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Effects of nonlinearity on cell-ECM interactions

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Abstract

Filamentous biopolymers such as F-actin, vimentin, fibrin and collagen that form networks within the cytoskeleton or the extracellular matrix have unusual rheological properties not present in most synthetic soft materials that are used as cell substrates or scaffolds for tissue engineering. Gels formed by purified filamentous biopolymers are often strain stiffening, with an elastic modulus that can increase an order of magnitude at moderate strains that are relevant to cell and tissue deformation in vivo. This review summarizes some experimental studies of nonlinear rheology in biopolymer gels, discusses possible molecular mechanisms that account for strain stiffening, and explores the possible relevance of non-linear rheology to the interactions between cell and extracellular matrices.

1. Introduction

Many soft biological materials stiffen as they are increasingly deformed either in simple shear or extensional strains [1]. A strain-dependent increase in elastic modulus is often essential to the proper functioning of tissues that are subjected to large and time-dependent stresses. Perhaps the most dramatic and best-characterized example is the elastic response of the aorta to pressure change during beating of the heart [2]. If the blood vessel wall were made from a linear elastic material, the change in vessel diameter with blood pressure change would be too large to permit efficient flow of blood down the vessel, and the vessel wall would distend to strains large enough to increase the risk of rupture. Extracellular matrices (ECM) formed by fibrin clots [3] or collagen gels [4] also increase stiffness with distension, allowing them to be compliant to small forces that occur during normal conditions, but resistant to excessive deformation or rupture at large stresses. The property of increasing stiffness with increasing deformation is usually called “strain-stiffening” to distinguish it “strain-hardening” which was used in some studies of biomaterials, but which more accurately refers to changes in the mechanical properties of materials such as metals that are processed under strains that alter their structure and therefore cause their hardening. In contrast, strain-stiffening is a reversible property and does not cause or require permanent changes in the material structure.

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The stiffness of tissues or bioengineered scaffolds for cell regeneration is increasingly recognized to be important for proper cell function, as numerous studies have shown how altering substrate stiffness changes cell structure and function in vitro [5–7]. Most such studies have used flexible substrates formed by polyacrylamide, hyaluronan, alginate, or polydimethylsiloxane (PDMS) as the complaint scaffold on which specific adhesion proteins are attached, and the linear rheology of these materials allows accurate assessment of traction forces from the displacements of markers placed within the substrates [8]. However, linear elastomers do not mimic the more complex rheology of tissues and ECM, and cell respond differently to linear and non-linear elastic substrates [9].

The molecular structures within tissues and the physical mechanisms that enable strain-dependent stiffening are beginning to be identified. Several different models have been proposed to account for non-linear rheology in biological materials. Defining the mechanisms used in different tissues would help in the design of improved scaffolds for tissue engineering and wound healing, and provide a rational basis for design of new classes of soft materials. This review summarizes examples of non-linear rheology in tissues and purified biopolymer networks, discusses different mechanisms by which strain-stiffening can arise, and suggests how non-linear rheology might be exploited by cells to enable intercellular interactions and organize tissue formation and repair.

2. Measurements of non-linear rheology

For purely elastic materials, non-linear response is the deviation of a plot of stress vs. strain from a line. In this case, the effective elastic modulus at any strain state is the slope of the stress-strain plot at that strain. Measurements such as these are the most common type of measurement in the bioengineering literature, and are generally done using a constant rate of strain in compression or extension, and less often, during a constant rate of shear strain in parallel plate or cone-and plate geometries [10].

Soft tissues and biopolymer gels generally have a large solvent constant, usually non-permanently crosslinked polymer chains, and multiple relaxation modes and are therefore viscoelastic or viscoplastic. As a result simple measures of stress vs. strain depend on the rate of strain imposition as well as on the magnitude of strain. Therefore, measurements are often made sign oscillatory rheometry, often in shear, and these methods lead to many issues and ambiguities in defining the actin-dependent elastic modulus. If stress and strain are not linearly related, the imposition of a sinusoidally varying stress (or strain) will not result in a sinusoidal response in strain (or stress). Under such conditions the elastic modulus is not strictly defined, and the effective value derived from some ratio of stress to strain depends on how the measurements are done and how the data are analyzed.

In stress-controlled rheometers used to impose a sinusoidal stress on a strain-stiffening material, the resulting strain is a flattened periodic function in which the maxima are difficult to define [11]. If, on the other a strain-controlled rheometer is used to impose a sinusoidal strain, then the resulting stress function is a sharply peaked periodic function, and the maximum can be accurately measured. In the latter case the loss function (G''/G') can be accurately determined, but neither G' nor G'' are clearly defined since their calculation assumes purely sinusoidal stimulus and response. Nevertheless, fitting the response functions to sinusoidal function and higher terms has frequently been used to derive quantitative measures of strain-stiffening [11].

Two variations of traditional oscillatory rheometry have been highly useful for characterizing strain-stiffening, materials. In one method, a constant static shear stress is imposed on the material and then a small amplitude oscillatory stress is superimposed to obtain the differential modulus, often denoted as K [12]. Some early measurement of the

strain stiffening of fibrin [3], actin [13], and neurofilament [14] gels were made with a torsion pendulum to apply a large, variable, constant stress over which small amplitude free oscillations were imposed. The differential modulus can also be measured using commercial stress-controlled rheometers which more accurately control both the static stress and the oscillatory stress amplitude, and have revealed fundamental insights into the strain stiffening of cytoskeletal networks [12]. A significant issue in measurement of the differential modulus is that since the materials are viscoelastic they creep during imposition of the static stress [14], and so the strain at which the differential modulus is measured changes systematically during the measurement. Measuring biomaterials under static stress mimics qualitatively the effects of gravity or muscle tone, for example, on a matrix in which cells also exert stresses, and so the effective stiffness that the cell responds to is the differential modulus of the matrix in the strained state.

3. Nonlinear elasticity of biopolymer networks

Nonlinear rheology characterized by strain stiffening is observed in vitro in gels formed by purified polymerize derived from both the cytoskeleton and the extracellular matrix.

3.1. Cytoskeleton components

There are three major types of cytoskeleton filaments: actin filaments—microfilaments, intermediate filaments, and microtubules. Each type of filaments has its unique molecular structure, function and mechanical properties. Microtubules are hollow tubes of 25 nm in diameter composed of approximately 13 parallel protofilaments arranged in a staggered manner to form a hollow tube. Actin filaments can be regarded as two parallel protofilaments that twist around each other into a right-handed helix with a diameter of 8 nm. Intermediate filaments have an average diameter of 10 nm which is larger than the actin filaments but smaller than the microtubules. IFs are held together by lateral hydrophobic and electrostatic interactions and a hierarchy of inter-subunit interactions results in the mature IF structure.

From a mechanic perspective the most striking difference among the cytoskeletal filament types is their bending stiffness (Fig. 1). The stiffness of polymer filaments can be quantified by their persistence length, ℓ_p , which is defined as the length scale for the decay of the tangent-tangent correlation along the filament, and is proportional to the stiffness of the polymer. If a polymer can be modeled as a uniform cylinder, $\ell_p = k_B/kT$, where k_B is the bending modulus of the filament [15].

The ratio between persistence length (ℓ_p) and the contour length (l_c) differentiates the polymer filaments into three regimes. When $l_c \gg \ell_p$, the filaments are flexible, i.e. thermal energy can cause large transverse fluctuations so that a filament in solution looks like a Gaussian polymer chain with a very small projected area. In the limit of $\ell_p \gg l_c$, filaments are stiff and do not show transverse thermal undulations but move in solution as rigid rods driven by thermal energy. Semi-flexible filaments lie between these two limits, and l_c and ℓ_p are on the same order of magnitude.

For filaments with micrometer long contour length, the three types of cytoskeleton filaments fall in these three stiffness regimes respectively. Intermediate filaments are the softest among the three major types of cytoskeleton filaments. Their ℓ_p is reported to range from 200 nm to slightly over 1 μm and is IF type dependent [16, 17]. The very small persistence length of the 10 nm diameter IF containing many coiled-coil dimer subunits per cross-section is surprising since the persistence length of a single coiled-coil dimer such as tropomyosin is itself approximately 50 nm [18]. The thinner actin filament is an order of magnitude stiffer than IFs and has a persistence length ranging from 3–17 μm which is

comparable to the typical filament length. Microtubules are stiff polymers with $\ell_p > 1\text{mm}$ [19]. Compared to IFs, MTs and F-actin are relatively brittle filaments and rupture under elongational strains less than approximately 10%. Intermediate filaments, in contrast, can bear much larger extensional strain than MTs and F-actin. Several types of intermediate filaments have been stretched to over 200% of their resting length without breaking [20, 21]. This large extensibility is attributed to the hierarchical structure of IFs that allows partial unfolding of subunits without breakage of the filament.

3.2 Extracellular Matrix

The extracellular matrix (ECM) that fills the space between cells within most soft tissues is, like the cytoskeleton, composed largely of filamentous polymers attached to each other and to cells by a series of complex molecular links. The ECM provides not only mechanical support for tissue cells but also biochemical and biomechanical signals that required for tissue homeostasis, morphogenesis and cell differentiation.

Collagen is the most abundant fibrous protein within the interstitial ECM and constitutes up to 30% of the total protein mass of a multicellular animal. Collagen filaments are long and stiff fibers composed of three collagen polypeptide chains wounding around each other in a ropelike superhelix. As the main structural element of the ECM, the fibrous collagen networks provide tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development [22].

Elastin is also an abundant ECM constituent that provides recoil to tissues that undergo repeated stretch. Elastin fibers are often tightly associated with collagen fibrils. Under external load, the stiff collagen fibers protect the elastic elastin fibers from being overstretched [23]. Elastin fibers are often covered by glycoprotein microfibrils, mainly fibrillins, which are also essential to maintain the integrity of the elastin fiber [23]. Unlike collagen, elastin is a flexible polymer and gels formed by purified elastin generally have near linear rheology.

Fibrin constitutes a different class of extracellular matrix polymer that form transiently during blood clotting and forms the provisional matrix though which inflammatory cells and fibroblasts move to repair tissue dam age and begin the regeneration of a collagen-based ECM. Collagen and fibrin fibers fall either in the semiflexible or stiff regime. Thick fibers that can be modeled as stiff filaments with ℓ_p on the order of hundreds of micrometers are observed when polymerizing collagen and fibrin at lower pH values, and/or with existence divalent cations such as calcium and magnesium [24, 25].

3.3. Elasticity of cytoskeletal, ECM, and synthetic polymer gels

The strain-dependent rheological properties of several of the most abundant cytoskeletal and ECM polymers are summarized in Figure 2. Networks formed by purified actin crosslinked by filamin A or purified vimentin increase their shear moduli as the shear strain increases to relatively modest strains of a few per cent for actin and 10-20% for the softer vimentin filaments. The magnitude of the shear modulus of vimentin network increases by more than an order of magnitude between 1% and 80% strain. The strain-stiffening of these networks is fully reversible, with the modulus retuning to low levels when the shear strain is reduced, unless the networks are strained to large enough magnitudes to break crosslinks between filaments. ECM proteins also form strain-stiffening gels, as shown by the increase in shear moduli of collagen and fibrin networks (Fig 2C,D) which stiffening at strains similar to those at which cytoskeletal networks stiffen. Gels of collagen filaments which are stiffer than fibrin fivers stiffen at smaller strain, similarly to the differences between actin and vimentin gels. Not all ECM components stiffen with strain, and numerous rheological

studies of elastin and elastin-related synthetic polymer networks show that elastin maintains a constant elastic modulus even at strains approaching 100%, consistent with the more flexible configuration of elastin polypeptides. In contrast to the non-linear elasticity of native cytoskeletal and ECM networks, the soft substrates most often used for cell culture studies, polyacrylamide and matrigel, (Fig 2E,F) show very little or no strain stiffening over the same range of strains where native polymer gels change stiffness by an order of magnitude or more.

Studies of purified synthetic and biopolymer systems such as those summarized in Figure 2 have led to a general model that hydrogels formed by flexible random coil polymers exhibit linear elasticity and relatively low elastic modulus compared to hydrogels with the same volume fraction of stiffer, more extended polymers such as those of the cytoskeleton or the ECM. An important limitation to this general conclusion is that strain stiffening requires strong crosslinks between the filamentous polymers that survive the forces concentrated on them as the strain in the networks increases. Gels of rigid polymers such as surfactant-stabilized carbon nanotubes or the filamentous bacteriophage Pf1 have large shear moduli when measured at small strain, but these systems soften with increasing strain in a manner consistent with the dynamic breaking and reformation of transient crosslinks formed by non-covalent bonds mediated by van der Waals interactions of counterions [26, 27].

4. Mechanisms of strain stiffening

The molecular mechanism for strain-stiffening in biopolymer gels has been the subject of extensive theoretical work. Two different models have been proposed, as summarized in Figure 3. Depending on the filament stiffness, network deformation can be accommodated by bending, stretching, and reorientation of individual filaments.

The linear elasticity of flexible polymer networks can be understood from the classical theory of rubber elasticity, which assumes affine (uniform) deformation due to similar stretching of each polymer in a network of flexible polymers. In this model, network elasticity comes from the entropic elasticity of individual Gaussian-like coiled filaments, and the linear force-extension relation of Gaussian coils gives rise to the linear elasticity of flexible polymer networks. The strain-stiffening of semiflexible polymer networks can, within a limited range of strain, also be accounted for by an entropic model which assumes that network deformation is uniform throughout the sample due to affine stretching of each semiflexible polymer. The elasticity comes from the resistance of each thermally writhing polymer against the stretching, which effectively reduces the amplitude of thermal fluctuation and thus the thermal entropy of the filament. MacKintosh et al. [28] derived a nonlinear relationship between the stretching force and extension for semiflexible polymers based on this entropic model. The nonlinear force-extension of individual polymers leads to strain-stiffening of the semiflexible polymer networks [28, 29].

An alternative explanation for strain-stiffening based on filament bending and enthalpic stretching has also been proposed for stiff filament networks [30–32]. In this model, the amplitude of thermal undulation is negligible in stiff filaments, and it is easier to bend the stiff filaments than to stretch them. Therefore, filaments initially bend at small network deformations and the network stiffness is mainly derived from the enthalpic bending of filaments. With increasing strain, the bent filaments reorganize so that more and more filaments align along the direction of shear. The network deformation then comes mainly from the enthalpic stretching of the aligned stiff filaments. This transition from bending to stretching gives rise to strain-stiffening. The bending, buckle and reorganization of filaments cause non-affine deformations in the network [32].

These two strain-stiffening models predict different network deformations: entropic model predicts uniform affine deformation; and enthalpic model predicts heterogeneous nonaffine deformation. Which theory works depends on the network structure and filament stiffness. It has been demonstrated that as crosslinker concentration decreases, the deformation of F-actin network transits from affine to nonaffine [33]. This change indicates that dense isotropic networks follow predictions of the entropic model, and the sparse networks follow predictions of enthalpic model. Significant non-affine deformations have been observed in collagen and fibrin gels [4, 34]. Collagen and fibrin gels are usually a mixture of protofibrils and large filament bundles. It is likely that both entropic model and enthalpic model contributes to the strain-stiffening of these gels [4]. Further details of theory and experimental studies of non-linear elasticity are summarized in recent reports [35–38].

5. Strain stiffening in tissues and biological constructs

The strain stiffening character of many biopolymer gels has many potential consequences in vivo.

The stiffness of tissues is tightly controlled under normal conditions [39]. Changes in tissue and organ stiffness occur during development and are frequently symptoms of diseases such as cancer [40, 41], fibrosis [42], and cardiovascular disease [43, 44]. Over the last 10 years, the importance of ECM stiffness as a mechanism for modulating cell shape and functions has become increasingly studied, and there is now good evidence that changes in stiffness not only correlate with disease, but might contribute to its development. For example, increased liver stiffness due to lysyl oxidase-mediated collagen crosslinking resulting from intoxication precedes cellular changes that produce fibrosis in the liver [45], and arterial stiffening predicts cardiovascular and renal outcomes in patients with diabetes [46]. The stiffness of tissue is not a simple constant as it is for linear elastic material, but depends on the time and extent to which the material is deformed. Also, since cells generate forces within the tissue, its stiffness can be altered both by changes in internal cell tension and in the imposition of external forces such as those from gravity or blood pressure.

5.1. Aorta stiffening

Perhaps the most thoroughly characterized strain-stiffening tissue is the artery. As the pressure within a blood vessel increases, the vessel diameter increases as it complaint walls distend, but the relation between pressure and diameter in an artery strongly differs from that of a tube made from a linear elastomer such as PDMS. Figure 4 shows how the Young's modulus (a measure of elastic resistance in a geometry appropriate for tube inflation [10]) changes as a function of the pressure within the lumen. For a linear elastic material, the quantity E would be independent of pressure, but for aortae, the Young's modulus increases by more than an order of magnitude over a range of pressures relevant to blood pressure in vertebrates. The importance of this non-linear response is shown by its conservation in animals with mean blood pressures that vary over a wide range, but when stiffness is plotted against pressures normalized by the mean pressure in each species, the data superimpose. Strain-stiffening in arteries is likely to arise from a combination of effects because this complex structure is composed of collagen, elastin, smooth muscle cells, and many other components arranged in a complex geometry, but the essential involvement of collagen fibers suggests that mechanisms such as those in Figure 3 are likely to contribute.

5.2. Fibrin-platelets

A structurally simpler example of cell-generated strain stiffening is shown in Figure 5, where the strain-dependent shear modulus of fibrin network (similar to that in Figure 2D) is shown when the fibrin network is formed by itself or with activated platelets within it. The

function of platelets is to extend long acto-myosin containing projections that attach to fibrin strands and then exert contractile forces that apply tension to the fibrin fibers. As a result the cells exert an internal strain within the network. The internal strain results in the same level of stiffening as does experimentally-imposed external strain. Fibrin network that have been pre-stressed by platelets have much larger shear moduli at small externally-imposed strains than do fibrin gels without platelets, but the elastic modulus of platelet-stressed fibrin no longer strain stiffens, presumably because the stretching and alignment of fibers needed for strain stiffening have already occur at zero imposed strain due to the activity of the cells. Similar stiffening of collagen networks by contractile cell has also been reported [9] and the effect requires actomyosin contractility and not only attachment of cells to the fibrin network [9, 47].

6. Effect on non-linearity on cell-cell interactions

The potential differences between linear and non-linear substrates on the response of mechanically sensitive cell are related to the resistance of the ECM to cell-generated forces and to the strain field that cells can generate within the ECM. Figure 6 illustrates the strain dependent differences between linear polyacrylamide gels and fibrin gels formed under conditions where both gels have the same shear modulus at small strains. Cell types such as neurons that are derived from very soft tissues and that exert relatively small forces on their substrates would respond similarly to mechanical signals from polyacrylamide and fibrin gels because they do not deform the gels enough to produce strain-stiffening in the fibrin gel. Previous studies have shown similar effects on the preferential growth of neurons on soft material formed by polyacrylamide and fibrin gels in vitro [48]. In contrast cells like fibroblasts or mesenchymal stem cells that can exert much larger traction forces might strain their substrates to levels great enough to stiffen a fibrin gel, and therefore any differential stress on fibrin in the strained state would meet with much larger resistance than from a polyacrylamide gels strained to the same extent. This difference in differential stiffness in the deformed state might account for the different responses of mesenchymal cells to fibrin monomer-coated polyacrylamide compared to intact fibrin [9].

A related difference between linear and nonlinear substrates is the pattern of the strain field exerted on these gels by contractile cells. Unlike linear polyacrylamide gels where the strain field decreases uniformly and approximately linearly with distance from the cell edge and becomes negligible within a few microns [49], the strain fields around collagen or fibrin or collagen gels extends significantly farther and can be highly non-uniform [9, 50, 51]. Figure 7 shows that the strain field within a fibrin network generated by a single contractile mesenchymal cell extends hundreds of microns away from the cell edge. The contraction of the network alters its properties in ways that might be sensed by neighboring cells. Since internal strain within a fibrin network can increase its shear modulus (Figure 5) a neighboring cell that moves into the stiffened region would respond to larger stiffness than a cell far away. The force generated by a cell could also be transmitted to the neighboring cell directly through the rigid fibers of a filamentous network. These mechanical changes can affect the distribution of cells as they move and proliferate in initially sparse cultures. As shown in Figure 7, cells on fibrin gels align their long axes toward each other even when they are several hundred microns apart, and likely too far to respond to any reciprocal chemical cues. Many cell types extend protrusions and move in the direction of increased stiffness [52], and the mechanisms of durotaxis combined with the local stiffening of nonlinear substrates by cell contraction might account for this effect, as suggested by a recent theoretical analysis [53]. However, a direct role for forces or matrix stiffness in these cell-cell interactions has not yet been unambiguously demonstrated in vitro. A possible aligning effect could also result not from direct force or stiffness changes, but from the change in geometry in a filamentous network under strain. As cells put fibrin or collagen

gels under strain, they also align the fibers in a direction parallel to the direction of force generation, and this alignment can extend very long distances from the contractile cells. Therefore, neighboring cells might use the aligned matrix fibers as tracks to guide their movement independent of any differences in local stiffness or force. Earlier studies concluded that matrix geometry rather than forces controlled cell movements [51, 54], and a recent study employing strain-stiffening composites made without rigid polymers comes to similar conclusions [55]. Therefore that extent to which non-linear rheology directly influences cell movements remains to be fully investigated.

7. Conclusions

Both the cytoskeleton and the extracellular matrix contain networks of semiflexible or rigid polymers that become stiffer with increasing strain, in contrast to linear elastic materials that maintain a constant stiffness independent of strain. The strain stiffening of biopolymer gels enables cells and tissue to rapidly alter stiffness by generating internal contractions by such mechanisms as activation of bipolar myosin filaments within the cytoskeleton or the contraction of fibrin or collagen gels by platelets or other contractile cells. The filamentous structure and non-linear rheology of some biopolymer networks also enables contractile cells to generate strain fields that extend much farther from their surface compared to the strain fields in linear elastomers, and the resulting changes in matrix stiffness and filament alignment can have effects on cell structure and movement that influence interactions between cells.

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Microtubule $\ell_p > 1 \text{ mm}$

F-actin $\ell_p \sim 10 \mu\text{m}$

IFs $\ell_p < 1 \mu\text{m}$

Polyethylene $\ell_p \sim 0.3 \text{ nm}$

Figure 1. Stiffness and structure of cytoskeleton filaments. Microtubule, F-actin and intermediate filaments of 10 micrometers are drawn on the same scale. Stiffer filaments with larger persistence length appear to be straighter. In comparison, a 10 μm flexible synthetic polyethylene chain is a random coil with a much smaller projected length.

Figure 2.

Shear storage modulus measurements of F-actin crosslinked by filamin (A), vimentin (B) collagen (C), fibrin (D), Matrigel (E), and polyacrylamide (F) measured at frequencies near 1Hz and a range of strain magnitudes. The solid symbols in C show results for G , the static shear modulus for a collagen gel of the same composition as used for oscillatory measurement of storage modulus. Data for A-E from [29], Data for E unpublished results, and F from [56].

Figure 3.

Entropic and enthalpic model for strain-stiffening. (a) Semiflexible polymers in the entropic under shear deformation. The red filament is stretched and resist to the shear stress; the yellow filament is being compressed with negligible contribution to the gel stiffness. (b) Stiff polymers in the enthalpic model under shear deformation. At small deformations, the red polymer is bent and contributes mainly to the gel stiffness at small deformations; the yellow filament rotates without contribution to the network stiffness. At large deformations, the red polymer buckles and the yellow filament is enthalpically stretched.

Figure 4.

Elastic moduli of aortae from different species as a function of inflation pressure. The data are normalized to the mean blood pressure for each species. Adapted from [2].

Figure 5. Effect of platelets on shear modulus of fibrin gels. Purified fibrinogen (3 mg/ml) was polymerized by addition of U/ml thrombin in solutions without (open circles) or with (closed symbols) platelets at a density approximately 50% that in blood plasma. Rheological measurements were done at 1 rad/s over range of strains. Data from [57].

Figure 6.

Comparison of strain-dependent shear moduli of polyacrylamide and fibrin gels, two commonly used soft substrates constructed to have comparable stiffness in the limit of small strains. Cells that apply little force on the matrix, such as neurons, would detect similar levels or resistance from both substrates, but cells such as mesenchymal stem cells that pull harder would deform the substrates so that fibrin would provide a larger resistance to additional stress than would polyacrylamide gels at large strains. Adapted from [9].

Figure 7.

Cell generated traction fields in fibrin gels, and the resulting effect on cell alignment. The left panel shows the direction and relative magnitude of strains generated by a single cell on the fibrin matrix surrounding it. The right panel shows that when cells are plated sparsely on fibrin gels, the long axes of spontaneously polarized cells are significantly oriented toward each other when cells are within 400 to 600 microns of each other, and a random orientation of cell direction occurs only at distances greater than 600 μm . Adapted from [9].