

Differences between the effect of anisotropic and isotropic laminin and nerve growth factor presenting scaffolds on nerve regeneration across long peripheral nerve gaps

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Abstract

Anisotropic scaffolds of agarose hydrogels containing gradients of laminin-1 (LN-1) and nerve growth factor (NGF) molecules were used to promote sciatic nerve regeneration across a challenging 20 mm nerve gap in rats. Step and continuous gradient anisotropic scaffolds were fabricated and characterized, and regeneration was compared to that in isotropic scaffolds with uniform concentrations of LN-1 and NGF and sciatic nerve grafts harvested from syngenic rats. Polysulfone tubular guidance channels were used to present the agarose-based scaffolds to the nerve stumps. Four months after implantation, regenerating axons were observed in animals implanted with anisotropic scaffolds with gradients of both LN-1 and NGF molecules and nerve grafts, but not in animals with isotropic scaffold implants. Also, the scaffolds with gradients of either LN-1 or NGF, with the other component being uniformly distributed in the scaffold, did not elicit axonal regeneration. The total number of myelinated axons was similar for the anisotropic scaffold and the nerve graft conditions, with the anisotropic scaffolds having a higher density of axons than the nerve grafts. Axonal diameter distribution was similar for the anisotropic scaffolds and the nerve grafts. The nerve grafts and anisotropic scaffolds resulted in better functional outcome compared to isotropic scaffolds as measured by the relative gastrocnemius muscle weight (RGMW). Additionally the state of neuromuscular junctions as assessed by pre- and post-synaptic staining revealed that both the anisotropic scaffolds performed as well as nerve grafts.

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1. Introduction

Injuries to peripheral nerves may occur due to trauma or surgical procedures, which can result in the loss of muscle function, impaired sensation, and/or painful neuropathies. The standard technique for repair is to transplant autologous nerve grafts from uninjured sites to the injured site. Even though nerve grafts are considered as the “gold standard” for nerve repair, they do not result in complete nerve regeneration and recovery [1,2]. The nerve graft also suffers from additional drawbacks, such as sacrifice of

healthy functional tissue, additional surgery time, and size mismatch.

As an alternative to nerve grafts, synthetic tubular nerve guidance channels (NGCs) have been used with promising results [3–6]. Performance of the NGCs can be enhanced by varying their porosity [7], electrical activity [8–10], and surface roughness [11]. Silicone NGCs promote spontaneous axonal regeneration over 10 mm nerve gaps in rats, when the distal end of NGC is sutured to the distal nerve stump. However, in almost all cases of NGC use, regeneration fails if the nerve gap is increased to 15 mm or longer [12,13]. Additional filler materials (such as hydrogels), growth factors, extracellular matrix (ECM) proteins, and fibers have been used to enhance the performance of NGCs [14–17].

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ECM protein laminin (LN), present in the basement membrane of most cells, is a potent stimulator of neurite outgrowth in neurons from peripheral and central nervous systems. LN also enhances attachment and survival of neuronal cells [18–21]. Nerve growth factor (NGF) is a neurotrophic factor produced by the target organs of sympathetic and sensory nerves [22]. NGF has been shown to stimulate neurite outgrowth and promote the survival of sensory ganglia, including those which give rise to spinal sensory nerves and sciatic nerves [23–25]. Due to their potency, LN-1 (an isoform of LN) and NGF have been used successfully for neurite extension *in vitro* and for nerve regeneration *in vivo* [26–30]. Hence, in this study we have used LN-1 and NGF to promote sciatic nerve regeneration.

Various hydrogel scaffolds have been used for neuronal cell culture *in vitro* and for axonal regeneration *in vivo* [31–33]. In this study, we have used agarose hydrogels to promote axonal regeneration. Agarose is a polysaccharide derived from red algae. *In vitro*, SeaPrep[®] agarose hydrogel has been shown to support neurite extension from a variety of neurons in a non-immunogenic manner [34–37]. Various proteins and glycosaminoglycans can be covalently linked to the agarose gels through functional groups on their polysaccharide chains. For example, LN-1 protein or fragments of LN-1 can be covalently coupled to SeaPrep[®] agarose gels to further enhance their ability to support neurite extension [30]. Photochemical conjugation can be used to photoimmobilize gradients of LN-1 in agarose gels to promote enhanced neurite extension *in vitro* [38].

NGF and other neurotrophic factors used to promote nerve regeneration have short half-lives, and regeneration over long nerve gaps occurs over a period of weeks, so the use of slow release delivery vehicles is important. For slow release of various molecules, such as growth factors and plasmid DNA phosphatidyl choline-based lipid microtubules (LMTs) have been developed [39–41]. The LMTs are ideal for such sensitive molecules because they can be loaded into the LMTs in an aqueous environment without exposure to organic solvents. LMTs can also be easily embedded in agarose scaffolds, facilitating slow release of trophic factors, without physically impeding the growth cones that navigate the scaffolds. By adjusting the amount of protein loaded into the microtubules, the number of microtubules used, and the length of microtubules; it is possible to control the duration of release of protein and the amount of protein released [42]. In this study we have used LMTs for slow release of NGF, and by loading different amounts of LMTs in different parts of the scaffold, we created NGF gradients to promote axonal regeneration.

Polymer guidance channels with uniform (*isotropic*) distribution of LN-1 and NGF promote nerve regeneration comparable to that of nerve autografts over 10 mm nerve gaps in rodents [43]. However, we hypothesized that for longer nerve gaps, 15 mm or more in rodents or over 80 mm

in humans, *isotropic* scaffolds might not be able to match the performance of nerve autografts. *Anisotropic* agarose hydrogel scaffolds, with gradients of LN-1, have been shown to promote enhanced neurite extension from chick dorsal root ganglia (DRG) *in vitro*, as compared to *isotropic* scaffolds [38]. Similarly, gradients of NGF can initiate turning of neurites towards the NGF-source [44], and guide the neurite growth [28,45]. We hypothesized that *anisotropic* scaffolds with gradients of both LN-1 and NGF will promote enhanced axonal regeneration and better functional outcome than *isotropic* scaffolds over long nerve gaps. Here, we describe the design and fabrication of *anisotropic* scaffolds containing gradients of LN-1 and NGF to bridge a challenging 20 mm nerve gap in rats, and compare anatomical and functional outcomes as compared to the clinical gold standard of nerve grafts as well as isotropic scaffolds with uniformly distributed LN-1 and NGF.

2. Materials and methods

2.1. Design of the anisotropic and isotropic scaffolds

2.1.1. Preparation of the polysulfone nerve guidance channels

Tubular Polysulfone NGCs (Koch Membrane System, Ann Arbor, MI) were used as carriers of LN-1 and NGF-containing agarose hydrogel scaffolds for sciatic nerve regeneration in rats. Polysulfone NGCs had an inner diameter of 1.6 mm, an outer diameter of 3.2 mm, and a length of 22 mm. Before filling the NGCs with the agarose scaffold, they were sterilized by immersion in 70% ethanol solution for 1 day, dried under a sterile laminar flow hood, and washed with sterilized phosphate-buffered saline (PBS) (Mediatech Inc., Herndon, VA). The NGCs were kept hydrated in 0.1 M PBS (pH 7.4) until they were filled with the agarose scaffolds.

2.1.2. Fabrication of LN-1 coupled agarose hydrogels and LMTs for the slow release of NGF

For spatially and temporally controlled presentation of ECM and trophic factors *in vivo*, the agarose hydrogel scaffolds were designed with three components: 0.5% (w/v) agarose hydrogel, immobilized LN-1, and NGF-loaded microtubules. First, thermo-reversible SeaPrep[®] agarose hydrogel (BMA, Rockland, Rockland, ME) was covalently coupled with ECM protein LN-1 using photochemical conjugation technique reported previously [38]. Briefly, LN-1 (BD Biosciences, Bedford, MA) was first conjugated to a bi-functional photochemical crosslinker, Sulfo-SANPAH (Sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate) (PIERCE, Rockford, IL) through the amine groups on the LN-1 molecule. An agarose solution was then added to this LN-1-Sulfo-SANPAH conjugate such that final concentration of agarose was 1% (w/v). The solution mixture was then exposed to ultra-violet light, binding LN-1 to agarose through the photocrosslinker. The solution was then solidified into a gel by cooling in a refrigerator at 4 °C for 20 min. Over the next 2 days, the gel was washed using 1 × PBS, with repeated changes, to remove uncoupled LN-1 molecules. The LN-1 conjugated agarose hydrogel was then liquefied by heating at 45 °C, and cooled to room temperature. The amount of LN-1 conjugated to agarose hydrogel was quantified by Bradford protein assay (BIO-RAD, Hercules, CA). The LN-1-agarose solution was then mixed with an equal volume of NGF-loaded microtubules and injected into polymer guidance channels while in liquid state, followed by cooling for 20 min at 4 °C, causing the gels in the NGCs. Preparation of NGF-loaded LMTs is described below.

2.1.3. NGF release from NGF-loaded LMTs

A drug delivery system using LMTs of 1,2-bis (tricoso-10,12-diyomoyl)-*sn*-3-phosphocholine (DC8,9PC; Avanti Polar Lipids, Inc., Alabaster, AL) was prepared by ethanol deposition method, and used for slow release of NGF, as described in other studies [30,42]. The LMTs had an average length of $45 \pm 20 \mu\text{m}$ (Fig. 1). NGF is slowly released by diffusion from the two open ends of the LMTs.

A study was designed to assess the long-term release of NGF from LMTs. Lyophilized LMTs (10 mg) were rehydrated with NGF solution (0.4 ml of 120 $\mu\text{g}/\text{ml}$ solution) overnight at 4 °C. The solution was then

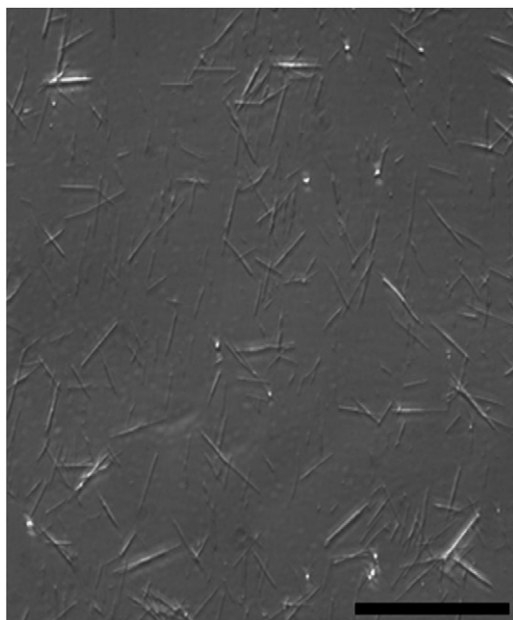


Fig. 1. Lipid microtubules. Phase-contrast image of lipid microtubules from DC8, 9PC lipid. Scale bar = 100 μm .

centrifuged to precipitate LMTs, and the supernatant containing free NGF was removed. The LMTs were mixed with equal volume of 2% agarose solution (w/v) to form a 1% (w/v) LMT-embedded agarose solution. A volume of 300 μl of the mixture was added to a 24-well—plate (Corning Inc., Corning, NY) and cooled at 4 °C for 20 min, to allow it to solidify. About 500 μl of PBS was added on top of the gel block, and the 24-well—plate dish was maintained at 37 °C for the duration of the study. NGF, slowly released from the two open ends of LMTs, diffused into the PBS solution. The PBS solution was replaced with fresh PBS daily, and the amount of NGF in the supernatant was quantified using NGF ELISA kit (Chemicon International, Temecula, CA) to determine the amount and duration of NGF release from the LMTs.

2.1.4. Synthesis of isotropic scaffolds

Isotropic scaffolds with uniform distribution of LN-1 and NGF within the hydrogel scaffolds were designed. LN-1 conjugated agarose solution (66 μg of LN-1/ml of 1% agarose) was synthesized as described above, and mixed with equal volume of NGF-loaded LMTs in PBS solution (9.6×10^8 LMTs/ml of PBS, loaded with 120 $\mu\text{g}/\text{ml}$ of NGF). This resulted in 0.5% agarose solution with LN-1 (33 $\mu\text{g}/\text{ml}$) and NGF-loaded LMTs (4.8×10^8 LMTs/ml). The agarose solution mixture was then injected into 22 mm long polysulfone NGCs using a 1 ml syringe fitted with 22G needles (Becton Dickinson & Co., Franklin Lakes, NJ) and gelled by cooling at 4 °C for 20 min. These scaffolds were kept hydrated in 0.1 M PBS until implantation in rats, on the same day.

2.1.5. Synthesis of step-gradient anisotropic scaffolds

Two kinds of anisotropic scaffolds were designed, one with step-gradients, and the other with continuous-gradients of LN-1. Both step-gradient and continuous-gradient scaffolds had four layers of gels, from one end of the tube to the other, each with a higher concentration of NGF than the previous layer when going from the proximal to distal end. However, in the step-gradient scaffolds, the LN-1 concentration increased in step-wise manner from one layer to another (Fig. 2), while in the continuous-gradient scaffolds, the LN-1 concentration increased smoothly from one end of the tube to the other (Fig. 3).

Agarose solution (0.5% w/v) with immobilized LN-1 (33 $\mu\text{g}/\text{ml}$) and mixed in NGF-loaded LMTs (called Solution 4) was prepared as described

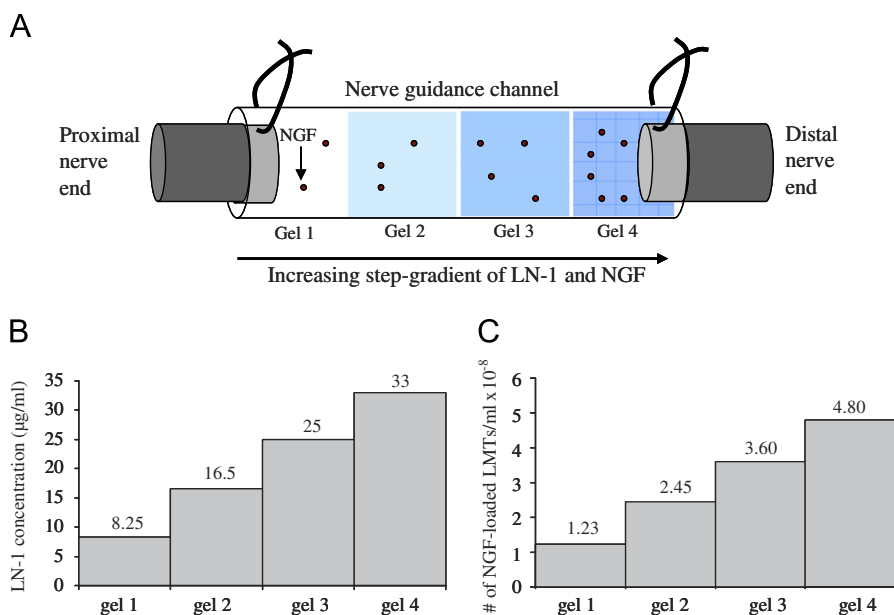


Fig. 2. LN-1 and NGF distribution in step-gradient anisotropic scaffolds. Fig. 2A is a schematic of an NGC connected to nerve ends, with four layers of gels (A). The darker shades of gel represent increasing concentration of LN-1. Gel 4 has higher concentration of LN-1 (Fig. 2B) and NGF-loaded LMTs (Fig. 2C). Gel 3, and so on, while LN-1 gradient is immobilized, NGF will diffuse and form a smooth gradient.

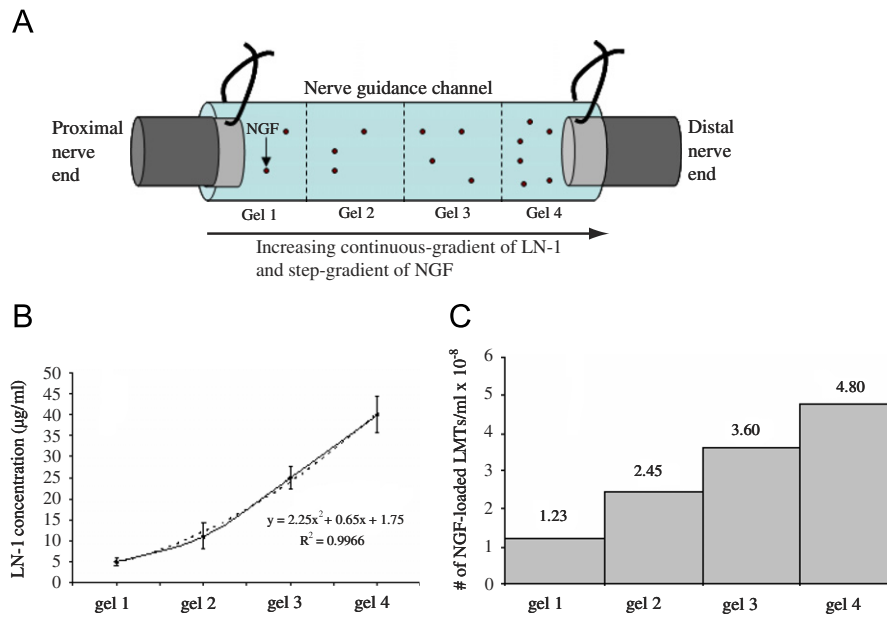


Fig. 3. LN-1 and NGF distribution in continuous-gradient anisotropic scaffolds. Fig. 3A is a schematic of a NGC connected to nerve ends, with four layers of gels in it. LN-1 gradient is smooth (Fig. 3B), as determined by LN-1 ELISA, while NGF-loaded LMTs are distributed in a step-gradient fashion (Fig. 3C). With time, NGF will diffuse out of LMTs and form a smooth gradient.

above. Solution 4 was then diluted with 0.5% agarose solution to form Solution 3, with lower concentration of LN-1 (25 µg/ml) and NGF-loaded LMTs (3.6×10^8 LMTs/ml). Similarly, Solution 2 (16.5 µg/ml LN-1 and 2.45×10^8 LMTs/ml) and Solution 1 (8.25 µg/ml LN-1 and 1.23×10^8 LMTs/ml) were made. Solution 4 was first injected into the polymer guidance channel to fill 25% of volume and allowed to gel by cooling at 4 °C for 10 min. The solutions 3, 2 and 1, were then injected into the polymer guidance channel, one after the other, to make four layers of gels. The four layers resulted in increasing concentrations of LN-1 and NGF from one end of tube to the other end. Fig. 2A shows schematic of a step-gradient NGC connected to the two nerve ends. The LN-1 and NGF-loaded LMTs distribution in a step-gradient scaffolds are also shown (Fig. 2B and C).

2.1.6. Synthesis of continuous-gradient anisotropic scaffolds

To build anisotropic scaffolds with continuous-gradients of LN-1 and NGF, first a step-gradient of NGF-loaded LMTs was synthesized and a LN-1 gradient was made later. A 0.5% agarose solution mixture (solution 4) was first made by mixing 1% agarose solution (no LN-1) with equal volume of NGF-loaded LMTs (resulting in 4.8×10^8 LMTs/ml of PBS). Solution 4 was diluted with 0.5% plain agarose solution to make Solution 3 (3.6×10^8 LMTs/ml). Similarly, solution 2 (2.45×10^8 LMTs/ml) and solution 1 (1.23×10^8 LMTs/ml) were prepared. Solution 4 was first injected into the 22mm long polymer guidance channel to fill 25% of volume, and allowed to gel by cooling. Solutions 3, 2, and 1 were then injected into the polymer NGCs, sequentially, to make four layers of gels, with increasing concentrations of NGF-loaded LMTs (no LN-1). A LN-1 gradient was then made by allowing controlled diffusion of LN-1-sulfo-SANPAH solution (0.68 µg/ml of LN-1) into the guidance channel through one end for 20 h (justification for 20 h is provided in the results, Section 3.1). The LN-1 gradient was then immobilized by UV-photocrosslinking. Fig. 3A shows a schematic of a continuous gradient NGC connected to the nerve ends. The actual distribution of LN-1 and NGF-loaded LMTs is also shown (Fig. 3B and C). To make *anisotropic* scaffolds with gradient of LN-1 but uniform NGF concentration, the entire NGC was first filled with Solution 4, and then a diffusion gradient of LN-1 was made.

To determine the concentration profile of LN-1 in continuous-gradient scaffolds, the tubes were cut transversely into 4 parts, 5 mm each. The total amount of LN-1 in each section was determined by LN-1 ELISA.

2.1.7. Experimental groups for *in vivo* experiments

Nerve implants were separated into 10 groups depending on their constituents, as described in Table 1. Control group I (saline) contained PBS solution. Control group II (plain agarose) contained 0.5% agarose solution without any LN-1 or NGF. Control group II (LN(U)) contained LN-1-coupled 0.5% agarose gel (33 µg/ml of LN-1 uniformly distributed in the gel volume). Control group IV (nerve graft) consisted of 20 mm long nerve grafts harvested from isogenic Fischer inbred rats. Experimental group I (LN(U)-NGF(U)) contained uniform concentration of LN-1 and NGF. Experimental group II (LN(U)-NGF(SG)) contained uniform concentration of LN-1 and step-gradient of NGF. Experimental group III (LN(SG)-NGF(U)) contained step-gradient of LN-1 and uniform concentration of NGF. Experimental group IV (LN(SG)-NGF(SG)) contained step-gradient of both LN-1 and NGF. Experimental group V (LN(CG)-NGF(U)) contained continuous-gradient of LN-1 and uniform concentration of NGF. Experimental group VI (LN(CG)-NGF(SG)) contained continuous-gradient of LN-1 and step-gradient of NGF. After preparation, these implants were stored at 4 °C in PBS until implantation *in vivo*, on the same day.

2.2. *In vivo* implantation of NGCs

A sciatic nerve transection injury model in rats was used. Adult Fischer inbred male rats (Harlan), weighing between 300 and 350 g, were used. The rats were anesthetized using inhaled isoflurane gas (3–4% v/v for induction, 1.5–2% for maintenance) (Aircor, Inc., Madison, WI). The right thigh region was shaved and the skin was sterilized by alternately applying chlorhexidine solution (2%, First Priority, Inc., Elgin, IL) and 70% ethanol, 2–3 times. A 25 mm long skin incision was made along the femoral axis. The thigh muscles were separated and the sciatic nerve was dissected free. Using micro-scissors, the nerve was transected and a 5 mm nerve segment was explanted. The proximal and distal nerve stumps were secured 20 mm apart in a 22mm long polymer guidance channel

Table 1
Notation of experimental and control groups

Groups	Notation of groups	Components	No. of rats
Control I (negative)	Saline	PBS solution	5
Control II (negative)	Plain agarose	0.5% agarose gels (no LN-1)	5
Control III (negative)	LN(U) ¹	Isotropic LN-1-agarose gels	5
Control IV (positive)	Nerve graft	Nerve grafts explanted from other Fischer inbred rats	8
Experimental I	LN(U)-NGF(U)	Isotropic LN-1-agarose gels embedded with isotropic NGF-loaded LMTs	10
Experimental II	LN(U)-NGF(SG) ²	Isotropic LN-1-agarose gels embedded with anisotropic NGF-loaded LMTs	9
Experimental III	LN(SG)-NGF(U)	Anisotropic LN-1-agarose gels embedded with isotropic NGF-loaded LMTs	11
Experimental IV	LN(SG)-NGF(SG)	Anisotropic LN-1-agarose gels embedded with anisotropic NGF-loaded LMTs	9
Experimental V	LN(CG) ³ -NGF(U)	Anisotropic LN-1-agarose gels embedded with isotropic NGF-loaded LMTs	7
Experimental VI	LN(CG)-NGF(SG)	Anisotropic LN-1-agarose gels embedded with anisotropic NGF-loaded LMTs	8

(U)¹ = uniform concentration, (SG)² = step-gradient, (CG)³ = continuous-gradient.

containing the experimental or control gel formulations, using a 10-0 nylon monofilament suture (Ethicon Inc., Somerville, NJ). In case of the positive control group, the nerve gap was bridged using a 20 mm long syngenic nerve graft obtained from another Fischer inbred rat. The standard procedure of using nerve graft from the same rat could not be used, because cutting a 20 mm long nerve section and suturing it back together created tension at the suture lines. The muscles were then closed using a 4-0 vicryl suture (Ethicon Inc., Somerville, NJ) and the skin was closed using wound clips (Braintree Scientific, Inc., Braintree, MA). Marcaine (0.25% w/v, Hospira, Inc., Lake Forest, IL) was administered subcutaneously for pain relief (0.2 ml/rat). The explanted 5 mm nerve was fixed in para-formaldehyde and prepared for histological analysis to evaluate the native nerve prior to injury. After surgery the rats were housed separately with access to food and water *ad libitum* in a colony room maintained at constant temperature (19–22 °C) and humidity (40–50%) on a 12:12 h light/dark cycle. Animals were maintained in facilities approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

In the *anisotropic* scaffolds, the tubes were sutured to the nerve such that the concentration of LN-1 and NGF-releasing LMTs increased from the proximal end to the distal end. The rats were under observation for 4 months before nerve regeneration was evaluated.

2.3. Evaluation of nerve regeneration

2.3.1. Histological analysis for nerve regeneration

Four months post-implantation, the rats were administered an intraperitoneal overdose of anesthetic cocktail (consisting of ketamine at 65 mg/kg of rat weight, xylazine at 7.5 mg/kg, and acepromazine 0.5 mg/kg). The rats were then perfused intracardially with saline, followed by cold 4% paraformaldehyde and 0.25% glutaraldehyde (both from Sigma-Aldrich, St. Louis, MO) in 0.1 M PBS. The site of nerve injury was opened and the implant (polymer NGC or nerve graft) was removed for histological analysis. Along with the nerve implant, the gastrocnemius muscle from the right (experimental side) and left (control side) limb were also explanted. All the harvested tissues were post-fixed, overnight, in 4% paraformaldehyde.

The nerve explants were cut into three equal parts: proximal, middle and distal, and additionally post-fixed, overnight, with 1% osmium tetroxide in PBS. After washing with PBS¹ and dehydration in graded ethanol series, the three parts were separately embedded in LX112 resin (Ladd Research Industries, Inc., Burlington, VT). Semi-thin sections (0.5 μm) of nerve explants were cut using microtome, stained with Toluidine blue (0.1%, Sigma, St. Louis, MO) and observed under a light microscope. Ultra-thin sections (100 nm) were observed under electron microscope.

Nerve regeneration was evaluated at the center (10 mm) and distal end (17 mm) of the NGC/nerve graft by: (a) percentage of guidance channels with successful nerve regeneration, indicated by presence of myelinated axons, (b) the total number of myelinated axons, (c) the area of axonal regeneration, (d) the number of myelinated axons per unit area (density); and (e) the diameter distribution of regenerated axons for each group. For quantification, images were captured using a Sony digital photo camera (Japan) attached to a Nikon Eclipse TE 300 microscope (Japan) running ImagePro[®] software (Media Cybernetics, L.P., Silver Spring, MD). First, images were captured using 4× or 10× objective lenses to determine the area of axonal regeneration, and then a 100× objective lens to count the number of myelinated axons. In the regenerated tissue the axons were evenly distributed, so the area for analysis were selected randomly. 4–5 images (at 100×) were captured for each nerve implant, so that 15–50% of the area of regeneration was used for analysis and the total number of myelinated axons was computed for each implant. One-way ANOVA was used for statistical comparison of the various groups, and a *p*-value < 0.05 was considered as statistically significant.

2.3.2. Relative gastrocnemius muscle weight measurement

The gastrocnemius muscle is innervated by the sciatic nerve, and starts to atrophy after the nerve injury. The gastrocnemius muscle (not including the Soleus muscle) from the right and the left limb were harvested after the rat was sacrificed. In order to account for any effects on muscle weight due to perfusion, rats from control as well as experimental groups were sacrificed by perfusion. All the rats with implants, in each group, were used to calculate relative gastrocnemius muscle weight (RGMW). The RGMW, which is defined as the ratio of the weight of muscle from the experimental side to the control side, was used as one of the parameters for motor function recovery. The RGMW should increase following the sciatic nerve regeneration and successful reinnervation of the muscle.

2.3.3. Neuromuscular junction (end plates), synaptic vesicles and neurofilament staining

Gastrocnemius muscles explanted from the experimental rats were post-fixed overnight in 4% paraformaldehyde, washed with saline solution and left overnight in 30% sucrose solution. The muscle tissue was then cryoembedded. Longitudinal sections, 25 μm thick, were cut using a Microtome (Cryo-star HM 560MV, Microm, Waldorf, Germany). The 25 μm thick sections of gastrocnemius muscle were collected every 800 μm. The mass and thickness of gastrocnemius muscle varied for different groups depending upon extent of regeneration, and hence the number of sections obtained from each rat varied from, 5 (minimum) to 9 (maximum). All the sections were processed for the following markers: *neurofilament 160* (NF160, Sigma-Aldrich, St. Louis, MO), *synaptic vesicles 2 protein* (SV2, Developmental Studies Hybridoma Bank, Iowa City, IA), and *acetylcholine receptors* (using alpha-Bungarotoxin-tetramethylrhodamine,

Sigma-Aldrich, St. Louis, MO) to identify new neuromuscular synapses formed as a result of nerve regeneration.

3. Results

3.1. LN-1 and NGF distribution in isotropic and anisotropic scaffolds

3.1.1. LN-1 concentration in isotropic scaffolds

Bradford assay (BIO-RAD, Hercules, CA) was used to determine the amount of LN-1 coupled to agarose gel. Efficiency of the conjugation technique was about 10–15%. Using a LN-1 solution of 0.51 mg/ml as starting solution, LN-1-agarose gel with LN-1 concentration of 66 µg/ml of 1% agarose solution could be made, and this solution was used for the *in vivo* experiments.

3.1.2. NGF release study

In vitro NGF-release from LMTs was followed up to 18 days (Fig. 4). The cumulative amount of NGF released over the 18 days was 60 ng. Since this amount was released into 500 µl of PBS supernatant, the NGF concentration in the supernatant would be 120 ng/ml after 18 days. For the first 6 days, the cumulative amount of NGF released was linear and rapid, and for the next 12 days the cumulative release still increased linearly, but less rapidly. The NGF-release curve did not reach a plateau after 18 days. The amount of NGF released on 18th day was still much above the detection limit for the NGF ELISA kit, indicating that NGF can be released for more than 18 days from the LMTs.

3.1.3. LN-1 distribution in anisotropic scaffolds

A continuous-gradient of LN-1 was made by allowing controlled diffusion of LN-1 into the anisotropic scaffolds through one end of the polysulfone tubes. By varying the duration of diffusion, different LN-1 distribution profiles were obtained. The continuous-gradient anisotropic

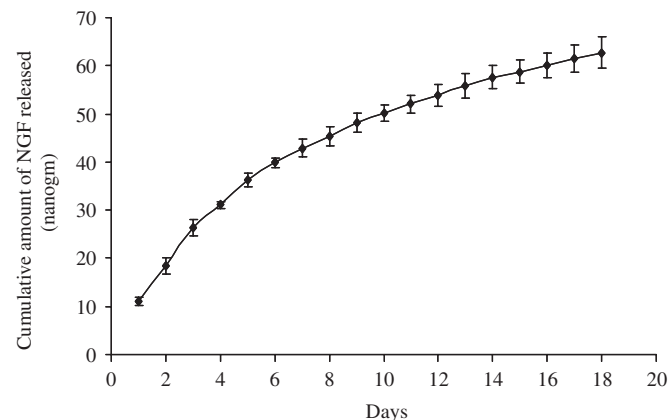


Fig. 4. NGF-release study *in vitro*. NGF-release from LMTs was followed up to 18 days. For the first 6 days, the release profile was almost linear and rapid. For the next 12 days, the release was less rapid but still increased linearly with time. All error bars indicate standard error of mean ($n = 3$).

scaffolds with LN-1 distribution close to that of step-gradient anisotropic scaffolds (4 layers of gel with 33, 25, 16.5, and 8.25 µg/ml, respectively, Fig. 3B) were used for *in vivo* studies, so that their performance can be compared. To obtain such LN-1 distribution, LN-1 solution (0.68 µg/ml) was allowed to diffuse through one end of the polysulfone tube for 20 h, and then immobilized by photocrosslinking.

LN-1 distribution in continuous-gradient anisotropic scaffolds was determined by LN-1 ELISA (Fig. 5). The 20 mm scaffolds were cut transversely into 4 parts, 5 mm each, the LN-1-agarose gel was pipetted out, and LN-1 ELISA was performed. In Fig. 5, LN-1 concentration distribution is plotted along the length of NGC. The average LN-1 concentration in a 5 mm section has been plotted at mid-length (i.e. at 2.5, 7.5 mm, etc.). Since these scaffolds had LN-1 distribution close to that of step-gradient anisotropic scaffolds, they were used for *in vivo* studies, so that their performance could be compared.

3.2. Histological analysis for nerve regeneration

3.2.1. Presence of regenerating axons in nerve implants

A total of 10 groups were studied. Only 3 groups had axonal regeneration through the implants, as determined by histological analysis (Table 2). The 3 groups were rats with 1) nerve grafts, 2) LN(SG)+NGF(SG) (step-gradient of LN-1 and NGF), and 3) LN(CG)+NGF(SG) (continuous-gradient of LN-1 and step-gradient of NGF). The success rate was highest for nerve grafts, with 83.3% of the rats showing regeneration, followed by LN(SG)+NGF(SG) (44.4%) and LN(CG)+NGF(SG) (37.5%) (Table 2). The rest of the histological analysis (axonal area, number of myelinated axons, etc.) could be done for these 3 groups only.

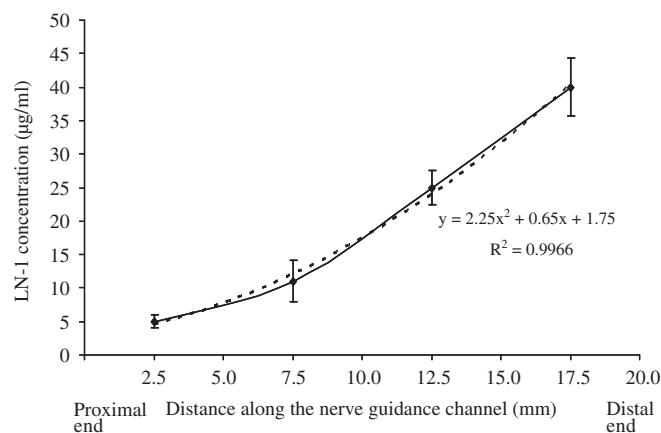


Fig. 5. LN-1 concentration distribution in continuous-gradient anisotropic scaffolds. 20 mm long nerve guidance channels with continuous-gradients of LN-1 were cut into 4 parts, each 5 mm long, and their LN-1 content was determined. The average value for each 5 mm section is plotted at mid-length. LN-1 distribution followed a second-order polynomial. All error bars indicate SEM ($n = 3$).

Table 2
Summary of nerve regeneration in control and experimental groups

Groups	N, total no. of rats in group	No. of rats with regeneration	% regeneration
Saline	5	0	0
Plain agarose	5	0	0
LN(U) ¹	5	0	0
LN(U)+NGF(U)	10	0	0
LN(U)+NGF(SG) ²	9	0	0
LN(SG)+NGF(U)	11	0	0
LN(SG)+NGF(SG)	9	4	44.4
LN(CG) ³ +NGF(U)	7	0	0
LN(CG)+NGF(SG)	8	3	37.5
Nerve graft	6	5	83.3

(U)¹ = uniform concentration, (SG)² = step-gradient, (CG)³ = continuous-gradient.

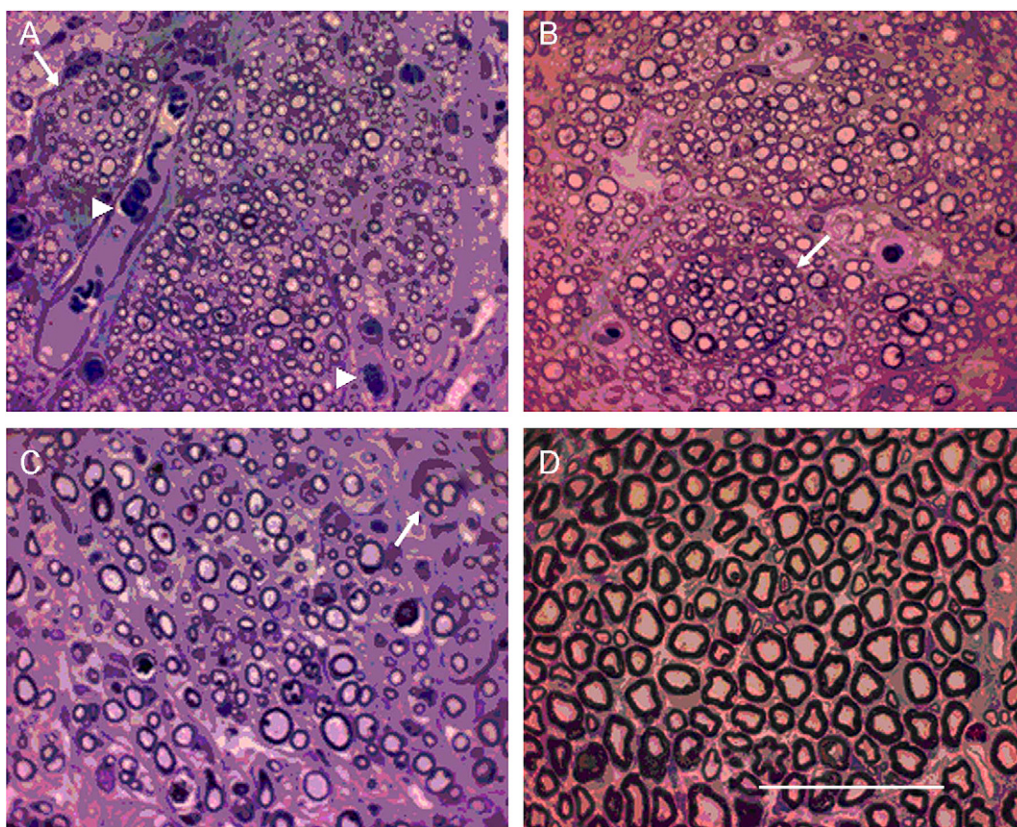


Fig. 6. Light micrographs of nerve sections from groups with successful axonal regeneration. Toluidine-blue stained cross-sections at mid-length (10 mm) are shown here. Anisotropic scaffolds with step-gradient (A) and continuous-gradient (B) of LN-I, have higher density of axons and smaller-diameter axons than nerve grafts (C) and normal nerve (D). Note the fiber grouping in small fascicles (indicated by arrow) and presence of red blood cells (arrowhead). Scale bar = 50 μ m.

The Photographs of histological cross sections at the mid-point of NGCs/autografts/normal nerve are shown in Fig. 6 (A–D). The gross structure of the regenerated nerves was similar to the normal sciatic nerve. Regenerated axons were packed in bundles in thin perineurial-like sheaths to form fascicles, and several fascicles were enclosed in the connective tissues of epineurium. There were abundant blood vessels in the epineurium of regenerated nerves. The implanted agarose gels were completely resorbed without

any trace. Electron microscopy of ultra-thin sections of implants revealed presence of many unmyelinated axons and large numbers of nuclei from Schwann cells clearly surrounding the myelinated axons (Fig. 7). The thickness of the myelin sheath around the axons was greatest for the native nerve (Fig. 7A), followed by the nerve grafts (Fig. 7B) and then the anisotropic scaffolds (Fig. 7C and D). The step-gradient and continuous-gradient scaffolds had similar thickness for the myelin sheath.

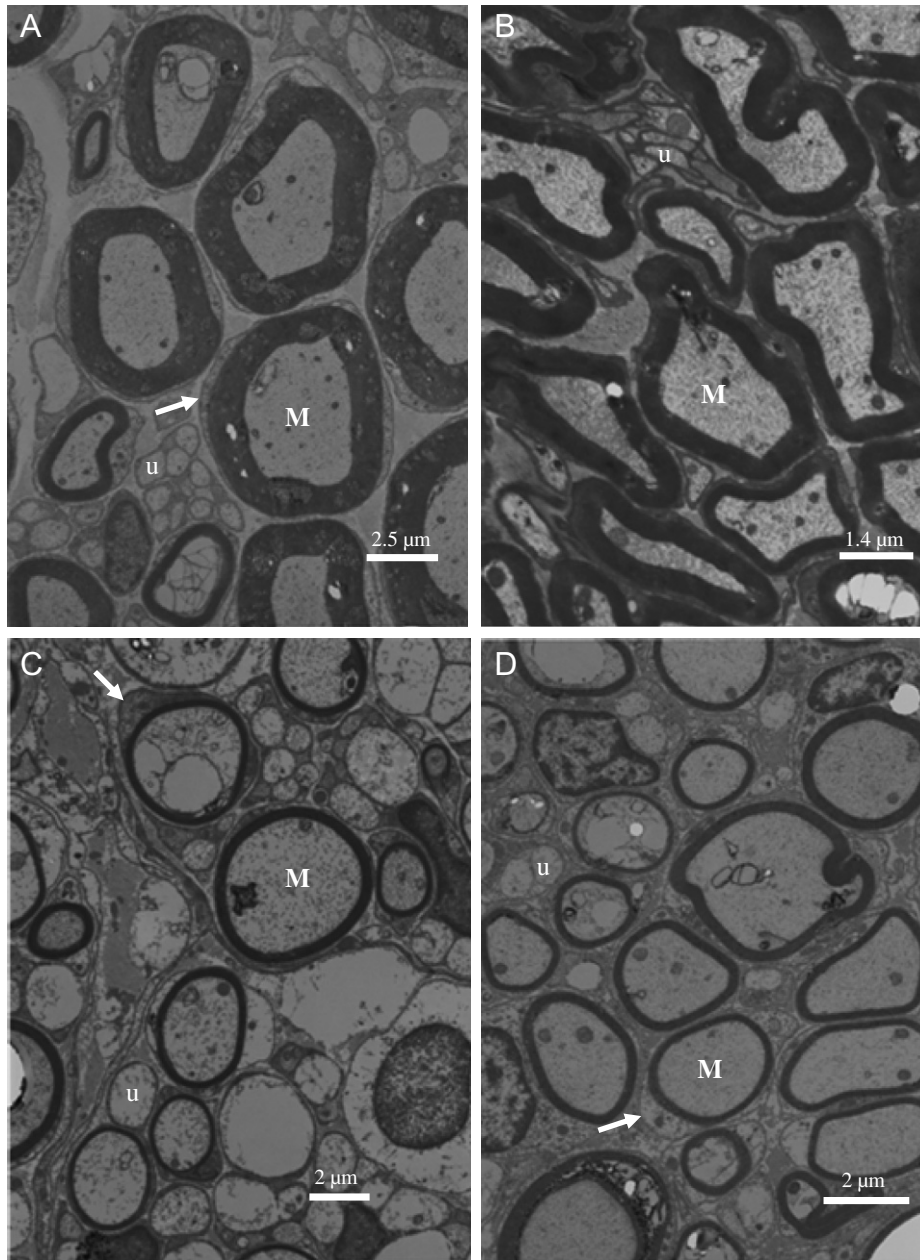


Fig. 7. Transmission electron micrographs of nerve sections at mid-length (10mm) of implants. Myelinated axons (M), unmyelinated axons (u) and Schwann cells surrounding the myelinated axons (arrow) can be seen clearly in all four conditions. The myelin sheath thickness was greatest for the native nerve (A), followed by nerve grafts (B) and then followed by the anisotropic scaffolds (C and D).

3.2.2. Axonal area of regeneration

The area of regeneration was measured at mid-length (10mm) and distal end (17mm) of the implants for the 3 groups which showed nerve regeneration. At mid-length, as well as the distal end, the area was significantly larger for the nerve grafts than the anisotropic scaffolds. However, there was no significant difference between the two anisotropic scaffolds, LN(SG)+NGF(SG) and LN(CG)+NGF(SG) group (Fig. 8). For each of the 3 groups, there was no significant difference between the area of regeneration at mid-length (10mm) and at distal end (17mm).

3.2.3. Total number of myelinated axons and the density of myelinated axons

For each of the 3 groups with regeneration, there was no significant difference between the total number of myelinated axons at mid-length (10mm) and at distal end (17mm) (Fig. 9). At mid-length, the total number of myelinated axons in the nerve grafts and the LN(SG)+NGF(SG) (step-gradient) implants was not significantly different. However, at mid-length, the number of myelinated axons in the LN(CG)+NGF(SG) (continuous-gradient) implants was less than that of nerve grafts. At the distal end (17mm), there was no significant difference

between the 3 groups. In the anisotropic scaffolds, the higher number of myelinated axons at the distal end could be due to higher concentrations of LN-1 and NGF at this end, resulting in branching of the existing axons. The number of myelinated axons in a normal sciatic nerve has also been plotted in Fig. 9. The total number of myelinated axons in the nerve grafts and the LN(SG)+NGF(SG) group is comparable to a normal sciatic nerve. However, the LN(CG)+NGF(SG) group has a lower number of myelinated axons than a normal nerve.

The regenerated axons were more densely packed in the anisotropic scaffolds than in the nerve grafts or in a normal nerve (Fig. 10). The fiber density in anisotropic scaffolds is almost twice that of nerve grafts and five times that of a normal nerve.

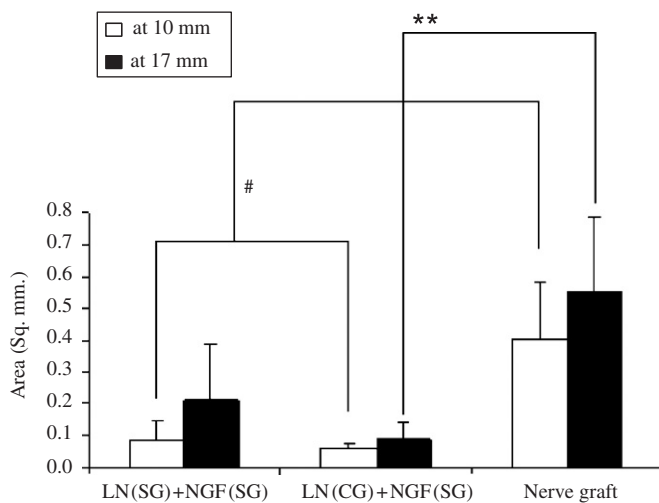


Fig. 8. Area of nerve regeneration. For all three groups, there was no significant difference between area of regeneration at distal end and at the middle. At midpoint, nerve graft has higher area of regeneration than either anisotropic scaffold group ($\#p < 0.05$). However, at distal end, area in nerve graft is higher compared to continuous-gradient group only ($**p < 0.05$). All error bars indicate standard error of mean.

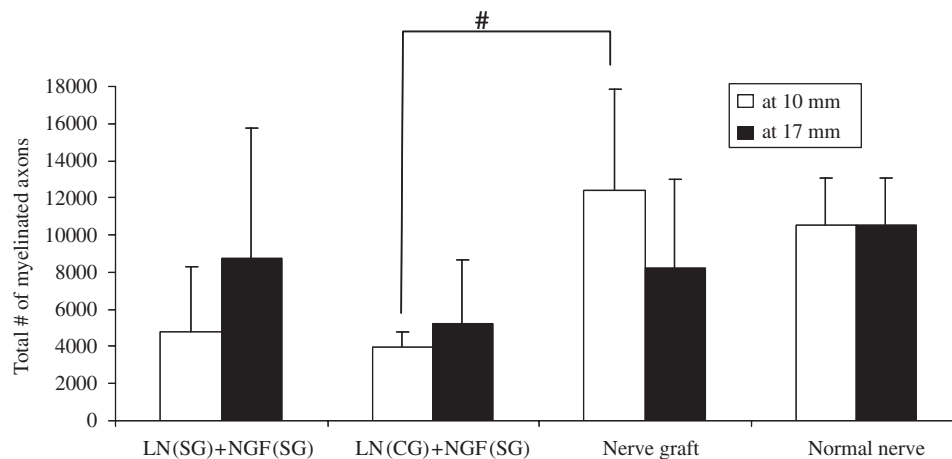


Fig. 9. Total number of myelinated axons. There was no significant difference between step-gradients and nerve grafts. There was no significant difference between the two gradient scaffolds themselves. At mid-length, the continuous-gradient scaffolds had lower number of myelinated axons than nerve grafts ($\#p < 0.05$). All error bars indicate standard error of mean.

3.2.4. Axonal diameter distribution

Regenerating axons were measured using ImagePro[®] software to determine their diameter distribution at mid-length and distal end. The diameter distribution followed a bell-shaped curve (Fig. 11A). The peak of the curve, i.e., the maximum number of axons, was in the 1–2 μm diameter range for the nerve grafts as well as the anisotropic scaffolds. However, the axon-diameter distribution in a normal nerve, was a bell-shaped curve with a much wider spread. In a normal nerve, the maximum number of axons had a diameter range from 2 to 5 μm . The axon diameter distribution pattern at the distal end (17 mm) was similar to that at mid-length (10 mm) (Fig. 11B). The nerve implants had more regenerated axons in the smaller diameter range than a normal nerve, and fewer axons in the larger diameter range than a normal nerve.

3.3. Relative gastrocnemius muscle weight

The gastrocnemius muscle starts to atrophy after sciatic nerve injury, and hence, its mass is directly proportional to the extent of sciatic nerve re-innervation. There was no significant difference in RGMW values between LN(SG)+NGF(SG) (step-gradients) and nerve grafts (Fig. 12). The other groups had significantly lower RGMW values. Although, LN(CG)+NGF(SG) (continuous-gradient) group showed RGMW values close to that of nerve grafts, they were still significantly lower. The step-gradient scaffolds resulted in better functional outcome than continuous-gradient scaffolds. It is possible that the LN-1 and NGF concentrations in the step-gradient scaffolds are more ideal than in the continuous gradient scaffolds, resulting in higher number of myelinated axons (Fig. 9) and higher RGMW (Fig. 12).

3.4. Neuromuscular junction, synaptic vesicles and neurofilament staining

Twenty-five micrometer thick sections of gastrocnemius muscle from normal (uninjured) rats and the treatment

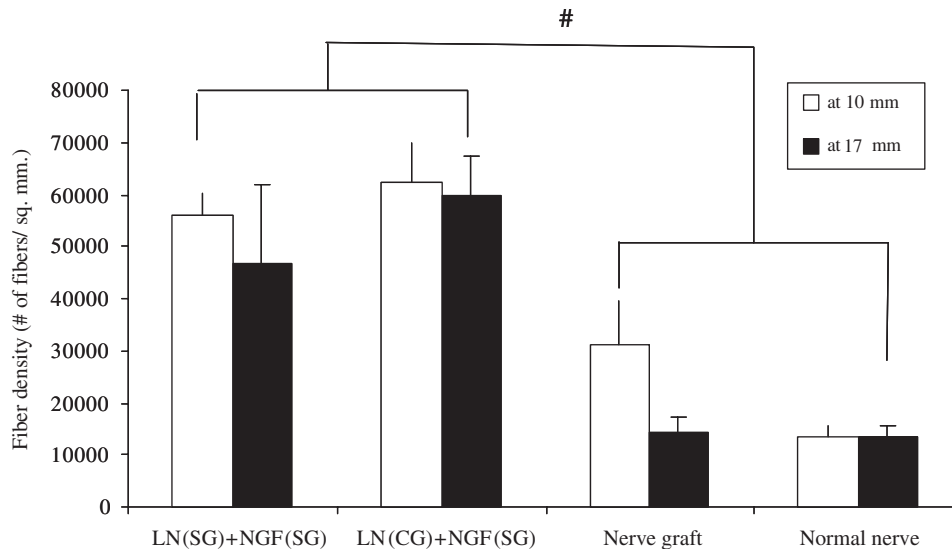


Fig. 10. Density of myelinated axons. The regenerated nerve fibers are more densely packed in anisotropic scaffolds than nerve grafts and normal nerve ($\#p < 0.05$). All error bars indicate standard error of mean.

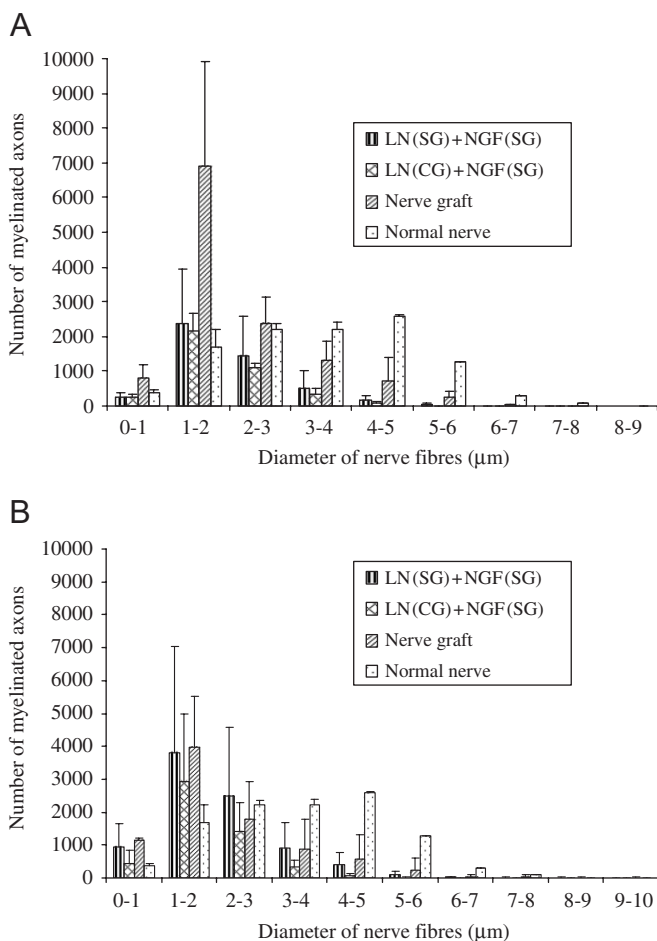


Fig. 11. Diameter distribution of nerve axons at (A) mid-length (10 mm), and (B) distal end (17 mm). For each group the diameter distribution is a bell-shaped curve. For all the nerve implants, the maximum numbers of axons have a diameter of 1–2 μm . However, in a normal nerve the maximum diameter distribution is broader, from 1 to 5 μm . All error bars indicate SEM.

groups (nerve graft, anisotropic and isotropic scaffolds) were stained for acetylcholine receptors (neuromuscular junctions, NMJ), neurofilament (NF) and synaptic vesicles. NMJs (red, Fig. 13) that were positive for NF staining (green, Fig. 13) and/or synaptic vesicles (green, Fig. 13) were counted and compared between the various groups. In normal rats, 96.4% of the NMJs were positive for both pre- and post-synaptic structures. However, in the nerve grafts and the anisotropic scaffolds, only about 15% of the NMJs were positive for both pre- and post-synaptic structures. There was no significance difference in the number of NMJs between the anisotropic scaffolds and the nerve graft. In case of isotropic scaffolds, there were no NMJs that were positive for both pre- and post-synaptic structures, indicating lack of formation of new NMJs.

4. Discussion

During embryonic development of the nervous system, the developing axons use a variety of haptotactic and chemotactic cues to find their target organs [46]. Some of these cues are presented in a gradient fashion in order to direct the axons towards their target tissues [47,48]. ECM proteins, such as laminin and fibronectin, and neurotrophic factors, such as NGF and BDNF, have been used in several studies to promote nerve regeneration *in vivo* [5,26,49]. However, these neuro-stimulatory molecules have not been presented in a gradient fashion as observed *in vivo*. To mimic *in vivo* conditions for axonal guidance and regeneration, we designed novel anisotropic scaffolds with gradients of ECM proteins and neurotrophic factors for nerve regeneration *in vivo*. Here we show that anisotropic agarose gel scaffolds with gradients of LN-1 and NGF promote enhanced axonal regeneration, as compared to isotropic scaffolds with uniform concentrations of LN-1

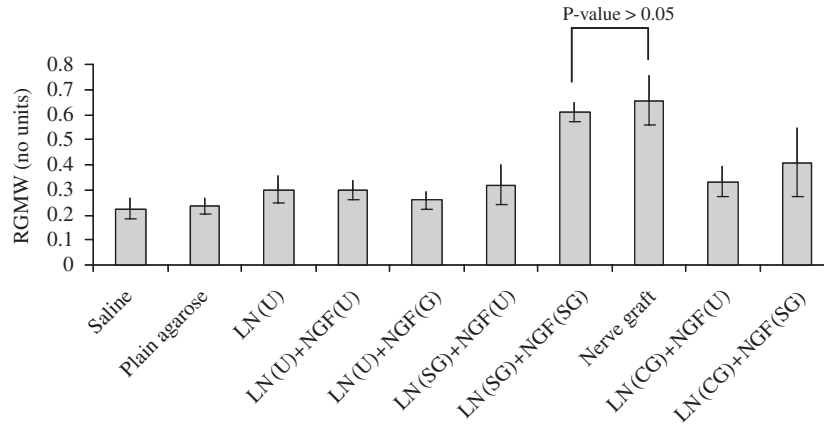


Fig. 12. Relative gastrocnemius muscle weight. Implants with step-gradients and nerve grafts had comparable RGMW ($p > 0.05$). The rest of the implant groups had significantly lower RGMW. All error bars indicate standard error of mean.

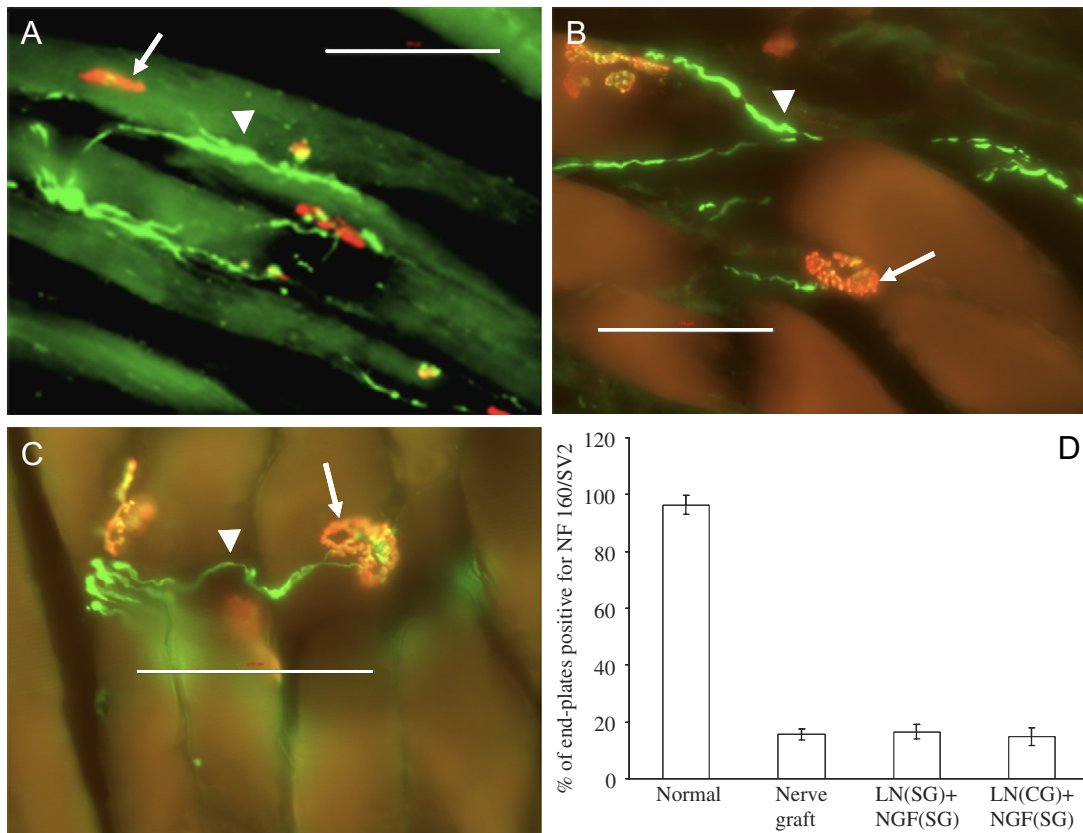


Fig. 13. Staining for acetylcholine receptors (AChR), synaptic vesicles (SV2) and neurofilaments (NF). All the three conditions (step-gradient scaffolds (A), continuous-gradient scaffolds (B) and nerve grafts (C)); had neuro-muscular junctions positive for AChR (arrow), NF 160 (arrow-head) and SV2. All the NMJs in the figure panels are positive for SV2, to different degrees. The percent of positive NMJs in the three conditions was similar, but much lower than that of a normal rat (D). Scale bar = 100 μ m.

and NGF, across a challenging 20 mm nerve gap in rats. In this injury model, axonal regeneration is limited and does not occur unless the NGCs have gradients of both LN-1 and NGF (step- or continuous-). Scaffolds with gradients of either LN-1 or NGF, with the other component at uniform distribution, did not promote axonal regeneration across the 20 mm nerve gap.

LN-1 is an appropriate candidate as the protein of choice to promote PNS regeneration because it has been shown to be a potent stimulator of neurite outgrowth [21,27,50]. 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 LN facilitates all of these

processes in several neural systems [51–53]. The spatio-temporally controlled expression of LN-1 in the developing peripheral nervous system, as well as in the visual pathway and the cerebellum, suggests that LN plays a role in axon outgrowth and guidance [54–56]. The LN expression has been found to be essential for ventral turning of axons during development. Blocking the nitrogen-binding motifs on LN prevents turning of axons, although it does not affect growth [48], indicating that LN might play a role in the orientation of axons in some systems. NGF has been shown to stimulate and promote the survival of sensory ganglia [23,24]. Griffin and Letourneau [57] have shown concentration-dependent neurite extension/retraction with NGF levels. NGF has been shown to prevent the complete death of axotomized sensory neurons following exogenous administration [58]. There is enough evidence to show that gradients of neurotrophins help in guiding developing as well as regenerating axons in a wide variety of neuronal systems [59,60]. Although many studies *in vitro* have shown influence of gradients of LN-1 and NGF for neurite extension, to our knowledge, there are no studies *in vivo* with gradients, due to the difficulties in making and sustaining gradients *in vivo*.

In this study, we present a diffusion technique to first make gradients of LN-1 in agarose gel, and then immobilize it using photochemical cross-linking. Immobilization of LN-1 imitates conditions *in vivo* where LN-1 is tethered to ECM. Gradients of NGF were designed using slow release LMTs, which imitates conditions *in vivo* where neurotrophins are released by target organs and taken up by nerve ends. Two kinds of LN-1 gradients were designed to promote nerve regeneration, one step-gradient and the other a continuous-gradient. However, the NGF gradient was always a continuous-gradient due to the diffusion of NGF. The step- and continuous-gradient scaffolds of LN-1 were designed such that the average LN-1 concentration in both conditions is very close. However, the actual concentration of LN-1 that the regenerating axons encountered dynamically in each condition were different because of the difference in gradient profile. For example, in step-gradients, from 0 to 5 mm distance, axons saw uniform concentration of LN-1 (8.25 µg/ml), whereas, in a continuous-gradient, axons saw increasing concentration profile of LN-1, but with an average LN-1 concentration of 5.1 µg/ml. The LN-1 concentration range selected for making LN-1 step-gradients was based on the maximum concentration of LN-1 that could be immobilized. However, this concentration range may not be the optimal range. A more thorough study will be needed to determine the optimal LN-1 concentration range for nerve regeneration.

Nerve regeneration over a 20 mm nerve gap in rats is challenging, and the failure of axonal regeneration in 7 out of the 10 experimental groups is a testament to the degree of difficulty of this model. Nerve regeneration was observed only when the scaffolds had gradients of both LN-1 and NGF. Scaffolds with gradient of only LN-1 or

only of NGF, with the other component at uniform concentration, were not able to promote nerve regeneration. This suggests a synergistic effect between LN-1 and NGF, as mentioned in a previous study [43]. Also, it suggests that while scaffolds with uniformly distributed LN-1 and NGF matched the performance of nerve autografts in our previous studies while bridging 10 mm nerve gaps, it is very important to test longer nerve gaps, as these might be more representative of those occurring in patients who might be candidates for such a procedure.

In the RGMW study, the anisotropic scaffolds with step-gradients of LN-1 were able to match the autografts for RGMW. The rest of the groups had significantly lower RGMW. Lower RGMW outcome with the continuous-gradient scaffolds, as compared to the step-gradient scaffolds, could be due to non-optimal concentration range of LN-1 in the continuous-gradient scaffolds. Even though both groups, nerve grafts and step-gradient scaffolds, show significant improvement in RGMW, they were still lower than the native, well-innervated muscle. It is possible that the regenerating axons take longer than 4-months to traverse the 20 mm nerve gap and the distal nerve stump, to form new NMJs and cause muscle regeneration. A study with longer duration of regeneration may result in higher values of RGMW for both groups.

To determine formation of new NMJs, gastrocnemius muscle sections were stained for pre-synaptic structures (synaptic vesicles and NF) and post-synaptic structure (acetylcholine receptors). In normal rats, 96.4% of post-synaptic structures were also positive for staining of pre-synaptic structures. However, in the nerve grafts and the anisotropic scaffolds, only about 15% of the NMJs were positive for both pre- and post-synaptic structures. In the implant groups, apart from the NFs making synapses at NMJs, additional NF staining was also observed. It was not possible to determine if these were sensory axons or motor axons, or if they would lead to formation of more NMJs in the future. Although there is no significant difference in % of NMJs stained between the nerve grafts and the anisotropic scaffolds, the total number of positive NMJs is lower with LN(CG) + NGF(SG) scaffolds than with nerve grafts and LN(SG) + NGF(SG). This could be the reason that the RGMW of LN(CG) + NGF(SG) is lower than that of the nerve grafts and LN(SG) + NGF(SG).

5. Conclusions

In conclusion, we report the novel design, implementation and evaluation of anisotropic hydrogel scaffolds with embedded gradients of ECM proteins and neurotrophic factors for directed peripheral nerve regeneration. Our data demonstrates that nerve regeneration across a 20 mm nerve gap occurs only when gradients of both LN-1 and NGF are present, but not when these proteins are presented in an isotropically distributed manner. However, while the step-gradient anisotropic scaffolds elicited axonal regeneration

and RGMW comparable to that observed with the clinical gold standard, nerve autografts, the continuous-gradient scaffolds were unable to match the performance of autografts. This suggests that more careful optimization of ECM and trophic factor gradients, and of their concentrations and slopes, maybe necessary to fully realize the promise of anisotropic scaffolds such that they match the performance of nerve grafts in bridging long peripheral nerve gaps. Nevertheless, our data leads one to unequivocally conclude that anisotropic distribution of trophic and ECM proteins in 3D hydrogels represents a significant step forward in the design of tissue engineered nerve bridges, when compared to isotropic 3D scaffolds, where growth promoting proteins are uniformly distributed with no embedded directional cues.

Acknowledgments

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