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## Student Award Winner in the Ph.D. Category for the Society for Biomaterials 31<sup>st</sup> Annual Meeting, Pittsburgh, PA, April 26–29, 2006

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### Anisotropic scaffolds facilitate enhanced neurite extension *in vitro*

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Received 30 January 2006; accepted 2 February 2006

Published online 24 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30747

**Abstract:** Tissue engineering (TE) techniques to enhance nerve regeneration following nerve damage have had limited success in matching the performance of autografts across short nerve gaps (< 10 mm). For regeneration over longer nerve gaps, TE techniques have been less successful than autografts. Most engineered scaffolds do not present directional cues to the regenerating nerves. In our efforts to design a TE scaffold to replace the autograft, we hypothesize that anisotropic hydrogel scaffolds with gradients of a growth-promoting glycoprotein, laminin-1 (LN-1), may promote directional neurite extension and enhance regeneration. In this study we report the engineering of three-dimensional (3D) agarose scaffolds with photoimmobilized gradients of LN-1 of differing slopes. Dorsal root ganglia (DRG) from chicken embryos were cultured in the agarose

scaffolds and their neurite extension rate was determined. DRG neurite extension rates were significantly higher in the anisotropic scaffolds, with a maximal growth rate in an anisotropic scaffold twice that of the maximal growth rate in isotropic scaffolds of LN-1. We suggest that these anisotropic scaffolds, presenting an optimal gradient of LN-1, may significantly impact nerve regeneration. Such anisotropic scaffolds may represent a new generation of tissue engineered materials with built-in directional cues for guided tissue or nerve regeneration. © 2006 Wiley Periodicals, Inc. *J Biomed Mater Res* 78A: 213–221, 2006

**Key words:** agarose, laminin, neurite extension, nerve regeneration, protein gradients

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### INTRODUCTION

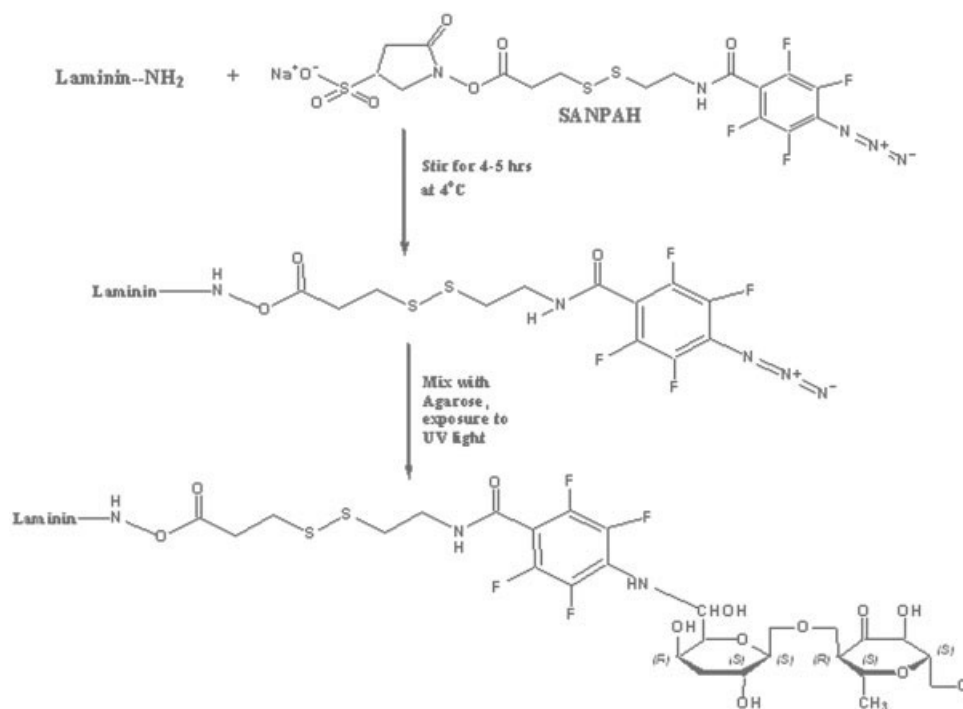
Techniques to repair damaged peripheral nerves include coaptation by suturing the two ends of a severed peripheral nerve together. When the gaps are large, autografts are used as the bridge materials. However, gaps greater than 3 mm cannot be repaired through coaptation and procuring autografts is often difficult because of lack of availability. This has necessitated the pursuit of alternatives to the use of autografts.<sup>1,2</sup> One such alternative approach is to design

a three-dimensional (3D) scaffold containing growth-promoting extracellular matrix (ECM) proteins, such as laminin-1 (LN-1), to promote peripheral nerve regeneration.<sup>3,4</sup> Laminins (LN) are a family of ECM-bound glycoproteins found in basement membrane of most tissues, and are expressed in developing nervous system as well as the adult peripheral nervous system (PNS).<sup>5</sup> Several studies have shown LN as potent promoters of neurite growth in several types of cultured neurons,<sup>6–8</sup> as directional cues for axonal growth *in vitro*<sup>9–12</sup> and as important guidance molecules for steering and pathfinding for developing axons *in vivo*.<sup>13,14</sup>

The ability of LN to stimulate neurite outgrowth *in vitro*, along with the known role of LN in the developing nervous system,<sup>15,16</sup> has led to the hypothesis that LN will contribute positively to peripheral nerve regeneration after injury. Even though regeneration in the PNS is more extensive than regeneration in the central nervous system

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Contract grant sponsor: National Institute of Health; contract grant number: 1R01 NS44409



**Figure 1.** Schematic of photocrosslinking chemistry. LN-1 first reacts with Sulfo-SANPAH via its amine group. The LN-1-SANPAH conjugate is exposed to UV light, activating SANPAH and initiating reaction with C—H bonds in the agarose backbone resulting in the crosslinking of LN-1 to agarose.

(CNS), successful regeneration over large nerve gaps in the PNS is rare.

During development, the guidance of growth cones to their target tissues is mediated by multiple factors, such as chemoattractant, chemorepellant, contact-attractive and contact-repulsive molecules,<sup>17</sup> and some of these growth cone guidance cues may be distributed in a graded fashion.<sup>18</sup> There is considerable evidence for gradients of soluble factors orienting the locomotion of non-neuronal cells and growth cones.<sup>19,20</sup> Although the influence of gradients of soluble substances on neuronal behavior has been extensively studied and has been used to unravel the molecular and cellular mechanisms of axonal guidance,<sup>15</sup> much less is known about gradients of substratum bound substances,<sup>21</sup> such as LN. We hypothesize that anisotropic scaffolds presenting a gradient of LN may enhance neurite extension relative to isotropic scaffolds, potentially leading to higher success rates in large nerve gap regeneration.

In this study we report the fabrication of anisotropic 3D agarose hydrogel scaffolds with gradients of coupled LN-1 and examine the effects of these gradients of LN-1 on neurite extension in 3D. We suggest that these scaffolds represent a new generation of tissue engineered scaffolds with built in directional cues for enhanced nerve regeneration.

## MATERIALS AND METHODS

### Generation of isotropic and anisotropic 3D hydrogels scaffolds

#### Isotropic LN-1 scaffolds generated using photochemistry

Photochemical coupling was used to immobilize soluble, photosensitive LN-1 conjugate.<sup>22</sup> LN-1 (BD Biosciences, Bedford, MA) was coupled to the agarose scaffold using a photocrosslinker, SANPAH (Sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate) (PIERCE, Rockford, IL). SANPAH is a hetero-bifunctional crosslinker with a Sulfo-NHS group, that chemically reacts with amine groups, and a photoreactive perfluoro arylazide group that replaces the hydrogen atom in C—H bonds when triggered with UV light,<sup>23</sup> as schematically depicted in Figure 1. When added to an aqueous solution containing LN-1, SANPAH immediately hydrolyzes and its reactive groups are exposed. The Sulfo-NHS group reacts with the amine group of LN-1 to form an LN-1-SANPAH conjugate. When the LN-1-SANPAH conjugate is added to an agarose solution and exposed to UV light, the perfluoro arylazide group of SANPAH facilitates the coupling of LN-1 to agarose, which has an abundance of C—H bonds. Briefly, SANPAH was weighed and added to a solution of LN-1 in phosphate buffered saline (PBS; pH 7.4) solution so that SANPAH:LN-1 molar ratio is 100:1. The mixture was incubated with stirring at 500 RPM for 4–5 h at 4°C to allow conjugation of SANPAH with LN-1. The LN-1-SANPAH conjugate was

dialyzed against PBS using Spectrum® dialysis blocks (MWCO 8 kDa, Spectrum Lab, Rancho Dominguez, CA) to remove unreacted SANPAH molecules. To this conjugate solution, an equal volume of 2% (w/v) SeaPrep® agarose (BMA, Rockland, ME) solution was added. Agarose solution was prepared by dissolving agarose in PBS solution (pH 7.4) by heating at 60°C and stirring until the solution became clear. The resulting 1% (w/v) agarose scaffold with LN-1–SANPAH conjugate was then exposed to UV light from a BP-100AP lamp (100 W, 365 nm, 12.5 mW/cm<sup>2</sup> at a distance of 3", UVP, Upland, CA), held 3" from the sample, for 60 s, to trigger the coupling of LN-1 to the agarose solution via the SANPAH photocrosslinker. The solution was then allowed to gel by cooling it at 4°C for 20 min. Unbound LN-1 was removed from the gel by washing with PBS solution, with repeated changes, for 2 days. The gel was then heated, below 40°C, degelled and the amount of LN-1 coupled to the gel was determined by Bradford protein assay (BIO-RAD, Hercules, CA). As a control, agarose gel mixed with LN-1 (no SANPAH) was used under conditions identical to those for coupling SANPAH–LN-1.

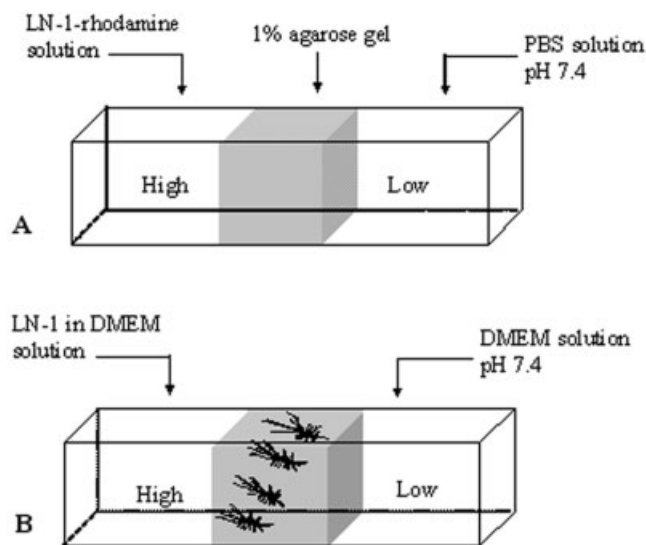
#### Determination of efficiency of coupling LN-1 to agarose scaffold

In order to test the efficiency of photochemical coupling of LN-1 to agarose, different concentrations of LN-1–SANPAH conjugate were mixed with agarose solutions so as to obtain 1% (w/v) agarose solutions with a range of LN-1 concentrations 85 µg/mL, 113.33 µg/mL, and 170 µg/mL. These solutions were then exposed to UV light, at 3" from the UV lamp, for 60 s. The solutions were then allowed to gel by cooling at 4°C for 20 min. As a control, agarose scaffold mixed with LN-1 (no SANPAH) was used under conditions identical to those for coupling SANPAH–laminin. The scaffolds were then washed with PBS solution for 2 days to remove any unbound LN-1. The amount of LN-1 coupled to these scaffolds was determined by Bradford protein assay.

#### Generation of anisotropic LN-1 concentrations in 3D agarose hydrogels

Eight-well chamber slides (Fisher Scientific, Pittsburgh, PA) were used for making anisotropic LN-1 scaffolds and for culturing chick DRG. A 2.5 mm thick block of agarose hydrogel was formed in each well of the chamber slide by adding 1% (w/v) agarose solution between two custom-built teflon bars separated by a distance of 2.5 mm [Fig. 2(A)]. The chamber slide was placed at 4°C for 20 min to allow the agarose solution to gel. After the agarose gelled, the teflon bars were removed and the well was divided into two compartments by the agarose scaffold.

To visualize LN-1 gradient in agarose scaffold, LN-1 molecule was labeled with a fluorescent molecule, rhodamine, using EZ-Label Rhodamine protein labeling kit (PIERCE, Rockford, IL). One of the compartments was filled with 100 µL of LN-1–rhodamine solution (henceforth referred to as the "high" concentration compartment, [Fig. 2(A)]) and the other compartment was filled with 100 µL of PBS solution with no LN-1 (henceforth referred to as the "low" concentration compartment, [Fig. 2(A)]). The scaffolds were then stored in an incubator at 37°C and 95% humidity for 6 h and LN-1 was allowed



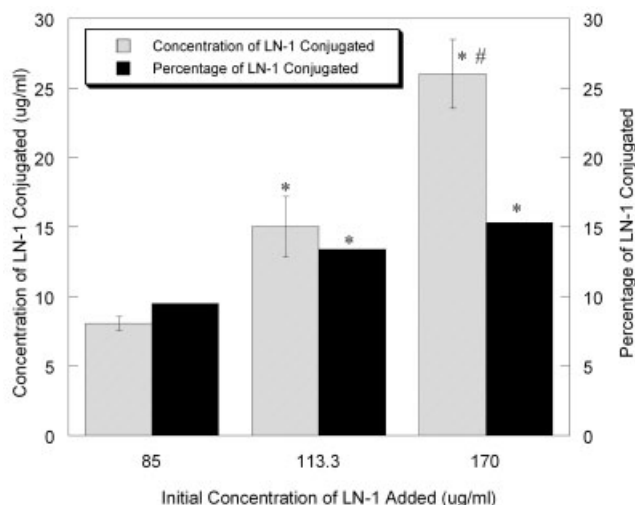
**Figure 2.** Schematic of experimental setup for gradient studies and DRG cultures. A: To synthesize and characterize anisotropic LN-1 scaffolds an agarose scaffold block was prepared in the middle of a slide chamber well. LN-1 solution was added in the compartment marked "high" and PBS solution was added in the compartment marked "low." B: For studying neurite extensions in LN-1 gradients, DRG were first mixed with agarose and then the LN-1 gradient was made.

to diffuse through the agarose scaffold from the "high" concentration compartment to the "low" concentration compartment. After 6 h, the solutions from the two compartments were removed and the scaffold block was immediately exposed to UV light from a BP-100AP lamp (12.5 mW/cm<sup>2</sup> at a distance of 3") for 60 s to photoimmobilize LN-1–rhodamine. Uncoupled rhodamine–LN-1 was removed by washing the agarose scaffold block with PBS solution for 2 days. To test whether LN-1 was coupled to the scaffold in a gradient fashion, fluorescent images were captured using MicroFire™ digital camera (Olympus, Melville, NY) attached to an upright microscope (Model Axioskop 2, Carl Zeiss, Germany). Images were taken at three different depths within the scaffolds, to check for uniformity of coupling through the thickness of the gel. The fluorescence intensity of the images was measured using ImagePro™ software (OPTRONICS, Coleta, CA). A relationship between fluorescence intensity and rhodamine–LN-1 concentrations was obtained by mixing known concentrations of rhodamine–LN-1 in 1% agarose scaffold and then measuring its fluorescence intensity.

#### Determining response of chick dorsal root ganglia neurites to LN-1 gradients

##### DRG culture in agarose hydrogel

Dorsal root ganglia (DRG) were harvested from 9-day old chicken embryos (E9) into PBS solution. Each DRG explant was split into 2–3 pieces and suspended in 1% agarose solution. An agarose scaffold block containing DRG was made as described above for plain agarose [Fig. 2(B)]. LN-1 solution diluted in Dulbecco's Modified Eagle Medium (DMEM) was added to



**Figure 3.** Photochemical immobilization of isotropic LN-1-concentrations in agarose scaffolds. Note that the concentration of LN-1 coupled to agarose scaffold increased when higher concentrations of LN-1 were added. The percentage of LN-1 coupled to agarose also increased with concentration but saturated around 15.31%. \* represents  $p < 0.05$  vs. 85 µg/mL data, # represents  $p < 0.05$  vs. 133.33 µg/mL data; student's paired  $t$ -test was performed for  $n = 3$  samples/group.

the "high" concentration compartment and DMEM by itself was added to the "low" compartment. LN-1 was allowed to diffuse through the agarose scaffold for 6 h and then the hydrogel was exposed to UV light for 60 s at a distance of 3". DMEM with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS) and 50 ng/mL nerve growth factor (NGF) was added to each of the wells. Neurite extension from DRG was visualized using an inverted microscope (Model Axiovert 200M, Carl Zeiss, Germany). The lengths of specific, marked neurites were quantified on day 2, day 3, and day 4 of culture to help determine the rate of neurite extension. Extreme care was taken to measure the length of the same neurites for these measurements, with appropriate landmarks in the culture. The choice of neurite was not random, however, the longest neurites were identified by measuring their lengths at day 2, and subsequently these neurites were followed until day 4 of culture. To test the influence on neurite outgrowth due to gradients of LN-1 as compared to uniform concentrations of LN-1, three conditions were studied. DRGs were cultured in 3D scaffolds with either uniform concentrations of LN-1 (0.2, 0.5, 0.8, 1.02, 3.4, and 10.21 µg/mL) or gradients of LN-1 (0.017 µg/mL/mm, 0.051 µg/mL/mm, and 0.121 µg/mL/mm) or plain agarose (0 µg/mL).

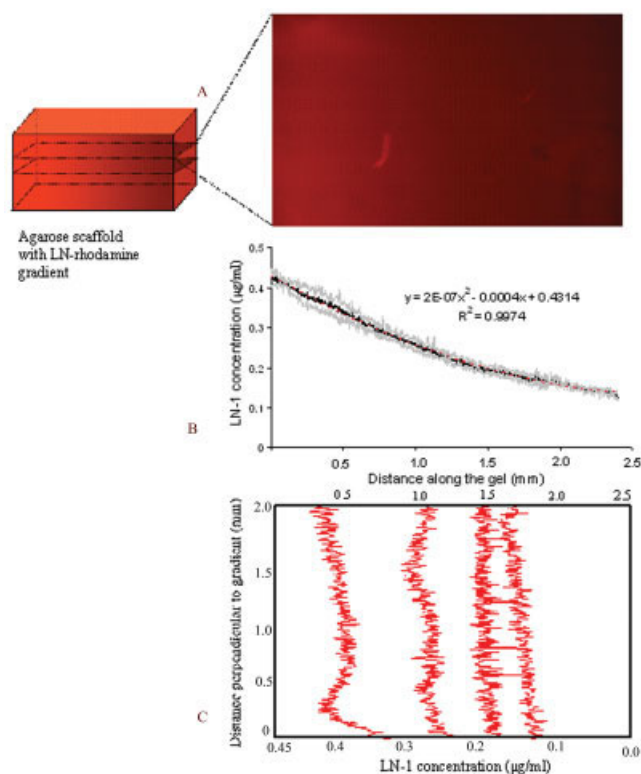
## RESULTS

### Characterization of 3D hydrogels with controlled, anisotropic LN-1 concentrations

#### Generation of isotropic LN-1-agarose scaffolds

Increasing the amount of LN-1-SANPAH conjugate initially added to the agarose solution led to propor-

tionally higher amounts of LN-1 being coupled to the agarose scaffold (Fig. 3). When the amount of LN-1 initially added to agarose scaffold was doubled from 85 µg/mL to 170 µg/mL, the amount of LN-1 coupled to the scaffold increased more than three times (from 8.07 to 26.02 µg/mL) suggesting that the relationship between the amount of LN-1 added and amount of LN-1 coupled to scaffold is nonlinear. The coupling efficiencies ranged from 9.5% (w/w) for the 85 µg/mL LN-1-agarose solution to 15.3% (w/w) for the 170 µg/mL LN-1-agarose solution (Fig. 3). The coupling efficiencies for 133.33 µg/mL (13.41%) and 170 µg/mL (15.31%) were not significantly different (as compared by student's paired  $t$ -test). This suggests that the coupling efficiency had saturated at around 15% (w/w) for the range of LN-1 concentrations used in our study.



**Figure 4.** Concentration profile of LN-1-rhodamine bound to agarose scaffold. The gradient shown here was obtained when 282 µg/mL of LN-1 solution was added to the "high" compartment and PBS was added to the "low" compartment. The concentration profiles in the direction along (B) and perpendicular (C) to the gradient were obtained by averaging intensity profiles from three images at different depths from the same scaffold block. In (B), the dark line indicates the average concentration, gray lines indicate standard deviations from the average values, and the red line indicates the polynomial fit to the average concentration profile. A second-order polynomial fit gives a close approximation of the concentration distribution.

### Characterization of LN-1 gradients in agarose scaffolds

Anisotropic LN-1 scaffolds with three different concentrations ranges, 0.430–0.126  $\mu\text{g}/\text{mL}$ , 0.250–0.123  $\mu\text{g}/\text{mL}$ , and 0.162–0.120  $\mu\text{g}/\text{mL}$ , having different slopes of LN-1 concentration gradients, 0.121  $\mu\text{g}/\text{mL}/\text{mm}$ , 0.051  $\mu\text{g}/\text{mL}/\text{mm}$ , and 0.017  $\mu\text{g}/\text{mL}/\text{mm}$ , respectively, were obtained by adding different concentrations of LN-1 in the “high” compartment. Figure 4 depicts the distribution of immobilized rhodamine–LN-1 across a 2.5 mm wide agarose scaffold block along the direction of gradient [Fig. 4(B)] and also perpendicular to the gradient [Fig. 4(C)]. This LN-1 distribution was obtained when 282  $\mu\text{g}/\text{mL}$  LN-1 solution was added to the “high” concentration compartment of the scaffold and PBS solution was added to the “low” concentration compartment. UV immobilization was done after 6 h of diffusion. An average intensity profile was obtained by taking fluorescence intensity images at three different, fixed levels within the same agarose scaffold block and averaging the intensity values. The LN-1 distribution in the anisotropic scaffolds could be approximated by a second-order polynomial as shown in Figure 4B.

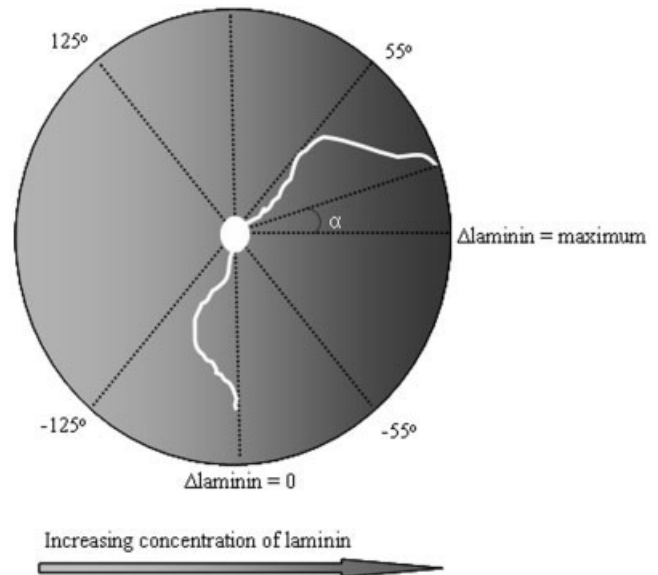
### Neurite growth from DRGs cultured in gradients of LN-1

#### Neurite extension in isotropic LN-1 scaffolds

Three culture wells were used for DRG culture for each of the different isotropic LN-1 concentration scaffolds: 0, 0.2, 0.5, 0.8, 1.02, 3.4, and 10.21  $\mu\text{g}/\text{mL}$ . For each condition, images of DRGs and their neurites were captured on day 1, day 2, day 3, and day 4. Of all the dozens of neurites imaged, around 30–35 neurites from 5–6 different DRGs, which could be reliably followed over the 4 days of culture, were chosen for growth rate measurements. The rate of neurite extension was calculated by measuring neurite lengths at different time points. Care was taken to see that the length of the same neurite was measured at different time points by using fixed landmarks in the culture well. Chick DRG showed maximum neurite growth rate for the LN-1 concentration range of 0.2–0.5  $\mu\text{g}/\text{mL}$  and the rate decreased as the LN-1 concentration was increased [Fig. 7(B)].

#### Neurite extension in anisotropic LN-1 scaffolds

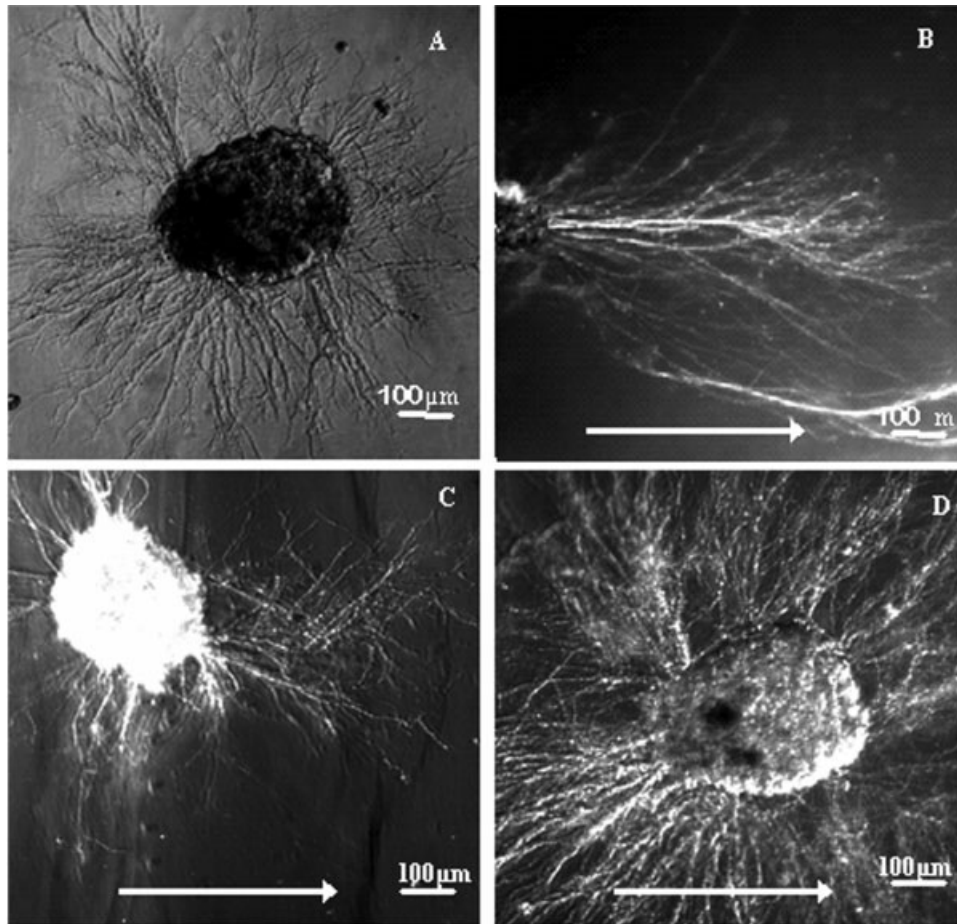
DRG neurite growth rate in three different LN-1 gradients: 0.121  $\mu\text{g}/\text{mL}/\text{mm}$  (from 0.430–0.126  $\mu\text{g}/\text{mL}$ , over 2.5 mm thick agarose scaffolds), 0.051  $\mu\text{g}/\text{mL}/\text{mm}$



**Figure 5.** Schematic of an axonal process on a LN-1 gradient and how the angle  $\alpha$  is defined. The angles that form the basis for the definition of neurites growing “up the gradient,” “down the gradient,” and perpendicular to the gradient” are indicated.

$\text{mL}/\text{mm}$  (0.250–0.123  $\mu\text{g}/\text{mL}$ ) and 0.017  $\mu\text{g}/\text{mL}/\text{mm}$  (0.162–0.120  $\mu\text{g}/\text{mL}$ ) (Fig. 6), was determined similar to isotropic LN-1 culture conditions. In all the three LN-1 gradients, similar initial concentration of LN-1 for the lower end was applied (around 0.120  $\mu\text{g}/\text{mL}$ ), so that the neurites were exposed to identical initial concentration of LN-1. The number of neurites and DRGs considered for rate measurements were identical to the isotropic LN-1 scaffold conditions. The neurite growth rate was highest for the lowest slope of LN-1 gradient, 0.017  $\mu\text{g}/\text{mL}/\text{mm}$ . As the slope of the LN-1 gradient was increased, the neurite growth rate decreased. Neurites were considered as growing “up the gradient” when the angle ( $\alpha$ ) between the line connecting the point where it emerged from the DRG and the tip of the neurite, and the line following the steepest increase in the concentration of LN-1 ( $\Delta\text{laminin} = \text{maximum}$  in Fig. 5) was such that  $-55^\circ < \alpha < 55^\circ$ . The  $55^\circ$  angle was selected because the neurites that grew within  $\pm 55^\circ$  experienced 50% of the maximum slope of change in LN-1 concentration ( $\Delta\text{laminin}$ ) of that gradient. The neurites were considered as growing “perpendicular to the gradient” if  $55^\circ < \alpha < 125^\circ$  and  $-125^\circ < \alpha < -55^\circ$ ; and neurites were considered as growing “down the gradient” if they were extending in the direction of decreasing concentration of LN-1 ( $125^\circ < \alpha < -125^\circ$ ). A schematic illustration of these definitions is shown in Figure 5. One-way ANOVA test was used for statistical analysis and a  $p$  value of less than 0.05 was considered as statistically significant.

For 0.017  $\mu\text{g}/\text{mL}/\text{mm}$  gradient, neurites growing



**Figure 6.** DRG cultured in anisotropic LN-1-agarose scaffolds. (A) plain agarose, (B) 0.017  $\mu\text{g/mL/mm}$  (C) 0.051  $\mu\text{g/mL/mm}$  and (D) 0.121  $\mu\text{g/mL/mm}$  scaffold. The white arrow indicates the direction of increasing concentration of LN-1 (Scale bar = 100  $\mu\text{m}$ ).

“up the gradient” grew significantly faster than neurites growing “down the gradient” ( $p < 0.01$ ), which in turn grew faster than neurites growing “perpendicular to gradient” ( $p < 0.05$ ). For 0.051  $\mu\text{g/mL/mm}$  gradient, neurites growing “up the gradient” were significantly faster than neurites growing “down the gradient” and neurites growing “perpendicular to the gradient,” but the neurites growing “down the gradient” did not grow faster than neurites growing “perpendicular to the gradient.” For 0.121  $\mu\text{g/mL/mm}$  gradient, there was no significant difference between growth rate of neurites growing “up the gradient,” “down the gradient,” and “perpendicular to the gradient” [Fig. 7(A)].

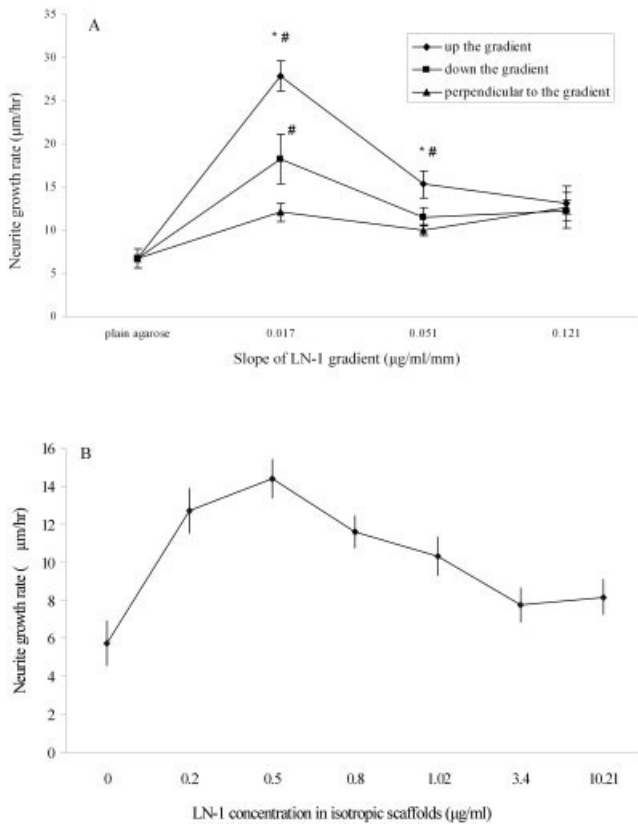
The best neurite growth rate in anisotropic scaffolds was compared to the best neurite growth rate in isotropic scaffolds (Fig. 8). Fastest neurite growth rate in anisotropic scaffolds was at a LN-1 gradient of 0.017  $\mu\text{g/mL/mm}$  (0.120–0.162  $\mu\text{g/mL}$ ) and in isotropic scaffolds was at 0.5  $\mu\text{g/mL}$  LN-1 concentration. In anisotropic scaffold neurite extension “up the gradient” (27.8  $\mu\text{m/h}$ ) and “down the gradient” (18.2

$\mu\text{m/h}$ ) were significantly faster than neurite extension in 0.5  $\mu\text{g/mL}$  isotropic LN-1 scaffold (14.38  $\mu\text{m/h}$ ).

In order to test if LN-1 gradients orient the direction of neurite outgrowth, the total number of neurites growing “up the gradient,” “down the gradient,” and “perpendicular to the gradient” were counted for each culture condition. No significant differences between these numbers were observed for any of the conditions. It was also noticed that neurites which had started growing “down the gradient” or “perpendicular to the gradient” did not try to reorient themselves “up the gradient,” but continued growing along the same direction.

## DISCUSSION

A technique to generate diffuse gradients of nerve growth factor (NGF) in 3D scaffolds has been previously described by Cao et al.<sup>22</sup> As a strategy to promote regeneration across long gaps, immobilized gra-



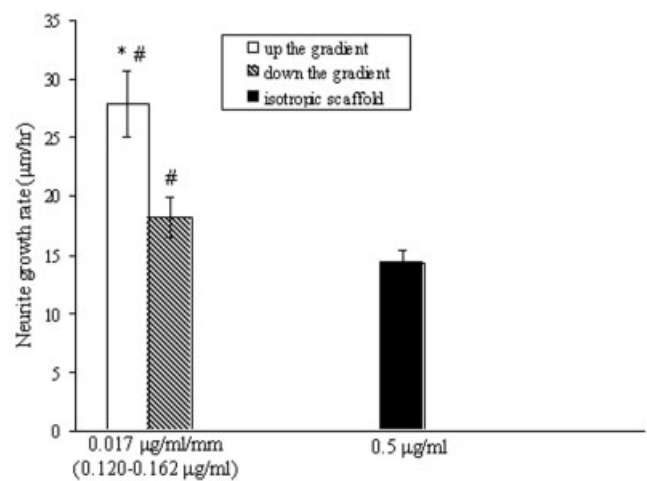
**Figure 7.** Neurite growth rate data from DRG explants in LN-1-agarose scaffolds. A: Neurite growth rate in anisotropic LN-1 scaffolds. One-way ANOVA was performed for  $n = 30$ – $35$  neurites/group. \* indicates  $p$ -value  $< 0.05$  compared to neurites growing “down the gradient,” # indicates  $p$ -values  $< 0.05$  compared to neurites growing “perpendicular to the gradient.” B: Neurite growth rate in isotropic LN-1 scaffolds with different LN-1 concentrations. All error bars indicate standard error of mean (SEM).

dients may be more sustainable than diffuse gradients over the period of several weeks during which PNS regeneration occurs. This study presents a technique to fabricate anisotropic 3D scaffolds with immobilized gradients of LN-1 and quantitatively evaluates the influence of these scaffolds on chick DRG neurite extension. Isotropic LN-1 scaffolds and plain agarose scaffolds without LN-1 were used as controls. The results demonstrate that particular concentration gradients of LN-1 promote faster neurite extension than the highest neurite growth rate observed with isotropic LN-1 concentrations. This suggests that gradients of LN-1 may be a more important parameter to optimize for maximal neurite extension than the absolute concentrations of isotropically distributed LN-1. When DRGs were cultured in anisotropic LN-1 scaffolds with a steep slope of LN-1 gradient ( $0.121 \mu\text{g}/\text{mL}/\text{mm}$  or 241% concentration difference across 2.5-mm scaffold), there was no difference in neurite growth rate “up the gradient,” “down the gradient” and “perpendicular to gradient.” When the steepness

of the gradient was reduced to  $0.051 \mu\text{g}/\text{mL}/\text{mm}$  (103.3% concentration difference across 2.5-mm scaffold), there was difference between neurite growth rate “up the gradient” and “down the gradient” ( $p$ -value  $< 0.05$ ). When the steepness of the gradient was further reduced to  $0.017 \mu\text{g}/\text{mL}/\text{mm}$  (or 35% concentration difference across 2.5-mm scaffold), there was even more difference between neurite growth “up the gradient” and “down the gradient” ( $p$ -value  $< 0.01$ ).

LN-1 is an appropriate candidate as the protein of choice for PNS regeneration since it has been shown to be a potent stimulator of neuronal cell migration and neurite outgrowth.<sup>6–8</sup> In most neuronal tissues, development of the axons and formation of new synapses is preceded by migration of the neuronal cell bodies to the appropriate regions of the brain. It has been proposed that laminin facilitates these processes in several systems.<sup>24–26</sup> The spatiotemporally controlled expression of LN-1 in the developing peripheral nervous system, as well as the visual pathway and the cerebellum, suggests that LN play a role in axon outgrowth and guidance.<sup>27–29</sup> LN expression has been found to be essential for ventral turning of axons during development. Blocking nidogen-binding motifs on LN prevents turning of axons although it does not affect growth,<sup>14</sup> indicating that LN might play a role in orientation of axons in some systems.

Most previous studies have examined the effects of sharp boundaries of LN itself or LN with other molecules to direct neurite outgrowth<sup>30,31</sup> in 2D cultures. Even in other studies that have examined the directionality of axonal elongation on gradients of substratum bound molecules, 2D cultures have been



**Figure 8.** Comparison of fastest neurite growth rate between anisotropic and isotropic LN-1 scaffolds using one-way ANOVA. \* indicates  $p$ -value  $< 0.01$  compared to decreasing gradient group, # indicates  $p$ -value  $< 0.01$  compared to isotropic LN-1 condition. The numbers in the bracket for  $0.017 \mu\text{g}/\text{mL}/\text{mm}$  scaffold show the range of LN-1 concentration in the scaffold. Error bars indicate SEM.

used.<sup>9,12,32</sup> In our study we have used a 3D culture model with immobilized gradients of LN-1, which may be of greater physiological relevance as, *in vivo*, the axons have to navigate a 3D ECM environment, and the changes in LN concentrations are more likely to be gradual than sharp. Previous studies have examined the directionality of axonal elongation on gradients of substratum-bound molecules. McKenna et al.<sup>32</sup> have shown that growth cones of sympathetic neurons show no directional response to substratum-bound laminin gradient. On the other hand, Halfter has shown that elongation of retinal axons was affected by gradients of merosin (laminin-2) or a basal lamina extract.<sup>9</sup> Axons growing down a gradient were shorter and more fasciculated, while axonal turning was noted for axons growing up a shallow gradient. Adams et al. have reported that axonal growth cones can turn and migrate up a substratum-bound gradient of peptide from the LN-1  $\alpha 1$  chain on 2D substrates.<sup>12</sup> But once the growth cones were oriented along the gradient, whether “up the gradient” or “down the gradient,” they grew rapidly in either direction. In the anisotropic scaffolds used in our studies, the extending neurites were exposed to either increasing or decreasing gradient of LN-1. The neurites that were exposed to decreasing gradient did not try to reorient themselves “up the gradient,” but continued to grow “down the gradient.” Similarly, neurites exposed to increasing gradient of LN-1 did not try to reorient themselves “down the gradient.” However, the steepness of the gradients influenced the rate of neurite extension, as described earlier in this section. In this study, the steepest gradient of 0.121  $\mu\text{g}/\text{mL}/\text{mm}$  yielded a 2.41% concentration change across 25  $\mu\text{m}$ , which is the mean diameter of a growth cone. In the 0.051  $\mu\text{g}/\text{mL}/\text{mm}$  gradient, there was a 1.03% concentration change across the growth cone, and in the 0.017  $\mu\text{g}/\text{mL}/\text{mm}$  gradient, there was a 0.35% concentration change across the growth cone. The percentage of change in concentration across the growth cone, at which we observed differences in neurite growth rates in these studies, was generally lower than that used by other studies (10% by Adams et al., and 3.5% by McKenna et al.). However, the amounts of LN-1 in gradients used in this study were generally higher compared to the above mentioned studies, which could have enhanced our ability to observe changes in the neurite growth rates. Interestingly, it was observed that when the steepness of LN-1 gradients (consequently the % change in concentration across growth cone) was decreased from 0.121  $\mu\text{g}/\text{mL}/\text{mm}$  (2.41%) to 0.051  $\mu\text{g}/\text{mL}/\text{mm}$  (1.03%) to 0.017  $\mu\text{g}/\text{mL}/\text{mm}$  (0.35%), the neurite growth rate increased, and the rate of neurite growth “up the gradient” was faster than rate of neurite growth “down the gradient.” This could be due to regulation of receptors for LN-1 by chick DRG. Condic et al. have shown that

chick DRG down-regulate receptors for LN-1 when cultured in high concentrations of LN-1 and up-regulate the receptors when cultured in low concentrations of LN-1.<sup>33</sup> It is possible that in the 0.017  $\mu\text{g}/\text{mL}/\text{mm}$  LN-1 gradient scaffolds, DRGs express more receptors for LN-1 (at the growth cones) than in the 0.051  $\mu\text{g}/\text{mL}/\text{mm}$  and the 0.121  $\mu\text{g}/\text{mL}/\text{mm}$  gradient scaffolds. Substrate–cytoskeletal coupling in the growth cone could transmit mechanical forces to pull the growth cone or its contents toward the anchored adhesive site. Therefore, in the anisotropic LN-1 scaffolds with lower slopes, higher density of receptors might lead to faster neurite growth rates by haptotactic mechanism.

In summary, a novel technique to fabricate anisotropic LN-1 containing hydrogel scaffolds was fabricated and characterized. The results clearly demonstrate that scaffolds with gradients of LN-1 can promote faster neurite growth rate than maximal growth rate possible in isotropic LN-1 scaffolds. The results also demonstrate that there exists an optimal “slope” for the gradient of LN-1, with the lowest slope in the range tested being the most optimal for the highest neurite growth rate. Although some studies have been done to understand how neural cells regulate their integrin expression in response to different isotropic concentrations of LN,<sup>33</sup> in future studies it would be interesting to investigate how neurites adapt to changing concentrations of LN as in the case of gradients. Such anisotropic scaffolds, with directional cues, are likely to enhance peripheral nerve regeneration *in vivo* when presented across nerve gaps inside carrier polymer nerve guidance channels.

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