The Impact of Laminin on 3D Neurite Extension in Collagen Gels

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Abstract

The primary goal of this research was to characterize the effect of laminin on three dimensional (3D) neurite growth. Gels were formed using type I collagen at concentrations of 0.4-2.0 mg/mL, supplemented with laminin at concentrations of 0, 1, 10, or 100 µg/mL. When imaged with confocal microscopy, laminin was shown to follow the collagen fibers; however, the addition of laminin had minimal effect on the stiffness of the scaffolds at any concentration of collagen. Individual neurons dissociated from E9 chick dorsal root ganglia were cultured in the gels for 24 hours, and neurite lengths were measured. For collagen gels without laminin, a typical bimodal response of neurite outgrowth was observed, with increased growth at lower concentrations of collagen gel. However, alteration of the chemical nature of the collagen gel by the laminin additive shifted, or completely mitigated, the bimodal neurite growth response seen in gels without laminin. Expression of integrin subunits, α1, α3, α6, and β1, were confirmed by PCR and immunolabeling in the 3D scaffolds. These results provide insight into the interplay between mechanical and chemical environment to support neurite outgrowth in 3D. Understanding the relative impact of environmental factors on 3D nerve growth may improve biomaterial design for nerve cell regeneration.

Keywords: Collagen scaffolds, neurite length, dorsal root ganglia, mechanical properties, laminin, integrin receptors
Introduction

When peripheral nerves regenerate, they reach their target cells by navigating a complex extracellular environment [1-3]. One tissue engineering approach to nerve regeneration has focused on the development and optimization of 3D scaffolds to guide a growing nerve to its target. To date, these in vivo 3D scaffolds have been designed around a variety of materials, including synthetic polymeric systems [4-6] and extracellular matrix proteins such as collagen [7-10], laminin [7], or fibrin [11, 12] (also see reviews for further references [13, 14]). As neurites project to their target cells, they encounter various chemical, mechanical, and electrical stimuli that guide them to their ultimate destination [15-18]. An important source of chemical and mechanical cues for the extending nerve is the environment within the scaffold, where structural and adhesive proteins, such as collagen I and laminin (LN) are incorporated. Understanding the interactions between neurites and the extracellular matrix (ECM) in vitro is therefore an important component to promoting nerve regeneration in vivo.

Previous work has characterized the in vitro extension of neurites within 3D gels of collagen I, examining both the effect of adhesion sites and gel mechanical properties [16, 19, 20]. These effects are not unique to neurite growth in collagen gels, and the mechanical:chemical balance has been studied in other 3D materials such as agarose [21], poly(ethylene glycol) [22, 23], Matrigel® [24-26], and chitosan [27]. Like these materials, collagen I is not considered a primary adhesive substrate for neural cells, but rather acts as the structural component of the ECM [28]. In mimicking the natural environment, collagen I can act as a scaffold for the evaluation of other ECM proteins, such as LN, in 3D.

LN is a basement membrane protein important in the development of the neural system. The LN protein has been shown to have a direct effect as a neuronal cue [29], influencing adhesion, neurite outgrowth, and growth cone movement [30]. While LN has been widely investigated and utilized as a
two-dimensional substrate or surface for neural growth, few studies have examined the interplay between protein and neuron in three dimensions. In a study by Deister et al (2007), gels made with collagen I of concentrations between 1.0 and 2.5 mg/mL had no impact on whole rat dorsal root ganglia (DRG) outgrowth or overall neurite length [20]. Further, LN positively impacted both whole rat DRG outgrowth and length, which increased with LN concentration (0.5, 1.0, and 1.5 mg/mL). LN, however, has been found to alter collagen fibrillogenesis [31] and fiber diameter [32] when incorporated into 3D scaffolds. Typically, these types of scaffold changes alter the mechanical properties of the gel. Rheological tests have indicated that the addition of LN in a range of collagen gel concentrations (0.4 mg/mL, [32] – 1.5 mg/mL [20]) had little effect on the gel modulus, but 100 µg/ml of LN was found to have a more significant effect on higher concentration gels (~2.4 mg/mL) [31].

Extending upon these data, in this study we evaluated individual neurite extension from dissociated DRG in gels of low collagen concentration (0.4 mg/mL – 2.0 mg/mL) that have been shown previously to support enhanced neurite growth [16, 33]. Examination of the neurite length over a range of collagen gel concentrations provided a more complete characterization of the effects of ECM component addition to gels. By adding 1, 10, or 100 µg/mL of LN to this range of low concentration collagen gels, a spectrum of 3D scaffold properties and the impact on neurite extension in three dimensions was examined. In general, the outcomes of this work can be applied to the development of biomaterials for \textit{in vivo} nerve regeneration.

\textbf{Materials and Methods}

\textit{Materials:} Phosphate buffered saline (PBS), HEPES, acetic acid, sodium hydroxide (NaOH), sodium bicarbonate, trypsin-EDTA, Hoescht 33258, Kaughn’s F12 media (F12K), LN, bovine serum albumin (BSA), Triton X-100, and formalin were purchased from Sigma-Aldrich (St. Louis, MO). Collagen type
I was isolated from rat tails as described previously [34, 35] or purchased (BD Biosciences). Nerve growth factor (NGF) and fetal bovine serum (FBS) were also purchased from BD Biosciences (San Jose, CA). Fertilized white leghorn Specific Pathogen Free (SPF) eggs were purchased from Sunrise Farms (Catskill, NY). The rabbit anti-rat $\alpha_1$ polyclonal antibody and rabbit anti-human $\beta_1$ polyclonal antibody were obtained from Abcam (Cambridge, MA). The rabbit anti-human $\alpha_3$ subunit polyclonal antibody and mouse anti-chick $\alpha_6$ monoclonal antibody were obtained from Millipore (Billerica, MA). The mouse anti-chick neurofilament antibody (3A10) was obtained from Developmental Studies Hybridoma Bank (DSHB, Iowa). Anti-mouse or anti-rabbit AlexaFluor secondary antibodies (488 nm or 568 nm), AmpliTaq 360 Mastermix, Trizol, and an Alexa Fluor 594 protein labeling kit were purchased from Life Technologies (Carlsbad, CA).

**Collagen Gel Fabrication:** Collagen gels were fabricated at physiological pH using a standard protocol [36]. The following components were mixed in order, on ice, for each collagen concentration: sodium bicarbonate (0.5 mg/mL in gel), ASTM Type I water, NaOH (0.1 M), collagen (rat tail type I in 0.02 M acetic acid), 10X F12K media, HEPES (10 mM in gel), and LN. After mixing, the final concentration of media was 1X. For neurite extension studies and antibody staining, cells were added as the final component of the suspension prior to gelation, maintaining the media concentration at 1X. Gels were composed of 0.4, 0.6, 0.8, 1.0, 1.25, 1.5, or 2.0 mg/mL collagen, and 0, 1, 10, or 100 $\mu$g/mL additive (LN). All solutions gelled at 37°C within 30 min and were within a pH range of 7.2-7.6.

**Neuron Isolation:** DRG were extracted from E9 chick and dissociated in 1x trypsin-EDTA for 20 minutes. Dissociated cells were resuspended in unsupplemented 1X F12K media prior to suspending them in the collagen gel solution. Individual DRG cells were seeded in gels at $1.2\times10^5$ cells/mL and cultured in 1X F12K media supplemented with 20% FBS and 50 ng/mL NGF, with a final concentration
of 25 ng/mL NGF in the gel:media system [19, 37]. Cells were cultured for 24 hours prior to fixing gels for experimentation.

**Microscopy of Gel Scaffolds:** Acellular gels were formed as indicated above except that the LN was pre-labeled with Alexa Fluor 594 via a protein labeling kit. Gels were hydrated with buffer for 1 min, coated with Vectashield, and imaged 24 hours after gelation with an Olympus confocal microscope (FluoView 1000) at 60x magnification using reflective light to detect collagen fibers and fluorescence to detect the Alexa Fluor 594 labeled LN.

**Mechanical Properties:** Viscoelastic properties of acellular gels were investigated via oscillatory shear rheometry (RFS3, Rheometric Scientific). Acellular gels were prepared as described above and allowed to gel for 30 min at 37°C on the rheometer prior to testing. The mechanical properties were measured for at least five gels of each concentration combination using oscillatory shear rheometry with a 50 mm, 0.04 radian stainless steel cone and plate, using a frequency sweep of 1-100 rad/sec at a constant 2% strain [16]. The strain percent was first determined using a strain sweep to maintain the entangled network within the linear viscoelastic regime. The complex modulus (G*, Pa) was determined for each gel from the storage and loss moduli (G’ and G”, respectively) in the linear viscoelastic region at 10 rad/s (n>5). Two-way ANOVA with Tukey post-hoc test (p<0.05) was used to determine statistical significance. All data are represented as mean ± standard error of the mean.

**Neurite Extension:** Gels with cells encapsulated were fixed 24 hours after seeding with 10% formalin and nuclei were labeled with Hoechst 33258. Gels were imaged and analyzed using AxioVision LE 4.8 (AxioVision Software, Carl Zeiss, Germany) to trace the length of the neurite. As previously described, several conditions were imposed to define a measurable extension: cell and neurites must be suspended
within the gel (not on top or bottom), extension must be longer than one cell body, extension must be clearly defined as being from an individual cell (not from a group of cells), and entire extension must fit in one field of view [16]. The neurite lengths (n>100 per condition) were calculated based on scaling factors in the measurement program. A two-way ANOVA with Tukey post-hoc test was completed in SAS (p<0.05) to determine statistical significance [16].

**Integrin Receptor Expression:** DRG cells were screened for integrin receptors via reverse-transcriptase-PCR using RNA isolated from neurons grown on 2D collagen I or laminin surfaces, or grown within 0.4 mg/mL collagen gels that either had 100 µg/mL LN added or no LN added. PCR was performed with primers designed to match the α1, α3, α6, and β1 subunit of the integrin receptors with AmplitTaq 360 Mastermix reagents according to manufacturer instructions. PCR reactions were performed with 40 cycles of 95°C for denaturing (30 sec), 55°C for annealing (30 sec), and 72°C for elongating (1 min). A final elongation step was run for 7 min at 72°C. The following forward and reverse primers (5’ to 3’)

- α1, GAAAGTTGTGCACCGCGC and CATGCCTGGGCTCCTGCGAG;
- α3, ACTTCTTCCAGCCGACGCG and TGTCCCCACGTGCTCCTGCGAG; α3,
- α6, ACCTGGACGCGGAGAACGTGAT and TGCTCTGCAACCCTTT; and β1,
- β1, CCTGCCTGCCACCGACGGA and TCACCGGCAGTGCTTCCTT, respectively. The housekeeping genes RNA polymerase II and GADPH were used for positive controls and to normalize the expressed transcript of the integrin receptor subunits. Three independent PCR reactions were performed, and density was determined using ImageJ from the agarose gel, and normalized to RNA polymerase II.

Using similar surface and gel conditions, confocal imaging of integrin protein-level expression was performed with isolated DRG neurons that were seeded on 2D collagen I or LN surfaces, or within 3D
collagen gels (1 mg/mL). The 3D collagen gels included LN at either 0 or 100 µg/mL, with cells seeded at a density of 2 x 10^4 cells/gel. Cells were fixed after 24 hrs with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 2 mg/mL BSA. For cells plated on surfaces, the primary antibody was incubated while rocking for 2 hours, and for cells in gels, the primary antibody was incubated overnight at 4°C while rocking. Cells were washed and incubated with a secondary AlexaFluor-conjugated antibody (1:200), nuclei stained with DAPI (1:1000), and stored with Vectashield (Vector Laboratories, Burlingame, CA) at 4°C. Images were acquired using a confocal microscope (Olympus FluoView 1000) with a 4x zoom on a 60x oil-immersion lens (NA 1.42).

Results

Microscopy of Gel Scaffolds: To image LN incorporation into collagen gels without any effects of drying, gels with concentrations of 0.4, 1, and 2 mg/mL collagen were made with fluorescently-labeled LN at 0, 1, 10, or 100 µg/mL concentration via confocal microscopy. The images show that LN is present along the collagen fibers (Figure 1). Fluorescent LN was not noted between the collagen fibers and was only localized near the fibers. The higher collagen gel concentration (2.0 mg/mL), and therefore higher fiber density gels exhibit brighter LN fluorescence compared to gels with lower collagen density.

Mechanical Properties: For collagen gels without LN additive, the mechanical stiffness, as defined by G*, significantly increased as collagen gel concentration increased (Figure 2). In general, the increase in collagen concentration increased the stiffness of the gels. In contrast, upon addition of LN to any of the concentrations of collagen gel, the stiffness was not altered compared to similar collagen concentrations with no LN added, for all gels studied.

Neurite Extension: Gels were prepared for a range of collagen concentrations (0.4-2.0 mg/mL) as well as a range of LN additive (1-100 µg/mL) to characterize both chemical and mechanical effects on
neurite extension. For neurite extension, all experiments were performed in parallel using gels with LN additive and those without LN to reduce the inherent variability in these types of neurite measurements.

Collagen Gels Without LN: In general, as the collagen concentration increased in the gel composition, the lengths of neurites decreased (Table 1). Similar to previous results [19], lengths of neurite outgrowth in 0.4 mg/mL collagen gels were statistically increased over gels with 2.0 mg/mL collagen (Figure 3A).

Addition of LN: The addition of LN had mixed results dependent upon the concentration of the base collagen gel (Figure 3B-D, Table 1). Within the 1 µg/mL LN group, neurite lengths in 0.6, 1.0, and 1.25 mg/mL collagen gels were statistically higher than 2.0 mg/mL. With 10 µg/mL of LN added to the collagen gels, neurite lengths in 0.4 mg/mL were statistically lower than 0.6, and 1.0 mg/mL; whereas, lengths in 0.6, 0.8, 1.0, and 1.25 mg/mL were higher than 2.0 mg/mL. In complete contrast to either the 1 or 10 µg/mL LN, 100 µg/mL LN added to the collagen gels resulted in neurite lengths that were markedly reduced in length compared to collagen gels with no added LN. Neurite lengths measured from within the 100 µg/mL LN gels have no statistical differences between collagen concentrations.

The neurite lengths within each collagen concentration were compared to characterize LN additive effects (Figure 4). To compare the effects of LN on neurite extension within each collagen gel concentration, neurite lengths measured in LN gels were normalized to the neurite lengths measured in collagen gels without added LN (Figure 4). Three broad categories of LN effects were observed from low concentration (0.4 mg/mL collagen), mid-concentration (0.6-1.25 mg/mL collagen), and high concentration (1.5-2 mg/mL collagen) gels. In the low concentration collagen gels (0.4 mg/mL), LN additive caused neurite lengths to decrease as LN concentration increases. LN added to these 0.4 mg/mL collagen gels caused a statistically significant decrease in neurite lengths of 27% for 10 µg/mL LN and 44% for 100 µg/mL LN compared to the 0.4 mg/mL collagen gel with no LN added. The higher
concentration collagen gels (1.5-2 mg/mL) all show similar slight reductions in neurite length that are less than the normalized lengths of neurites without LN, but none are statistically different, unlike the 0.4 mg/mL collagen gels. In contrast, the mid-concentration (0.6-1.25 mg/mL) collagen gels all show a similar trend to one another that is quite different than the low and high end of the collagen concentration gels.

**Integrin Receptor Expression:** We investigated whether integrin receptors are expressed by the DRGs to determine whether the structural aspects of LN could be causing the neurons to exhibit reduced neurite outgrowth in scaffolds with 100 µg/mL LN concentrations. First, we determined which transcripts are expressed in DRGs grown on 2D surfaces of collagen or LN, and expressed in DRGs grown within 3D scaffolds of collagen alone, or collagen scaffolds with added LN (100 µg/mL). Reverse transcriptase-PCR was performed with primers unique to three different α subunits and one β subunit that are each known to bind to collagen and/or LN: α1, α3, α6, and β1 [38-40]. Each receptor subunit was tested for expression on and within each surface/scaffold, and normalized to expression of the housekeeping gene, RNA polymerase I. Figure 5A shows a representative set of images acquired from the results of PCR for the α3 DNA on the collagen and LN surfaces as well as the collagen gels with and without LN. Relative to the RNA polymerase I transcript, each of the subunits was expressed in cells grown on the substrate surfaces or within the scaffolds at levels that were significantly different than zero (Figure 5B).

To determine whether each of these integrin receptor subunits are expressed at the protein level, immunocytochemistry was performed on DRGs grown on 2D collagen and LN surfaces, and within 3D gels (1 mg/mL collagen and 1 mg/mL collagen with 100 µg/mL LN). All subunits are expressed on both surfaces, and within both gels. The α1, α6, and β1 subunits were expressed in neurons only, whereas the α3 subunit was expressed within neurons and support cells of the DRG preparation (Figure 6).

**Discussion**
The ECM of developing nerves has long been recognized as a primary source of tropic and trophic support. Unfortunately, the role of specific ECM components in the development of neurites remains largely uncharacterized, particularly in 3D scaffolds. LN, a well-established adhesive protein for neural cells, has been previously shown to support and encourage neurite outgrowth on 2D substrates [25, 41, 42]. In addition, collagen scaffolds for nerve regeneration have been studied [8, 43-46] and several chemical additives have been evaluated in tissue-engineered nerve guidance constructs including LN [7, 43, 44, 47-49], fibronectin [43, 44, 47, 50] and chondroitin sulfate [51, 52]. For this study, neurite growth within 3D collagen gels was studied because 3D culture allowed for a more clinically applicable environment than outgrowth on a 2D substrate, yet still enabled examination of extension \textit{in vitro}.

Primary differences in 2D versus 3D neurite growth arise when investigating overall length, which is reduced on 2D substrates [53, 54], and when studying the impact of environmental factors, such as protein concentration and soluble peptides [19]. By providing a spectrum of 3D mechanical and chemical environments, this study examined the effect of LN on neurite growth.

Changes in collagen I fibrillogenesis have recently been noted with LN addition [31]. Previous studies have demonstrated that LN, up to 100 µg/ml, was found to evenly coat collagen fibers within collagen gels [32]. However, a report where collagen had been pepsin-treated showed non-homogeneous binding of LN, emphasizing the importance of the source and treatment of the collagen [31]. In this study, the effects of LN addition on gel properties were examined by microscopy and rheology.

Specifically, confocal microscopy was used to examine the distribution of LN within the collagen scaffolds. The images, particularly at 100µg/ml, had a relatively homogeneous distribution of LN in the region of the collagen fibers, with little to no fluorescence located within interfiber spacing. This result agrees with previous reports with similar collagen types (non-pepsin treated) [32], where LN seemed to associate with the collagen fibers homogeneously.
The mechanical properties were investigated to determine if LN coating would impact the overall moduli. Previous work demonstrated that LN had no impact on the shear storage modulus of low (0.4 mg/mL) [32] or high (1.5 mg/mL) [20] concentration collagen gels. In contrast, reduced mechanical properties in pepsin-treated collagen gels was found with added LN (10 µg/mL – 100 µg/mL), likely due to the non-homogenous binding of LN [31]. This data agrees with Deister [20] and Kuntz [32], where collagen gel stiffness was unchanged with added LN and the type of collagen (e.g., non-pepsin treated vs pepsin treated) was consistent.

To determine if LN altered cell behavior, we investigated dissociated DRG neurite extension in collagen gels with added LN. Dissociated DRG cells rather than whole DRG explants were used in this study because they allowed for direct measurement of the length of neurite extension from individual neuron bodies. The lengths of neurites reported here were similar to previous studies using dissociated chick DRG [16, 19, 21]. Also, neurite outgrowth was greater at lower collagen concentrations than higher collagen concentrations [16]. The addition of LN had a varied effect on neurite extension, although the trends indicated similar responses for LN compared to other additives within 3D collagen environments [19, 20]. Therefore, the changes in neurite extension at each collagen concentration can be isolated to the 3D chemical environment experienced by the cell.

Both concentration of binding sites and ligand affinity have been implicated in neurite extension in 3D scaffolds, and in particular, intermediate densities have resulted in enhanced growth [55, 56]. Within the collagen scaffold, the LN was found through confocal microscopy to be available on or near the surface of the collagen fibers. Therefore, the fiber surface, where binding sites for the growth cone would be expected to reside, appeared to be altered with LN addition. In addition, no background fluorescence was found between the fibers with confocal microscopy (Figure 1), suggesting that the LN is located only in the vicinity of the fibers. Even with these changes to the scaffolds, differences with
integrin gene expression by PCR or protein expression by immunochemistry within the various materials were not found (Figures 5 and 6). While we did not see differences with the addition of LN, previous results obtained with cells in 2D culture showed reduced RNA and total protein expression of \( \alpha 6 \) when cells were cultured on low versus high levels of LN, although the amount of \( \alpha 6 \) expressed on the surface increased with decreasing LN concentration [40]. These differences could be due to either disparities in 2D versus 3D culture, or to the sensitivity of the assays employed with 2D culture that are difficult to utilize in 3D culture.

We show that increasing concentration of LN had varying effects on neurite length (Figure 4). LN has been repeatedly shown as a supportive substrate for neural outgrowth in 2D [57-59] and 3D [7, 20, 60] systems; however, few reports characterize concentration dependent changes [7, 20]. Previous work has demonstrated that the adhesion between the DRG growth cone and LN is increased over other neurite to protein interactions due to high affinity receptors for LN in addition to the low affinity receptors for both LN and collagen type I [57]. The changes in neurite length with increasing amounts of LN were dependent upon collagen concentration. For example, addition of LN to the lowest collagen concentration, 0.4 mg/mL, which demonstrated the longest lengths without added LN, decreased with increasing LN concentration. Examining the lengths within individual LN concentrations shows a clear shift of longest average neurite to increasing collagen concentrations until the highest concentration of LN, 100 \( \mu \)g/mL. This decrease in neurite length with 100 \( \mu \)g/mL LN across all collagen concentrations can be attributed to the altered fiber composition within a collagen scaffold with 100 \( \mu \)g/mL LN over a collagen-only scaffold. It is also interesting to note that not only are the neurite lengths (except within 2 mg/mL collagen gels) reduced with the addition of 100 \( \mu \)g/mL LN, but also the profile of the lengths (relative to stiffness) is flat. While the flat profile is in contrast both to previous work and the collagen-only gels reported here that show lower stiffness gels supporting longer neurite growth, the flattened
profile at 100 µg/ml LN also agrees with work that shows that LN concentration has a greater impact on growth than stiffness [20].

Competitive inhibition of free protein or peptide in a 3D system may cause changes in overall length of the neurites, as has been shown previously [33]. Typically, inhibition studies add small molecules to the supernatant, which readily diffuse in 3D systems, to determine the impact of a particular cell receptor on the growth process. One possibility for reduced growth with high LN concentration could be that free LN inhibited the growth through competition with the fiber surface. For studies with peptide competition within collagen gels, 1mM cyclic Arg-Gly-Asp reduced the growth uniformly across all concentrations of collagen gels. Equivalent molar amounts of free LN would be approximately 200 µg/mL. The confocal images clearly showed increased LN near the fiber surfaces with increasing LN concentration, making it unlikely that free LN is available at concentrations of 1 or 10 µg/mL LN, even after limited washing. In addition, free LN was not noted in the images at any LN concentration and the effect of competitive inhibition was not noted in previous work with collagen-LN scaffolds at higher LN concentration [20]. Therefore, while interactions between growing neurites and free laminin cannot be unequivocally ruled out, they are unlikely to play a role for the LN concentrations studied here.

Conclusion

This study elucidated the physical and chemical effects of LN on DRG neurite growth in 3D collagen gels. Previous research has established the role of collagen I as a structural component of the ECM that also supports neurite outgrowth by providing sites for cellular adhesion. Initially, LN may provide more adhesive contacts with the growing DRG than other proteins within the ECM, but neurites would have enhanced growth in those environments. However, we report in this study that the addition of LN
reduced neurite growth without impacting the stiffness of the gels. Therefore, we have demonstrated that the impact of low concentrations of LN on chick DRG neurite growth depends on the mechanical stiffness of the 3D scaffold. At higher concentrations, the growth becomes independent of the gel stiffness. Because many neural tissue engineered scaffolds in use today utilize LN, these results indicate that LN concentration within varying stiffness scaffolds plays an important role for neurite outgrowth.

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Figure 1: Laminin deposition follows the collagen fibers. Representative confocal images are shown from collagen gels (2 mg/mL) with 0, 1, 10, or 100 µg/mL LN. The collagen was imaged with total internal reflection and the fluorescently labeled LN was imaged with 594 nm excitation wavelength. Minimal fluorescent LN was noted between the fibers (scale bar = 50 µm).
Figure 2: Mechanical stiffness increases with increasing concentration of collagen; however, the addition of LN did not alter the stiffness. Mechanical stiffness of collagen gels with added LN was measured by the overall modulus, $G^*$ with oscillatory shear rheometry within the linear viscoelastic range. Significance (p<0.05) is noted by the symbols for plain gels only: * compared to 0.4 mg/mL collagen gels, # compared to 0.6 mg/mL collagen gels, @ compared to 0.8 mg/mL collagen gels, + compared to 1.0 mg/mL collagen gels, % compared to 1.25 mg/mL collagen gels, and & compared to 1.5 mg/mL collagen gels. No differences were noted with added LN within any concentration of collagen.
**Figure 3:** Neurites extend processes grown within collagen gels. Neurite length was measured from collagen gels without LN (A) and with 1 (B), 10 (C), or 100 (D) µg/mL LN added. Without LN, the neurite length is maximal in the gels of lower collagen concentration. As LN is added, the length plateaus at higher concentrations of collagen. With the addition of 100 µg/mL LN, no differences are noted in the length of the neurites across the range of collagen concentrations. Greek letters indicate a significant difference between the samples marked; no differences were noted between collagen concentrations with 100 µg/mL LN.
Figure 4: Neurite length extension is shifted with stiffness of scaffolds. Neurite lengths grown in collagen with LN scaffolds are normalized to neurite lengths in collagen-only scaffolds. The addition of LN to the collagen matrix impacts the neurite growth, shifting the peak extension from lower to higher stiffness. Greek letters indicate significant differences, with $\alpha$ compared to collagen-only gels, $\beta$ compared to 1 $\mu$g/mL, and $\gamma$ compared to 10 $\mu$g/mL within the same collagen concentration.
Figure 5: Integrin receptor subunits are expressed in the neurons growing on 2D surfaces and within 3D scaffolds. (A) Representative set of PCR products for the α3 subunit is shown with the DNA ladder (L) on the left and the sample (S) loaded on the right. (B) Relative RNA amount compared to RNAPol (n≥3) for surfaces or gels as indicated.
Figure 6: Representative confocal images for DRG neurons growing on a 2D LN surface (A) or within 3D collagen (1mg/mL) gels with or without 100 µg/mL LN (B) are shown. In A, the neurons growing on the LN surface were stained for α3 (red), neurofilament (green) and nuclei (blue), with the merge shown on the right. The images show that the α3 subunit is expressed within neurons and glial/fibroblast
support cells. In B, neurons growing within the 3D scaffolds were stained with antibodies that are specific to the integrin receptor subunits, α1, α3, α6, and β1 (green) and neurofilament antibody specific for neurons (red), with the merge shown on the right. Scale bar is 20 μm.
References


