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Microscale mechanisms of agarose-induced disruption of collagen remodeling

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Abstract

Cells are strongly influenced by the local structure and mechanics of the extracellular matrix (ECM). We recently showed that adding agarose to soft collagen ECMs can mechanically stiffen these hydrogels by two orders of magnitude while limiting 3D cell motility, which we speculated might derive from agarose-mediated inhibition of collagen fiber deformation and remodeling. Here, we directly address this hypothesis by investigating the effects of agarose on cell-collagen interactions at the microscale. Addition of agarose progressively restricts cell spreading, reduces stress fiber and focal adhesion assembly, and inhibits macroscopic gel compaction. While time-of-flight secondary ion mass spectrometry and scanning electron microscopy fail to reveal agarose-induced alterations in collagen ligand presentation, the latter modality shows that agarose strongly impairs cell-directed assembly of large collagen bundles. Agarose-mediated inhibition of cell spreading and cytoarchitecture can be rescued by β-agarase digestion or by covalently crosslinking the matrix with glutaraldehyde. Based on these results, we argue that cell spreading and motility on collagen requires local matrix stiffening, which can be achieved via cell-mediated fiber remodeling or by chemically crosslinking the fibers. These findings provide new mechanistic insights into the regulatory function of agarose and bear general implications for cell adhesion and motility in fibrous ECMs.

Keywords

brain; cell adhesion; hydrogel; ECM (extracellular matrix); mechanical properties; elasticity

1. Introduction

The ability of the extracellular matrix (ECM) to present localized biomechanical and biochemical cues to resident cell populations is as critical to proper cell and tissue function

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as the structural support it provides. Indeed, it is now well established that the geometry, elasticity, and dimensionality of the ECM can act through specific cell-matrix adhesion receptors to control a wide variety of cell behaviors central to tissue homeostasis [1-5]. However, it has only recently begun to emerge that the microscale context of this signaling plays a critical role in modulating these interactions. For example, native ECMs are often structurally and mechanically anisotropic on the cellular length scale, and cells may locally remodel ECM fibers and other microcomponents to create microenvironments that instruct or permit cell behavior in ways that are not intuitively predictable from the bulk properties of the matrix [6-8]. Thus, the development of biomaterial systems that facilitate detailed interrogation of cell-ECM interactions at the microscale have the potential to yield fundamental insight into the nature of normal and perturbed crosstalk between cells and the surrounding ECM. This in turn could inform the design of material scaffolds that manipulate these interactions in tissue engineering and regenerative medicine applications.

A growing number of biomaterial systems allow manipulation of one or more microscale, matrix-linked cell-instructive cues, including microenvironmental mechanics, topography, and biochemistry [9-15]. Reconstituted collagen hydrogels have become one of the most commonly used 3D cell culture models for studying the effect of these cues, largely because type I collagen is the most abundant protein in the human body and can be readily reconstituted to form a 3D scaffold in vitro [16]. Importantly, the nonlinear elasticity of collagen and other fibrillar ECMs can render cellular responses to matrix biophysical properties dramatically different from those observed when similar matrix-linked cues are presented in linearly elastic biomaterial systems such as polyacrylamide and other highly crosslinked polymer networks [17, 18]. For example, the strain-stiffening behavior of 3D collagen gels can allow mammalian cells to spread on the surface of ECMs of low bulk elastic moduli that would otherwise only be expected to support a rounded cell morphology [8, 19]. This occurs because as cells deform individual collagen fibers by even modest amounts, the fibers stiffen dramatically and present an effective high-elasticity microenvironment even though the bulk elasticity of the material remains relatively low. Understanding cellular remodeling in these matrices is of particular physiological relevance given the ubiquity of type I collagen and other fibrous proteins in connective tissue matrices. Moreover, many if not most soft tissues in the body are comprised of fibrillar collagen meshed with non-fibrillar collagens and other components such as laminin and fibronectin; while our understanding of how cells interact with single-component fibrous matrices is slowly beginning to advance, relatively little is understood about how cell-ECM interactions and resulting cell behaviors are modified by the presence of both fibrillar and non-fibrillar ECM components.

Composite collagen-agarose hydrogels have recently been developed in which fibrillar collagen is co-assembled with the linear marine polysaccharide agarose, which is biocompatible but bioinert to mammalian cells [19-22]. We previously reported the creation of a collagen-agarose composite hydrogel system in which the mechanical properties of 0.5 mg/mL native collagen I hydrogels are directly modulated via incorporation of agarose [19]. We showed that adding agarose to soft collagen ECMs can mechanically stiffen these hydrogels by two orders of magnitude while limiting 3D cell motility, which we speculated might derive in part from agarose-mediated inhibition of collagen fiber deformation and remodeling. Through a combination of single-cell immunofluorescence and electron microscopy imaging, mass spectrometric analysis, and measurements of cell contraction, we now address these mechanistic hypotheses.

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2. Materials and Methods

2.1 Synthesis and characterization of ECM substrates

Collagen and collagen-agarose matrices were prepared as previously described [19], except PBS was used for matrix reconstitution instead of DMEM as noted. Pre-digestion of molten 2% agarose in PBS was accomplished via incubation with β-agarase (Lonza, Rockland, ME) for 2 h at 45°C according to manufacturer's instructions; the solution was heated to 95°C to fully inactivate the β-agarase prior to synthesis of collagen-agarose hydrogels. Matrix crosslinking was accomplished following full gelation via overnight incubation with a superlayer of 0.2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA). Glutaraldehyde-treated hydrogels were washed extensively with PBS and soaked in 8% L-glutamic acid, pH 7.4, for 48 h to quench residual glutaraldehyde [23] prior to extensive washing and cell seeding on the gel surface.

2.2 Cell culture

U373-MG human glioma cells were obtained from the Tissue Culture Facility at the University of California, Berkeley and cultured in Dulbecco's Modified Eagle medium (DMEM, Life Technologies, Grand Island, NY) supplemented with 10% Calf Serum Advantage (J.R. Scientific, Woodland, CA) and 1% penicillin/streptomycin, MEM nonessential amino acids, and sodium pyruvate (Life Technologies).

2.3 Electron microscopy

Silicon wafers were functionalized with 0.1 N NaOH, 3′aminopropyltriethoxysilane, and 0.5% glutaraldehyde and washed extensively with deionized water prior to sterilization with 70% ethanol. Collagen-agarose solutions were dispensed onto treated silicon wafers prior to gelation, and incubated at 37°C at high humidity for at least 45 minutes prior to cell seeding. Cells were cultured for 48 hours prior to fixation, dehydration, and sputter-coating for electron microscopy as previously described [19]. We note that β-agarase-digested agarose oligomers are alcohol-soluble, as per manufacturer's information; therefore, when imaging ethanol-dehydrated SEM samples from our digestion studies, we selected regions of the gels in which the agarose oligomers were not completely washed away during sample processing.

2.4 Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) measurements and Principal Component Analysis (PCA)

Collagen-agarose solutions were prepared in PBS, polymerized as thin films on glass slides and dried at 37°C. ToF-SIMS measurements were carried out with a TOF-SIMS V instrument (ION-TOF GmbH, Germany), by using 25 keV Bi_3^{2+} primary ions. The analysis area of 150 × 150 μm^2 was randomly rastered by the primary ions and was charge-compensated for glass-slide samples by low-energy electron flooding. The primary ion dose density was maintained below 1.0×10^{12} ions/cm^2 to ensure static SIMS conditions. Mass resolution was higher than 5000 at $m/z < 500$ in both positive and negative modes. To conduct PCA analysis, 3 positive and 3 negative spectra were acquired from each sample, respectively. For internal mass calibration, CH_3^+, $C_2H_3^+$ and $C_3H_5^+$ peaks were used for positive spectra and CH^-, C_2H^- and C_3H^- peaks were used for negative spectra. PCA analysis was performed on SIMS spectra using a PLS_Toolbox (version 5.2, Eigenvector Research, Manson, WA) for MATLAB (version 7.1, MathWorks Inc., Natick, MA). Each peak was selected by using the auto-peak searching algorithm in the Ion-Specs software (ION-TOF GmbH, Germany) and normalized by using the summation of the intensity of the selected peaks to eliminate systematic differences between the spectra, and then mean-centered before PCA [24,25].

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2.5 Phase contrast and fluorescence microscopy

All phase contrast and fluorescence imaging was performed using an inverted Nikon TE2000-E2 microscope equipped with a motorized, programmable stage (Prior Scientific, Inc, Rockland, MA), an incubator chamber to maintain constant temperature, humidity, and CO_2 levels (In Vivo Scientific, St. Louis, MO) during live-cell imaging, a digital camera (Photometrics Coolsnap HQ II, Roper Scientific, Tucson, AZ), and SimplePCI software (Hamamatsu Corporation, Sewickley, PA). Immunofluorescence staining for F-actin, nuclear DNA, and the focal adhesion protein vinculin was accomplished as previously described in detail [18].

2.6 Hydrogel contraction assays

Collagen-agarose matrices were prepared with U373-MG cells fully embedded in 3D or plated on the 2D surface of fully polymerized gels as noted. After 24 hours of culture, gels were carefully detached from the edges of each well with a thin, sterile blade and allowed to contract for at least 48 hours prior to imaging with a handheld Canon PowerShot SD780 IS digital camera set in macro mode. Percentage gel contraction was measured using ImageJ software to manually identify the edge of the well and contracted gel, respectively [26]. Data reported are mean ± standard deviation for 4 gels per condition in one experiment. Experiments were repeated in triplicate with qualitatively consistent results.

2.7 Parallel plate rheometry

Collagen-agarose matrices were prepared in PBS and gelled at 37°C and high humidity for at least 2 h prior to rheological analysis. All measurements were taken with an Anton Paar Physica MCR-301 rheometer (Anton Paar USA, Temecula, CA) at 37°C and high humidity using a 25 mm parallel plate geometry and Rheoplus software. Frequency sweeps at 5% strain over the range 0.1–10 Hz were used to extract the storage modulus, loss modulus, and complex modulus of each sample as previously described [19]. At least five samples were measured for each gel formulation; reported shear moduli represent mean ± standard deviation at 1 Hz.

3. Results

3.1 Cell-mediated bundling of collagen fibers

We began by directly visualizing associations between cells and collagen fibers in the presence of varying concentrations of agarose. In our previous report, we used live-cell Nomarski differential interference contrast (DIC) microscopy to image glioma cells and surrounding collagen fibers simultaneously, and saw evidence of enhanced collagen bundling in low-agarose content gels [19]. However, the limited spatial resolution of DIC imaging makes it difficult to visualize all but the largest collagen bundles or to colocalize bundles with cellular migratory processes. Thus, to investigate this finding more deeply, we began here by imaging glioma cells on collagen and collagen-agarose matrices using scanning electron microscopy (SEM). SEM of glioma cells adherent to the surface of native 0.5 mg/mL collagen I hydrogels revealed clear cell-directed bundling of collagen into large fibers, which was especially pronounced at the tips of cell protrusions into the matrix (Fig. 1). When agarose was introduced at low concentrations, the intercalating agarose network modestly restricted glioma cell spreading and collagen fiber bundling (Fig. 2). However, at high agarose concentrations, cells on the gel surface were rounded and appeared to have restricted capacity for remodeling, as evidenced by a decrease in the number of collagen fiber bundles oriented radially away from the cell body (Fig. 3). In these cases, the random orientation of collagen fibers located on the gel surface a short distance away from the cell body supports our previous speculation that the presence of agarose restricts long-range

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transmission of cell-generated forces through collagen matrices [19]. We note that the apparent change in the surface density of collagen fibers as agarose content is increased can be partially attributed to incidental compaction of the gels during SEM sample preparation [27] and partially attributed to apparent differences in localized cell-mediated compaction of collagen fibers during culture, a process that is strongly regulated by the pericellular microenvironment [28]. We also note that our previous in situ imaging studies of cell-free matrices revealed no appreciable difference in collagen microstructure between 0.5 mg/mL collagen gels containing 0% and 0.25% w/v agarose [19].

3.2 Molecular structure of collagen

Cell adhesion and remodeling of collagen-agarose matrices can be affected not only by matrix mechanics but also by the surface chemistry of exposed collagen binding motifs in the matrices. Thus, we investigated changes in the molecular conformation of collagen fibers at the surface of dried collagen-agarose matrices with time-of-flight secondary ion mass spectrometry (ToF-SIMS). ToF-SIMS can characterize the surface chemistry of protein films because of its chemical selectivity and surface sensitivity with a sample depth of ~1 nm [29]. We obtained ToF-SIMS positive spectra from collagen-agarose matrices of varying composition (Fig. 4A). As described previously [24, 25], by applying the multivariate principal component analysis (PCA) technique, we were able to reduce a complex set of ToF-SIMS spectra to quantitatively interpretable scores and loading plots, which reflect the relationship among samples as well as the relationship between original variables (spectral peaks) and new variables (principal components), respectively.

We completed PCA for peak lists selected by an auto peak search of the positive ToF-SIMS spectra acquired from collagen-agarose hydrogels, as well as pure collagen and pure agarose. Scores and loadings for the first principal component (PC 1) from this analysis show that the surface chemistry of 0.5 mg/mL collagen gels does not vary significantly across matrix formulations as the agarose content is increased to 0.4% w/v (Fig. 4B,C). The loading plots for PC 1 reveal differences in the compositions of the outer surface of the matrices. For peaks loading positively on PC 1 such as 30 (cysteine), 44 (alanine/cysteine/lysine), 70 (arginine/leucine/proline/threonine) and S containing SO_3H, C_2SO_2, and C_3SO_2 peaks, the intensities contribute to positive PC 1 scores. Thus, the relative concentrations corresponding to these peaks should be higher in samples with positive PC 1 scores. Conversely, for peaks loading negatively on PC 1 such as Na containing C_2HNa and $HSNa_2$ peaks and agarose fragment peaks of *m/z* 165, 180, 186, 196, 289, and 305, the corresponding surface species should be more prevalent in agarose. The scores on PC 1 of collagen and agarose mixture gels with agarose content up to 0.4% w/v (0.5C/0.4A) are similar to that of pure collagen fibers. This suggests that the addition of agarose does not modify the surface composition or molecular configuration of collagen fibers in 0.5 mg/mL collagen matrices up to 0.4% w/v agarose.

3.3 Cellular adhesion and contractility

Our imaging and ToF-SIMS data suggest that the observed differences in glioma cell spreading and bundling of collagen fibers are not attributable to initial differences in exposed collagen as the concentration of agarose is increased. Because engagement of ECM fibers by adhesion receptors is a necessary first step for generation of contractile forces against these fibers, we next asked whether an increasing agarose concentration also compromises the ability of cells to mobilize the intracellular adhesive and contractile machinery. To assess microscale cell contractility, we used immunofluorescence imaging to visualize actin cytoskeletal assembly and focal adhesion maturation. We found that glioma cells on low-agarose content gels form actomyosin stress fibers and robust vinculin-positive focal adhesions, and that these structures become progressively less prominent as the

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agarose content of the matrix is increased (Fig. 5A, B). We then evaluated macroscale contractility by conducting bulk gel contraction assays in which U373-MG cells were fully embedded inside gels with varying agarose content. Here, measurements of projected gel area were taken 48 h after the gels were detached from the edges of the wells as a macroscopic measure of the ability of embedded cells to remodel and contract the ECM. We found that gel contraction is inversely related to agarose content, with cells compacting gels by 40-50% at low agarose concentrations and <5% at high agarose concentrations (Fig 5C). Together, these two data sets support the notion that cells on agarose-poor gels are in a more highly contractile state and are able to deform and remodel individual collagen fibers to a much greater extent than cells in agarose-rich gels.

3.4 Chemical crosslinking and agarose digestion

The above results indicate that incorporation of agarose into collagen ECMs reduces cell contractility. This is somewhat surprising given that we previously showed that agarose increases the elasticity of entangled 0.5 mg/mL collagen networks by several orders of magnitude without appreciably altering the collagen microstructure [19]; traditionally, one would expect increasing matrix stiffness to be associated with increased cell spreading and contractility [18]. We reasoned that this apparent contradiction could be explained by considering the non-affine mechanical properties of fibrillar matrices, where the non-linear propagation of applied stresses in collagen gels stands in stark contrast to the propagation of stresses through linearly elastic culture materials such as polyacrylamide [8, 19]. In other words, even though agarose increases the *bulk* stiffness of collagen hydrogels, it reduces *microscale* stiffness because it interferes with the strain-stiffening and remodeling properties of collagen. To directly test this hypothesis, we investigated the effects of two additional modifications to our collagen matrices: disruption of collagen fiber remodeling via covalent crosslinking and partial disruption of the agarose network via enzymatic digestion. We reasoned that because covalent crosslinking stiffens the matrix on all length scales but reduces the ability of cells to remodel and compact the collagen network, it should rescue cell spreading and elaboration of stress fibers and focal adhesions without rescuing collagen bundling or bulk gel contraction. Conversely, partial digestion of the agarose should rescue both cell spreading and cell-mediated matrix remodeling for all gel formulations because it disrupts the intercalating agarose network, restores the collagen to a nonlinearly elastic regime, and liberates it for cell-directed remodeling.

Indeed, we found that cells are able to spread on the surface of glutaraldehyde-crosslinked collagen-agarose matrices, including gels containing 0.5% w/v agarose that would otherwise result in cell rounding (Fig. 6; Supplementary Fig. 1). Importantly, these glutaraldehyde-crosslinked gels lack the prominent cell-directed fiber bundles characteristic of pure collagen gels, showing that cells can spread on these matrices without requiring substantial matrix remodeling. It also confirms that the agarose does not simply act by sterically blocking adhesion sites on collagen, as glutaraldehyde crosslinking should in principle be unable to overcome such barriers. Similarly, we find that partial enzymatic pre-digestion of agarose with β-agarase rescues cell spreading and matrix reorganization on high-agarose content matrices (Fig 6; Supplementary Fig. 2). Under these digestion conditions, the agarose still lightly coats the collagen fibers without forming a continuous network throughout the hydrogel, indicating that the presence of an intact agarose network, and not merely the constituent agarose oligomers, is required to induce the cell rounding and decrease in cell contractility observed in high-agarose content matrices. We note that doubling the collagen concentration to 1 mg/mL also enhances cell spreading on the surface of gels containing 0.5% w/v agarose (Fig 6), illustrating that a substantial increase in initial ligand availability can also rescue cell spreading on high-agarose content matrices.

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To gain additional insight into the mechanism governing the observed rescue of cell spreading on these modified matrices, we first used parallel plate rheology to evaluate the bulk shear modulus of each gel formulation. As expected, we found that glutaraldehyde crosslinking stiffens low-agarose content gels; with increasing agarose content, the elastic moduli of crosslinked and non-crosslinked matrices converge, presumably as the gels transition from a regime in which gel mechanics are dominated by the collagen network to one in which the agarose network is the main determinant of gel mechanics (Fig 7A). Likewise, β-agarase digestion abrogated agarose-induced gel stiffening in all gel formulations. We note that a decrease in measured shear moduli here compared to our previous measurements [19] is not unexpected since these samples were subjected to more extensive handling and washing over multiple days following glutaraldehyde and L-glutamic acid treatment, and the slightly higher ionic strength of PBS (0.193) than that of DMEM (0.169) [30] would be expected to result in softer gels as well [31]. Finally, we evaluated the effect of collagen crosslinking and agarose digestion on cell-mediated matrix remodeling. To do so while avoiding glutaraldehyde cytotoxicity, we modified our contraction assay to plate cells on the 2D surface of fully polymerized gels. We found that glutaraldehyde restricts the ability of cells to contract gels at all agarose concentrations, whereas β-agarase digestion rescues this ability (Fig. 7B). Immunofluorescence imaging of cellular cytoskeletal and adhesive structures on glutaraldehyde-crosslinked 0.5C-0.5A gels reveals actomyosin stress fibers, indicating that cells are in a highly contractile state despite their inability to contract the gels (Fig. 7C).

4. Discussion

We previously demonstrated that incorporation of agarose can increase the shear modulus of soft 0.5 mg/mL type I collagen ECMs by two orders of magnitude while limiting 3D glioma cell invasion, which we speculated might be partly derived from agarose-mediated inhibition of collagen fiber deformation and remodeling [19]. Here, we have provided direct mechanistic support for this hypothesis. Despite the fact that agarose does not affect collagen presentation in matrices containing up to 0.4% w/v agarose, it strongly decreases glioma cell contractility and matrix remodeling. Either chemical crosslinking of these composite matrices with glutaraldehyde or enzymatic digestion with β-agarase rescues agarose-induced inhibition of cell spreading and cytoarchitecture, whereas only β-agarase digestion (but not glutaraldehyde crosslinking) rescues cell-mediated bulk matrix contraction. Thus, cell spreading and motility on collagen requires local matrix stiffening, which can be achieved either by chemically crosslinking the fibers or by allowing the cells to remodel the fibers into large bundles. Agarose precludes this local stiffening because it prevents bundling while also failing to mechanically couple the fibers and offer resistance to cellular contractile forces. However, whereas cell-mediated bundling is a local phenomenon that cumulatively leads to gel contraction on the macroscale, chemical crosslinking stiffens the gel globally and therefore makes the gel resistant to macroscopic contraction. In other words, progressive incorporation of agarose increases the macroscale stiffness of collagen hydrogels while interfering with the ability of embedded cells to locally stiffen their environment, resulting in an effective *softening* of the cell-scale microenvironment in high agarose-content gels.

In addition to providing new insight into the mechanisms by which agarose interferes with cell-mediated collagen remodeling, our findings have general implications for the micromechanics of composite fibrillar-nonfibrillar ECMs and the use of such matrices in tissue engineering and regenerative medicine applications. First, our results indicate that cell spreading, local matrix remodeling, bulk matrix contraction, and cellular contractility are not necessarily correlated in matrices containing both fibrillar and non-fibrillar components. This disparity underscores the inherent complexity of 3D culture systems; as engineered

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biomaterial platforms become increasingly sophisticated in an effort to recapitulate the complexity of native tissues, associated design principles must anticipate and account for incongruities across length scales in cellular responses to fibrillar, nonfibrillar, and hybrid fibrillar-nonfibrillar matrix components. Second, these findings lend insight into the function of collagen and other fibrillar ECM components in defining matrix mechanics in vivo and in 3D culture systems. Specifically, it is important to understand the degree to which non-fibrillar components facilitate or interfere with microscale cell-directed remodeling of fibrillar components, as this remodeling may critically underlie establishment of polarity, migration, and other behaviors. Similarly, these nonfibrillar components may alter bulk matrix properties in ways that are uninformative or even misleading with respect to predicting cell responses; microscale matrix properties are much more relevant. A contemporaneous and independent study involving high-resolution rheological and optical characterization of cell-free collagen-agarose hydrogels seems broadly supportive of these ideas and specifically supports the notion that although collagen is traditionally thought of as the primary load-bearing element in most tissues, nonfibrillar ECM components can play a significant role in modulating cell-fiber interactions and overall tissue mechanics [32]. With this in mind, additional methods will be needed to quantify microscale matrix mechanics in three-dimensional systems, such as those based on particle tracking microrheology [33] and inference of collagen fiber mechanics from image correlation spectroscopy [34].

To expand upon this last point, our observation that agarose can increase the bulk stiffness of collagen networks while effectively decreasing the local stiffness of the cell-scale microenvironment corresponds quite well to a theoretical study by Chandran and Barocas in which the underlying affine mechanics of a collagen network model was seen to be markedly different than a model of individual collagen fibril kinematics, which was instead found to be non-affine in nature [35]. Because cells interact with collagen ECMs on the length-scale of individual fibers or bundles of fibers, it seems intuitive that cellular behavior should be correlated more with individual fiber mechanics than with macroscale hydrogel mechanics. This concept is also consistent with the recent observation that cellular compaction of collagen gels is limited to a zone that extends only tens of micrometers from the cell surface, which strongly implies that cell-directed remodeling produces a local microenvironment with dramatically different mechanical properties than the macroscale material [28].

We may also place our findings in the context of other recent studies of nonlinear matrix elasticity and its effect on cell-ECM interactions. Our observations of cell spreading and matrix remodeling in nonlinear collagen ECMs are in good agreement with a recent report by Winer et al., in which local strain-stiffening and non-linear propagation of cell-generated forces were seen to give rise to long-distance cell-cell communication and cell-fiber alignment during fibroblast or human mesenchymal stem cell culture on fibrin matrices [8]. Importantly, pharmacologic inhibition of cellular contractility was seen to disrupt strain-stiffening behaviors on fibrin gels, underscoring the interdependence of cell mechanics and matrix mechanics, and reinforcing the link between cellular force generation and strain-stiffening of fibrillar matrices. These ideas have important implications for the design of tissue engineering constructs and for our understanding of the relationship between tissue stiffening and disease progression in a variety of contexts. For example, Provenzano and colleagues have observed distinctive patterns of collagen fiber reorganization into radially-oriented bundles at the tumor-stromal interface in vivo and in vitro that have been associated with tumor cell invasion [36]. More recently, they have shown that the presence of these “tumor associated collagen signatures” both depends on generation of cellular contractile forces and is necessary to facilitate efficient invasion, suggesting that the ability to manipulate nonlinear ECMs is a critical early step in tumor progression [37]. As a second example, the initiation of liver fibrosis has been associated with early-stage tissue stiffening

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resulting from modifications to the native collagen ECM [38], suggesting that the disease implications of these ideas extend beyond tumorigenesis.

Finally, while our collagen-agarose hydrogel system is not intended to be a physiological mimic of native tissues, we anticipate that this system will be valuable for further interrogation of cell-ECM interactions and the interactions between collagen fibers and nonfibrillar matrix components. Indeed, progress on this problem will depend on the creation of innovative 3D biomaterial systems that are collectively capable of deconstructing the rich biochemical and mechanical diversity of the cellular milieu into experimentally tractable numbers of distinct interactions [39]. We anticipate that the complementary insights gleaned from such 3D cell culture systems will ultimately bring us closer to the realization of 3D culture methodologies that truly can bridge the gap between current 2D platforms and animal models, as well as a maturation of our understanding of cell-ECM interactions that will facilitate creation of theoretical models with predictive power in complex tissue environments.

5. Conclusions

We have evaluated the microscale mechanisms of agarose-induced disruption of collagen remodeling by human glioma cells in composite collagen-agarose matrices. By combining high-resolution and macroscale measures of cellular contractility and matrix remodeling, we have provided direct support for the hypothesis that incorporation of agarose plays dual roles by increasing the bulk stiffness of collagen hydrogels while reducing microscale stiffness via disruption of the strain-stiffening and remodeling properties of collagen. Our results have general significance for the micromechanics of composite fibrillar-nonfibrillar ECMs in vivo and the use of such matrices in tissue engineering and regenerative medicine applications, and we anticipate that the approaches described in this study will prove valuable for detailed investigation of cell-ECM interactions in both fibrillar and composite fibrillar-nonfibrillar matrices.

Supplementary Figure Captions

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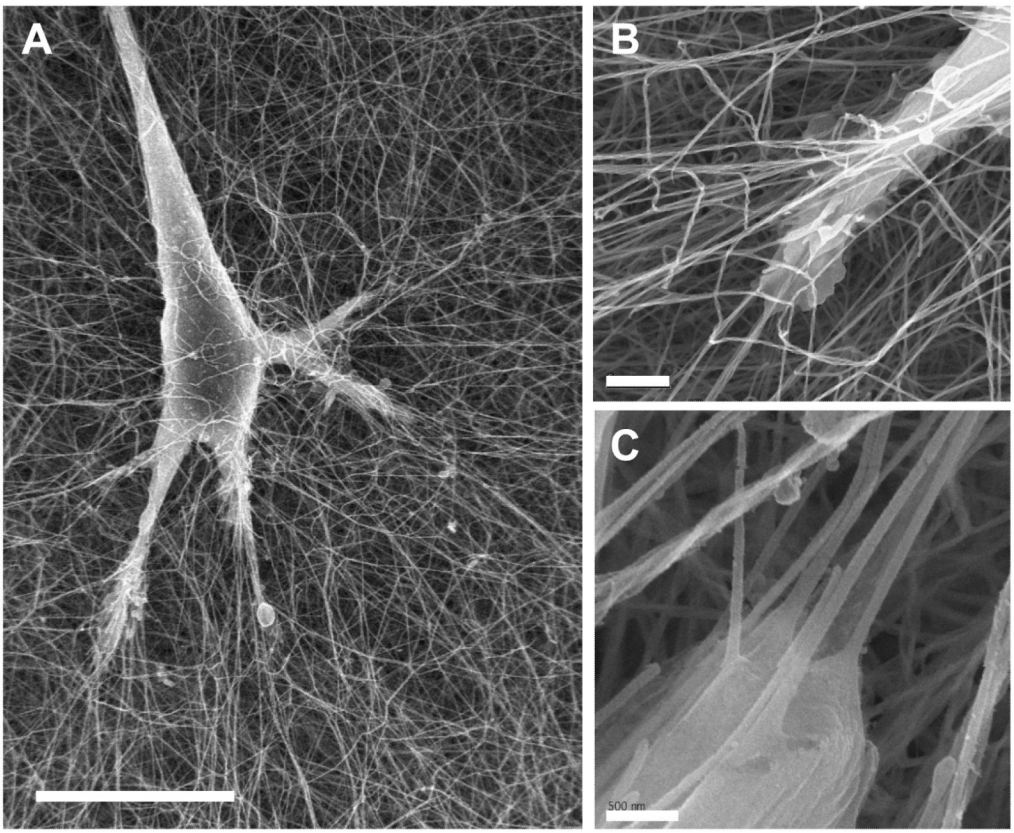

Figure 1.
Scanning electron microscopy images of a U373-MG glioma cell on a 0.5 mg/mL collagen matrix. A. Low magnification image of a single cell. Bar is 20 μm. B. High magnification image of a cell protrusion in the collagen matrix. Bar is 2 μm. C. The tip of a cell protrusion with bound collagen fibers. Bar is 500 nm.

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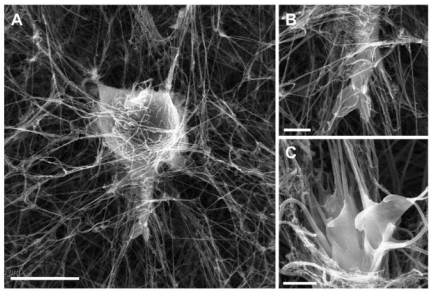

Figure 2.

Scanning electron microscopy images of a U373-MG glioma cell on a 0.5 mg/mL collagen matrix containing 0.125% w/v agarose. A. Low magnification image of a single cell. Bar is 10 μm. B. High magnification image of a cell protrusion in the matrix. Bar is 2 μm. C. The tip of a cell protrusion with bound collagen fibers. Bar is 1 μm.

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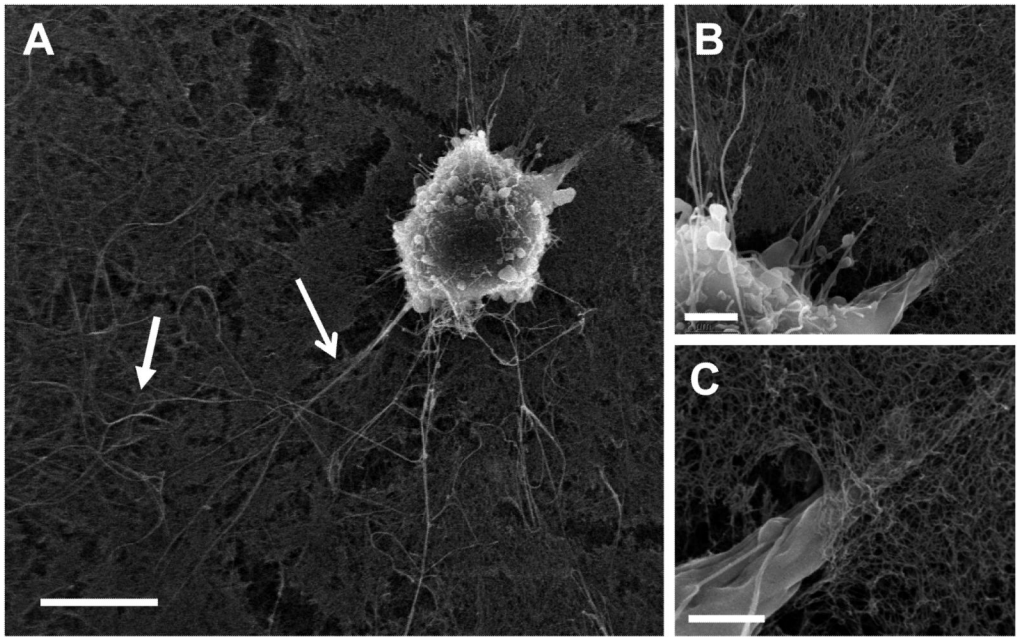

Figure 3.
Scanning electron microscopy images of a U373-MG glioma cell on a 0.5 mg/mL collagen matrix containing 0.5% w/v agarose. A. Arrows highlight randomly oriented native collagen fibers in the matrix (closed), in contrast to the aligned collagen fibers found near glioma cells (open). Bar is 10 μm. B. Aligned collagen fibers extending into the matrix at the cell edge. Bar is 2 μm. C. A cell protrusion extends through the agarose matrix. Bar is 1 μm.

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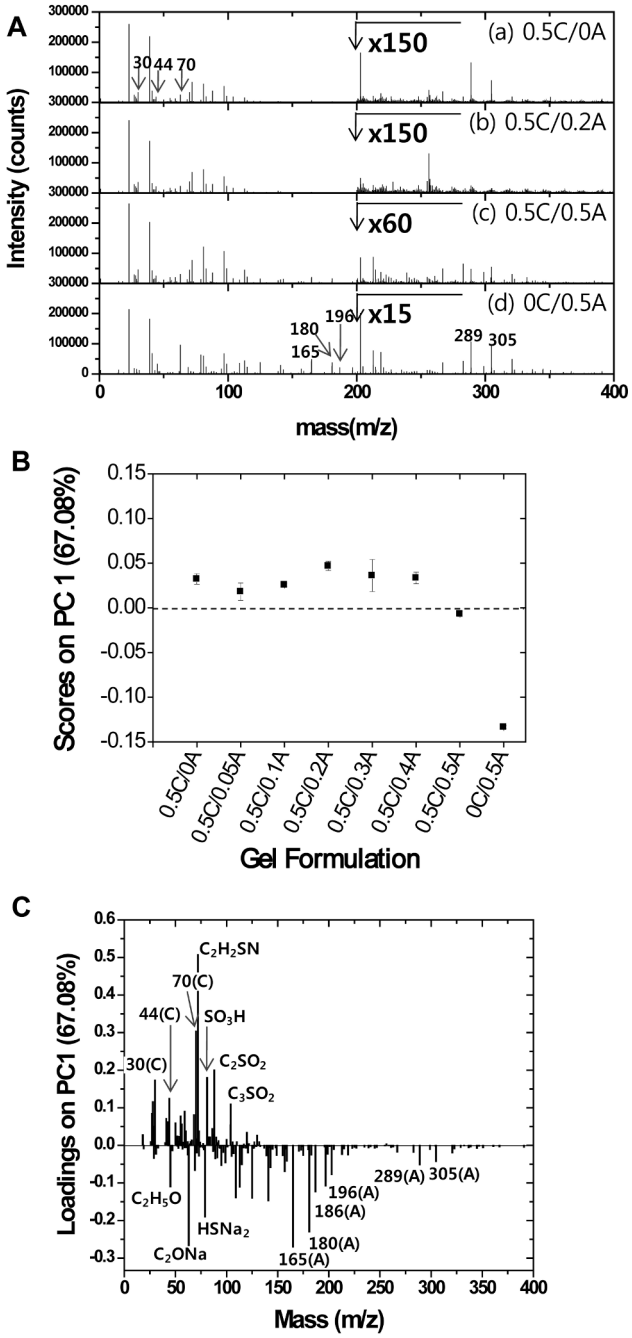

Figure 4.

Effect of agarose content on the molecular conformation of collagen fibers in matrices containing 0.5 mg/mL collagen (0.5C) and 0 – 0.5% w/v agarose (0A – 0.5A). A. Positive ToF-SIMS spectra obtained from samples containing (a) 0.5C, (b) 0.5C/0.2A, (c) 0.5C/0.5A, and (d) 0C/0.5A. B,C. Plots of PC 1 (B) scores and (C) loadings from PCA of positive ion spectra obtained from collagen-agarose gels with indicated compositions. Peaks were selected by auto peak searching.

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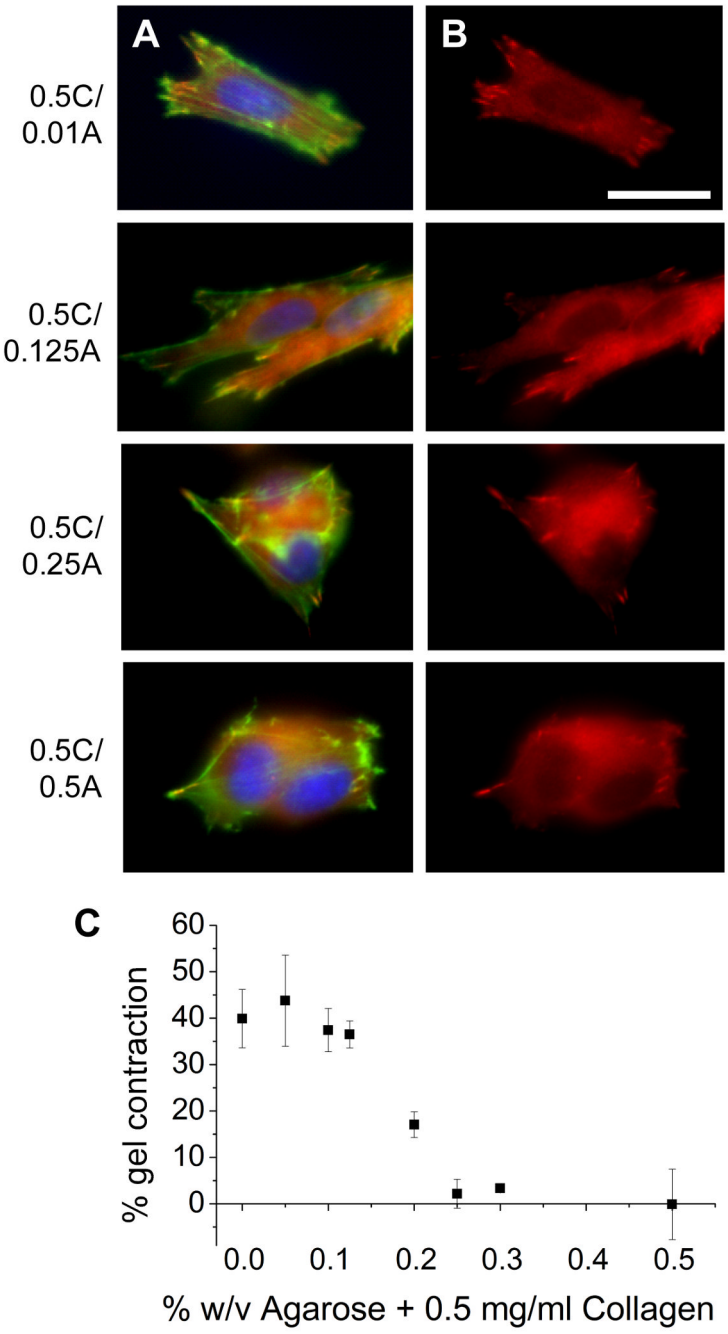

Figure 5.

Effect of agarose content on cellular contractility. A. Fluorescence imaging of cytoskeletal and adhesive structures of U373-MG cells cultured on the surface of 0.5 mg/mL collagen gels (0.5C) containing 0 – 0.5% w/v agarose (0A – 0.5A). Cells were stained for F-actin (green), nuclear DNA (blue) and the focal adhesion protein vinculin (red). B. Isolated view of vinculin signal only. Bar is 25 μm. C. Percentage gel contraction for U373-MG glioma cells cultured within the 3D matrix, measured 48 hours after detachment.

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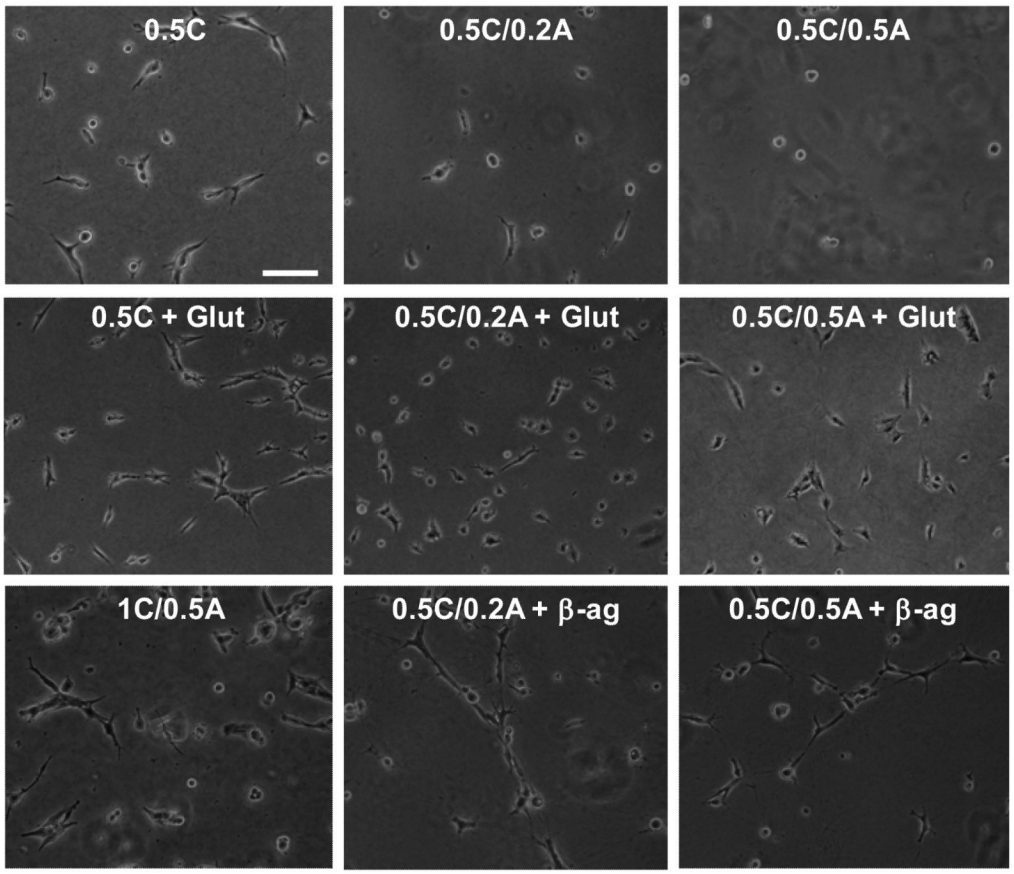

Figure 6.

Effect of agarose degradation or collagen crosslinking on glioma cell morphology. Live-cell phase contrast imaging of U373-MG cells cultured for 24-48 hours on the surface of matrices containing 0.5 – 1 mg/mL collagen (0.5C – 1C) and 0 – 0.5% w/v agarose (0A – 0.5A). Crosslinking was accomplished via overnight incubation with 0.2% glutaraldehyde following gel polymerization (+Glut). β-agarase was used for pre-digestion of agarose prior to gel synthesis (+β-ag). Scalebar is 100 μm.

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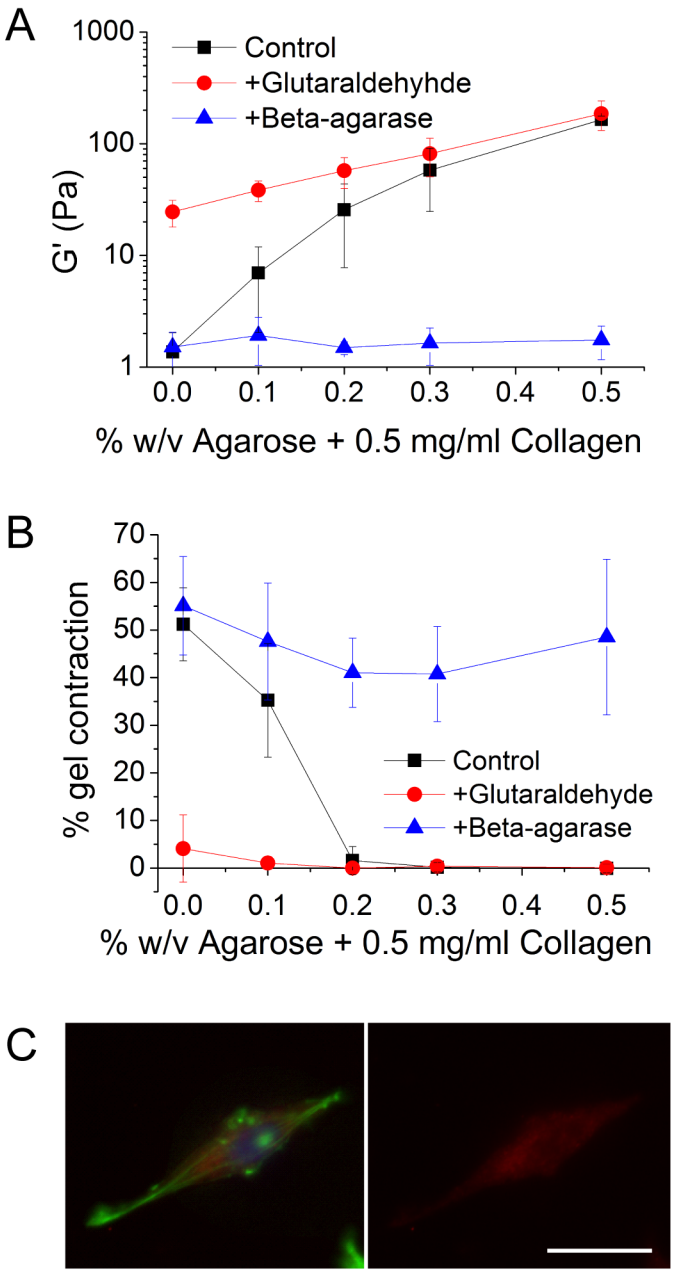

Figure 7.

Effect of agarose degradation or collagen crosslinking on glioma cell contractility. A. Elastic modulus of 0.5 mg/mL collagen gels containing 0 – 0.5% w/v agarose that were treated with 0.2% glutaraldehyde overnight following gel polymerization (+Glutaraldehyde) or that were created with agarose that had been pre-digested with β-agarase prior to gel synthesis (+Beta-agarase). B. Percentage gel contraction for U373-MG cells cultured on the gel surface, measured 72 hours after gel detachment. C. Fluorescence imaging of cytoskeletal and adhesive structures of U373-MG cells cultured on the surface of 0.5C/0.5A + Glutaraldehyde gels. Cells were stained for F-actin (green), nuclear DNA (blue) and the focal adhesion protein vinculin (red). Bar is 25 μm.