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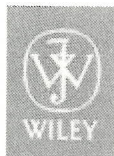

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Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence

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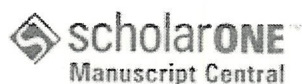

Journal of Biomedical Materials Research: Part A

Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence

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Genipin-induced changes in collagen gels

Abstract

Controlled crosslinking of collagen gels has important applications in cell and tissue mechanics as well as tissue engineering. Genipin is a natural plant extract that has been shown to crosslink biological tissues and to produce color and fluorescence changes upon crosslinking. We have characterized the effects of genipin concentration and incubation duration on the mechanical and fluorigenic properties of type I collagen gels. Gels were exposed to genipin (0, 1, 5, or 10mM) for a defined duration (2, 4, 6, or 12hrs). Mechanical properties were characterized using parallel plate rheometry, while fluorigenic properties were examined with a spectrofluorimetric plate reader and with a standard, inverted epifluorescent microscope. Additionally, Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize and track the crosslinking reaction in real-time. Genipin produced significant concentration- and incubation-dependent increases in the storage modulus, loss modulus, and fluorescence intensity. Storage modulus was strongly correlated to fluorescence exponentially. Minimal cytotoxicity was observed for exposure of L929 fibroblasts cultured within collagen gels to 1mM genipin for 24 hrs, but significant cell death occurred for 5mM and 10mM genipin. We conclude that genipin can be used to stiffen collagen gels in a relatively short time frame, that low concentrations of genipin can be used to crosslink cell-populated collagen gels to affect cell behavior that is influenced by the mechanical properties of the tissue scaffold, and that the degree of crosslinking can be reliably assayed optically via simple fluorescence measurements.

Genipin-induced changes in collagen gels

Keywords: crosslinking, collagen, tissue engineering, rheology, mechanotransduction, FTIR

Genipin-induced changes in collagen gels

Introduction

It is now clear that in many tissue systems, the mechanostructural properties of the extracellular matrix contribute to the regulation of cellular functions in addition to the mechanical functions of the tissue [1,2]. To investigate these phenomena, and to properly design bioartificial, tissue-engineered replacements, it is frequently desired to control the mechanical properties of biomaterials. Collagen-based tissue equivalents are of special interest, largely because collagen is a primary mechanostructural element in many connective tissues, including dermis, blood vessels, tendons, and ligaments [3-6]. Additionally, collagen's superior biocompatibility and nearly ubiquitous bioactivity have made it one of the most extensively investigated biomaterial scaffolds for engineering the tissues listed above, and others, including hepatic [7] and neural tissues [8]. It is, therefore, critical to maintain the ability to manipulate the mechanical properties of collagen gels, both to study mechanotransduction and to improve the properties of bioartificial tissues. While the properties of a collagen scaffold can be altered by merely changing the concentration of collagen monomers prior to self-assembly, thereby making a more concentrated gel, most often a crosslinking mechanism is implemented.

A variety of methods exist to crosslink collagen. In vivo, tissues are naturally crosslinked by enzymes such as lysyl oxidase [9,10] and transglutaminase [11,12]. However, use of these enzymes for bulk changes in mechanical properties in cultured tissue mimics is cost prohibitive. Chemical treatments with aldehydes are often used to preserve and stiffen tissues. However, for cell-populated collagen gels (often termed "tissue equivalents"),

these treatments are highly toxic. Non-enzymatic glycation has been used to improve the mechanical properties of bioartificial blood vessels in vitro by including a reducing sugar, such as ribose, in the culture medium [13]. However, the concentrations necessary to achieve sufficient crosslinking to significantly affect the mechanical properties in a timely manner (<1-2 weeks) are toxic, requiring longer incubations at lower concentrations. Irradiation with ultraviolet (UV) light has also been used to crosslink collagen [14], but has limited use in cellular tissues and tissue equivalents because of the potential for UV-mediated DNA degradation. Furthermore, UV light may crosslink thicker tissues non-uniformly. Non-enzymatic nitration, which is linked to many age-associated changes, including alterations in collagen connective tissues consistent with nitrite end-products of nitric oxide, has been shown to increase type I collagen crosslinking and deplete tyrosine residues, and is not immediately cytotoxic [15]. Nitrites can also alter the structure of other proteins and enzymes to affect their regulatory functions [16].

Recently, genipin, a compound extracted from the fruit of the *Gardenia Jasminoides*, has been shown to crosslink cellular and acellular tissues [17-21], as well as biomaterials including gelatin microspheres [22], alginate-chitosan composites [23], and poly(ethylene)-glycol hydrogels [24]. Additionally, results suggest that genipin is cell-tolerated [25]. For these reasons, genipin has been offered as an alternative crosslinking agent for improving the mechanical properties of bioartificial tissues.

Genipin-induced changes in collagen gels

Genipin has been found to crosslink gelatin through nucleophilic attack by primary amine groups on lysine and arginine residues on the C3 atom of genipin [26], subsequently embedding a tertiary nitrogen in the six-membered ring in place of an oxygen atom [27]. We expect a similar mechanism for the reaction of collagen and genipin. In addition to crosslinking collagen and increasing mechanical strength, treatment with genipin, which is blue in crystalline form but produces a clear solution when dissolved in water or saline, has two unique outcomes: (1) following crosslinking with genipin, normally opaque collagen turns blue [28]; and (2) these crosslinks emit fluorescence at 630nm when excited at 590nm [29]. Thus, genipin crosslinking generates a molecular fingerprint that may be probed optically in situ to evaluate the degree of crosslinking and, possibly, the mechanical properties of collagen. Herein, we characterize the effects of genipin exposure on the mechanical properties of acellular collagen gels, and we correlate these properties to fluorescence intensity. We examine the molecular changes during crosslinking with Fourier Transform Infrared Spectroscopy (FTIR) in situ. We also assess the cytotoxic effects of direct exposure of genipin to cells in collagen tissue equivalents. These data provide a valuable blueprint for future studies applying genipin for efficient crosslinking in vitro to evaluate mechanotransduction and to assist in the design of bioartificial tissues for a variety of tissue systems.

Methods

Collagen gels

Type I collagen gels were prepared as previously described [30] by mixing 20 μl 1M Hepes buffer, 140 μl 0.1N NaOH, 100μl of 10X PBS , 60 μl of PBS (Invitrogen, Carlsbad, CA),

and 677 μl of 2.0 mg/ml collagen (Elastin Products Company, Owensville, MO) to make a 2.0 mg/ml collagen solution. The collagen solution self-assembles into a gel upon incubation at 37°C. For mechanical testing and fluorescence studies, acellular type I collagen gels were incubated in 0mM, 1mM, 5mM, or 10mM genipin (Challenge Bioproducts Co., Taiwan) in phosphate buffered saline (PBS) for 2, 4, 6, or 12hrs. Samples were placed on a rocker to ensure adequate diffusion and equilibration of genipin through the gel.

Mechanical testing

Mechanical testing was done using a Rheometrics SR-2000 parallel plate rheometer with a temperature-controlled incubation chamber set to maintain 37°C (TA Instruments, New Castle, DE). A 25mm diameter hole was punched in a 4mm thick layer of poly(dimethyl siloxane) (PDMS). Collagen solution (800μl) was pipetted into the well and transferred to a 37°C incubator to induce self-assembly. Following gel formation, 4.8ml of PBS with a defined concentration of genipin (0, 1, 5, or 10mM) was added to the Petri dish and the dish placed on a rocker to ensure complete mixing. Collagen gels were incubated in genipin for a defined period of time (2, 4, 6, or 12hrs), after which the solution was aspirated, and gels were rinsed generously with PBS. The gels were carefully removed with a spatula and transferred to the bottom plate of the rheometer. The top plate was lowered to a height of 0.8 mm. The dynamic storage and loss moduli of the gel were evaluated at 1% shear strain amplitude at frequencies ranging from 0.1 – 10Hz. Three samples prepared from separate batches of collagen were tested at each combination of genipin concentration/incubation duration. The data were analyzed statistically with

Genipin-induced changes in collagen gels

ANOVA with genipin concentration and incubation of duration as fixed effects. Significance levels were set at $P < 0.05$.

Fluorescence testing

Changes in fluorescence intensity due to genipin crosslinking were evaluated in gels prepared in a 96-well tissue culture plate. A 40μL aliquot of collagen was pipetted into each well. The plate was incubated at 37°C to induce self-assembly. PBS (240μl) with defined concentrations (0, 1, 5, and 10mM) of genipin was added to each well and the plate placed on a rocker plate to ensure equilibration of genipin throughout the gel. The gels were incubated in genipin for defined durations (2, 4, 6, 12hrs) that matched the conditions from the rheology studies. At the appropriate time point, the genipin solution was removed, and the gels were rinsed extensively with PBS.

Genipin-induced fluorescence was evaluated in two ways. Some plates were transferred to the computer controlled stage of an Olympus IX81 inverted microscope (Olympus, Melville, NY) to evaluate the feasibility of evaluating the fluorescence with standard epifluorescence microscopy for tissue engineering and mechanotransduction applications. An image of the fluorescence intensity of a representative field from each well (generally near the volumetric centroid of the gel) was captured digitally (Hamamatsu ORCA, Hamamatsu City, Japan) (590nm Exc, 630nm Em), and the mean intensity of the field was measured using Olympus Microsuite software (Olympus, Melville, NY). Identical exposure settings were used for all epifluorescent imaging. Each combination of genipin concentration/incubation time was tested in at least triplicate from at three replicates per

condition per experiment. Separate plates were read with a Cytofluor spectrofluorimetric plate reader (Applied Biosystems, Foster City, CA) with 590nm excitation and 645nm emission filters to demonstrate the ability to rapidly screen the degree of crosslinking based on fluorescence.

FTIR Characterization

The reaction between genipin and collagen was monitored in situ using FTIR for up to 4.5 hours in an Attenuated Total Reflection (ATR) geometry (Fig. 1). A type I collagen solution was pipetted onto a silicon plate (~1cm x1.5cm) with the longer side beveled at a 45° angle for entry and exit of the IR beam. The plate was sandwiched between two pieces of Teflon® with the top piece hollowed out to contain the collagen. The ATR setup was maintained at 37°C in a nitrogen-purged Magna-IR 760 FTIR Spectrometer (Thermo Electron Corporation, Waltham, MA) to facilitate self-assembly of the collagen. After self-assembly, a solution of 10mM genipin was deposited on top of the collagen and allowed to diffuse into the gel to the silicon-collagen interface. Only the highest concentration of genipin was studied with FTIR to see the most exaggerated response to crosslinking. The infrared beam entered the silicon wafer and was reflected internally (~8x in the top face) creating an evanescent wave that probed a depth of ~2 μm above the surface of the silicon wafer into the collagen gel. Spectra for collagen crosslinked with 10mM genipin for 12hrs (with genipin solution equilibrated throughout the gel as described above), and then rinsed extensively with PBS to remove all free genipin, which represents the most extreme condition characterized rheometrically and fluorimetrically, and the spectra for a pure 10mM genipin solution were similarly acquired. Spectra from

untreated type I collagen gels served as the reference for the crosslinked collagen, while the spectrum from water was used as the reference for the genipin solution.

Cytotoxicity

Cytotoxic effects of genipin were evaluated using Calcein-AM (Invitogen Corp, Eugene, OR) as an indicator of live cells. L929 fibroblasts were uniformly suspended in collagen solution at 50,000 cells/ml. Aliquots of collagen solution (40μl) were then pipetted into individual wells of a 96-well plate, which was transferred to a 37°C incubator to allow the gel to self-assemble. Genipin (0, 1, 5, or 10mM) was added to the media, and the plates were placed on a rocker to facilitate mixing. Gels were incubated in media with genipin for 12 or 24hrs. At the appropriate time point, gels were rinsed in PBS, and 20μl of 8μM Calcein solution was added to each well. Plates were transferred to the computer controlled stage of an Olympus IX81 inverted microscope operating in epifluorescence mode (480nm Exc, 535nm Em). Three representative areas from each well were imaged serially through the thickness of the gel. The images were stacked to project all of the cells through the imaged volume on to one plane, and the cells were counted manually. The number of cells was compared across conditions with ANOVA ($P < 0.05$).

Results

Mechanical testing

Rheological testing with parallel plate rheometry revealed that incubation in genipin increased the storage and loss moduli of acellular collagen gels (Fig. 2 and Fig. 3, respectively). No shrinkage of the gels was observed with crosslinking (data not shown).

Genipin-induced changes in collagen gels

Storage moduli increased gradually with frequency for all conditions, and then dropped off at higher frequencies for many samples. Inspection of gels revealed damage to the samples, which did not occur if experiments were run only at lower frequencies (data not shown), and we assumed that the damage was responsible for the apparent decrease in stiffness. In general, increased crosslinking delayed this damage. Loss modulus decreased gradually with frequency in all conditions, and generally began to increase concurrent with the decrease in storage modulus, which we again attribute to damage to the gel, though the increase in loss modulus was more gradual than the corresponding decrease in storage modulus. Increasing genipin concentration and the duration of incubation also produced significant increases in storage and loss moduli ($P < 0.001$). Cell-induced strain of tissue equivalents, such as the strains produced during cell-mediated gel compaction or cell migration, generally occurs at a low strain rate [31]. We therefore focused on storage moduli at 0.1Hz, which are shown for the different genipin concentrations and incubation durations in Fig. 4. Post hoc analysis (Fisher's Least Significant Difference test) revealed significant differences among all pairwise comparisons for the effects of genipin concentration on storage modulus (all $P < 0.001$) and all pairwise comparisons of loss modulus (max $P = 0.046$). For incubation duration, all pairwise comparisons of storage modulus were significantly different (max $P = 0.001$) except 4hrs vs. 6hrs ($P = 0.913$). Similar results were obtained for pairwise comparisons of the effects of duration on loss modulus at 0.1 Hz: all pairwise comparisons were significantly different (max $P = 0.013$), except 4hrs vs. 6hrs ($P = 0.655$). Nearly identical results were observed for comparisons of storage moduli at 2Hz, which represents a loading rate more consistent with functions of many load-bearing tissues. Comparisons of loss moduli at 2Hz showed significant

differences between all concentrations (max $P = 0.003$) except 0mM vs. 1mM ($P = 0.270$). Loss moduli at 2Hz were significantly different only between 12hrs and each of the other durations (max $P < 0.001$).

Fluorescence testing

Incubation of acellular collagen gels in genipin caused the normally opaque, non-fluorescing gels to turn blue and emit a red fluorescence. The fluorescence intensity of collagen gels was measured in separate samples in parallel to the mechanical testing (Fig. 5). Fluorescence intensity measured from digital images captured with epifluorescence microscopy increased significantly with genipin concentration ($P < 0.001$) and incubation duration ($P < 0.001$) (two-way ANOVA). Post-hoc analysis (Fisher's LSD) revealed significant differences among all pairwise combinations of concentration (all $P < 0.001$) and durations (max $P = 0.003$). Similar statistically significant trends were observed in measurements taken spectrofluorimetrically ($P < 0.001$). Post hoc analysis of plate reader fluorescence revealed significant differences (max $P = 0.033$) among all pairwise comparisons of concentrations except 5mM vs. 10mM ($P = 0.199$) and among all pairwise comparisons of duration except 4hrs vs 6hrs ($P = 0.240$). As with any fluorimetric (or colorimetric) optical assay, intensity measurements tended to saturate at high levels of fluorescence for both systems of measurement using a constant exposure setting.

The concurrent increase in fluorescence intensity with crosslinking presents a unique opportunity to assay the stiffness of the gels optically, if the fluorescence measurement

can be appropriately calibrated against a measure of the mechanical properties. The average storage moduli at 0.1Hz and at 2Hz (~largest frequency before a drop-off was observed) were plotted against the average fluorescence intensity at each combination of genipin concentration and duration of incubation (Fig. 6). For both fluorescence measurement techniques and both frequencies, stiffness was correlated exponentially to intensity (Table 1). The correlation coefficients were nearly identical for 0.1Hz and 2Hz (R^2 = 0.808 and 0.810, respectively, for measurements taken microscopically, and R^2 = 0.782 and 0.788, respectively for measurements taken spectrofluorimetrically). The exponential correlation curves shifted to the left slightly with increasing frequency, consistent with the increase in storage modulus. However, the resulting constants from the correlation were statistically indistinguishable.

FTIR Characterization

The FTIR spectra of 10mM genipin, 'fully' genipin-crosslinked collagen (exposure to 10mM genipin for 12hrs and extensively rinsed of free genipin), and collagen during in situ crosslinking with 10mM genipin are presented together in Fig. 7. The spectrum of the genipin solution is dominated by three modes at 990, 1080, and 1635 cm^{-1}, assigned to the ring C-H out-of-plane bend [32], ring C-H in-plane bend [32], and C=C double bond ring stretch modes [32,33] of the core of the genipin molecule, respectively. The absorption at 1080 cm^{-1} may also include the C-O stretch mode of the primary alcohol on the genipin molecule [32]. Additionally, the C-O-C asymmetric stretch and the CH_3 bend of the methyl ester are observed at 1300 and 1433 cm^{-1}, respectively. The 12hr crosslinked collagen spectrum features these modes, as well as bands at 1104 cm^{-1} and 1370 cm^{-1} that are

believed to be vibrational modes related to the formation of new bonds between genipin and the primary amines of lysine, hydroxylysine, or arginine residues in collagen. The band at 1370 cm^{-1} is assigned to the C-N stretch of the tertiary aromatic amine[32,34] of the crosslinked genipin nitrogen iridoid[35] that is bound covalently to the collagen. The broad, flat appearance of the crosslinking band at 1370 cm^{-1} in the 12hr spectrum is likely due to the flanking of two genipin molecule modes at 1360 and 1395 cm^{-1} (unassigned). The band at 1104 cm^{-1} is assigned to the C-N stretch of the tertiary nitrogen with the adjacent aliphatic carbon atom present in lysine or arginine residues [32,36]. An absorption near 1104 cm^{-1} is also present in the unreacted genipin molecule as a shoulder to the absorption at 1080 cm^{-1}. It is assigned to the vibrations of both the cyclic ether and secondary alcohol on the six-membered ring of the genipin molecule. When genipin reacts with collagen, both of these moieties are removed. Furthermore, the band at 1104 cm^{-1} in the 12hr crosslinked spectrum is significantly stronger than the corresponding band in the spectrum of pure genipin (relative to the band at 1080 cm^{-1}), suggesting that this absorption band is mostly associated with modes formed as a result of crosslinking.

To better identify the origin of features present in the spectrum of 12hr crosslinked collagen, the changes in the collagen spectrum were monitored in situ during the first 4.5hrs of crosslinking (Fig. 7). In this time-resolved experiment, spectral features were expected to increase due to: 1) diffusion of genipin into the region probed by the IR beam (the bottom surface of the collagen gel); and 2) crosslinking of collagen, leading to the appearance of new vibrational modes due to bonds formed during crosslinking. The in situ time-resolved spectra show the growth of several bands that are present in both crosslinked collagen and genipin, such as modes at 990, 1080, 1443, and

1633 cm^{-1}. In addition, the beginning of the growth of a band centered near 1370 cm^{-1} is observed. This feature is only seen in crosslinked collagen. Fig. 8 summarizes the time dependence of several absorbance features. Due to the proximity of the various genipin molecule and crosslinking bands, calculated band areas may include components of smaller bands adjacent to the dominant spectral feature. The 1080 cm^{-1} band area (spanning 1040-1180 cm^{-1}) likely includes the growth of a number of other smaller bands possibly including the crosslinking feature at 1104 cm^{-1}, although it is too small to contribute substantially to band area. The feature at 1370 cm^{-1} is adjacent to genipin bands as stated earlier, and all are included in the area calculation (band complex spanning 1344-1414 cm^{-1}). To facilitate comparisons between trends in band growth, area absorbance values of the weaker band at 1370 cm^{-1} were scaled by a constant (indicated in the inset). Band area growth also differed in absolute value from run to run. Therefore, final in situ values (t = 4.5 hours) of absorbance band areas amongst runs were normalized to a common value for each band, respectively. Similar increasing monotonic trends were observed for change in absorbance of genipin bands at 1080 and 1630 cm^{-1}, however, the growth of the crosslinking band at 1370 cm^{-1} appeared to slow down within several hours. Indeed, the small area of the crosslinking band at 1370 cm^{-1} and its apparent slowing in growth are likely due to the relatively small number of genipin-to-collagen crosslinks in the gel that can form compared with the amount of genipin that diffuses to the region. A description of the modes marked in the spectra of Fig. 7 is shown in Fig. 9.

Genipin-induced changes in collagen gels

Cytotoxicity Data

Most of the previous cytotoxicity studies of genipin had examined cell death following rinsing of genipin-crosslinked tissues or biomaterials prior to addition of cells. For tissue equivalent studies, knowledge of the cytotoxic effects of the genipin solution is required. Cytotoxicity studies using L929 fibroblasts indicated that genipin does cause significant cell death (ANOVA, $P < 0.001$) (Fig. 10). However, individual comparisons against the control condition (post hoc analysis – Fisher's LSD test) demonstrated that the adverse effects were limited to exposure 5mM ($P < 0.001$) and 10mM ($P < 0.001$); though lower, cell numbers from samples incubated in 1mM were statistically indistinguishable from controls ($P = 0.26$). The results at 12hrs were consistent with the 24hr results.

Discussion

We have characterized the effects of genipin-induced crosslinking of collagen gels on rheological properties, fluorescence, spectroscopic changes, and cytotoxicity. Rheological measurements were performed at 1% shear strain amplitude over a range of shear rates, which is consistent with previous characterizations of collagen and other biopolymeric gels using similar techniques [37-41]. We found that both the concentration and the duration of incubation in genipin significantly influenced the storage and loss moduli. The storage modulus measurements demonstrated a gradual increase with increasing frequency and were generally consistent with previous reports of collagen rheology [37-40]. Loss modulus showed a gradual decrease with frequency. We further found that genipin-mediated crosslinking produced significant changes in fluorescence that are well-correlated to the stiffness, and that genipin has marked cytotoxic effects at concentrations of 5mM and above. We conclude that genipin-induced crosslinking offers a simple alternative to improve the mechanical properties of tissue constructs, though caution must be taken to preserve cell viability.

We also observed a decline in the storage modulus (and increase in loss modulus) at larger frequencies that we associated with damage to the gel. In previous reports, where this trend was not observed, gels were generally prepared directly on the parallel plates [37-40]. In our case, the lengthy incubations in genipin precluded this possibility, and instead gels were transferred to the rheometer. It is possible that the adhesion to the plates was not as optimal as when gels are directly prepared or that the transfer increased the

potential for damage. Nonetheless, the observed decrease in storage modulus was consistent among samples and, interestingly, the 'failure' properties of the gel improved with increasing concentration and crosslinking duration, indicating that it is a stress-based phenomenon.

Previously, genipin has been used to crosslink biological tissues [21,42], chitosan-based tissue equivalents [43], and gelatin [44] with genipin concentrations ranging from 1-10mM. In several of these studies, the influence of genipin-mediated crosslinking on cytotoxicity and/or cell viability has been evaluated for different cell types in several different conditions, each in the context of development of a genipin-crosslinked biomaterial [44-46], and relatively low toxicity has been identified following rinsing of the crosslinked tissue. Unlike these studies, we have assessed the cytotoxic effects of genipin on cells directly exposed to controlled concentrations of genipin during crosslinking. We found that exposure to 1mM genipin for 24hrs was mildly cytotoxic to L929 fibroblasts, while exposure to 5mM and 10mM caused significant cell death. Thus, studies involving cellular collagen constructs should be limited to exposure to <= 1mM, while studies with acellular constructs, or at least ones that are initially acellular and rely on cells deposited post-crosslinking to migrate into scaffold, can employ higher concentrations, provided the free genipin is rinsed prior to exposure to cells.

The rheological studies presented herein provide a screening of the effects of genipin on stiffness, but not a direct indication of the utility of the chemical for tissue engineering applications, particularly because we have not yet evaluated the effects of genipin on

functional properties of cells. Additionally, the strains and rates used, while consistent with standard parallel plate rheology protocols, are insufficient to assess the utility of genipin for crosslinking bioartificial tissues that routinely experience finite deformations at accelerated rates. Future studies are aimed at identifying the appropriate timing and incubation durations to optimally influence the mechanical properties of cellular collagen constructs, including mechanical testing to larger strain levels at high rates more appropriate for tissue engineering applications. Previous characterizations with native tissues suggest that such treatments with genipin are plausible and may improve the mechanical properties of the constructs [17,19,20].

Beyond providing significantly improved mechanical stiffness of acellular collagen gels, the ability to manipulate the mechanical properties of cellular tissue equivalents on a reasonably short time scale affords the opportunity to study, and potentially exploit, the phenotypic response of cells to changes in mechanical properties within a 3D tissue construct, if it is shown that genipin does not adversely influence cell viability and/or function. The past decade has shown increased focus on quantifying the behavior of cells grown on substrates or in systems of varying compliance, beginning with Pelham and Wang's studies of fibroblast durotaxis using functionalized poly(acrylamide) gels [47]. This system has been adapted to study neural cells [48], endothelial cells [49], and smooth muscle cells [50-52]. In vivo, tissue cells reside within a three dimensional (3D) matrix, which presents a significantly different set of environmental cues than when cells are cultured on a 2D substrate. Quantitative studies of the effects of mechanical properties on cell behavior in 3D, where cells are uniformly distributed throughout the tissue equivalent,

rather than seeded on a gel and/or coaxed to invade the gel, have been limited to thin layers of collagen on top of poly(acrylamide) with controlled compliance to indirectly control the stiffness [53]. Genipin can be used to crosslink the 3D tissue equivalents to influence the mechanical properties of the tissue matrix directly, allowing more complex shapes and boundary conditions to be investigated. For example, culturing cells within collagen gels seeded on poly(acrylamide) membranes naturally mimics a constrained system, where stress is generated at the poly(acrylamide)-collagen boundary as cells exert traction and attempt to contract fibrillar network. However, it has been shown that the accumulation of network stress in a constrained system significantly affects the response of the resident cells [54]. Genipin could be used to stiffen unconstrained, free-floating tissue equivalents as well as constrained ones to distinguish between the influence of the intrinsic mechanical properties of the fibrillar, extracellular matrix network and the mechanostructural properties dictated by the network and its attachments/constraints to external entities.

Genipin also has the added novelty of producing crosslinks that appear blue and fluoresce allowing easy visualization and quantitation of crosslinking. The fluorigenic quality was first identified in forensics research that investigated genipin as a potential fingerprint reagent with increased sensitivity [29]. We hypothesized that the same properties could be used to differentially indicate the degree of crosslinking in collagen gels, which would potentially enable the optical evaluation of mechanical properties. We measured the fluorescence intensity in parallel to mechanical properties in extensively rinsed samples to remove all free genipin, and found strong correlations between the genipin-generated

fluorigenic properties and the mechanical properties of the collagen gels using both an epifluorescent microscope and a spectrofluorimetric plate reader. Spectrofluorimetric plate readers have advantages of high throughput and consistent measurement with no lag time between measurements of different samples. The plate reader will also capture the intensity signal through the thickness of the gel. However, only standard plate geometries can be used for measurement. Epifluorescent imaging is more cumbersome with each sample requiring manual focusing and measurement but could prove useful for samples that do not fit into standard well plate configurations, and for identifying any spatial variance in the degree of crosslinking. Traditional epifluorescent microscopy will also capture a signal through the thickness, but the signal will be strongest at the focal plane, while confocal microscopy could be used to pinpoint the fluorescence changes through the depth of the sample, as well.

The kinetics of the fluorescence changes roughly matched those in stiffness, but the intensity began to saturate at higher levels of crosslinking, leading to the exponential correlation of stiffness with fluorescence. In our experiments, fluorescence intensity was evaluated over a wide range of crosslinking regimens – from no crosslinking solution to incubation in 10mM genipin for 12hrs. To appropriately compare intensity values across this range, constant exposure settings were used for all conditions, and using the same settings necessary to measure low intensity levels at low levels of crosslinking can cause saturation at higher levels of crosslinking. A more sensitive calibration can easily be achieved by optimizing the exposure times for a narrower range of fluorescence.

Genipin-induced changes in collagen gels

The strong correlation of genipin-generated fluorescence to mechanical properties allows simple, non-invasive confirmation of mechanical properties for crosslinked gels/equivalents of various geometries that may not be particularly amenable to mechanical characterization to provide measures of within- and between-experiment variabilities. The ability to visualize crosslinks also enables direct observation of spatially varying crosslinking fields, such as defined patterns and gradients, and associated indirect assessment of the spatially varying mechanical properties. In all cases, the thickness of the actual sample and the ones used to generate a standard curve of fluorescence intensity vs. crosslinking (duration or concentration) must be carefully considered, as a thicker sample (of the same collagen concentration) will generate increased fluorescence; the correlation presented is specifically derived from the samples probed in this study. A separate calibration is necessary for other sample sizes and/or collagen concentrations. Moreover, the introduction of cells and subsequent compaction of the collagen gel will alter the observed fluorescence by increasing fiber density. The fluorescent labeling of collagen via genipin also presents interesting opportunities to observe and measure collagen degradation via lost fluorescence. Thus, while the quantitation of stiffness via fluorescence may be best applied for prescribing and screening initial conditions to evoke specific, stiffness-driven behavior, the fluorigenic potential of genipin may also be used to evaluate matrix remodeling.

The colorimetric and fluorimetric properties of the crosslinked collagen are associated with molecular changes produced during crosslinking. In our study, the FTIR spectra include features at 1104 cm^{-1}(C-N stretch) and 1370 cm^{-1} (C-N stretch), that are neither

characteristic of genipin nor collagen alone, and are therefore presumably associated with the crosslinked collagen and, perhaps, the color/fluorescence changes. The in situ FTIR demonstrated temporal changes in these features that paralleled the early changes in stiffness and fluorescence at 10mM. However, due to limitations in the FTIR set-up, the in situ spectroscopy could only be performed for ~4.5 hrs before evaporation began to introduce inconsistencies in the results, and a true correlation of stiffness-to-fluorescence-to-spectroscopy was not obtained. Interestingly, Touyama et al. examined the intermediate pigment changes that occur upon reaction of genipin with methylamine. Brownish-red intermediates were associated with 2-methyl-4-carbomethoxy-2-pyrindine derivatives, which had a spectroscopic feature at 1630cm^{-1} [33,55]. We also observed a peak at ~1630cm^{-1} in the rinsed, crosslinked collagen, which is shifted slightly to the left of a corresponding peak at ~1635cm^{-1} in the genipin solution. This shift may be artefactual due to water vibrational peak subtraction and/or due to double bonds, which contribute to the 1630/35 cm^{-1}, being slightly affected due to their proximity to the covalent bonding upon crosslinking and subsequently causing a small 5 cm^{-1} shift for the average of all double bonds.

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Genipin-induced changes in collagen gels

Figure Captions

Fig. 1: ATR setup showing genipin deposition for time-resolved study. Genipin solution (10mM) is deposited on top of and diffuses through the collagen gel. A 2μm region of the collagen gel is probed by the IR evanescent field during genipin-mediated crosslinking. The genipin solution (10mM) and collagen crosslinked with genipin for 33 hours are probed in a similar way.

Fig. 2: Storage moduli following parallel plate rheometry. (A) 2hr incubation; (B) 4hr incubation; (C) 6hr incubation; (D) 12hr incubation. Samples were subjected to 1% shear strain amplitude over a range of frequencies. Results are average +/- std err. Both genipin concentration and the duration of incubation significantly affected the storage modulus. Storage modulus tended to decline at higher frequencies, which was associated with damage to the gels.

Fig. 3: Loss moduli following parallel plate rheometry. (A) 2hr incubation; (B) 4hr incubation; (C) 6hr incubation; (D) 12hr incubation. Results are average +/- std err. Loss modulus tended to decline with frequency and then rise concurrent with the decline in storage modulus at higher frequencies. Both genipin concentration and the duration of incubation significantly affected the storage modulus.

Fig. 4: Average storage moduli (A) and loss moduli (B) (+/- std err) at 1% shear strain amplitude and 0.1 Hz vs. incubation time. Increasing genipin concentration and the duration of incubation in genipin significantly increased the storage and loss moduli of the collagen gels

(two-way ANOVA, $P < 0.001$). Fisher's LSD test revealed significant differences among all pairwise comparisons of concentration for storage modulus (all $P < 0.001$) and loss modulus (max $P = 0.046$). For incubation duration, all pairwise comparisons of storage modulus (max $P = 0.001$) and of loss modulus (max $P = 0.013$) were significantly different except 4hrs vs. 6hrs.

Fig. 5: Fluorescence intensity (average +/- std err) of genipin-crosslinked collagen measured using (A) epifluorescent microscopy (590nm excitation, 630nm emission) and (B) spectrofluorimetrically (590nm excitation, 645nm emission). For both, the intensity of fluorescence emission increased significantly with genipin concentration and duration of incubation ($P < 0.001$). Post hoc analysis (Fisher's LSD test) revealed significant differences among all pairwise comparisons of concentrations except 5mM vs. 10mM (max $P = 0.033$) and among all pairwise comparisons of duration except 4hrs vs. 6hrs ($P = 0.240$).

Fig. 6: Correlation of average storage modulus (+/- std err) with average fluorescence intensity (+/- std err) measured with epifluorescence microscopy (A) or spectrofluorimetrically (B) for 0.1Hz and 2Hz. In all cases, strong, exponential correlations were observed, indicating that stiffness can be assayed optically following appropriate calibration. Increasing frequency shifted the correlation curve to the left.

Fig. 7: IR Absorbance spectra of 10mM genipin referenced to a spectrum of pure water (top); collagen after 12 hours of crosslinking with 10mM genipin and extensive rinsing, referenced to a

spectrum of uncrosslinked collagen (second from top, scaled 2x) and collagen crosslinked with 10mM genipin *in situ*, 0.2, 0.4, 0.8, 1.7, 3.1, and 4.5 hours after adding genipin, referenced to the initially genipin-free collagen gel (bottom). Several spectral features that are present in the genipin solution alone, and/or the crosslinked and rinsed collagen are seen to evolve during the in situ crosslinking.

Fig. 8: Changes in absorbance band areas versus crosslinking time for some of the highlighted bands in Fig. 7: two genipin bands (triangle, 1080 $cm^{-1}$and circle, 1630 cm^{-1}), and a new genipin-to-collagen crosslinking feature (square, 1370 cm^{-1}). To allow all spectra to be viewed on a common plot, values of smaller band areas were scaled by a constant, as indicated in the inset. Crosslinking time began (at t = 0) when genipin reached the bottom of the collagen gel at the interface with silicon. Error bars combine two sources of errors: 1) baseline selection for area calculations, and 2) reproducibility of runs. End point area values of each band for different runs were normalized to a common value due to variation in absolute absorbance area possibly caused by variation in collagen density among samples.

Fig. 9: Description of the modes marked in the spectra of Fig. 7.

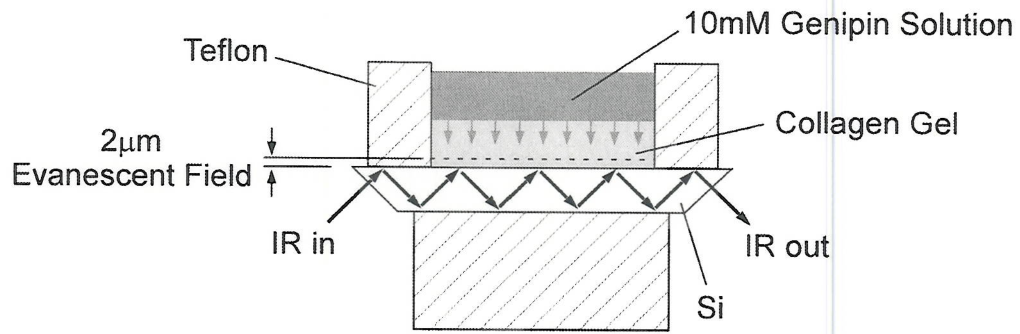
Fig. 10: Cytotoxic effects of genipin. L929 fibroblasts entrapped in collagen gels were exposed for 24hrs to culture medium with defined concentrations of genipin immediately upon completion of self-assembly. Live cells were labeled fluorescently with Calcein-AM, and the

Genipin-induced changes in collagen gels

average number of cells (+/- std err) in a vertical field through the thickness of the gel was determined by manually counting the cells in a stack of several images taken through the height of the gel. Genipin was cytotoxic (ANOVA, $P < 0.001$), but only a small fraction of cells were lost at 1mM, and these results were not statistically different than the 0mM control (Fisher's LSD post hoc test, $P = 0.26$).

Table 1: Results of storage modulus – fluorescence intensity correlations

$G' = A\exp(B \times Intensity)$				
Fluorescence Measurement	Frequency	*A* (+/- std err)	*B* (+/- std err)	R^2
Microscope	0.1Hz	22.9 +/- 7.33	3.05e-5 +/- 4.30e-6	0.782
Microscope	2Hz	24.3 +/- 7.82	3.12e-5 +/- 4.32e-6	0.788
Spectrofluorimeter	0.1Hz	33.2 +/- 8.36	8.34e-4 +/- 1.1e-4	0.808
Spectrofluorimeter	2Hz	35.7+/- 9.11	8.51e-4 +/- 1.1e-4	0.810



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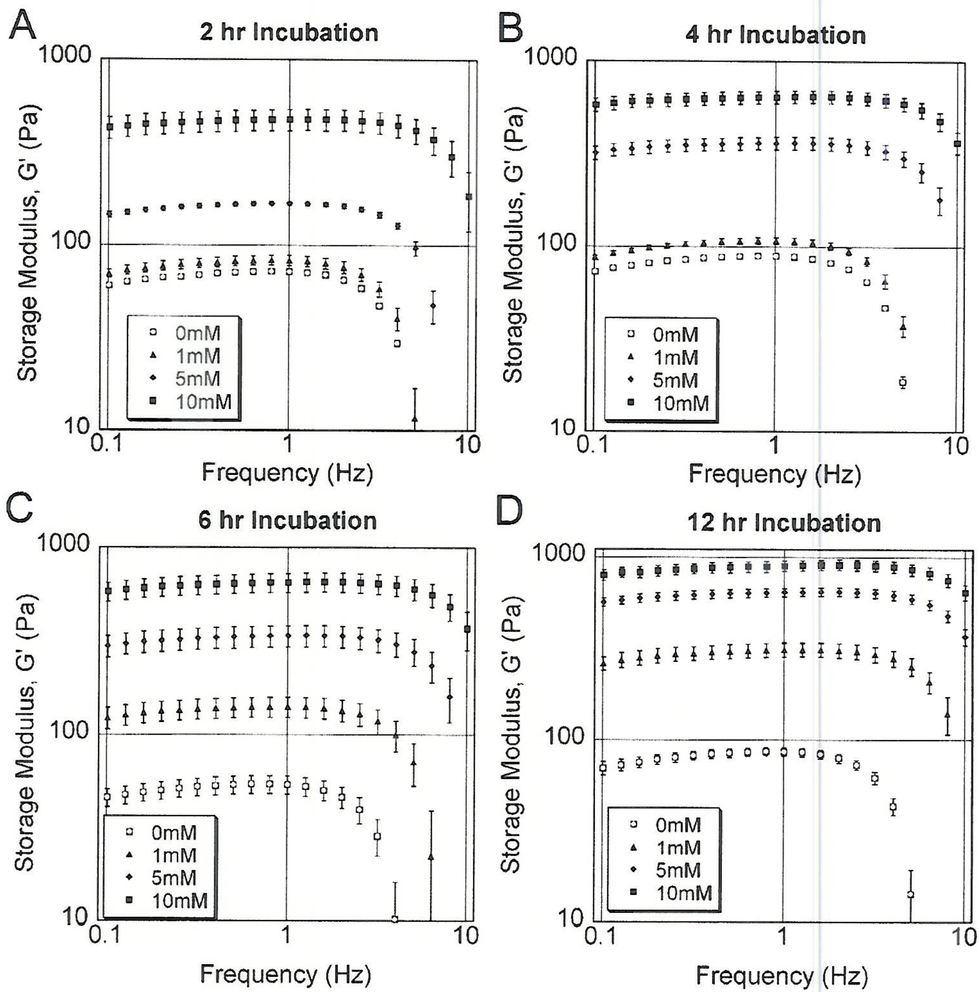
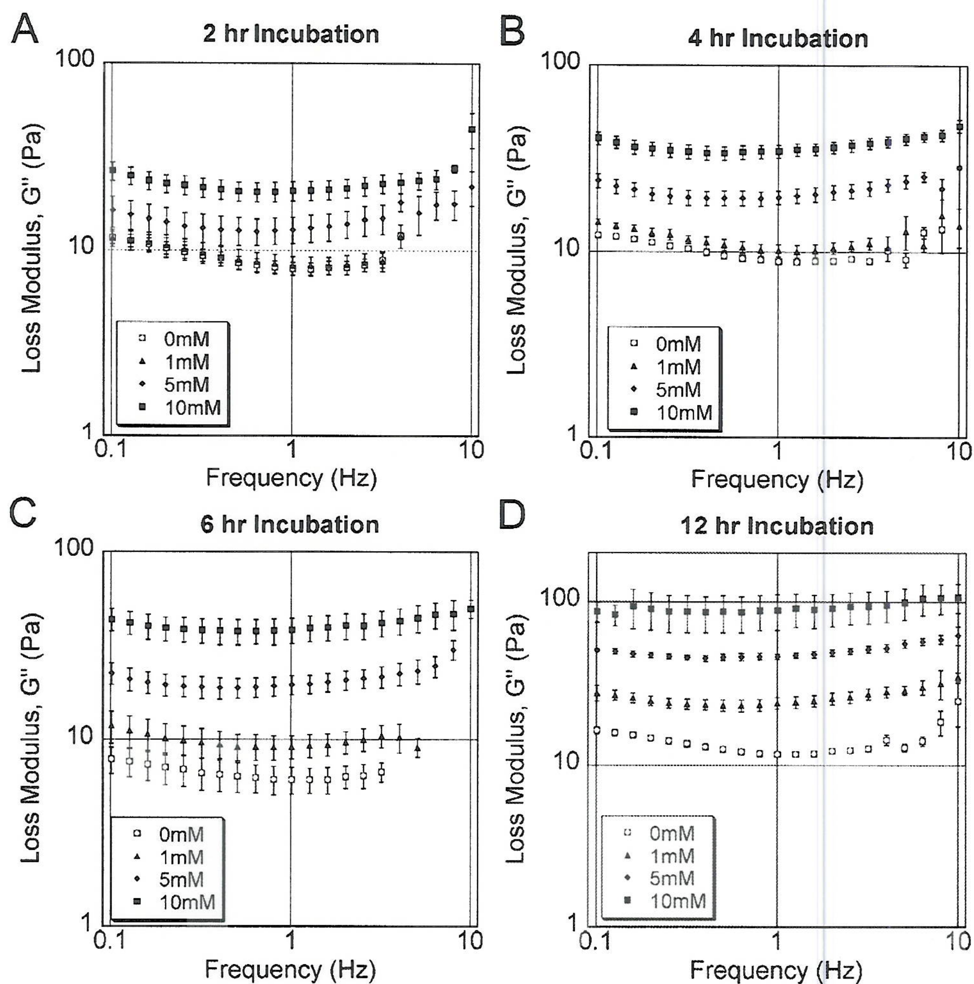
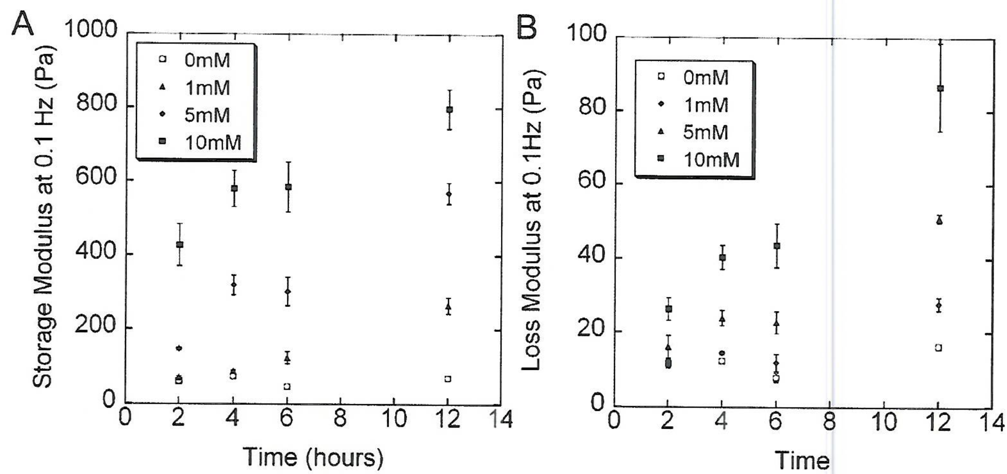

A
2 hr Incubation
B
4 hr Incubation
C
6 hr Incubation
D
12 hr Incubation
Storage Modulus, G' (Pa)
Frequency (Hz)
1000
100
10
0.1
1
10
0mM
1mM
5mM
10mM


A
2 hr Incubation
B
4 hr Incubation
C
6 hr Incubation
D
12 hr Incubation
Loss Modulus, G'' (Pa)
Frequency (Hz)
0mM
1mM
5mM
10mM
100
10
1
0.1
1
10



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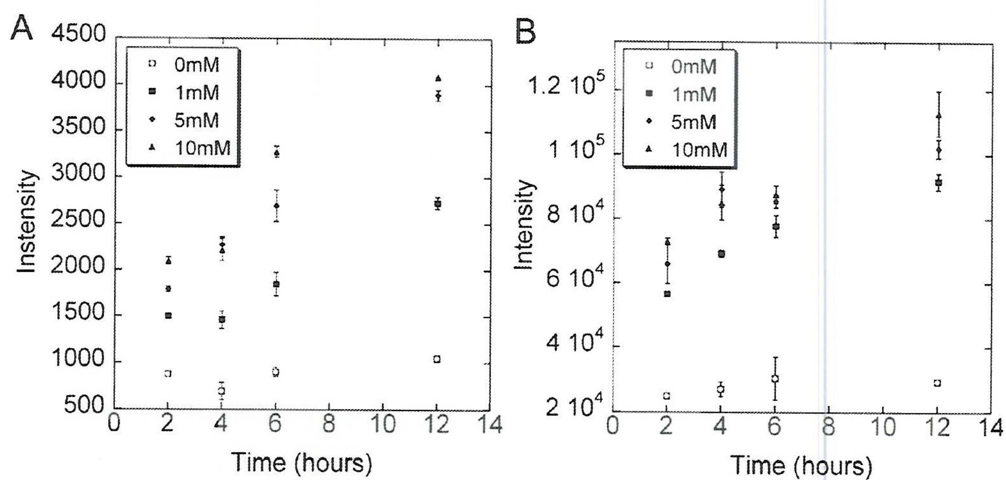

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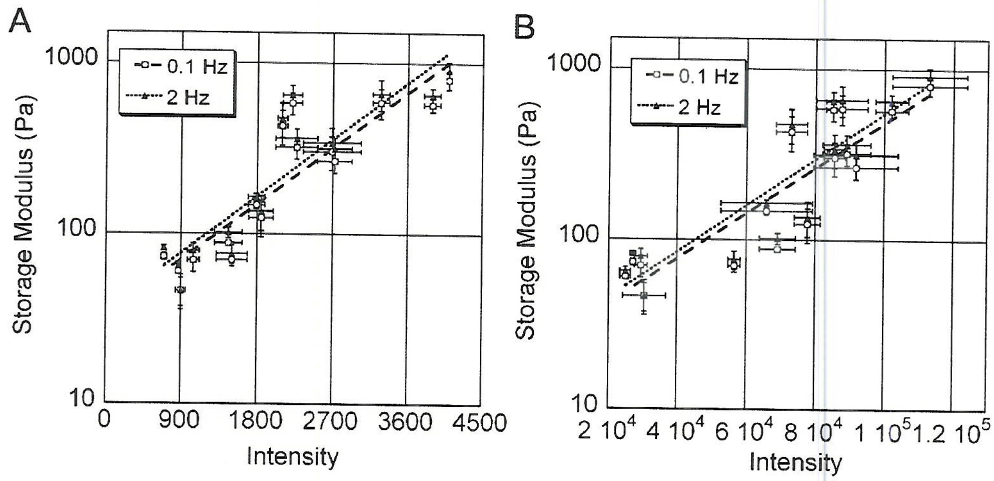

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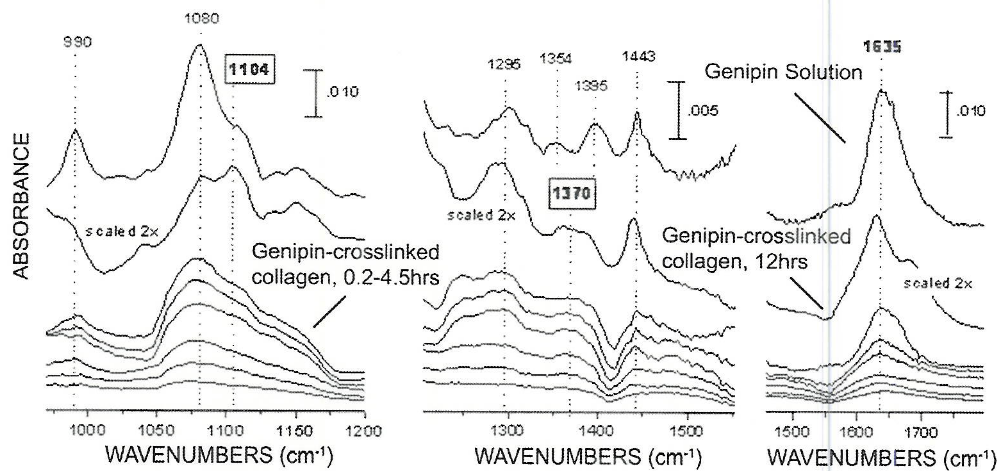

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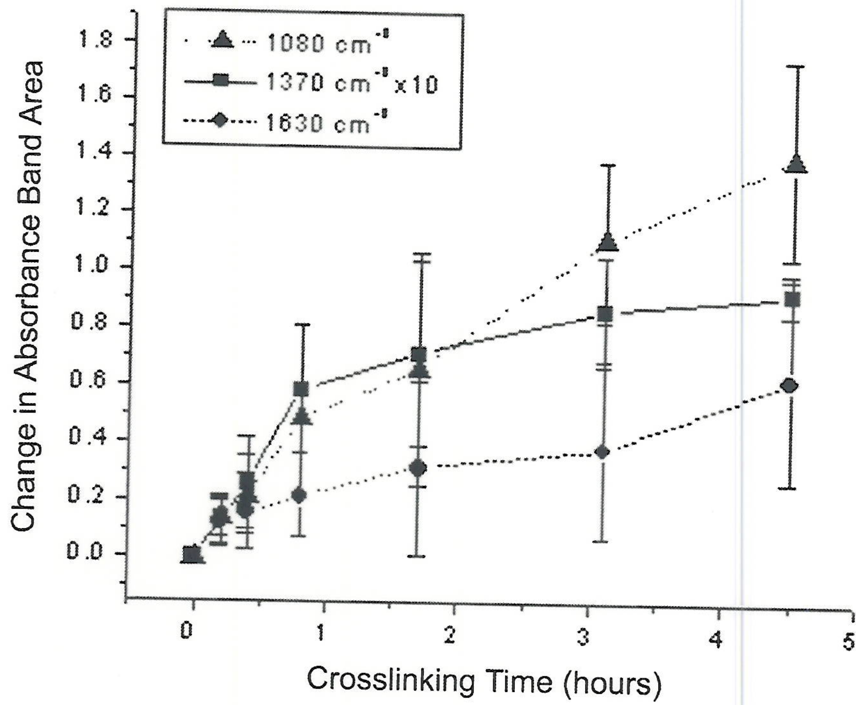

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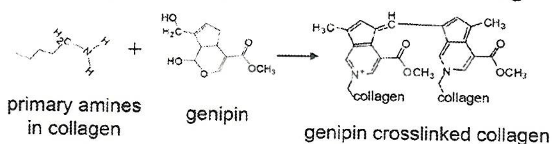
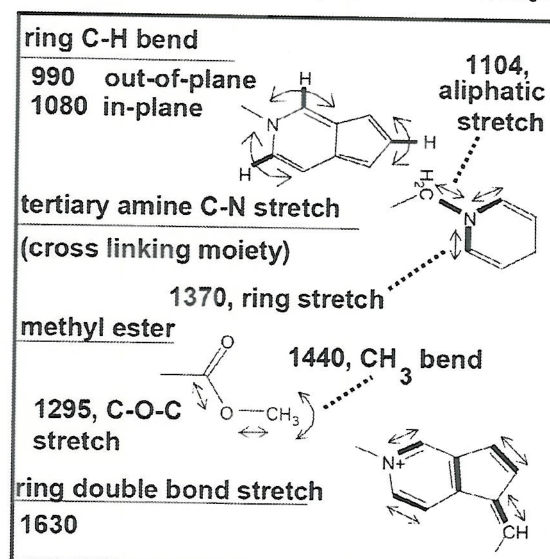
Vibrational modes (wavenumber, cm^{-1}) of post-reacted genipin in crosslinked collagen





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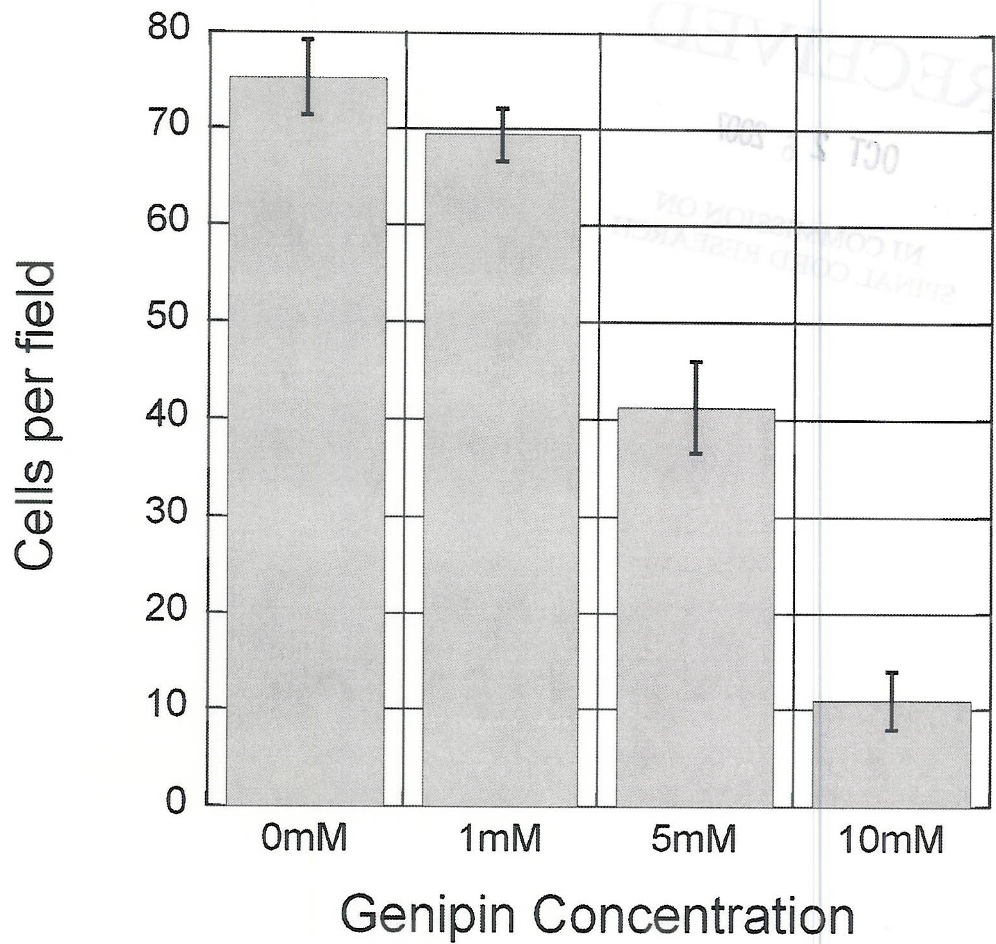

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