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## CHARACTERIZATION OF GENIPIN-CROSSLINKED COLLAGEN GELS

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### ABSTRACT

Controlled crosslinking of collagen gels has important implications in studies of cell and tissue mechanics as well as tissue engineering. Collagen gels can be crosslinked by many compounds including aldehydes and reducing sugars such as ribose. Though successful in altering the mechanical properties of collagen gels, these methods have inherent difficulties that often preclude their use in biomaterial applications. Aldehydes are toxic to cells and need to be washed out of the gels before cells can be added [1]. Even with thorough washing, there is the threat of toxicity. Though reducing sugars have been known to crosslink collagen in the body, the process is cumbersome and takes weeks to be effective in vitro [2]. Recently, a novel compound, genipin, has been found to crosslink biomaterials. Genipin is a naturally occurring crosslinking agent that can be extracted from the gardenia plant. Genipin crosslinks gels within several hours and has been shown in some studies to be nontoxic to cells [3]. We have conducted rheological studies to show the crosslinking ability of genipin. Genipin also has the unique property of generating crosslinks that fluoresce. Using this property, we correlated mechanical properties to fluorescence allowing for a non-contact way to measure mechanical properties. Cytotoxicity tests show high cell viability when using low concentration of genipin (1mM) with 95% cell viability. Thus, genipin is a versatile crosslinking agent that has many potential uses in cell and tissue engineering.

### METHODS

For materials testing and fluorescence studies, acellular type I collagen was prepared as previously described [4] and incubated in three different concentrations of genipin (1, 5, 10mM) for four different durations (2, 4, 6, 12 hours). Mechanical testing was done using a cone and plate rheometer. Fluorescence intensity was assessed by capturing images under epifluorescence microscopy (650nm excitation, 670nm emission). A standard curve for cytotoxicity studies was generated by serially diluting a L929 fibroblast cell suspension (from 400,000 to 20,000 cells/ml) in collagen solution with additional collagen. A total volume of 50 $\mu$ l the individual collagen solutions was added to the wells of a 96-well plate in triplicate. Collagen gels with 80,000cells/ml

were incubated in four different concentrations of genipin (0, 1, 5, or 10mM) in media for 6 hours. Cell viability was tested using Calcein AM staining and a fluorescent plate reader. Results were compared to cells incubated in collagen without genipin.

### RESULTS AND DISCUSSION

The objective of this study was to determine the influence of genipin concentration and exposure time on mechanical properties of acellular collagen gels, and the corresponding toxicity of genipin at those concentrations. Type I collagen gels were exposed to 0, 1, 5, or 10 mM genipin for 2, 4, 6, or 12hrs. Storage and loss shear moduli were measured with cone and plate rheometry. Increasing concentration and exposure time significantly affected the mechanical properties of collagen (Fig 1).

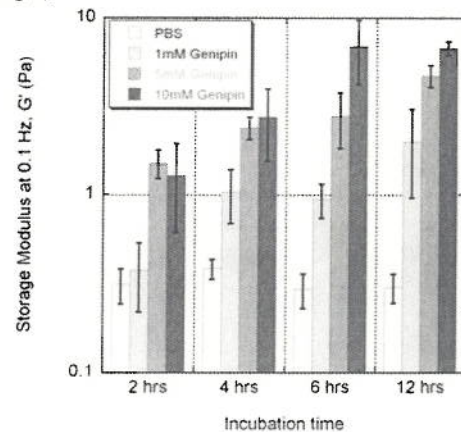


Figure 1: Storage modulus of acellular collagen gels when crosslinked with collagen

Both concentration and exposure duration significantly increased the storage modulus at low frequencies, which is indicative of the strain rates applied during typical cell-mediated traction (2-way ANOVA,  $P < 0.05$ ). Posthoc analysis (Scheffe's test) indicated that

exposure to at least 5mM genipin for a minimum of 6hrs was required to increase mechanical properties ( $P < 0.05$ ). Increasing concentration to 10mM increased the shear modulus ( $P < 0.05$ ), but increasing exposure to 12hrs did not ( $P = 0.11$ ), although the small number of experiments ( $n = 4$  per combination) limits the power of our conclusions.

Cytotoxicity studies were done to evaluate cell viability at different concentration of genipin at 6 hours of exposure. Cytotoxicity data shows that genipin is relatively nontoxic at low concentrations (1mM) but becomes increasingly toxic as genipin concentration increases, with close to half the cells dying at 10mM (Fig 2). Even at 10mM genipin concentrations, we still find that toxicity is lower than using aldehydes.

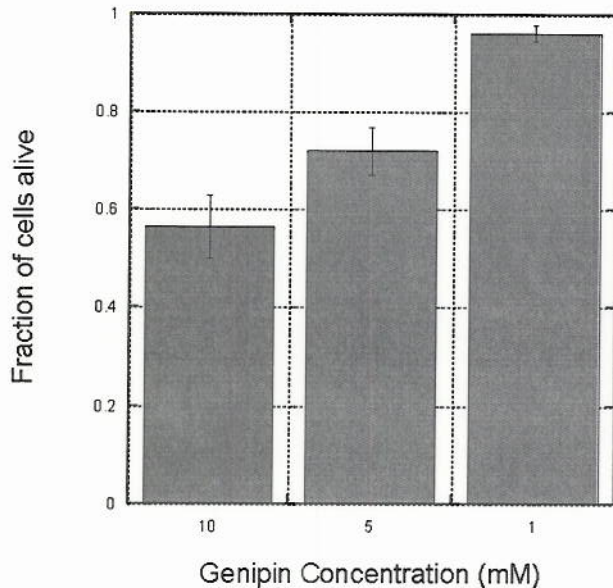


Figure 2: Cytotoxicity analysis of Genipin

Another distinctive characteristic of genipin is that the crosslinks fluoresce as they are formed (Fig 3). This potentially allows for a non-contact way to evaluate mechanical properties unlike conventional methods such as cone and plate rheometry, AFM, or nanoindentation. By using fluorescence microscopy, fluorescence can be correlated to mechanical properties.

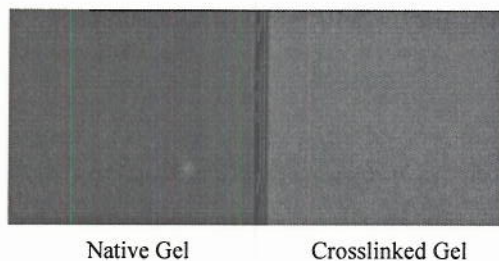


Figure 3: Genipin crosslinked gels emit fluorescence that increases with concentration and duration of exposure

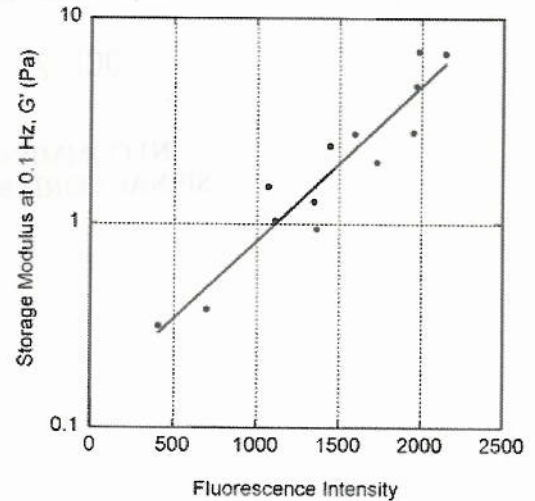


Figure 4: Correlation between fluorescence and storage modulus ( $R^2 = 0.92$ )

To determine if genipin-induced fluorescence correlated to changes in mechanical properties due to crosslinking, the storage modulus data was linearized with a log transformation and regressed against fluorescence intensity at the appropriate concentration and incubation duration (Fig 4). There was a strong correlation between fluorescence and mechanical properties, suggesting that genipin fluorescence can be used for non-contact evaluation of mechanical properties.

#### CONCLUSIONS

Results of our studies show that genipin is a valuable way to control the mechanical properties of collagen gels. Further testing needs to be done to determine the optimal genipin concentration and crosslinking duration time to allow for high cell viability with the minimum duration of exposure. We are using generating genipin-mediated gradients of stiffness with microfluidics to investigate influence of mechanical properties on neurite guidance for spinal cord regeneration. Genipin can be used for similar studies of mechanotransduction and cell mechanics for other anchorage-dependent cells, as well as tissue engineering applications.

#### ACKNOWLEDGEMENTS

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