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Modifying the Properties of Collagen Scaffolds with Microfluidics

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Abstract

It is now well accepted that the mechanical properties and cell adhesion profile of 2D and 3D extracellular matrix molecules combine to dictate cellular fate processes, such as differentiation, migration, proliferation, and apoptosis, through a process generally known as 'mechanotransduction', or the conversion of mechanical signals into a cellular response. The stiffness and adhesion density combine to affect the force balance that exists between an adherent cell and the surrounding substrate. We have established BioMEMS, microfluidic technology to alter the mechanical properties and cell adhesion profile of collagen scaffolds. Using soft lithography, we fabricate elastomeric networks that serve as conduits for the controlled mixing of type I collagen solutions. Our technology enables us to generate reproducible, controlled homogeneous and inhomogeneous microenvironments for 3D cell culture, assays of cell behavior in 3D, and the development of bioartificial tissue equivalents for regenerative and reparative therapies. The adhesivity of collagen is modulated by covalently grafting peptides (such as RGD) or proteins (such as albumin) to soluble collagen molecules with 1- ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), a hetero-bifunctional coupling agent. EDC activates the carboxylic group of collagen and forms an amine bond with the grafting molecule. The grafted collagen self-assembles into a fibrillar gel at physiological temperature and pH with no measurable changes in rheological properties compared to controls. A solution of peptide-grafted collagen is then mixed in microfluidic networks with unaltered collagen to form controlled gradients or other patterns of the two solutions, which immobilize upon self-assembly. Separately or in the same network, the mechanical properties of the collagen gel can be altered regionally by the microfluidic delivery a solution of a cell-tolerated crosslinking agent. We use genipin, which has the unique property of generating crosslinks that autofluoresce. The intensity of the fluorescence correlates with the degree of crosslinking (and thus the mechanical properties) enabling us to monitor and measure changes in mechanical properties dynamically and non-invasively. Lastly, though it requires constant delivery or recirculation, the same networks can be used to impose gradients of soluble factors, such as growth factors and cytokines. Thus, we have developed a platform to examine the response of cells to simultaneous chemotactic, haptotactic, and durotactic gradients in a 3D environment. We are employing this technology to examine the response of neural cells to gradients of biomaterial properties to optimize cues for spinal cord regeneration.

Introduction

It is now clear that in many tissue systems, the mechanostructural properties of the extracellular matrix are critical contributors and regulators of cellular functions in addition to the mechanical functions of the tissue. The mechanical properties and cell adhesion profile of ECM molecules, in 2D and in 3D, combine to dictate phenotypic behavior of cells, such as migration, traction, proliferation, and apoptosis through mechanotransduction mechanisms [1, 2]. The adhesion density and stiffness act together to affect the force balance that exists between an adherent cell and the surrounding substrate [3]. To investigate these phenomena, and to properly design

bioartifical, tissue-engineered replacements, it is frequently desired to control the mechanical properties and adhesivity of biomaterials. In particular, 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. Additionally, collagen's superior biocompatibility and nearly ubiquitious bioactivity has made it one of the most extensively investigated biomaterial scaffolds for engineering the tissue listed above, and others, including hepatic and neural tissues.

In vivo, precursors to collagen are synthesized in the endoplasmic reticulum and are packaged in the Golgi apparatus along with enzymes required for processing. The packages are excreted by fusion, and fibril formation begins extracellularly as short fibril segments in formation compartments, which move into bundle compartments, where they fuse linearly and laterally with other fibrils. The growing fibrils continue to mature and are coated with proteoglycans, and are crosslinked enzymatically and stabilized post deposition [4, 5]. Collagenous tissues, such as rat tail tendons and bovine skin, can be degraded enzymatically or with acids into molecular fragments that will reassemble into a collagen network at physiological pH and temperature. If the pH is adjusted first, cells can be added to the collagen solution, and become trapped as the temperature is raised and the collagen self-assembles into a hydrogel. These so-called 'tissue equivalents' have proven to be vital analogs for studying aspects of cell-tissue interactions in controlled, 3D, in vitro culture systems [6-10], and form the basis for many bioartifical tissues, including Apligraf™, which was the first 'living' tissue engineered product to receive FDA approval.

To enhance the functionality of tissue equivalents, it is desirable to manipulate the mechanostructural properties of collagen gels, both to study mechanotransduction and to improve the properties of bioartificial tissues. Moreover, spatial control of these properties could impart critical biomaterial cues for directing cell migration and control heterotypic cell-cell and cell-tissue interactions with a 3D hydrogel scaffold. Since these systems are inherently cellular – cells are entrapped in the gel instead of being induced to invade an acellular scaffold – all reactions and procedures must be cell tolerated. For 2D studies, patterning discrete regions and continuous gradients of adhesion and mechanical properties has become routine with the advent of simplified micropatterning and microfluidics devices [11, 12]. The biphasic ('solid' network, and 'fluid' solution phases), hydrogel nature of collagen soft tissues and tissue equivalents inherently implies that the solution phase can flow through the network phase [13]. Thus, microfluidics networks can be filled with a collagen hydrogel and solutions delivered through the hydrogel with spatial control.

Herein, we present our techniques to modulate separately the adhesion and mechanical properties of cell populated, collagen gels. Moreover, we employ these techniques in microfluidics networks that produce controlled gradients of adhesion and mechanical properties within 3D collagen gels. We demonstrate preliminary cell viability within the networks, and discuss implications for tissue engineering with emphasis on spinal cord regeneration.

Materials and Methods

Collagen gels

Collagen gels were prepared as described previously [10] by mixing 20 µl 1M Hepes buffer, 132 µl 0.1N NaOH, 100µl of 10X MEM, 60 µl of M199, 1 µl of pen-strep, 10µl of L-glutamine, 677 µl of Vitrogen (Cohesion Corp, Temecula, CA) in order to make a 2.0 mg/ml collagen solution. The collagen solution self-assembles into a gel upon incubation at 37°C.

Modifying adhesion of collagen gels

Peptides and proteins were grafted to type I collagen in solution using the carbodiimide heterobifunctional linker molecule, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) (Sigma). Three different grafted species were assayed: a FITC-labeled KRGDGD peptide sequence (flRGD) (Invitrogen, Carlsbad, CA), which includes a repeat of the RGD cell adhesion domain; bovine serum albumin (Sigma), which nominally suppresses cell adhesion [14], and GRDGS peptides (Sigma), which generally serves as a control, 'scrambled' peptide for RGD experiments, and in this case is used as a short peptide that we hypothesize will decrease adhesivity. A 1:1 mixture of 1M EDC and grafting adhesion molecule is incubated at room temperature overnight. The EDC/peptide/protein solution is added to the normal collagen recipe above, replacing M199 so as to maintain a final collagen concentration of 2.0mg/ml. The final concentration of the grafting molecules is 25μM for flRGD and GRGDS and 250μM for albumin. This stock solution of grafted, type I collagen can be mixed in different ratios with untreated, 'native' type I collagen solutions to establish solutions with intermediate levels of grafted molecules. To validate grafting, mixtures with 0%, 33%, 66%, and100% (0-25μM) of the flRGD peptide were prepared and allowed to self-assemble. Following extensive rinsing, the fluorescence intensity of the gels was recorded with a spetrofluorimetric plate reader.

Adhesion assay

The adhesion of L929 fibroblasts to the modified collagen hydrogels was evaluated by forming collagen gels with varying volume fractions of grafted collagen, seeding a known number of cells on the gel, allowing the cells to attach to the gels for 6 hrs, and then extensively rinsing [15]. The remaining cells are stained with calcein AM, and the fluorescence of each well read with a spectrofluorimetric plate reader. The intensity is converted to a cell number from a standard curve established in parallel with the experiment. The adhesion assay was performed for all 3 grafted molecules.

Modifying mechanical properties with genipin.

For mechanical testing and fluorescence studies, acellular type I collagen gels were incubated in three different concentrations of genipin (Challenge Bioproducts, Taiwan) (1, 5, 10mM) for four different durations (2, 4, 6, 12 hours).Mechanical testing was done using a Rheometrics SR-2000 cone and plate rheometer with a temperature controlled incubation chamber set to maintain 37°C (TA Instruments, New Castle, DE). A 1cmx1cm square was etched in a Petri dish with a glass scribe using a template. Collagen solution (500μl) was pipetted into the square and transferred to a 37°C incubator to induce self-assembly. Following gel formation, PBS with a defined concentration of genipin (0, 1, 5, or 10mM) was added to the Petri dish. Collagen gels were incubated in genipin for a defined period of time (2, 4, 6, or 12hrs), after which the solution was aspirated and the gels were rinsed with PBS. The gels were carefully removed with a spatula and transferred to the rheometer. The cone of the rheometer was lowered until it came into contact with the gel (measured as a minimal rise in until the axial force). The dynamic moduli – storage (elastic) modulus and loss (viscous) modulus – of the gel were evaluated at 1% shear strain at frequencies ranging from 0.1 – 10Hz. At least three samples were tested at each combination of genipin-concentration/incubation duration. A two-way ANOVA was used to evaluate influence of genipin concentration and incubation duration of the mechanical property data ($P<0.05$). Mechanical testing of collagen grafted with flRGD peptides to ensure that the grafting process did not alter the mechanical properties of the gels.

Fluorescence intensity of genipin crosslinked gels was evaluated through epifluorescence microscopy. Collagen (400 μL)was pipetted into each well of a 24 well plate. The plate was incubated at 37°C to induce self-assembly. PBS with defined concentrations (0, 1, 5, and 10 mM) of genipin was added to each well and incubated for defined durations (2, 4, 6, 12, hrs) that matched the conditions from the cone and plate rheometry studies. At the appropriate time point, the genipin solution was removed and replaced with PBS, and the plate was transferred to the computer controlled stage of an Olympus IX81 inverted microscope. An image of the fluorescence intensity of a representative field from each well was captured digitally (Hammamatsu ORCA) (620 Exc, 655 Em), and the mean intensity of the field was measured using Olympus Microsuite software (Olympus). Identical exposure settings were used for all imaging. Each combination of genipin concentration/incubation time was tested in at least triplicate.

Cell viability

A standard curve for cytotoxicity studies was generated by serially diluting a L929 fibroblast (ATCC) cell suspension (from 400,000 to 20,000 cells/ml) in collagen solution with additional collagen. A total volume of 50μl the individual collagen solutions was added to the wells of a 96-well plate in triplicate. Collagen gels with 80,000 cells/ml were incubated in four different concentrations of genipin (0, 1, 5, or 10mM) in media for 24 hours. Cell viability was tested using Calcein AM staining and a fluorescent plate reader. Results were compared to cells incubated in collagen without genipin.

Collagen fiber morphology

The influence of the EDC-mediated grafting and genipin-mediated crosslinking on collagen fiber morphology was qualitatively evaluated using scanning electron microscopy (SEM).

Generation of gradients of adhesion and mechanical stiffness

Gradients of collagen adhesion and mechanical properties were generated by adapting microfluidics networks used for chemotaxis studies – a 'tree' network developed by Li Jeon et al. [16], and a simpler, source-sink network, which we refer to as the 'H' model. The 'tree' model consists of 2 100μm wide, 100μm deep channels that become progressively mixed in branches, ultimately forming 6 inlets to a 600μm wide outlet 'trunk'. The 'H' model consists of two 100-200μm wide channels that mix statically in a 100μm wide, 500-2000μm long cross channel. All channels are 100μm deep. Diffusive gradients are first predicted computationally with CFD-ACE+ (ESI CFD, Inc., Huntsville, AL) For adhesion gradients, the source channel was filled with 100% flRGD-grafted collagen solution, while the sink conduit was filled with 100% native, untreated collagen solution using a syringe pump (Harvard Apparatus, Cambridge, MA). The source and sink solutions and were pumped through the microfluidic networks at a flow rate of 0.1ml/min for 30 min under a stereomicroscope. The network was transferred to a 37° incubator, and the flow rate was decreased to 0.01ml/min to reduce the tendency for the flowing collagen to disrupt formation of the neo-fibers. After 30 min, the flow was turned off, and the collagen-filled network was incubated for another 30 min at 37°. Networks were transferred to the microscope stage and the presence of a gradient was validated by epifluorescence microscopy.

For gradients of mechanical properties, the networks were first filled with untreated collagen solutions at 0.1ml/min, which was allowed to self-assemble as above. The source and sink solutions were then changed to medium with and without genipin, which were flowed through the networks at 0.01ml/min. The gradients form within the first minute after filling, and then are maintained for 6-12 hrs. The gradient of mechanical properties was evaluated using epifluores-

cence microscopy to evaluate the fluorescence signal generated by genipin-mediated crosslinking.

The viability cells subjected to the microfluidics procedure was verified morphologically by including dispersed Schwann cells, hippocampal neurons (kindly donated by Dr. Bonnie Firestein, Cell Biology and Neuroscience, Rutgers, the State University of New Jersey), and sensory neurons isolated from chick embryo dorsal root ganglia in the collagen solutions prior to filling of the microfluidics networks.

Results

Adhesion

The adhesion of L929 fibroblasts to collagen was modulated by grafting additional peptides and proteins to soluble collagen with EDC prior to self-assembly. The adhesion molecules/peptides were successfully incorporated on the collagen and remained following self-assmembly, as indicated by the increase in fluorescence with increasing volume fraction of grafted, soluble collagen (Fig 1). Fibroblast adhesion increased with increasing concentration of flRGD (Fig 2A), and decreased with increasing concentration of albumin (Fig 2B) and the scrambled RGD peptide, GRDGS (Fig 2C). Significant changes in adhesion were observed in all three cases (ANOVA, $P<0.05$).

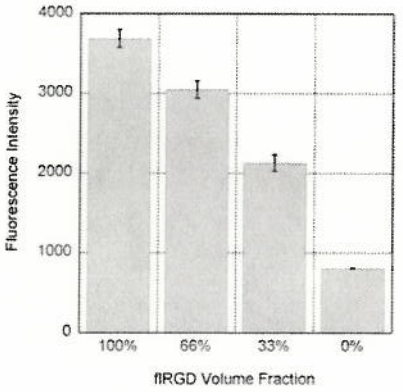

Figure 1: Intensity readings of flRGD-grafted collagen (average +/- standard deviation). Soluble type I collagen was grafted with flRGD peptides and mixed in different volume fractions with untreated soluble type I collagen, and the mixtures were allowed to self assemble. Following generous rinsing, the fluorescence intensity of the gels was recorded. The intensity of the gels increased with increasing volume fraction of the grafted collagen.

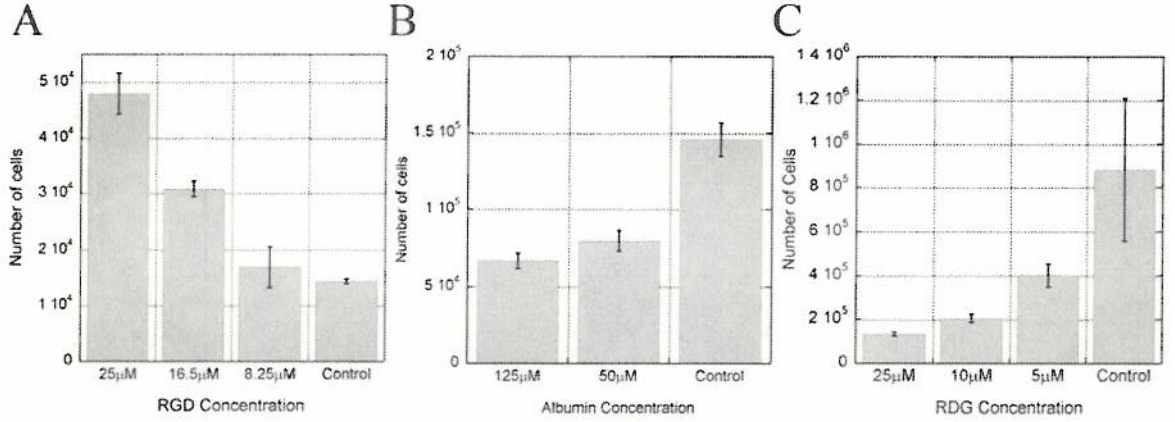

Figure 2: Modifying the adhesion properties of collagen (average +/- standard deviation). Increasing the amount of RGD peptide grafted to soluble collagen increased fibroblast adhesion to the collagen gel following self-assemble (A), whereas increasing the concentration of albumin (B) or the scrambled, RGD peptide (C) grafted to the collagen decreased adhesion.

Mechanical testing

As anticipated, rheological testing with cone-and plate rheometry revealed that incubation in genipin increased the storage modulus of acellular collagen gels. Storage moduli increased gradually with frequency at low frequencies and then quickly at higher frequencies (Fig 3A). Differences in storage moduli were most evident at low frequencies and became statistically indistinguishable at higher frequencies (Fig 3B). Both genipin concentration and the duration of incubation were significant factors ($P < 0.05$, two-way ANOVA). Mechanical testing also demonstrated no differences between flRGD-grafted collagen and native, untreated collagen (Fig 3C).

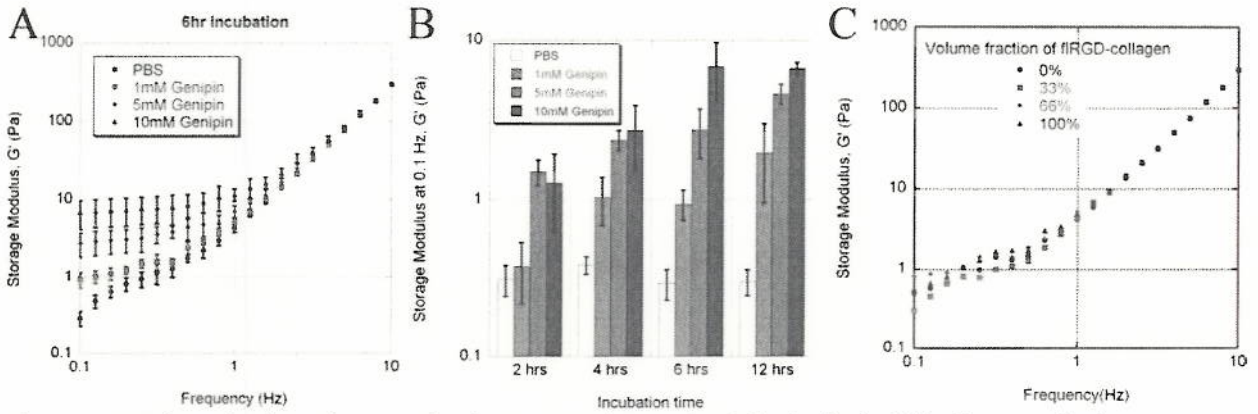

Figure 3: Mehcanical testing results (average +/- standard deviation). (A) Cone-and-plate rheometry demonstrated that increasing the concentration of genipin resulted in an increase in the storage modulus at lower frequencies. (B) Significant differences in storage modulus were observed at low frequency mechanical stimulation with increasing genipin concentration and with increasing duration of incubation in genipin. (C) No differences were observed in the mechanical properties of the flRGD-grafted collagen.

The fluorescence intensity of collagen gels crosslinked with genipin was measured in parallel samples. Similar to the storage modulus, fluorescence intensity increased significantly with genipin concentration and incubation duration ($P<0.05$, two-way ANOVA) (Fig 4A). As with any fluorimetric (or colorimetric) optical assay, intensity measurements saturated at high levels of fluorescence. Plotting the average storage modulus at 0.1 Hz average intensity measurements against the average fluorescence intensities for the different concentration-incubation duration combinations revealed a semi-log relationship with high correlation ($R^2=0.84$) (Fig 4B), thus providing a non-contact means of evaluating the change in stiffness due to genipin crosslinking.

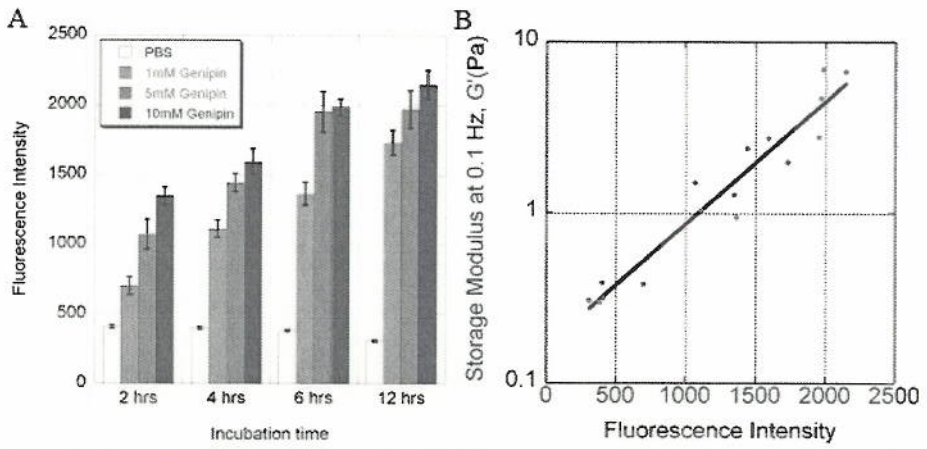

Figure 4: Mechanical properties correlated with fluorescence intensity (average +/- standard deviation). Genipin-mediated crosslinking of collagen produces crosslinks that emit fluorescence (620exc, 655em). (A) The intensity of the fluorescence significantly increases with genipin concentration and the duration of incubation. (B) The intensity was correlated to the storage modulus presented in 3B using a semi-log relationship (R^2=0.84).

Cell Viability

Cytotoxicity studies indicate that genipin has little effect on cell viability at 1mM genipin. However, unlike previous reports, incubation in higher concentrations of genipin (>= 5mM) caused a significant decrease in cell viability (Fig 5).

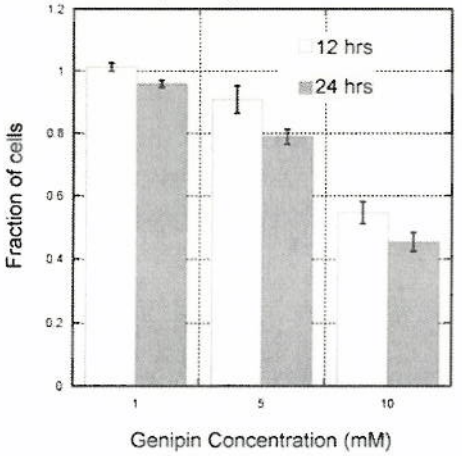

Figure 5: Cell viability following incubation in genipin (average +/- standard deviation). L929 fibroblasts were seeded in collagen gels, which were then exposed to 1-10mM genipin for 12 or 24 hrs. Cell viability began to suffer follow incubation in concentrations >=5mM.

Collagen morphology

SEM images of collagen gels grafted with additional peptides or proteins, or crosslinked with genipin, were examined qualitatively to evaluate the effects of the treatments on fiber morphology (Fig 6). Grafting with peptides (flRGD or GRDGS) did not cause obvious changes in collagen fiber structure. However, grafting the larger, albumin protein increased the apparent porosity and decreased fiber thickness compared to untreated gels. Treating with EDC alone appeared to also increase porosity and decrease fibril diameter.

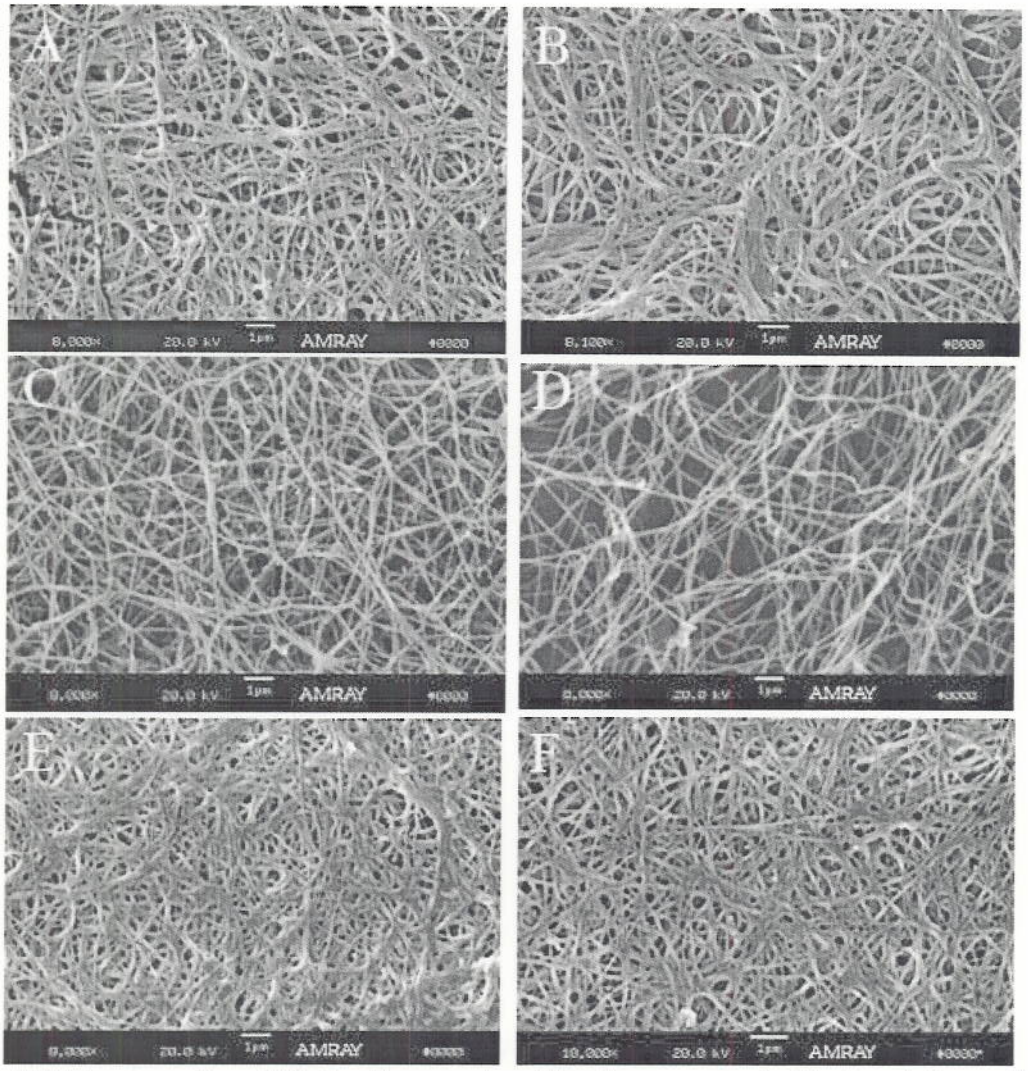

Figure 6: SEM images of modified collagen gels. All gels were nominally 2mg/ml type I collagen. (A) Untreated, type I collagen. (B) Grafting flRGD (25μM) had no overt effect on fibril morphology, however grafting albumin (125μM) (C) or using EDC alone (D) resulted in smaller fibers and greater porosity. Treating with 1mM (E) or 10mM (F) genipin for 12hrs increased lateral association of fibers, resulting in thicker fibers and decreased porosity.

Gradient generation

Gradients of adhesion peptides (Fig 7) and mechanical properties (Fig 8) were verified by observing gradients of fluorescence within microfluidics networks. The procedure to fill the networks proved to have negligible effects on cell viability (Fig 9).

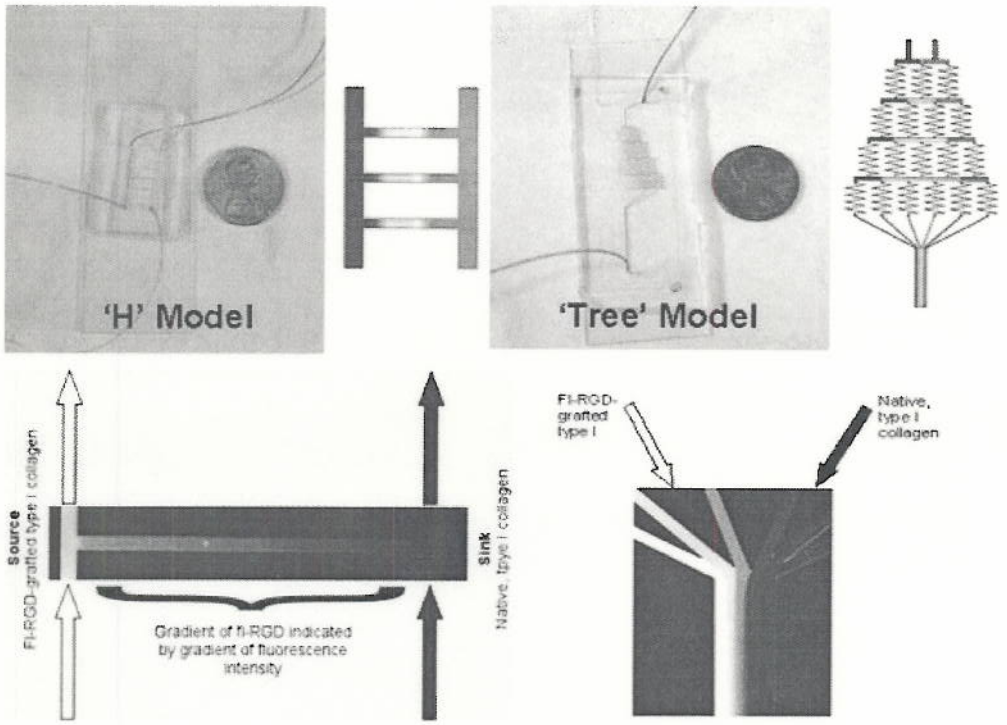

Figure 7: Gradients of adhesion in 3D collagen gels generated with microfluidics. Using our 'H' model and a 'Tree' model (adapted from [16]) of diffusive mixing in microfluidics networks, we generated stable, 'permanent' gradients of cell adhesion molecules by controlling the mixing of peptide-grafted and untreated type I collagen solutions, and then allowing the spatially defined mixture to self assemble and 'lock-in' the gradient. Gradients match those predicted computationally (CFD-ACE+, ESI CFD, Inc).

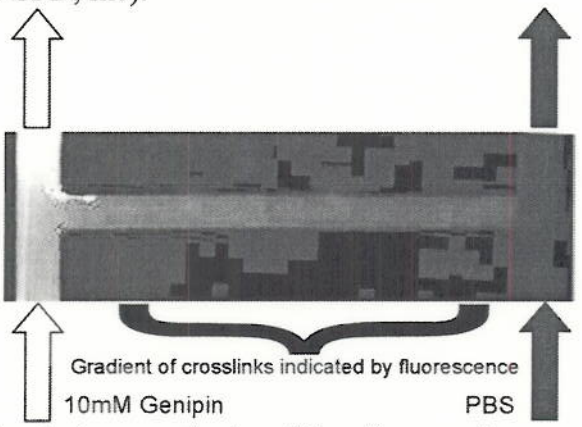

Figure 8: Gradient of mechanical properties in a 3D collagen gel generated with microfluidics. Using the 'H' model, he network is first filled uniformly with a type I collagen solution, which is allowed to self assemble into a hydrogel. PBS (or medium) with a defined concentration of genipin is pumped into one inlet, and PBS (or medium) without genipin into the other to create a stable gradient of genipin throughout the gel. The gradient is established in under a minute, and is maintained for 6hr. The gradient of stiffness is indicated as a gradient of genipin-induced changes in fluorescence, which we have shown to correlate to mechanical properties.

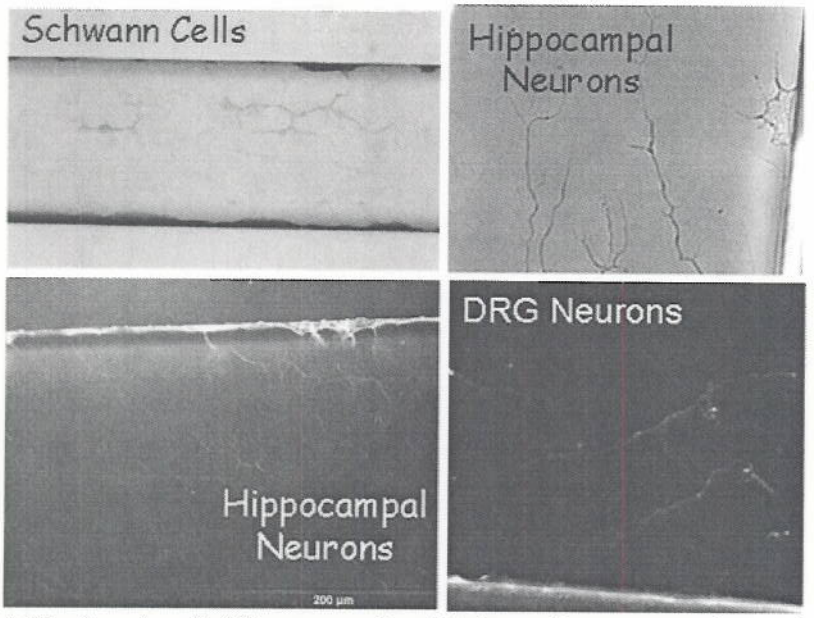

Figure 9: Cell viability in microfluidics networks. We have demonstrated sustained viability of Schwann cells and neurons entrapped in the collagen gels within the microfluidics networks for up to a week, which is the longest duration we have assayed thus far. We are currently quantifying the response of neurons to gradients of mechanical and adhesive properties.

Discussion

The adhesive properties of collagen are easily modified by grafting other molecules to the collagen with heterobifunctional linker molecules, such as N-hydroxysuccinimide (NHS) esters and EDC, or by biotinylating the collagen and grafting the desired molecule to avidin. However, many of the species used in these reaction chemistries are cytotoxic, and in other cases, the procedures require excessive rinses or exposure to temperatures that, while not cytotoxic, significantly affect cell viability. We have presented a protocol that allows for the modification of collagen adhesion properties while in the soluble form with no cytotoxicity, and to therefore safely entrap tissue cells in the modified collagen following self-assembly to form specialized tissue equivalents. Moreover, the technique demonstrates minimal influence on the gel structure or mechanical properties, thus allowing us to have more distinct control over adhesion and mechanical properties

A variety of methods exist to crosslink collagen to increase the mechanical stiffness. In vivo, tissues are naturally crosslinked by enzymes such as lysyl oxidase[17, 18] and transglutaminase [19, 20]. However, use of these enzymes for bulk changes in mechanical properties is cost prohibitive. Chemical treatment with aldehydes are often used to preserve and stiffen tissues. However, for cell-populated collagen gels (often termed "tissue equivalents"), these treatments are 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 [21]. 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 [22], 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. Recently, genipin, a compound extracted from the fruit of the *Gardenia*

Jasminoides, has been shown to crosslink cellular and acellular tissues[23-26], as well as biomaterials including gelatin microspheres[27], alginate-chitosan composites, and poly(ethylene)-glycol hydrogels. Additionally, genipin had been reported to be cell-tolerated [26]. For these reasons, genipin has been suggested as an alternative crosslinking agent for improving the mechanical properties of bioartificial tissues.

The exact mechanism of genipin crosslinking remains unclear, but includes two unique outcomes. First, crosslinking with genipin causes the collagen to turn blue, and the intensity of the color correlates to the degree of crosslinking. Moreover, it has been recently shown that these crosslinks emit fluorescence. Thus, genipin crosslinking generates a molecular fingerprint that may be probed non-destructively in situ to evaluate the degree of crosslinking and, therefore, the mechanical properties of collagen. Herein, we have characterized the effects of genipin exposure on the mechanical properties of acellular collagen gels, and we have demonstrated that these properties correlate to the emitted fluorescence intensity. We also confirmed cell viability of fibroblasts during incubation in genipin, but only at concentrations less than 1mM. These data provide a valuable blueprint for future studies applying genipin-mediated crosslinking in vitro to evaluate mechanotransduction and assist in the design of bioartificial tissues for a variety of tissue systems.

It is now clear that that cell-tissue interactions dictated by the type and density of adhesion sites and the intrinsic mechanical properties of extracellular matrix molecules that provide the tissue structure. The ability to spatially dictate these properties in cell-populated collagen gels with limited effects on cell viability had broad reaching applications for comprehensive in vitro analysis of cell behavior associated with wound healing, development, and tumorigenesis, as well as tissue engineering. We are actively pursuing using this technology to determine the optimum gradients of adhesion and mechanical properties to enhance and direct the growth of spinal cord axons, pattern supporting glia, and stimulate stem cell differentiation, all working towards a multi-modal therapy for spinal cord regeneration.

Acknowledgements

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References

1. Geiger, B., et al., *Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk.* Nat Rev Mol Cell Biol, 2001. **2**(11): p. 793-805.
2. Bao, G. and S. Suresh, *Cell and molecular mechanics of biological materials.* Nat Mater, 2003. **2**(11): p. 715-25.
3. Engler, A., et al., *Substrate compliance versus ligand density in cell on gel responses.* Biophys J, 2004. **86**(1 Pt 1): p. 617-28.
4. Christiansen, D.L., E.K. Huang, and F.H. Silver, *Assembly of type I collagen: fusion of fibril subunits and the influence of fibril diameter on mechanical properties.* Matrix Biol, 2000. **19**(5): p. 409-20.
5. Silver, F.H., L.M. Siperko, and G.P. Seehra, *Mechanobiology of force transduction in dermal tissue.* Skin Res Technol, 2003. **9**(1): p. 3-23.
6. Devireddy, R.V., et al., *Cryopreservation of collagen-based tissue equivalents. I. Effect of freezing in the absence of cryoprotective agents.* Tissue Eng, 2003. **9**(6): p. 1089-100.

7. Shreiber, D.I., V.H. Barocas, and R.T. Tranquillo, *Temporal variations in cell migration and traction during fibroblast-mediated gel compaction.* Biophys J, 2003. **84**(6): p. 4102-14.
8. Enever, P.A., D.I. Shreiber, and R.T. Tranquillo, *A novel implantable collagen gel assay for fibroblast traction and proliferation during wound healing.* J Surg Res, 2002. **105**(2): p. 160-72.
9. Bromberek, B.A., et al., *Macrophages influence a competition of contact guidance and chemotaxis for fibroblast alignment in a fibrin gel coculture assay.* Exp Cell Res, 2002. **275**(2): p. 230-42.
10. Shreiber, D.I., P.A. Enever, and R.T. Tranquillo, *Effects of pdgf-bb on rat dermal fibroblast behavior in mechanically stressed and unstressed collagen and fibrin gels.* Exp Cell Res, 2001. **266**(1): p. 155-66.
11. Whitesides, G.M., et al., *Soft lithography in biology and biochemistry.* Annu Rev Biomed Eng, 2001. **3**: p. 335-73.
12. Kenis, P.J., et al., *Fabrication inside microchannels using fluid flow.* Acc Chem Res, 2000. **33**(12): p. 841-7.
13. Barocas, V.H. and R.T. Tranquillo, *An anisotropic biphasic theory of tissue-equivalent mechanics: the interplay among cell traction, fibrillar network deformation, fibril alignment, and cell contact guidance.* J Biomech Eng, 1997. **119**(2): p. 137-45.
14. Thompson, D.M. and H.M. Buettner, *Schwann cell response to micropatterned laminin surfaces.* Tissue Eng, 2001. **7**(3): p. 247-65.
15. Garcia, A.J. and N.D. Gallant, *Stick and grip: measurement systems and quantitative analyses of integrin-mediated cell adhesion strength.* Cell Biochem Biophys, 2003. **39**(1): p. 61-73.
16. Li Jeon, N., et al., *Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device.* Nat Biotechnol, 2002. **20**(8): p. 826-30.
17. Casey, M.L. and P.C. MacDonald, *Lysyl oxidase (ras recision gene) expression in human amnion: ontogeny and cellular localization.* J Clin Endocrinol Metab, 1997. **82**(1): p. 167-72.
18. Quaglino, D., et al., *Extracellular matrix modifications in rat tissues of different ages. Correlations between elastin and collagen type I mRNA expression and lysyl-oxidase activity.* Matrix, 1993. **13**(6): p. 481-90.
19. Piacentini, M., et al., *"Tissue" transglutaminase in animal development.* Int J Dev Biol, 2000. **44**(6): p. 655-62.
20. Nurminskaya, M.V., et al., *Transglutaminase factor XIIIA in the cartilage of developing avian long bones.* Dev Dyn, 2002. **223**(1): p. 24-32.
21. Girton, T.S., T.R. Oegema, and R.T. Tranquillo, *Exploiting glycation to stiffen and strengthen tissue equivalents for tissue engineering.* J Biomed Mater Res, 1999. **46**(1): p. 87-92.
22. Weadock, K.S., et al., *Physical crosslinking of collagen fibers: comparison of ultraviolet irradiation and dehydrothermal treatment.* J Biomed Mater Res, 1995. **29**(11): p. 1373-9.
23. Chang, Y., et al., *Acellular bovine pericardia with distinct porous structures fixed with genipin as an extracellular matrix.* Tissue Eng, 2004. **10**(5-6): p. 881-92.
24. Liang, H.C., et al., *Effects of crosslinking degree of an acellular biological tissue on its tissue regeneration pattern.* Biomaterials, 2004. **25**(17): p. 3541-52.
25. Sung, H.W., et al., *Stability of a biological tissue fixed with a naturally occurring crosslinking agent (genipin).* J Biomed Mater Res, 2001. **55**(4): p. 538-46.
26. Sung, H.W., et al., *Crosslinking of biological tissues using genipin and/or carbodiimide.* J Biomed Mater Res, 2003. **64A**(3): p. 427-38.
27. Liang, H.C., et al., *Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: in vitro and in vivo studies.* J Biomed Mater Res, 2003. **65A**(2): p. 271-82.