results in reorganization of the gel and expulsion of unbound water which reduces the gel volume by an order of magnitude and increases the collagen density. The *compaction* process is often termed *contraction* of the gel, although it is best to reserve the term *contraction* for cell contractile behavior which utilizes muscle-like cell machinery (see grey terminology box below).

Typically, cell-populated collagen gels are produced by rapidly combining *stock solutions* of purified monomeric collagen (collagen extraction discussed in Sect. 2.4), cells, and concentrated cell culture medium. The stock collagen solution is stored in chilled weak acid to avoid premature fibrillation thus NaOH (typically 0.1 M) is used to neutralize pH of the solution. Concentrated medium (e.g., 5× DMEM) and serum are utilized to obtain a normo-osmotic solution with the desired serum concentration for culture. For example, for 10 ml of an initial solution with 2 mg/ml collagen, 0.5 million cells/ml, and 10% serum, stock solutions of the following volumes can be utilized: 4 ml of 5 mg/ml collagen, 5 ml of 1 million cells/ml with 20% serum, and 1 ml of 5× concentrated medium (the NaOH volume is minimal) [13]. The solution is quickly pipetted into a culture dish with desired shape, placed in a warm stable location, and allowed to gel for 30–60 min before further manipulation.

Compaction and Concentration Terminology

Compaction: short-term cell-mediated reorganization of protein fibers in a hydrogel where the majority of the fluid is expelled resulting in a dramatic reduction of gel volume.

Contraction: active exertion of tension on the protein matrix by stationary cells (distinct from forces imposed during migration during compaction).

Stock concentration: concentration of in the stock solution before forming a hydrogel.

Initial concentration: protein concentration in the cast hydrogel which including dilution by media and cells.

Final concentration: protein concentration at end of culture duration (post compaction, remodeling, stretching, etc.)

2.2 Effects of Protein Concentration

The initial concentration of the protein alters both the mechanics of the gel and the ligand presentation (number of sites for cell adhesion). Typical concentrations range from 0.3 to 5 mg/ml collagen (although some report up to 30 mg/ml) [37, 58, 94, 142]. These hydrogels typically contain <1% protein and are >99% water since water is 1,000 mg/ml and the gels typically contain <10 mg collagen per ml solution. For comparison, most native connective tissues have hundreds of mg/ml protein and 70–80% water; a table of values for gels and various tissues can be found in [100]. When comparing studies, one must be careful to differentiate between stock concentration, initial concentration, and final concentration (see terminology text box), as many groups use “final” concentration for the protein concentration in the gel after it is cast, before the start of culture. The cells within the gels interact with the collagen fibrils (Fig. 1a) and remodel, crosslink, and synthesize ECM proteins. Thus, after standard culture duration (>6 h) the initial protein concentration does not represent the local environment of the cell accurately which confounds the study of mechanobiology as the stiffness and collagen (and thus ligand) density change over time. Further, the protein concentration is higher around the cell due to local densification (see Fig. 1b).

Interestingly, the degree of compaction decreases with an increase in initial concentration, and the average final concentration of a collagen gel is actually higher for gels with lower initial concentration [58, 94, 142]. For example, Zhu

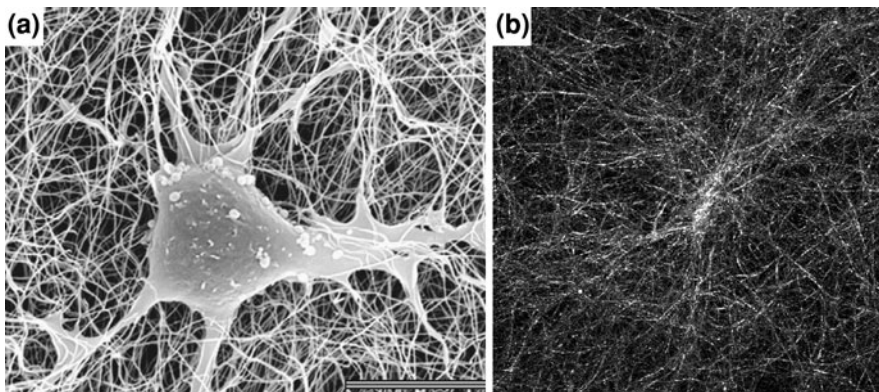


Fig. 1 **a** Scanning electron micrograph of a human fibroblast within a collagen gel demonstrating the multiple adhesions to the collagen fibers. Note the small size of the fibers and pores relative to the cell size and the penetration and entanglement of cell extensions and collagen matrix. Reprinted from Ref. [108] with permission from Elsevier. Bar, 10 μm **b** Confocal reflectance image of the collagen network surrounding a fibroblast cultured within a collagen gel after 4 h in culture showing the local densification produced by fibroblast-mediated compaction. Low passage neonatal human dermal fibroblasts (5×10^4 cells/ml) and 2% FBS were utilized; shear storage modulus ~ 50 Pa. Image width = 180 μm . Image generously provided by Dr. Sherry Voytik-Harbin

et al. [142] varied the initial collagen concentration from 0.75 to 2.5 mg/ml and after 21 days compaction by human fetal lung fibroblasts measured final collagen concentrations of ~ 50 mg/ml to ~ 8 mg/ml, respectively; Helary et al. [58] found similar a monotonic relationship between initial and final collagen concentration for human dermal fibroblasts (0.66–3 mg/ml initial concentration resulting in 37–11 mg/ml final concentration, respectively). Evans and Barocas [37] found this pattern only with initial cell density >0.15 million cells/ml; they also found the final mechanical properties could not be predicted by the final collagen concentration alone (see Sect. 3.2) although they only cultured the gels for 24 h. Collagen degradation occurs during culture due to matrix metalloproteinase (MMP) secretion, although total collagen after substantial culture duration (>2 weeks) is relatively constant [142] even with a high initial cell density (2×10^6 cells/ml) [1]. The collagen in the gel inhibits collagen synthesis by the resident cells [25] but some collagen is secreted along with other proteins and polysaccharides, notably fibronectin and hyaluronic acid.

2.3 Effects of Cell Density and Activity

The cells entrapped within collagen gels (Fig. 2a) have striking dendritic morphology not observed in those cultured on the surface of collagen-coated coverslips (Fig. 2b). The morphology of the cells is also dependent upon the cell density, an indication that the cells actively interact and can sense the traction and/or soluble factors from adjacent cells (compare Fig. 2c, d with low and high cell density, respectively).

A wide range of initial cell densities are utilized in collagen gels depending upon the application from $<10^4$ [10] to $>10^6$ cells/ml [1]. As with stock, initial, and final protein concentrations, definitions of cell density can differ between studies in a similar manner. At low initial density (e.g., 10^4 cells/ml) the cells do not initially interact with each other as there is, on average, approximately 500 μm between cells (assuming even distribution of cells and cuboidal volume elements). At high density (e.g., 10^6 cells/ml with approximately 100 μm between cells) the cells can sense their neighbors and interact even at short culture durations; a more extended cell morphology can be observed 1–4 h after casting than seen in cells seeded at low density. In general, the final cell density is determined by the extent of gel compaction by the cells as proliferation is very slow in 3D gels compared to standard (stiff) 2D culture. For example, it is commonly reported that fibroblast cell doubling time is on the order of a week in a standard density collagen gel compared to 12–14 h on tissue culture plastic. Grinnell [50] suggests that proliferation is inhibited by increased collagen fibril-cells contacts; however, in compliant 2D systems the regulation of cell proliferation is strongly controlled by matrix stiffness [32] and recent studies demonstrate that cell proliferation increases with initial collagen concentration (at high concentrations, which correlates to initial stiffness) [56, 58]. Alternatively, Simmons and colleagues [138] recently

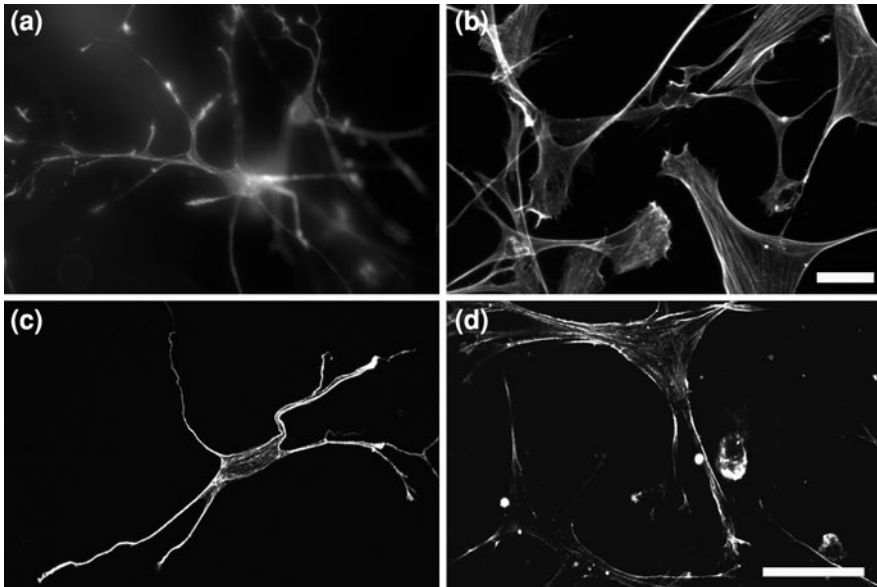


Fig. 2 Fluorescent images of human fibroblasts cultured within collagen gels (**a**, **c**, **d**) show dendritic shapes compared to the flat, extended morphology of cells on collagen-coated glass (**b**). Cells within gels cultured at low density (10^5 cells/ml) have shorter extensions when gels are unrestrained (**a**) than when anchored (**c**). The cells more closely resemble the stiff glass control when cultured at high density (10^6 cells/ml) where they interact heavily (**d**). All cells stimulated with PDGF for robust development of stress fibers in a short time period (1 h for **a** and **b** and 4 h for **c** and **d**) and visualized by phalloidin staining of the actin cytoskeleton. *Scale bars* indicate 25 μm ; images in *same row* have same magnification. Images generously provided by Frederick Grinnell and reprinted from *Mol Biol Cell*, 13, Tamariz, E. and Grinnell, F. Modulation of fibroblast morphology and adhesion during collagen matrix remodeling, 3915–3929, 2002 (**a** and **b**) and from Ref. [51] (**c** and **d**) with permission from the American Society for Cell Biology

report that valvular interstitial cells proliferated more rapidly on compliant matrices (thick collagen gels) than stiff matrices (thin gels on glass). Regardless, the cell density increases with culture duration as the cells compact the matrix resulting in increased cell–cell interactions and collective behavior even with low initial density and slow proliferation. Thus, if it is desired to study how an individual cell is affected by its local mechanical environment in a 3D gel, e.g., the effect of matrix stiffness on migration speed [118], low cell density and short culture duration should be chosen. Although collagen gels are used for these types of “single cell” studies, models with higher initial cell density are utilized in the majority of research, and the collective response of the cells to chemical and physical stimuli are studied with extended culture duration.

Not surprisingly, the extent and rate of compaction is strongly affected by cell density. In their seminal paper, Bell et al. [10] demonstrated dose-dependent increases in free gel compaction from 10^4 to 10^5 fibroblasts/ml, with differences

being most prominent at low cell densities and little difference in final compaction at higher cell densities (4×10^4 to 1×10^5). Allen and Schor [4] observed a linear increase in the initial (2 h) rate of compaction with cell density from 5×10^4 to 5×10^5 fibroblasts/ml. In collagen gels rigidly attached to an isometric force transducer (a.k.a. culture force monitor), the force during compaction has been shown to increase monotonically with cell density although the relationship can be complex [31]. These trends have been confirmed in further systematic studies by others [37, 58, 94, 106]. Further, a high degree of compaction which occurs with free gel boundaries and low initial collagen concentration leads to higher final cell density but also apoptosis [142]. If higher initial collagen concentration or an anchored configuration is used, lower levels of apoptosis are observed, apparently due to the cells' ability to generate tension against the matrix. However, due to the complex interaction between the cells and collagen (including increasing collagen density with compaction), more work is needed to determine if cells alter their behavior (phenotype) as the cell density increases during compaction due to soluble factors, cell contact, or mechanical factors.

2.4 Collagen: Types, Sources, and Extraction Methods

Collagen constitutes one quarter of the total protein mass of animals and is thus the most abundant protein in vertebrates [3]. Procollagen is secreted from the cell and is soluble under physiological conditions and able to diffuse through intercellular space. The ends are enzymatically cleaved, leaving triple-helical rod-like tropo-collagen monomer [3]. Collagen monomers fibrillate outside of the cell laterally then aggregate further laterally and longitudinally to form interconnected branching fibrillar networks. The creation of collagen gels exploits this self-assembly process (described in Sect. 3.1), and the ability to recapitulate this native process in vitro provides rationale for utilizing collagen gels as models of tissue development and healing.

However, when adopting a collagen gel model for mechanobiology, it is important to understand that all collagen is not equal. Most obviously, there are many types of collagen (29 at last count [47]) which are tissue specific, e.g. type II predominates in cartilage, type IV is found in basal lamina and blood vessels, and large amounts of type III (relative to type I) are observed in healing wounds and developing skin [3, 47]. Of these, type I collagen is the major collagen type found in skin and bones, and it accounts for about 90% of the collagen present in the body. Accordingly, type I is most often used for creating collagen gels and is the focus of this review. Yet it is important to note that the collagen type will likely alter gel physical and biochemical properties, and what is sold as "type I collagen" may include a significant portion of other collagen types, most likely type III when young animals are utilized. Further, the animal source, method of extraction, purity, and processing of the collagen all have major impacts on the fibrillation and resulting mechanics of collagen gels.

Purified type I collagen is most often obtained from tendons due to its high collagen content and relative purity, although reticular dermis is also a popular source. For lab research, collagen is often obtained from rat tail tendons (RTT) which are abundant in many laboratory settings and are readily soluble under acidic conditions (processing described below). But for commercial scale, calf hide (dermis), bovine Achilles tendon, or porcine skin are most often used. Collagen undergoes crosslinking over an animal's life-time, thus, unlike tail tendons from young rats, the extraction efficiency using dilute acid alone is very low for mature bovine tissues and enzymes are often utilized. Dilute acid extracts only recently synthesized collagen as aldimine type inter-molecular crosslinks can be dissociated by dilute acids, but not the keto-imine type which form with age. Thus, the age of the animal is an important determinant of the quality and amount of the collagen and an important consideration of choice of collagen source [141].

To extract the collagen, tendons are dissected from surrounding fascia, washed in cold neutral salt solution, frozen, minced with ice to keep cold, and finally suspended in weak acid (e.g., 1–10 mM HCl) for a few days at 4°C. The collagen slurry is then filtered (e.g., through a 1 mm nylon screen) to separate the “soluble” (acid-extractable) and insoluble fractions (see Zeugolis et al. [140, 141] for a clear flow chart of the entire process). The acid-extracted collagen fraction is purified by repeated salt precipitation and centrifugation then dialyzed (~8,000 Mw cut-off filter) against weak acid (0.01–0.05 M). To obtain a usable solution of collagen from the insoluble fraction, the insoluble fraction is treated with pepsin under acidic conditions for a few days, filtered, then purified by salt precipitation, centrifugation, and dialysis as described above to yield pepsin-extracted collagen. It is important that all steps, including mincing, centrifugation, and storage, are performed cold (4°C) to preserve the native (triple-helical) structure of the collagen molecules. The remaining insoluble residue cannot be used to form collagen gels as defined herein; it contains large aggregates of crosslinked collagen fibrils. Despite apparently similar processing, the purity of the resulting “monomeric” collagen solutions can be variable and may contain a substantial fraction of oligomers (more with acid-soluble than pepsin-soluble collagen) which significantly alters fibrillation kinetics [42].

Acid- and pepsin-extracted collagen are both commonly utilized for creating collagen gel models. They are often used interchangeably, yet there are substantial differences in the fibrillation kinetics and the resulting physical properties. Pepsin cleaves the telopeptide region of collagen, which, in addition to allowing high extraction efficiency (>90% from calf tendon as opposed to <5% of wet weight for dilute acid alone [75]) also alters the ability to form a collagen gel by self-assembly. The telopeptide region is important for the rate and extent of fibrillation and crosslinking both in vivo [24] and in vitro [75]. For example, the strength of gels made from acid-extracted collagen is >10-fold higher than for pepsin-extracted collagen [75], and the stiffness of gels made from acid-extracted collagen [110] are significantly higher than that of gels made from pepsin-extracted collagen [96]. On the nano-scale, extruded collagen fibers (similar to gels) created from acid-extracted collagen contain thick quarter-staggered fibrils, whereas

pepsin-extracted collagen fibers have both thin non-banded and thick banded fibrils [141]

Acid-extracted, pepsin-extracted, and relatively unprocessed “fibrous” collagen extracted from skin and tendons from cows and pigs are available packaged either in dilute acid or freeze dried for long-term storage. Acid-extracted versions include “DM6 Bovine Dermal Collagen Acid Soluble” from Devro Medical (3 mg/ml, pH 2). Pepsin-extracted bovine dermal collagen products (~ 3 mg/ml in ~ 0.01 N HCl) include “Purcol” from Advanced Biomatrix (previously named “Vitrogen 100” from Inamed/Cohesion/Collagen Corporation), “Collagen from Calf Skin” from Sigma–Aldrich, “Collagen 04902” from Stem Cell Technologies (Canada), “DM1 Atelo” from Devro (USA), and “Porcogen” from Sunmax (Taiwan), the latter being porcine-derived. Freeze-dried pepsin-extracted bovine dermal collagen products are supplied by Kensy Nash (US), Koken (Japan), and Symatase Biomateriaux (France). Freeze-dried non-purified collagen products are also available including “Calf Skin Collagen” from Sigma–Aldrich, “Bovine Achilles Tendon Collagen” from Fluka, and “Bovine Achilles Tendon Collagen” from Yi Erkang (China). As they are largely unprocessed, they are less expensive though they must be purified as described above to obtain acid- and pepsin-extracted type I collagen for making collagen gels. The subsequent processing and storage of collagen for distribution can also alter the collagen material without changing its composition, e.g., by rendering it insoluble with poor temperature control during freeze drying [140]. More recently, recombinant human collagen has become available, developed in large part to avoid interspecies disease transmission but also to reduce lot-to-lot variability and provide higher purity, albeit at a high cost [95].

Further, other proteins, such as the components in serum, are generally considered necessary for the cells to adhere to the matrix, actively generate forces, and remodel the collagen. The need for serum is shown quantitatively using culture force monitoring [31, 71], although some researchers report compaction of gels by fibroblasts in the absence of serum [6, 66]. Thus, serum proteins further complicate the biochemical environment, and simple “one-component” collagen gels generally contain a small fraction of other collagen types, e.g., “Semed S” contains $\sim 5\%$ type III collagen. Some consider this a benefit since native collagen fibers are heterotypic to varying extents [47]. Finally, it is important to remember that even with the purest collagen material, cells will create their own surrounding matrix when allowed to culture for sufficient time which complicates the composition of the gel.

Collagen Source Terminology

Acid-extracted collagen: collagen fraction that can be extracted by dilute acid alone; a.k.a. “telo collagen” as the non-helical telopeptide regions remain intact. It is also called “acid-soluble,” but this terminology is not specific since pepsin-extracted collagen is also soluble in weak acid.

Pepsin-extracted collagen: the collagen fraction that can be extracted by pepsin treatment in dilute acid (~ 0.5 M). Also termed pepsin-soluble collagen and atelocollagen since pepsin solubilization cleaves the telopeptide region.

Soluble collagen: both “acid-extracted” and “pepsin-extracted” collagen are termed “soluble” collagen in the literature. Both are extracted by weak acid and are insoluble under physiologic conditions.

Insoluble collagen: collagen that is not soluble in dilute acid; most appropriately used to refer to residue remaining following acid extraction with or without pepsin treatment. Raw collagen before extraction is sometimes referred to as “insoluble” although a small fraction of this collagen is soluble in acid and should be termed “fibrous collagen.”

3 Characterization: Structure and Physical Properties of Collagen Gels

3.1 Collagen Self-assembly and Architecture

The *in vitro* progression of collagen fibril formation is similar to the native process in the extracellular environment, and thus collagen gels have been used extensively to study this process [11, 39, 69, 105, 119]. Acid-soluble collagen self-assembles into fibrils and bundles of fibrils (fibers) by an entropy-driven process; that is, collagen molecules exclude water and lower surface energy by aggregating [119]. The monomers (~ 1.5 nm diameter and 300 nm long) first form aggregates of 5–17 molecules, so called subfibrils 4–5 nm in diameter and 2–3 molecules long, which further simultaneously assemble both linearly and laterally into fibrils (20–300 nm wide). These fibrils bundle into fibers to form a 3D interpenetrating and interconnected fibrillar network [23, 119, 137]. The sigmoidal shape of standard turbidity and rheometric measurements with time during gelation suggests that a nucleation-and-growth mechanism consistent with percolation theory governs collagen fibril assembly [39].

The monomer backbone of the fibrils is stabilized by noncovalent, hydrophobic bonds and electrostatic interactions which results in a physical gel. The importance of noncovalent interactions has been demonstrated by the modification of the self-assembly process by the addition of ions, alcohols and other substances [23]. Further, covalent bonds (crosslinks) can form between the helical crosslinking sites and nonhelical monomer ends; however, these crosslinks are sparse compared to those contributed by cell-mediated crosslinks. Further, in pepsin-extracted collagen the nonhelical (telopeptide) region is removed thus this crosslinking mechanism is lost resulting in a slowing of aggregation and wider fibers [119]. Incubation of self-assembled collagen solutions for long periods (up to 150 days) increases mechanical and thermal stability [30] possibly due to an increase in the number of

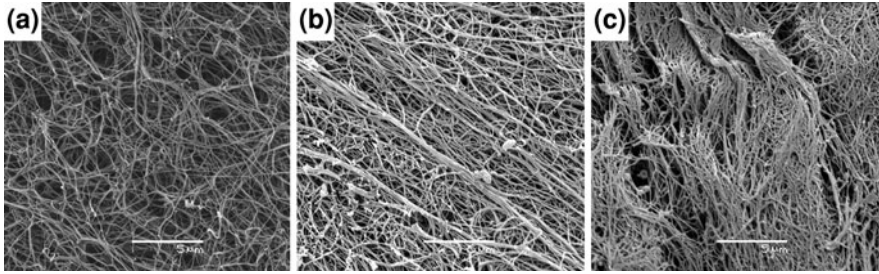


Fig. 3 Scanning electron micrographs of the collagen gel fibrous structure showing the aggregation and decrease in pore size which occurs with fibroblast-mediated compaction over one (*left*), two (*middle*) and three (*right*) weeks in floating culture. Initial concentrations: 3.4×10^4 cells/ml human dermal fibroblasts; 5% FBS; 0.66 mg/ml collagen. Images generously provided by Dr. Christophe Helary; see [58] for methods. *Scale bars* 5 μ m

crosslinks that form spontaneously over time. In the absence of cells, the fibrils mainly interact by entanglement resulting in a physical gel rather than crosslinked gel, despite the lack of thermal reversibility characteristic of physical gels [39].

Cells entrapped in the matrix further condense and bundle the fibrils into larger structures and provide additional stabilization methods, most notably lysyl-oxidase-mediated crosslinks [61]. Contraction by fibroblasts gives rise to bundles of collagen fibrils; the collagen network becomes heterogeneous and large fibril bundles ($\sim 5 \mu\text{m}$ fibers) appear from initial small fibrils ($\sim 50 \text{nm}$) with time in culture (Fig. 3). Condensation of collagen is observed around cells locally (shown clearly in Fig. 1b) creating local heterogeneity with contraction [98]. Yet even without cells, the initial gel is shown to be very inhomogeneous both in structure and local mechanical properties [83].

The parameters of the 3D fibrillar network (fiber diameter, spacing, length, etc.) are dependent upon the initial collagen density, pH, and temperature [110]. In general, lower temperature and pH lead to larger fibers as they affect the balance of forces (hydrophobic, electrostatic, and hydrogen bond) between fibrillating collagen monomers and fibrils. Lower temperature weakens attractive hydrophobic forces favoring lateral fibril and fiber growth [105]. Despite the wide range of these variables studied in acellular preparations, the pH and temperature ranges are quite limited when utilizing cells.

Electron microscopy is the most common imaging modality for characterization of the collagen fibrillar network structure, although confocal reflectance microscopy and second harmonic generation (SHG) with two-photon microscopy are proving to be useful techniques for visualizing the fibril architecture in the hydrated state [105, 110]. The latter techniques allow direct visualization of the evolution of collagen fibrils with gelation and have the advantage of being non-invasive (Fig. 4). Recently, SHG and two-photon excited fluorescence (TPF) have been combined and correlated with mechanical properties; SHG reflects fibril, monomer and α -chain configurations whereas TPF is created by fluorescent crosslinks which offers superior correlation to mechanical properties than either

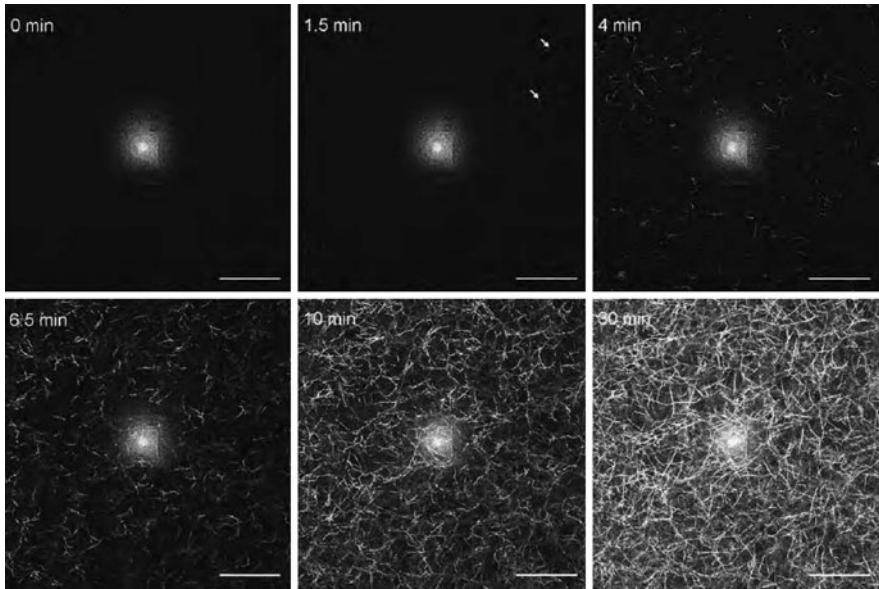


Fig. 4 Time-lapse confocal reflectance images showing the evolution of collagen fibrillation (1 mg/ml collagen at 37°C). The first fibers can be visualized at 1.5 min with more fibers evolving with time and clear interconnections at longer times. *Bright spot* at center is due to reflection off of optical elements. *Scale bar* 50 μm . Reprinted from Ref. [137] with permission from the Biophysical Society

modality alone [105]. With standard SEM and TEM, the fibrillar network collapses with drying and application of vacuum [4], thus the fiber spacing, pore structure, and interconnections between fibers are difficult to quantify accurately with these techniques. Fiber diameters observed by SHG have recently been reported to be an order of magnitude larger than observed by SEM [105]. Despite the processing limitations, electron microscopy remains the standard for measuring fibril size due to its high spatial resolution ($\sim 5\text{--}10$ nm). For comparison, the lower limit of confocal is ~ 200 nm [100], the Rayleigh resolution limit for SHG images is ~ 415 nm [105], and the effective lateral resolution is ~ 1 μm in many standard confocal systems. Estimating pore size remains even more problematic than fibril size; from a combination of modeling and microscopic measurements, pore sizes in collagen gels appear to be on the order of 5–10 μm [81].

3.2 Mechanical Properties

3.2.1 Micromechanics of Acellular Gels

Due to the similarity of the collagen self-assembly process in vitro and in vivo, the mechanics of acellular collagen gel models have been studied extensively for a

better understanding of native collagen fibrillogenesis and self-organization, e.g. [119]. As with matrix architecture, and due to this architecture in large part, the mechanical properties of collagen gels are dependent upon a number of parameters including collagen density, pH, and temperature, but one must not forget the profound effects of cell remodeling. Even with low cell density and short culture duration (minutes to hours) cells profoundly alter their local environment leading to pronounced densification of the surrounding gel which undoubtedly alters mechanical signaling. With higher cell density and longer term culture (hours to days), the cells alter the global properties dramatically with greater than 10-fold compaction, reorganization (bundling of fibrils), and crosslinking of fibers. Thus, when describing mechanobiology in collagen gels, it is useful to consider separately short-term (hours) and long-term (hours-days) studies. In the short-term, the initial properties of the gel are similar to those of an acellular gel and are critical as cells apply traction to migrate through and reorganize the matrix—processes which are mechanically regulated. Over the long-term, it is useful to characterize the mechanics of the gel both to better understand the mechanical environment of the cell and to assess the cell-mediated remodeling and resulting changes in the functional mechanical properties of the more tissue-like material. In this section we first examine the general features of collagen gel mechanics gleaned from combined uniaxial stretch, confocal imaging, and modeling. Changes in the mechanics with cell-mediated remodeling are then considered. Finally, we discuss more general mechanical characterization of gels with a focus on terminology, methods, and the effects of initial parameters to aid the reader in selection of a test and comparison between studies.

As mentioned previously, in the short-term collagen gels can be considered physical gels with few crosslinks and >90% water. In terms of bulk behavior, a collagen gel can be considered an entropic spring such as an elastomeric polymer, being extended from its highest entropy state and losing organizational entropy (and excluding water) when stretched [100]. Although collagen monomers are not rigid as originally thought and have been shown to be flexible in bending [119], they are very long and axially stiff (~ 1 GPa) compared to globular proteins (e.g., albumin) and thus do not uncoil when loaded, but rather rotate and bend. In their pioneering work combining confocal reflectance imaging with uniaxial stretching, Voytik-Harbin and colleagues [109, 110, 133] describe the collagen fibril matrix to be an interconnected network of tensile elements (collagen fibrils connected by hinges). In response to low levels of loading, the fibrils within a gel reorient, slide, bend and buckle, rather than becoming extended themselves. This non-affine fibril behavior (discussed in Sect. 6.1) leads to an increase in density and consolidation of fibrils locally, high distensibility, low stiffness, and surprisingly large decreases in transverse dimensions (“Poisson’s ratio” >2). Models predict bending predominates over slipping at intersections of collagen fibrils, at least for confined compression [20], and this is supported by confocal reflectance imaging observations of buckling of fibrils perpendicular to uniaxial tensile direction [111]. The relative proportion of each mechanism of fibril deformation likely depends upon collagen concentration and crosslinking. Whether slipping or bending, collagen

fibrils align in the loading direction and as the applied strain increased a “scissoring” effect of the “hinged” fibrils resulting in a reduction in angle between fibrils [111]. Understanding failure mechanisms in collagen gels is even more complex than analysis of sub-failure mechanics, and little is known. Observed correlations between tensile strength and fibril length in acellular gels suggests that the strength of self-assembled collagen is dependent on the presence of end-to-end crosslinks between molecules [119], although in non-aligned gels failure is likely due to inter fibril slippage between fiber entanglements.

While study of acellular collagen gels with combined stretch and imaging modalities provides substantial insight into collagen fiber kinematics, small fibrils and bundles (<200 nm) surrounding the cells cannot be observed, and with cell compaction the mechanical environment surrounding the cells changes substantially. Utilizing quantitative measurements and mathematical modeling, Evans and Barocas [37] show that the equilibrium modulus after 1 day of reorganization by cells in culture cannot be explained by final collagen concentration; gels that start at a low collagen concentration remain less stiff than higher initial-concentration gels. The initial cell density is an important determinant of final properties due to extreme densification near cells (Fig. 1b) leading to a heterogeneous final gel structure that can be modeled more accurately as stiff inclusions within a soft matrix than by standard composite models of stiff fibers in parallel with a soft matrix. The effects of both non-affine and non-uniform deformation on transfer of stress and strain to embedded cells are discussed in Sect. 6.

Mechanics terminology

Strain: deformation normalized to the sample dimensions; typically change in length divided by initial length. Less than 10% strain is considered “infinitesimal” or small strain.

Stiffness: slope of stress–strain curve typically referred to as Young’s modulus E , for linear elastic materials; often defined in low and high stress regions for nonlinear (J-shaped) stress–strain curves.

Storage modulus: elastic part of the dynamic stiffness, typically defined in shear as G' which can be related to E assuming linear elastic behavior.

Loss modulus: shear loss modulus, G'' , which indicates the proportion of the viscous behavior of the gel.

Compliance: inverse of stiffness, also functional measure for tubes (e.g., % change in diameter per change in pressure).

Strength: the maximum stress the gel can withstand, generally peak force normalized to initial cross-sectional area and termed ultimate tensile strength (UTS).

Elasticity: stress and strain are uniquely related thus loading and unloading curves are identical regardless of time and strain level.

Elastic return: ability to regain initial (prior to stretch) dimensions.

Hysteresis: energy lost between loading and unloading. Calculated as difference between loading and unloading curves.

Plastic deformation: non elastic return, note different mechanism than metals since fibrous—can often return with rehydration but with long time constant.

Viscoelasticity: time-dependent properties due to both elastic and viscous behaviors being present.

Preconditioning: cyclic loading before representative stress–strain behavior is recorded; it is necessary for repeatable stress–strain curves.

3.2.2 Terminology

Due to the complex network behavior described above, collagen gels do not behave as linear elastic solids but rather non-linear viscoelastic materials. To avoid confusion when describing the properties of collagen gels, it is important to utilize precise terminology especially considering the broad range of backgrounds of researchers utilizing collagen gel models. A grey text box with definitions of the terms most commonly used is provided. The most common misunderstandings are between stiffness and strength, between linearity and elasticity, and between plastic deformation and long-term viscoelastic behavior. Stiffness and strength are entirely separate concepts. *Stiffness* can be broadly defined as the change in stress for a given change in strain. Due to non-linearity in the stress–strain response different moduli are often reported for the “low stress” region and for the “high stress” region, e.g., maximum tangent modulus (MTM). A gel with a low modulus is often termed “soft.” The “Young’s modulus” (E) obtained from uniaxial testing can be related to the shear modulus (G) obtained from shear testing using the Poisson’s ratio (ν , ratio between axial and transverse strain) by $E = 2(1 + \nu)G$, with the caveat that this relationship assumes linear elasticity only remotely justified at small strain. *Strength* relates only to the maximum force per unit material (usually initial cross-sectional area) that the gel can withstand before breaking. Although these definitions are very basic, “strong” is often incorrectly used to describe a “stiff” gel. *Elasticity* refers to deformation that is reversible, that is, the unloading curve follows the loading curve. Elasticity does not imply linearity or a minimal level of stiffness, it precludes viscous behavior and plastic deformation. Due to the mobility of the collagen fibrils, collagen gels do not have particularly good elastic return (to original dimensions) following loading, thus elastic supports are generally needed for use in mechanobiological studies involving cyclic loading as discussed in Sect. 5. The proportion of the applied deformation that is not recovered following stretch is often termed plastic deformation or permanent set, but may also be due to long time-constant viscoelastic (i.e., time dependent) behavior. Plastic deformation may arise from sliding, reorientation, and buckling of fibers into new configurations; however, if allowed to equilibrate in a stress-free state for long enough, rehydration can often provide a sufficient restoring force to

recover much of the original configuration if the deformation was not excessive. Due to the high water content, collagen gels also exhibit other viscoelastic behavior including stress relaxation when held at constant strain, creep under constant or cyclic load, and hysteresis between loading and unloading. Gels are also loaded with sequential rapid ramps separated by relaxation periods to obtain both an instantaneous modulus (E_0) and an equilibrium modulus (E_∞). When cyclic loading is utilized, the stress–strain relationship is complex (literally), and the complex modulus can be separated into a storage modulus (real part, G' in shear) and loss modulus (imaginary part, G'' in shear). To obtain stable stress–strain behavior in tensile or compressive testing, cyclic preconditioning for 3–8 cycles is needed. Finally, due to their fibrous nature, collagen gels can exhibit anisotropy, i.e., different behavior along different directions. Although not often taken advantage of in studies of mechanobiology, the anisotropy of collagen gels represents a major advantage over other polymer systems towards understanding mechanical regulation of cell migration and orientation in three dimensions.

3.2.3 Mechanical Testing Methodologies

Many mechanical testing methods have been employed to study collagen gel mechanics, each with its own benefits and limitations. The choice of method depends and test parameters depend upon what information is needed. The strain or stress magnitude, strain rate, and test duration should be based on expected external and internal (cell-generated) loading, but the most relevant ranges of values are under debate. Methods used to characterize bulk and local gel properties are highlighted below. A table of values for key mechanical parameters is provided in Table 1.

Shear rheometry: Due to the high sensitivity of commercial instruments (seven orders of magnitude of torque) and the low stiffness of collagen solutions during and immediately after fibrillation, rotational rheometers have been utilized extensively to study collagen fibrillation kinetics and the bulk viscoelastic properties of collagen gels (see Table 1). Specifically, collagen fibrillation has been shown to start with a lag phase during nucleation in which turbidity and shear modulus are constant (with $G'' > G'$), followed by a growth phase in which turbidity and G' rapidly increase (with G' crossing over G''), and finally reaching a plateau where the available collagen monomers are fully incorporated into assemblies [39]. Strain, stress, and frequency sweeps over a range of values can be performed relatively easily without having to manually handle the gels, and most commercial rheometers are equipped with Peltier plates to accurately control temperature. From strain sweeps, the response is roughly linear under small strain (<5%) [105]. Typically the applied strain is very small (<1%) and the frequency set to 1 Hz to avoid slippage. Upon gelation, the storage modulus greatly exceeds the loss modulus ($G' > 10G''$) for collagen networks [137], thus the gel is considered predominantly elastic (rather than viscous). Due to the high water content the gels are often assumed incompressible and $\nu = 0.5$ is used to calculate Young's modulus. The mechanics of cell-populated collagen gels are not often

Table 1 Mechanical properties of collagen gels from rheometry, uniaxial loading, and biaxial loading

Test method	Metric (units)	Typical range	Min/max values	Collagen (mg/ml)	Strain (%)	Comments
Shear rheometry						
	G' (Pa)	0.3–50 ^{a-i}	0.1 ^a /290 ^g	0.4–5.0 ^{a-i}	0.1–5% ^{a-i}	See 1
	G'' (Pa)	1–29 ^{a-i}	0.02 ^a /44 ^g			
Uniaxial						
Acellular	E_{low} (kPa)	0.5–33 ^{j-z}	0.32 ^k /60 ⁿ	1–4 ^{j-z} (0.3, 200 ^p)	5–50% ^{j-z} (2% ⁱ , 70% ^l)	See 2
	MTM (kPa)	42–1,805 ^p	42 ^p /1,805 ^p			
Cell-populated	E_{low} (kPa)	100 ^r –500 ^r	25 ^r /2,100 ^w	0.32–2 ^{j-z} (0.065 ^q)	26–38% ^{q,t}	
	MTM (kPa)	23–1,800 ^{j-z}	16 ^u /3,400 ^w			
	UTS (kPa)	8–240 ^{j-z}	6 ^r /380 ^w			
Biaxial						
Cell-populated	E_{low} (kPa)	5.6 ^{c'}	0.2 ^{c'} /3,000 ^{b'}	1.92–2.2 ^{a'-c'}	5–30% ^{a'-c'}	See 3
	MTM (kPa)		46.9 ^{a'} /9,000 ^{b'}			

G' shear storage modulus, G'' shear loss modulus, E_{low} tensile or compressive modulus in low stress region, MTM maximum tangent modulus in high stress region, UTS ultimate tensile stress. Outliers are listed in *parentheses* and references are *superscripted*

Comment 1 G' generally increases with concentration and strain; cell-populated [118]; [86]; microrheology [58]; [118]

Comment 2 Dogbone, rectangle, cylindrical, and ring shapes all used; plastic compression [56]; rings used for all cases except for one linear shape [116]; 0.2 to 2×10^6 cells/ml typical; dynamic conditioning used [113], [114]

Comment 3 Circular samples inflated to failure [13]; varying sized cruciform samples [65]; square samples [124]

^a [81]; ^b [76]; ^c [131]; ^d [99]; ^e [105]; ^f [137]; ^g [58]; ^h [118]; ⁱ [86]; ^j [96]; ^k [76]; ^l [110]; ^m [104]; ⁿ [80]; ^o [136]; ^p [56]; ^q [21]; ^r [61]; ^s [6]; ^t [113]; ^u [46]; ^v [114]; ^w [63]; ^x [38]; ^y [116]; ^z [45]; ^{a'} [13]; ^{b'} [65]; ^{c'} [124]

studied by rheometry as extended culture within a rheometer is problematic in terms of sterility, metabolite availability, and cost of machine time. It is also difficult to utilize a rotational rheometer to study gels remodeled by cells in standard culture dishes due to slipping of gels not polymerized in situ and non-uniform strain in the parallel-plate configuration (strain is zero at the center and maximum at the outer edge for parallel plates, whereas a cone-and-plate configuration would compress the sample unevenly). Specialized microrheometric methods have been developed for cell-populated gels as described below under “Local Property Characterization.” As seen in Table 1, there is approximately a 1,000-fold difference between shear and tensile moduli which may be attributed to the difference in strain applied in the two modalities. At low strains fiber motility and rearrangement precludes substantial transfer of tension to the larger bundles of

fibrils as evidenced by the strong dependence of shear modulus on crosslinking which reduces fiber mobility [123].

Uniaxial testing: Uniaxial testing in various configurations is the most utilized testing modality for large deformation analysis and characterization of cell-remodeled collagen gels. As previously discussed, much of our knowledge of fiber kinematics during loading comes from uniaxial stretch experiments of “dogbone”-shaped specimens in concert with confocal reflectance microscopy [109]. Due to the time needed to obtain images, these studies utilize quasistatic loading, but for general mechanical characterization a range of strain levels and rates have been utilized and many studies include mechanical preconditioning [113]. Cyclic preconditioning is needed to obtain a stable state with water and to accurately assess stored energy. The “dogbone” sample shape is used to minimize the effect of stress concentrations at the grips as rectangular specimens invariably rip at the grips. An aspect ratio of at least 5:1 is required for homogeneous strain. For cell-remodeled gels, researchers generally utilize ring configurations, either made as rings [70, 134, 135] or cultured as tubes and cut into rings [113, 120], as they are relatively easy to make and to mount between pins for mechanical testing. Collagen gels tested under uniaxial tension generally exhibit a more linear response, lower stiffness, and less elastic return than native collagenous tissues likely due to a less mature and complex fiber architecture. Under uniaxial confined compression, the fluid phase is much more pronounced and extrusion of fluid through the matrix dominates early behavior [76]. Unconfined compression has been utilized to obtain structural stiffness, a relative measure of collagen gel properties (force/distance) but not intrinsic properties [6, 138].

Tube inflation: Inflation of tubular-shaped collagen gels is a popular method for obtaining “functional” properties (burst pressure and compliance) of tissue engineered blood vessels [46, 82]. This testing modality applies multiaxial stress to the gel which results in approximately pure uniaxial (strip biaxial) deformation in the absence of the application of axial stress and/or torsion which are controlled only in more sophisticated devices, e.g., Vorp et al. [132]. To obtain material properties, the dimensions of the sample including diameter and wall thickness must be known. As ring testing is easier and cheaper it is more popular.

Planar biaxial testing: To characterize remodeling of planar collagen gel samples, a few research groups have employed biaxial testing. Holmes and colleagues [124] hung weights of increasing mass from the edges of square samples to obtain the quasistatic mechanical properties and found anisotropic behavior in gels cultured under uniaxial constraint. More recently, cruciform sample geometry have been used to minimize edge effects and biaxial testing has been combined with powerful optical microscopy techniques to examine structure–function relationships in collagen gels [60, 65]. Tranquillo and colleagues [65] combined planar biaxial testing with polarized light microscopy to demonstrate that the initial prescribed fiber alignment of cell-remodeled collagen gels strongly affects the mechanical anisotropy of cruciform-shaped samples. Hu et al. [60] combined biaxial stretch with second harmonic generation to characterize development of the collagen architecture in planar gels.

Membrane inflation: Due to the fragility of collagen gels, it is difficult to assess their failure properties (strength, extensibility) accurately. Even with “dogbone” samples and rings, the samples often fail at the grips due to the high stress concentration and crushing. We have developed a fluid inflation method to characterize the biaxial mechanical properties of planar samples [13]. It is important to note that the burst pressure which is sometimes reported [75] is not a measure of the intrinsic strength of the gel due to the dependence on clamp diameter. The tension must be calculated from the radius of curvature and pressure.

Local property characterization: Indentation using an atomic force microscope probe is becoming a popular method for measuring the local properties of tissues and thin soft gels used for 2D cell culture, e.g., see table of values in Reilly and Engler [107]; however, this technique has not, to our knowledge, been applied to characterize the local properties of collagen gels (with the exception of glutaraldehyde-treated gels [36]). We have recently measured the surface stiffness of isoelectrically focused collagen gels using a microsphere-tipped AFM probe (unpublished data) and found little difference between fibrillated and non-fibrillated gels; however, such measurements only probe the top 500 nm of the gel and ignore the anisotropy of the material. Recently, Baaijens and colleagues [27] successfully combined deep microindentation and digital image correlation (DIC) to measure the resulting non-uniform strain field to assess the anisotropic stiffness of cell-remodeled collagen/matrigel composite gels. To measure local properties within collagen gels during cell-mediated remodeling, Tschumperlin and colleagues [86] developed an innovative microrheometric approach based on the rotation of ferromagnetic microbeads cast within the gel, and the authors utilized this method to demonstrate TGF- β 1 and interleukin-1 β enhanced matrix stiffness cooperatively. Optical tweezers have been used to probe the properties at the level of a single microbead deep within a collagen gel. The researchers show that the gels are highly inhomogeneous due to the gel having very low density areas locally, even in the absence of non-uniform cell-mediated compaction [83]. Two-dimensional laser traps have been employed to study local collagen gel anisotropy [98]. The latter three particle-based techniques for probing the mechanical environment surrounding cells during mechanical loading and cell-mediated remodeling hold tremendous promise for increasing our understanding of cell mechanobiology in 3D gels and tissues.

3.2.4 Effects of Fibrillation Parameters on Mechanical Properties of Collagen Gels

As mentioned at the start of this section, the mechanical properties of collagen gels are dependent upon a number of parameters including collagen density, pH, and temperature—not to mention cell compaction. It is instructive to examine a few of these dependencies to better understand reasons behind the large range of mechanical properties for collagen gels listed in Table 1.

Concentration: In a recent well-controlled study, Helary et al. [58] show the dependence of acellular gel stiffness on initial collagen concentration clearly with G' values of 13, 50, 290, and 189 Pa for 0.66, 1.5, 3, and 5 mg/ml, respectively at 2% strain. In this study the highest initial concentration hydrogels (5 mg/ml) tended to creep and to be thus less stiff due to non-homogeneity of these hydrogels (dense and loose regions scattered within), a condition that has since been rectified by handling the stock solutions at 20°C rather than 4°C to reduce viscosity (Christophe Helary, personal communication). For collagen gels “plastically compressed” to obtain extremely high density collagen the modulus by uniaxial tensile testing ranges from 42 to 1,805 kPa for 0.4–20% collagen (4–200 mg/ml hydrated) [56].

pH: In terms of solution acidity during fibrillogenesis, fibril diameters increase as pH decreases. Silver and colleagues [23] found that fibril diameter correlates positively with the low strain modulus (increasing from 0.5 to 5.5 MPa with pH decreasing from 8.5 to 6 at 37°C) but not with either the ultimate tensile strength or the high strain modulus. These studies were completed with extruded gels thus the modulus values are higher than found in isotropic cast collagen gels. For cast gels, the relaxation modulus measured by uniaxial compression has been found to correlate with pH; gelation pH between 5 and 8 alters stiffness from 5 to 25 kPa [136].

Temperature: The storage modulus has been shown to increase roughly two orders of magnitude with polymerization temperature within reasonable limits, from a G' of 0.3 Pa for collagen gels polymerized at 4°C to 22.7 Pa for 37°C-polymerized gels [105]. This finding is surprising since the mean fiber diameter for these gels, as measured by SHG using a two-photon microscope, were 216 and 62 nm for the 4 and 37°C groups, respectively, and larger fibers have been found to correlate with higher stiffness (e.g., with pH as discussed above). The authors attribute the higher stiffness in the 37°C group to a higher volume fraction of interconnected fibers; larger pore structures were observed in the low temperature group and a more interconnected and continuous network with higher fibril density was observed in the 37°C group. Interestingly, the loss modulus was approximately twofold higher than the storage modulus in this study indicating a more viscous than elastic response, an uncommon finding for collagen gels.

4 Static Boundary Conditions: Modulating the Effective Stiffness of the Gel

The stiffness of a material is related to the amount of force needed to deform a material a given amount, normalized to appropriate dimensions. To generate tension, cells require their surroundings to have a minimal level of resistance to deformation (effective stiffness). There are multiple ways to alter the ease by which cells deform the surrounding collagen matrix for a given amount tension generated by the cells. As discussed above, pH and temperature affect the stiffness, but ideally, the collagen gels should be polymerized with entrapped cells under

physiological conditions (neutral pH and 37°C). Collagen crosslinking and increases in collagen density can also be utilized to modulate the stiffness of the gel, yet it is generally held that the collagen gel should present the same ligand density (collagen concentration) to the cells and only the mechanical stimuli should vary between treatment groups. Thus, despite the range of stiffness values reported for collagen gels, we have quite little control over the initial intrinsic properties of collagen gel models when trying to isolate mechanical stimuli for mechanobiological studies. As an alternative approach, many researchers have capitalized on the fact that the mechanical environment of the cells within collagen gels is strongly dependent upon the external boundary conditions of the gel; many innovative approaches have been developed to alter these conditions. This section begins with a short discussion of approaches for modulating the intrinsic stiffness of collagen gels then focuses on means of modulating the effective stiffness of the gel by controlling the boundary conditions.

4.1 Concentration and Crosslinking

The intrinsic stiffness of a collagen gel can be directly modulated by crosslinking the collagen molecules. Biochemical additives including ribose glycation [46], genipin fixation [123], photo-crosslinking [14], and non-enzymatic nitrite modification [97] have all been used to increase the matrix stiffness. Both the stiffness of the collagen fibrils themselves and the strength and number of inter-fibrillar bonds (reducing slippage) likely increase with crosslinking and contribute to the stiffening, although the specific influence of each factor has not been elucidated. At low concentrations, these treatments effectively increase the matrix stiffness by twofold or greater and have been shown to be minimally cytotoxic (unlike glutaraldehyde treatment). However, such exogenous crosslinkers also modify the collagen biochemistry and are likely to alter the cell interaction with and remodeling of the matrix, confounding the assessment of the effects of mechanical factors. As such, they have not been widely adopted for mechanobiological studies.

The intrinsic stiffness can also be directly modulated by altering the collagen concentration (as described in the previous section), and this method has been widely adopted. When cells compact a collagen gel, the collagen concentration increases dramatically (10 to 20-fold increases are typical), and the stiffness rises with the collagen density. Although somewhat counterintuitive, with cell compaction lower initial collagen concentration actually leads to higher final concentration [142] although the final stiffness does not rise proportionally [37]. To obtain higher collagen concentration and reduce compaction, Helary et al. [58] created concentrated collagen hydrogels at up to 5 mg/ml initial concentration which, unlike the “normal” concentration of 0.66 mg/ml, favored cell growth that reached about 10 times the initial cell number at day 21. The concentrated gels had lower compaction in vitro and enhanced neovascularization in vivo. Brown et al. [16] have developed a method for “plastically compressing” gels and wicking the

excess fluid from gels to greatly increase collagen concentration (up to 200 mg/ml with only $\sim 15\%$ cell death). The compression results in a >40 -fold increase in Young's modulus and correlates with increased fibroblast proliferation. This method is gaining popularity with the technology licensed and semi-automated devices available commercially (RAFT system, Automation Partnership, UK). A dense skin (collagen membrane) is formed on the surface of the mechanically compacted gels whereas the inner region is relatively uniform. Examination of differences between cell-compacted and mechanically compacted gels of equivalent final concentration may provide clues towards the importance of cell-generated structures (crosslinks, bundles, local densifications) in cell mechanobiology. Although the collagen biochemistry is essentially the same in these high-concentration preparations, the cells are presented with altered ligand density (Nemir and West 2010) and differences in pore size. As collagen fibrils are not covalently bound, at low concentration the cells are able to migrate through the interfibrillar spaces, whereas motility in high concentration gels may require enzymatic degradation.

4.2 Floating, Anchored, and Released Gels

The most common means for examining the effect of the mechanical environment of cells within a collagen gel is to compare cells cultured in gels attached to a rigid culture dish with those released from the dish following polymerization and cultured freely floating in media. Altering the external boundary conditions has profound impact on the cell mechanical environment if cells are seeded at sufficient density to interact in a concerted manner (e.g., $>10^4$ cells/ml and >4 h). The terminology for these culture conditions is varied and the names used are often imprecise and/or misnomers. For example, anchored gels are often termed "loaded" since the cells can generate macroscopically measureable tension (see terminology text box for more precise terminology).

Regardless of terminology, cell phenotype and activity in the gels are strongly modulated by the boundary conditions of the gel shown schematically in Fig. 5. If the gel is cultured unconstrained and floating (zero force boundary condition), the matrix cannot sufficiently resist cell-generated tractional forces which results in slow compaction of the matrix. Concerted mechanical stress is not generated, the matrix remains highly compliant, the cell morphology becomes rounded and dendritic, and fibroblastic cells do not differentiate into myofibroblasts even in the presence of TGF- β 1, a known stimulant of myofibroblast activation for fibroblasts cultured on stiff substrates [15, 126]. If the gel is anchored around its periphery (zero displacement boundary condition), the matrix is able to resist the cell-generated tension, mechanical stress develops within the matrix, cell-mediated matrix remodeling occurs, and the matrix becomes relatively stiff [6]. Inside anchored matrices, fibroblasts initially and at low cell density appear similar to cells in floating matrices (personal communication, Fred Grinnell), and at short times

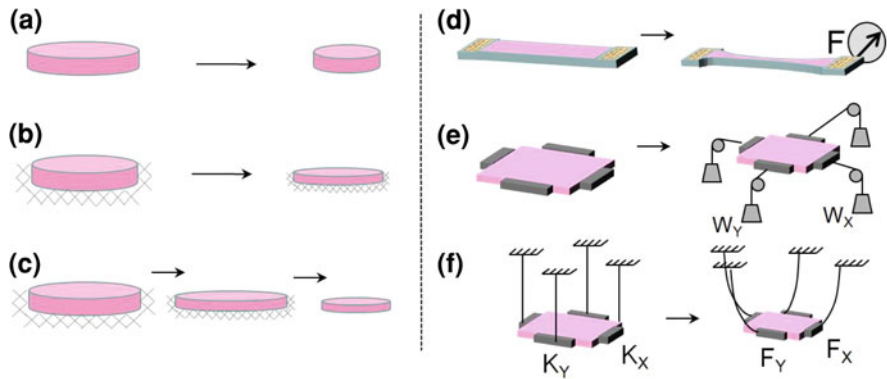


Fig. 5 Schematic diagrams of different static boundary conditions used to modulate the mechanical environment of the cells cultured within collagen gels. The standard culture conditions are shown on the *left* and include **a** floating gel, **b** anchored gel, and **c** released gel (following being anchored). More quantitative and specialized systems shown on the *right* include **d** the culture force monitor with an isometric force transducer, **e** the isotonic force device with calibrated hanging weights, and **f** the compliant anchor device with controlled stiffness springs at the boundaries

(<24 h) migratory forces dominate contractile forces [35]. At longer times (days), with cooperative cell remodeling of the gel, the cells become extended and stellate or bipolar [15, 51]. In the presence of TGF- β 1, after a few days in culture, the cells in anchored gels differentiate into myofibroblasts manifested by increased α SMA expression, remodeling, and force of contraction [6, 59].

To abruptly alter the mechanical environment of the cells within a gel, an anchored gel may be released after a period time (generally 3–5 days). This experimental condition is thought to represent an accelerated transition between active tissue repair and healed tissue where the cells are shielded from extrinsic stress [48]. Following release, the cells contract the matrix rapidly by a smooth muscle-like mechanism [127], profound changes in cell morphology occur, cell proliferation and collagen synthesis decline rapidly, and the cells appear to switch from an active to a quiescent phenotype [91]. Release of collagen gels has also been shown to trigger apoptosis of fibroblasts and myofibroblasts, although the factors that regulate this phenomenon are unclear [52]. Pre-compacted (floating) gels have also been “nested” within acellular gels to examine the migratory behavior across boundaries of different stiffness [90]. Cells are able to migrate out of the relatively stiff compacted gels and into the soft acellular gels. Time-lapse microscopic observations demonstrate “flow” of collagen fibrils from the acellular gel towards the compacted gel which is attributed to the forces associated with cell migration. Further characterization of the mechanics of this system may yield interesting information about mechanical regulation of migration in 3D.

Collagen gels are generally circular due to the shape of common cell culture dishes and for uniformity of the boundary attachment and thus compaction. Tubular

specimens are similarly symmetric and utilized extensively for the formation of “media equivalents” and for ease of mechanical stimulation and testing, although they require a mold for preparation. The use of different shapes (square, rectangular and annular gels) and mixed boundary conditions (some edges free, some anchored) highlights the importance of boundary conditions on cell-mediated remodeling of the gels as shown elegantly by Costa et al. [26]. Anchoring the ends of long rectangular samples and culturing annular gels results in a high degree of alignment (both cells and collagen fibrils) parallel to the free surfaces. Tranquillo and colleagues [65] further demonstrate that the degree of alignment can be controlled by the relative width (anisotropy) of the arms of cruciform gels.

In addition to the periphery, the bottom and top surface of anchored planar gels have very different boundary conditions (usually free on top and fixed below), although the effects of this asymmetry is generally ignored as most of the cells are sufficiently far from the surfaces in thick gels (>1 mm) and coordinated tension is assumed to be in the plane of the gel. Reducing the gel thickness substantially can be used to alter the effective stiffness of the gel. Simmons and colleagues [138] demonstrate that very thin gels have substantially higher compressive structural stiffness than thick gels (6,000 N/m for $\sim 10\ \mu\text{m}$ vs. 2,000 N/m for $\sim 2.5\ \text{mm}$) and show mechanical modulation of calcification potential by valve interstitial cells using these gels. The authors further demonstrate the relative difference in shear deformation between the thin and thick gels using finite element analysis. This analysis is focused on the cells cultured on the surface of these gels, and cells cannot be cultured in ultrathin gels; however, with further development this relatively simple but powerful method for mechanically manipulating the effective stiffness of the cell environment may prove useful for 3D mechanobiology.

Static Boundary Conditions Terminology

Floating gel: collagen is allowed to gel then released after 30 min–1 h (a.k.a. “free” or “unloaded gels”)—a zero force boundary condition.

Anchored gel: held rigidly along outer perimeter and generally lower surface as well (a.k.a., “fixed,” “loaded,” “restrained” or “stressed” gels)—a zero displacement boundary condition.

Released gel: cultured in anchored state (1–5 days generally), then released to free floating state (a.k.a. “stress relaxed” gel).

Isotonically loaded gel: weights are suspended (via pulleys) on the edges; these are generally square whereas other types are typically circular.

Controlled boundary stiffness: gels are cultured suspended by springs of known stiffness.

4.3 Other Isotonic and Isometric Boundary Conditions

Utilizing an innovative device involving hanging weights to produce constant force along each edge of a gel [78], researchers have recently demonstrated that different isotonic boundary conditions can be imposed on each axis of a collagen gel to control the anisotropy of the remodeled gel [124]. An additional advantage of this system is the ability to assess biaxial tensile properties by the use of optical tracking of fiduciary markers used to monitor deformation. Using this system, Costa and colleagues [84] demonstrate realignment of collagen with static loading (Fig. 6) and studied the mechanical regulation of cell alignment in 3D gels. The authors demonstrate that cell realignment precedes collagen realignment when the direction of load is altered. This finding indicates that the cells, when acting in concert at sufficiently high density, actively sense the external boundary and overcome the local contact guidance of the aligned collagen in which they reside.

Prestretching a gel 5–10% uniaxially has been described as a method for effectively increasing the stiffness of a collagen gel without altering the matrix biochemistry. Specific values of stiffness are not described in the development of the method [72], and it is unclear if the proposed increase in stiffness is due to moving to a higher modulus portion of a non-linear stress–strain curve (i.e., past the “toe-region”), or if the effective stiffness increase is analogous to that of a pre-stressed drum or guitar string. However, interesting changes in cell contraction and matrix remodeling are observed in this system [72, 73].

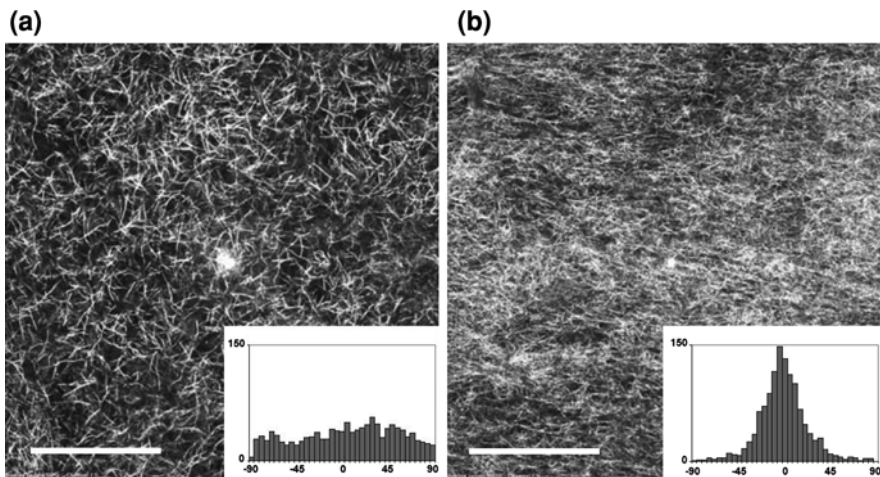


Fig. 6 Confocal reflectance images demonstrating collagen fibril alignment due to remodeling under static uniaxial constraint in the isotonic force device for 72 h. With kind permission from Springer Science + Business Media: from Ref. [84], Fig. 5a and b

4.4 Compliant Boundaries: Springs as Anchors

To modulate the effective stiffness that the cells experience in a graded and controlled manner without altering the physiochemical properties of the extracellular matrix, we have developed a method utilizing compliant anchors (0.048–0.64 N/m) to tune the boundary stiffness of suspended collagen gels (Fig. 7f) [67]. Using this system we find that increased boundary stiffness elicits enhanced basal tension and potassium-stimulated active contractile force from fibroblasts. Remodeling of the collagen matrix is also increased with boundary stiffness indicating stiffness-dependent phenotypic regulation of the cells. TGF- β 1 acts synergistically with boundary stiffness to enhance remodeling and α SMA expression (Fig. 7). A similar concept has been developed by Chen and colleagues [85] to study the effect of boundary stiffness on small collagen gel “microtissues” utilizing soft lithography methods. The authors report that mechanical stress increases with increased boundary stiffness, but decreases with increased collagen density (used to increase intrinsic stiffness); a finding that highlights the complex relationship between generation of tension and compaction of collagen matrices.

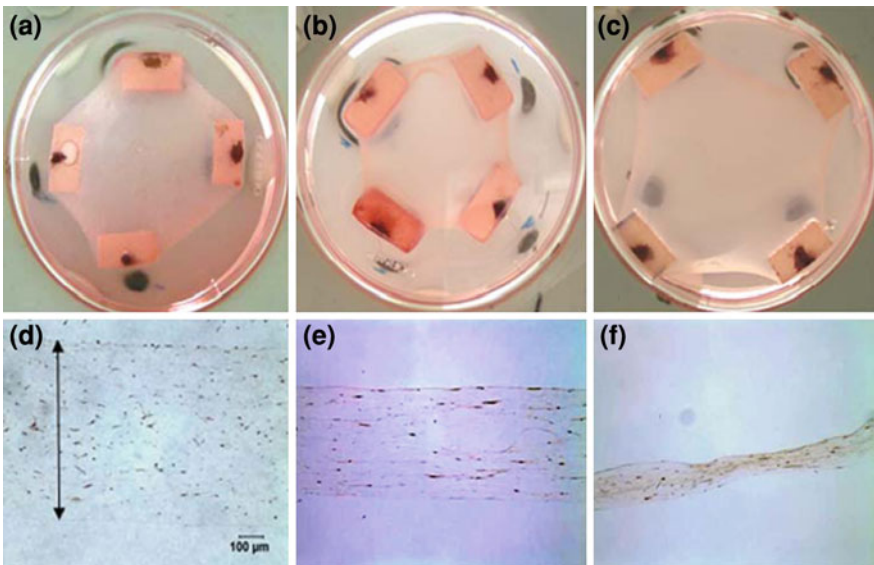


Fig. 7 Compaction of collagen gels after culturing for 3 days in the controlled boundary stiffness device with (a, d) compliant beams ($K = 0.048$ N/m) without 10 ng/ml TGF- β 1 (b, e) compliant beam with TGF- β 1 and (c, f) in presence of stiff beam ($K = 0.57$ N/m) and TGF- β 1. The diameter of the dish is 60 mm and original magnification is $200\times$ (d–f). With kind permission from Springer Science + Business Media: Ref. [67], Fig. 8

5 Dynamic Boundary Conditions: Cyclic Loading and Stretch

Whereas modulating the effective stiffness of the matrix can be used to probe “inside-out” cellular mechanotransduction, stretching the cells can be used to investigate “outside-in” signaling [32]. Dynamically stretching fibroblasts within collagen gels has been shown to regulate myriad aspects of cell behavior including alignment [70], morphology [34], altered MMP synthesis [114]. It has also been shown to modulate cell phenotype [2, 5] and gene regulation including those encoding for collagen [19] and matrix proteases [103]. Many systems have been developed to stretch collagen gel models as described below and schematically illustrated in Fig. 8.

5.1 Uniaxial Loading

The majority of studies of mechanobiology in collagen gels utilize cyclic uniaxial stretch. Samples are generally cast into either long rectangular molds with porous anchors at each of the ends to affix the samples to the stretch device [117], or into short tubular molds to create ring-shaped specimen [70, 134, 135]. Rectangular specimens become cord-like due to cellular compaction with larger area at the

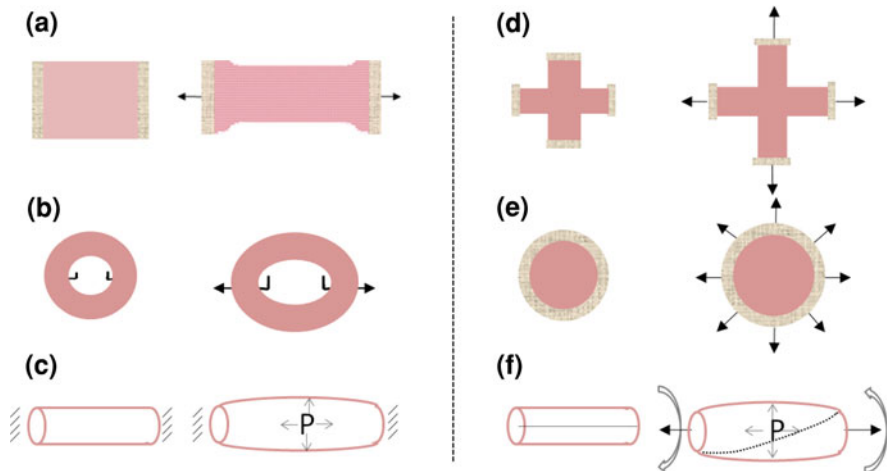


Fig. 8 Schematic diagrams of different dynamic boundary conditions used to modulate the mechanical environment of the cells cultured within collagen gels. The uniaxial culture conditions are shown on the *left* and include **(a)** rectangular-, **(b)** ring-, and **(c)** tube-shaped gels (note that the tube is inflated resulting in roughly pure-uniaxial circumferential stretch with little transverse contraction). The biaxial culture conditions are shown on the *right* and include **(d)** cruciform-shaped samples pulled along orthogonal axes, **(e)** circular samples stretched on silicone membranes, and **(f)** tube-shaped samples simultaneously inflated, stretched, and twisted

anchors which, similar to a “dogbone” shape, minimizes stress concentration. Rings have the advantage of being easy to stretch between pins without the need to grip the samples, thus not relying on integration into porous anchors and avoid crushing the ends. Using these systems, researchers have found that 10% stretch at 1 Hz results in increased contractile apparatus within smooth muscle cells relative to static controls [70], and that the mechanical effect is augmented when combined with growth factors including PDGF and TGF- β 1 [120]. Uniaxial stretch has also been combined with twist to simulate tendon loading and found to direct mesenchymal progenitor cell differentiation towards a ligament cell lineage [5].

If wide rectangular samples are utilized as is often the case with culture force monitors, the transverse compaction is minimized; in the center of the sample the strain field is pure uniaxial [15, 34]. In short-term stretching experiments Brown et al. [15] found that fibroblast populations within collagen gels have a homeostatic contractile force set point; when a gel is decreased in length and the tension removed, the fibroblasts contract the gel and increase the tension in the gel back to the previous level.

For long-term cycling, Flexcell International has developed a modification to their cell stretching device to apply uniaxial stretch to collagen gels (Tissue Train; Flexcell). In this commercial device, the silicone membrane provides elastic return to the attached ends, but lack of elastic return of the collagen gel itself may lead to buckling of the cord-like collagen gel especially at high frequency cycling (personal observation). Recently, collagen gels have been cast into macroporous elastic polymer substrates to provide local elastic return [128]. Cells within the collagen can be imaged and the authors report lung fibroblast-to-myofibroblast differentiation in the device (30% strain at 0.1 Hz) by α SMA-positive staining; however, the cell-remodeled collagen gel cannot be removed to assess functional remodeling or cell contractile force.

Tube inflation is also a popular method for applying cyclic stretch to collagen gels [63, 113, 120]. Due to the high permeability of the gels, a silicone support is generally utilized. Inflation of the silicone tube with fixed ends results in almost pure uniaxial (circumferential) stretch to the gel if it is well adhered to the silicone tube. For sufficient attachment, the silicone tubes can be pre-treated with acid to etch the surface and a thin pre-coating of collagen and/or chitosan can be dried onto the tube [113]. Without proper adherence to the silicone, the collagen gel lacks sufficient elastic recoil to follow the cyclic inflation and little dynamic strain is applied to the cells. Further, without adherence, the cells freely compact the gel laterally resulting in a short dense tube, although pulling off of the membrane can occur even with good adhesion if the contraction is strong, e.g., with TGF- β 1 stimulation combined with stretch [120]. Using tube inflation of rat smooth muscle cell-populated gels, Seliktar et al. [113] observed pronounced alignment of the collagen and cells (Fig. 9) and found that the stiffness and strength increased more than twofold after 8 days of 10% stretch at 1 Hz relative to static controls. In a similar study, the same group reports an increase in the production of MMP-2 and activation of latent MMP-2 with 4 days of dynamic culture and, importantly, that

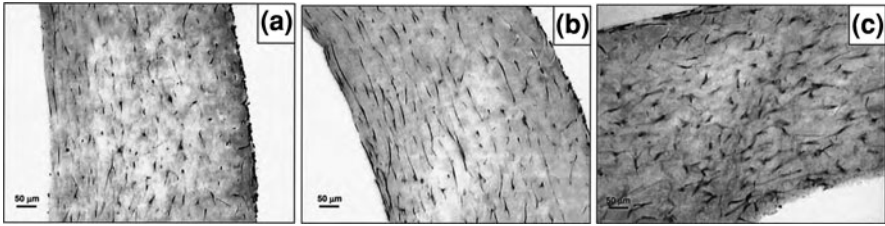


Fig. 9 Cross-sections of rat smooth muscle cell-populated collagen gel tubes following 4 days of **a** static culture on a rigid mandrel, **b** 10% cyclic uniaxial stretch, and **c** floating culture. Comparison of **a** and **b** illustrates the circumferential alignment of the cells and collagen with dynamic stretch and the complete lack of orientation in gels following floating culture. The collagen stains light and the cells dark with the hematoxylin and eosin stain in the histological sections. With kind permission from Springer Science + Business Media: from Ref. [113], Fig. 5

nonspecific inhibition of MMPs completely mitigate the stretch-induced changes in mechanical properties [114].

It is difficult to determine the precise effect of uniaxial stretch on alignment since, even in the absence of stretch, uniaxial samples become cord-like with a high degree of cell and collagen alignment due to the lack of resistance to cell traction along the free transverse boundary [26, 61, 135]. This limitation has led to the development of controlled biaxial stretch methodologies.

Dynamic Boundary Conditions Terminology

Uniaxial loading: loaded on only one axis (whether force or displacement controlled), with other two axes free to deform with zero stress at the boundaries.

Strip biaxial stretch: deformation restricted to only one direction, with the transverse strain held to zero (3rd axis free to deform) a.k.a. pure uniaxial strain.

Biaxial loading: controlled loading or stretch along two axes generally without shear (strip biaxial is a special case with zero transverse strain).

Shear loading: loading perpendicular to the axis of interest e.g., applying a lateral (x-direction) force on the upper (z) face of a gel; shear stresses and strains are also present in cases of non-uniform loading, e.g., near clamps, tethers, or rigid inclusions.

5.2 Biaxial Loading

For fibrous gels, equibiaxial stretch has the advantage that the overall fiber alignment in the plane of the sample does not change with stretch and fiber

realignment in the direction of stretch does not overshadow other remodeling events (compaction, protein accumulation, and crosslinking). Similar to uniaxial methods, biaxial methods are hindered by the poor elastic return of collagen gels and difficulty in attaching the gels to elastic polymeric substrates. We have utilized equibiaxial stretch of fibrin gels, taking advantage of the adhesive property of fibrin and utilizing anchors around the perimeter of the gel, and shown dramatic strain magnitude-dependent compaction and strengthening [8]; however, similar large magnitude dynamic stretch of collagen gels has proven unsuccessful with the Flexcell system. Considering the difficulty in keeping collagen gels adhered to static culture plates against the substantial cell traction during compaction, it is not surprising that external stretch causes the gels to detach from silicone membranes. Nerem and colleagues [17] report that small hemispherical collagen gel layers can be adhered to acid-etched silicone membranes with Cell-Tak, and that they can withstand being stretched to 10% area strain at 1 Hz for up to 2 days. The authors report that smooth muscle cells in these very thin stretched gels become less elongated and shift to a more synthetic phenotype.

Grande-Allen and colleagues [55] developed a device to equibiaxially stretch cruciform-shaped collagen gels populated with valvular interstitial cells up to 10% at ~ 1 Hz for 2 days and demonstrated that glycosaminoglycan and proteoglycan synthesis of these cells is regulated by cyclic stretching in a magnitude-dependent fashion. With minor modifications, this device has the potential to apply non-equibiaxial strain as well, although the sample dimensions are quite large (30 mm maximum dimension) and thus expensive. The goal of this and most other test systems has been to obtain a strain field that is as homogeneous as possible.

We have created a system to apply repeatable non-uniform strain fields to cells by utilizing rigid inclusions affixed to circular membranes that are pulled uniformly and radially [9]. In this system, there is a gradient of strain anisotropy from pure uniaxial near the inclusion to equibiaxial at the outer edge. Our preliminary results with dermal fibroblasts cultured within fibrin gels indicate that cells orient perpendicular to the direction of stretch in the pure uniaxial stretch region, in contrast to findings of alignment along the uniaxial stretch direction in systems described in the previous subsection. Additional work is needed with collagen gels in these biaxial systems to investigate cell responses to a range of strain anisotropy (not just pure uniaxial and equibiaxial) as well as cell responses to gradients of strain within a 3D environment.

6 Cell Environment

6.1 *Transfer of Stress and Strain to Cells within a Collagen Gel*

For the study of cell mechanobiology in collagen gels, it is important (and non-trivial) to determine the forces and deformations experienced by the cells.

The transfer of stress and strain to the cells within a collagen gel (and native tissue) depends not only upon the fibril microstructure immediately surrounding the cell and the how the cell is attached to the matrix elements [100, 109, 126], but also the matrix architecture far from the cells. Due to non-affine fiber behavior and non-uniform stiffness within collagen gels, local strains experienced by cells are generally lower than global (applied) strains as demonstrated by confocal microscopy [100, 111]. Collagen gels are inherently non-affine as they are made up of discrete fibers tangled together in a physical gel. These fibrils are also able to exhibit local reorientation, scissoring, and buckling as predicted by multiscale models [112] and confirmed experimentally [111]. We have quantified similar non-affine behavior of collagen fibers within native tissues [12], and bucking behavior has been observed in collagen/GAG sponges due contraction of single cells [57]. With affine fiber kinematics, all elements are stretch to the same degree as the bulk applied strain (Fig. 10a, b). With non-affine behavior, as shown in

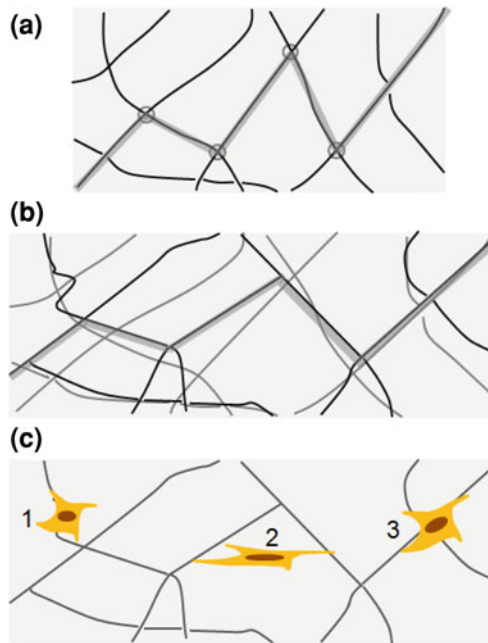


Fig. 10 Schematics of a hypothetical fiber arrangement within a collagen gel. **a** Undeformed state showing entanglements (circled) and highlighting one fiber path (wide grey lines). **b** Following 30% pure uniaxial stretch, the difference between affine (light grey lines) and non-affine (black lines) fiber kinematics is shown. In the affine case, all points in the material follow the bulk deformation. In the exaggerated non-affine case, the fiber segments between entanglements rotate and are not stretched at all (wide grey lines are the same length in **a** and **b**). **c** Depending upon how a cell is attached to the fibrous matrix, the cell can experience 1 unloading, 2 greater than bulk stretch, or 3 zero stretch. In a collagen gel, cells actually span many fibrils as shown in Fig. 1a, and thus the transfer to the cells is more likely similar to the affine case than the exaggerated non-affine case shown in schematic above

Fig. 10c, a fiber can remain unstretched due to rotational freedom, even with large bulk strains. Even with pure uniaxial applied strain, cells attached to a non-affine fiber matrix may experience a range of strains from compressive to highly tensile or no strain at all (cells 1, 2, and 3, respectively in Fig. 10c). Although these strains are theoretically possible, in collagen gels the fibrils are much smaller than the cells and are relatively dense (see Fig. 1a), thus these extreme cases are not likely to occur. Further, the relative impact of non-affine fiber kinematics on strain and stress transfer likely decreases in relative importance in very dense and/or highly crosslinked gels (e.g., after extensive cell-mediated remodeling) since the relative mobility of the fibers is low compared to sparse gels prior to compaction. As the gel is stretched to higher levels of strain, the impact of non-affine behavior is also reduced as the fibrils are reordered and lose entropy [20].

Under physiological loading levels and following localized remodeling, heterogeneous material properties likely dominate non-affine fibril behavior in governing the (lack of) strain and stress transfer to the cells. Although non-uniform deformation due to heterogeneous mechanical properties is by definition non-affine deformation (local deformation does not follow bulk deformation in a one-to-one manner), here we consider material heterogeneity separately from non-affine fiber kinematics in terms of their effects on strain non-uniformity within the collagen gel. Cells in a collagen gel are not necessarily attempting to “shield themselves” from the external loading, but rather they simply can reorganize their local matrix which leads to heterogeneous material properties. A few simplified cases shown schematically in Fig. 11 demonstrate the impact of heterogeneous material properties on stress and strain transfer. Figure 11a shows a material with alternating dark and light regions in series. Upon loading, the (engineering) stress in all locations is the same and if the material properties of the regions are similar the strains are similar (Fig. 11b), whereas if the dark material is twice as stiff as the light region, the strain is $\frac{1}{2}$ that of the light region (Fig. 11c). Alternatively, if the alternating regions are arranged in parallel as shown in Fig. 11d, when loaded the strain is the same in both materials regardless of stiffness (Fig. 11e) but the stress is higher in the more stiff material. This parallel arrangement is analogous to fibrous native tissues where stiff surrounding fibers bear the external loads and cells surrounded by softer matrix (e.g., proteoglycans) are stress shielded and can remain quiescent. With transection of the stiff bundles upon injury (Fig. 11f) the stress from external loading is transferred to the cells. Neither of these simplified structures are representative of collagen gels where local remodeling around each cell creates roughly spherical stiff inclusions within a compliant bulk matrix [37]. This heterogeneous structure leads to both strain and stress shielding of the cells as shown by preliminary measurements [100]. Regardless of the structure of a material, it is important to remember that the deformation must be conserved with the local deformations adding up to the overall applied displacement, thus low stiffness areas in series with stiff cell-remodeled areas must undergo exaggerated deformation (Fig. 11c). Simply stated, although strains measured locally within

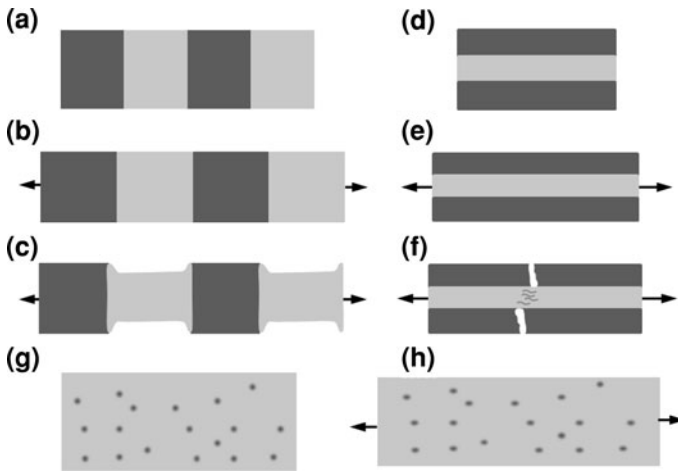


Fig. 11 Schematics of a hypothetical material with alternating regions arranged in series (a) demonstrating the resulting deformation if the regions have the (b) same stiffness ($E_1 = E_2$) or (c) different stiffness ($E_1 = 2E_2$). Schematic of parallel arrangement of materials (d) is analogous to native fibrous tissues with stiff fibrous bands (dark region) and cells within soft GAGs (light region). With stretch (e) cells experience the bulk stretch but are stress shielded by the stiff fibers. If the stiff “fibers” are transected to simulate injury, the soft regions are exposed to high stresses (f). The stiff inclusions (dark) in a soft matrix (light) show in (g) are more realistic for collagen gels. Cells within the stiff inclusions are shielded from both stress and strain applied externally

collagen gels have been shown to underpredict applied strain levels, the local strain of the matrix cannot be lower than the global strain in all locations.

The previous discussion considers the local strain and stress on the fibrils surrounding the cell rather than strain of the cell itself, and the cell is assumed to be attached to the local fibrils. Clearly, if a cell is not adhered to the collagen matrix it will not be strained, and no force transfer can occur to or from the cell. Due to the natural binding sites on the collagen molecule, adherent cells generally attach well to the fibrils (via integrins; see recent reviews [7, 22, 74]). Elson and colleagues [134] demonstrate, by treating cell-populated gels with cytochalasin-D, that active cell tension has a large impact on the overall stress–strain response under cyclic uniaxial extension. These results indicate that cells can be assumed to be attached firmly to the collagen fibrils in the absence of excessive forces or inhibited attachment, e.g., by blocking integrin subunits.

6.2 Calculation of Cell-generated Forces

In addition to external loading that is transferred to the cells through the matrix, the cells also exert tension on the matrix and on each other. Cell-generated tension

propagates through the matrix surprisingly far in soft collagen gels, hundreds of microns [130]. Propagation of cell tension is especially evident when combined with remodeling of the collagen fibers along lines of stress [121]. When cells are present in sufficiently high density and the matrix is sufficiently compliant, the force can be measured externally [15]. Most commonly, a culture force monitor (CFM) is utilized [33]. Similar to a blood vessel myograph, a CFM consists of a highly sensitive isometric force transducer attached to one end of a compacting collagen gel submerged in media while the other end is rigidly anchored. The force of compaction may also be measured simply by the deflection of compliant beams uniaxially [40] or biaxially [67], which also modulates the effective stiffness of the collagen gel system, or by measuring the frequency and damping of a mechanically pulsed gel cultured on a flexible membrane [129].

The measured force of compaction rises exponentially over the course of approximately 24 h due to the cells migrating through the matrix and compacting the collagen fibrils [87]. The force then levels out at a homeostatic level of basal cell tension which can be eliminated by a variety of agents such as cytochalasin-D. The cells can also be stimulated to contract more vigorously by various agents, the simplest being potassium chloride [67]. In long-term culture, the cells “shorten” the matrix and residual stress is developed within the matrix as evidenced by residual matrix tension following cytochalasin-D treatment.

The force per cell is generally estimated by dividing the total (uniaxial) contractile force measured by the number of cells in the gel [31, 33]. Using this calculation, dermal and cardiac fibroblasts generate 0.1–10 mN/million cells (i.e., 0.1–10 nN/cell) in uniaxial [31, 33] and biaxial [67, 78] systems. Although straightforward, this calculation assumes all cells act in parallel (see schematic, Fig. 12a) which is clearly inaccurate. Assuming that all of the cells act in series, the total force measured would be equal to the force generated by a single cell which is similarly flawed (Fig. 12b). Since the cells are distributed approximately uniformly throughout a cell-populated collagen gel, cells act in parallel with some cells and in series with others (Fig. 12c). Kolodney and Wysolmerski [79] divide the measured uniaxial force by the cell cross-sectional area measured from histological cross-sections which is an improvement for uniaxially constrained gels but not for biaxial configurations. As a simple solution for (equi)biaxially restrained gels, we utilize a representative volume element (RVE) containing one cell and the surrounding volume of ECM (Fig. 12c, inset), and integrate over the RVEs in the perpendicular cross-section and assume a uniform distribution of cell orientations to calculate the total force along each axis. Using this method, dermal fibroblasts generate 17–100 nN/cell for low to high stiffness boundary conditions, respectively, after 3 days in standard media. Comparable values have been reported by Chen and colleagues [85] for NIH 3T3 cells cultured within collagen gels attached to micro-pillars of different stiffness (14 and 24 nN/cell for low and high boundary stiffness, respectively). To more accurately estimate the force per cell in collagen gels, a computational model such as the anisotropic biphasic theory

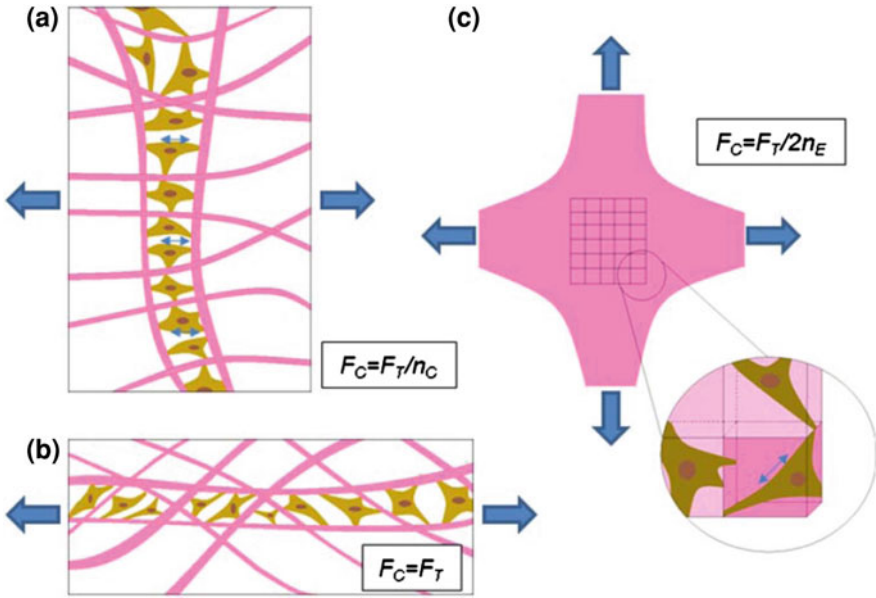


Fig. 12 Schematics representing idealized arrangements of cells in a collagen gels in **a** parallel and **b** series formations for the purpose of estimating the force per cell F_C , assuming uniaxial restraint. **c** Shows a biaxial configuration subdivided into representative volume elements (RVEs) with each RVE containing a single fibroblast in random orientation; n_C number of cells, n_E number of RVEs in parallel in a given cross section. Note that F_C is lowest in (a), highest in (b), and in between in (c). With kind permission from Springer Science + Business Media: from Ref. [67], Fig. 10

[77] would be superior, yet current models rely on assumptions of cell–ECM interactions that are not valid for long culture times.

For collagen-GAG sponges, Gibson and colleagues [57] developed an innovative method involving analysis of the bending of ECM “struts” by individual fibroblasts and found similar values as reported above (26 nN/cell average, maximum 450 nN). These forces are substantially higher than estimated by dividing force by total cell number in earlier studies using the same system (~ 3 nN/cell) [41]. Unfortunately the pore and strut size are too small in collagen gels to apply this method. Ideally, the cell force would be determined on a per cell basis using optical measurements of displacements combined with computational methods similar to traction force microscopy used extensively for 2D cell studies on compliant gels. Although not straightforward, 3D traction force microscopy is showing promise with preliminary results indicating that carcinoma cells (human breast) generate forces in excess of 100 nN (unpublished data described in [89]). It is only a matter of time, with new microscopy techniques and computational power, before researchers will be able to image bead/fibril displacements in 3D with sufficient resolution for accurate, routine quantification of cell forces in collagen gels.

7 Discussion

While it is clear that a 3D culture environment is necessary to study cell behavior in a biofidelic manner, there are many aspects that need to be considered when using the collagen gel model system. Collagen gels are complex compared to 2D substrates due to dynamic reciprocity, yet highly simplified both biochemically and structurally compared to native tissues. Dynamic reciprocity between cells and matrix confounds analysis of the specific effects of mechanical cues, e.g., stiffness of the gel changes with cell-mediated remodeling and gels become heterogeneous. Yet the interaction between the matrix and cells in collagen gels can be used to our advantage by measuring functional changes in cooperative cell behavior upon external modulation of the mechanical environment. These metrics, including contractile force and matrix remodeling, can be measured in collagen gels more readily than in standard 2D culture. Further, in 2D systems mechanical cues have been shown to alter gene expression for both ECM synthesis and degradation. Collagen gel models allow researchers to determine the functional outcome of a given set of physical stimuli, i.e., on balance, does the tissue grow or is it resorbed? While we limited our focus to mesenchymal cells entrapped within collagen gels and the effects of stiffness and stretch, it is important to note that interstitial flow within collagen gels is also a strong mechanical signal which alters both fiber alignment and cell behavior [93]. Further, epithelial cells (endothelial cells, keratinocytes, airway epithelial cells, etc.) which reside at the solid/fluid interface have been cultured on collagen gels (i.e., in 2D environment), and the interaction between cell types (e.g., endothelial and interstitial) can be studied in collagen gels as well [18].

This review only touches on complex combinations of proteins; for these the reader is referred to reviews and works regarding fibrin gels [100], Matrigel [139], and decellularized cell-derived matrix [28]. Many of same tools (modulation of boundaries) could potentially be applied to these protein gel models systems to study mechanotransduction in more biochemically relevant matrices. For specific aspects of fibroblast biology in collagen gels, the reader is also referred to excellent reviews by Grinnell and colleagues [49, 50, 108] which include discussions of cell migration, matrix remodeling, and cell morphology in collagen gels. Biomaterial-related reviews for matrices produced with higher order structures such as electrospun biopolymers [88, 115], extruded collagen microfibers [119], and crosslinked collagen sponges, e.g., lyophilized and crosslinked collagen/GAG scaffolds [41] are also available.

Finally, although extensively utilized for studies of cell response to substrate rigidity in 2D (mainly polyacrylamide-based substrates) and beginning to be used for 3D studies (mainly PEG-based gels) to afford independent control of mechanical and biochemical parameters, synthetic polymers are beyond the scope of this review. Their use is described in an excellent review by Nemir and West [92] which includes informative tables with stiffness ranges of tissues and model systems utilized, and future prospects are reviewed by Tibbitt and Anseth [125].

In the future, the field would benefit from a more quantitative and evidence-based analysis of stress transfer and strain shielding in collagen gels. At present, it is unclear if cells are able to sufficiently remodel the collagen matrix to shield themselves from external loading and become quiescent as occurs in native (uninjured) tissues; this important issue should be addressed with additional models and combined microscopic/mechanical studies. Clearly, quantitative measurements of cell and matrix deformations are needed for better understanding of strain transfer to the cells, and these are difficult to visualize in 3D. Although powerful microscopy tools are becoming available, the resolution limit is generally above that of the collagen fibrils and the time required for imaging is long for viable cell studies. A few local methods are described herein, but there is a clear need for additional methods for assessing mechanics of the collagen gel at the cell level including viscoelastic effects. Further, nonlinear collagen stiffness may make local stiffness much higher than bulk stiffness in 3D collagen [122], thus there is a need for large deformation measurements near the cells as well. Better understanding how cell populations are governed by boundary stiffness is also essential, as this condition involves a complex interplay between the boundary and the cells similar to that which occurs *in vivo*.

The collagen gel remains a powerful model for studying mechanobiology in a three-dimensional environment. Knowledge gained from mechanically controlled and properly characterized systems have had, and will continue to have, a significant impact on fields as diverse as physiotherapy, wound and fracture healing, hypertension, or tissue engineering.

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