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## Abstract

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## Keywords

biomedical engineering, biomedical sciences, lactic acid, laminin, polyglycolic acid, bioremediation, cell adhesion, drug effect, development and aging, tissue engineering, ultrastructure, cell isolation, chemical cue

## Disciplines

Biomedical Engineering and Bioengineering | Chemical Engineering | Polymer Science

## Comments

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# Synergistic Effects of Physical and Chemical Guidance Cues on Neurite Alignment and Outgrowth on Biodegradable Polymer Substrates

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and SURYA MALLAPRAGADA, Ph.D.<sup>3</sup>

## ABSTRACT

**This article demonstrates that directional outgrowth of neurites is promoted by applying a combination of physical and chemical cues to biodegradable polymer substrates. Films of poly-D,L-lactic acid and poly(lactide-co-glycolide) were micropatterned to form grooves on substrate surfaces, using novel indirect transfer techniques developed specifically for biodegradable polymers that cannot be micropatterned directly. Laminin was selectively adsorbed in the grooves. Whole and dissociated dorsal root ganglia were seeded on the substrates and neurite outgrowth and alignment along the microgrooves were measured. The microgrooves provide physical guidance, whereas laminin provides chemical cues to the neurons. The groove depth and spacing were found to significantly influence neurite alignment. The presence of laminin was found to promote neurite adhesion and outgrowth along the grooves. Using a combination of optimized physical and chemical cues, excellent spatial control of directional neurite outgrowth, with up to 95% alignment of neurites, was obtained. The synergistic effect of physical and chemical guidance cues was found to be more effective than individual cues in promoting directional outgrowth of neurites.**

## INTRODUCTION

**N**UMEROUS SURGERIES are attempted every year to repair peripheral nerve damage.<sup>1</sup> When a nerve is transected the axons and myelin completely degenerate distal to the injury, leaving the neurilemma, which forms a tube directing axonal growth, in the reparative phase. Axonal outgrowth is guided by the growth cone, which uses environmental cues. Laminin, a noncollagenous multidomain glycoprotein, is one extracellular matrix (ECM) component that is continuously synthesized after nerve injury. The ECM and its components, including laminin, cause neurite elongation during development and regeneration<sup>2,3</sup> and laminin molecules can self-assemble into feltlike sheets *in vitro*.<sup>4</sup> However, if the nerve gap due to the injury is large, the distal and proximal sides of the transected nerve cannot communicate well, and the natural regeneration process is hindered.

In such cases, nerve autografting procedures are commonly used. The problems associated with nerve

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autografting have led researchers to study alternative methods to engineer an artificial environment to mimic the physical and chemical stimuli that promote peripheral nerve regeneration *in vivo*. The entubulization method, in which a conduit is used to connect the nerve endings, has great potential as a repair method. But this method has enjoyed limited success as it does not provide guidance to regenerating axons at the cellular level.<sup>5,6</sup>

The overall purpose of this research is to use biodegradable polymer substrates to mimic, *in vitro*, the physical and chemical mechanisms that direct and guide peripheral nerve outgrowth at the cellular level *in vivo*. The hypothesis being tested in this article is that the synergistic effects of physical and chemical guidance cues can promote neurite alignment and outgrowth in the direction of the grooves better than the individual cues alone. Once this hypothesis is successfully tested, and the substrate dimensions are optimized for directional neuronal outgrowth, biological cues in the form of Schwann cells will also be provided to further enhance directional neurite outgrowth to enable peripheral nerve regeneration, as outlined in the second part of this article.

Previous work has shown that nerve cells recognize three-dimensional geometric configurations on substrates and use these topographical features to align and migrate along the structures.<sup>8-12</sup> These responses are believed to be probabilistic.<sup>13</sup> Deeper grooves have been found to reduce branching of neurites, which can lead to neurolemoma formation.<sup>12</sup> However, most of these studies involving neurites were conducted on nondegradable substrates such as glass or Perspex because most conventional micropatterning techniques cannot be applied readily to biodegradable polymers. The use of biodegradable substrates is crucial as it enables extension of this work to *in vivo* studies to investigate the effect of these cues on nerve regeneration.

Some studies of growth cone migration on two-dimensional substrates adsorbed with proteins in pattern shapes with highly permissive chemicals and less permissive regions have been conducted.<sup>14,15</sup> In these experiments, the growing neurites stayed in the highly permissive regions for widely spaced patterns, but crossed less permissive regions for narrowly spaced patterns. Our studies have focused on investigating the *in vitro* behavior of neurites when they are subjected simultaneously to both physical and chemical cues on biodegradable polymer substrates to promote neurite outgrowth and alignment in chosen directions at the cellular level.

Photolithographic techniques or laser ablation, commonly used to produce microgrooves on various substrates, cannot be used directly on biodegradable polymers as they accelerate the polymer degradation rates.<sup>16</sup> Microstamping techniques can be used to pattern proteins on biodegradable surfaces, but cannot be used to create microgrooves on substrates for physical guidance.<sup>17</sup>

We have developed novel patterning techniques to create microgrooves on biodegradable polymer substrates and attach adhesive proteins selectively in the grooves. We have previously tested the alignment of Schwann cells on these substrates,<sup>8</sup> and in this article we extend this approach to optimize these substrates for promoting alignment of neurites. To our knowledge, this is the first successful attempt to create microgrooves on biodegradable polymer substrates with adhesive proteins selectively attached to the grooves and examine the synergistic effects of physical and chemical cues on directional neurite outgrowth. Directional growth of axons is crucial for peripheral nerve regeneration and the results from this work could be extended to an *in vivo* situation to aid peripheral nerve regeneration after injury.

## MATERIALS AND METHODS

### *Microgrooved substrate fabrication*

Poly-D,L-lactic acid (PDLA) and poly(lactide-co-glycolide) (PLGA) (Birmingham Polymers, Birmingham, AL), at a copolymer ratio of 85:15 lactide:glycolide, were used to fabricate the substrates. These polymers were chosen as they are resorbable, biocompatible polymers that are used extensively in biomedical applications. Conventional microlithographic techniques accelerate the degradation process of biodegradable polymers and therefore cannot be used directly on these polymers. To overcome this drawback, novel techniques were developed to fabricate microgrooved PDLA and PLGA films by imprinting patterns onto the polymer surface from microgrooved quartz and silicon substrates.<sup>8</sup>

These patterned quartz and silicon substrates were produced by conventional photolithographic techniques and reactive ion etching. The patterns were transferred onto biodegradable polymers by compression molding or solvent casting.<sup>8</sup> Briefly, micropatterned compression-molded films of PDLA and PLGA were obtained by compressing polymer films over the quartz dies in a Carver press at a pressure of 500 lb/in<sup>2</sup> at 50°C for 5 min. Solvent-cast films were produced by spin-casting solutions of polymer in chloroform onto micropatterned silicon wafers. An aqueous polyvinyl alcohol (PVA) (Elvanol; Du Pont, Wilmington, DE) solution (6%, w/v) was used as a release agent and spun on a silicon wafer at 4000 rpm for 1 min and allowed to dry. The polymer solution in chloroform (30%, w/v) was then spun on the substrate at 1800 rpm for 1 min. After drying for 24 h, these films were floated off the silicon wafers onto the surface of water and used.

The groove dimensions that were found to be optimal for Schwann cell alignment<sup>8</sup> were used for the neurite alignment studies. For the ultimate use of these substrates for peripheral nerve regeneration, both the Schwann cells and neurites need to align along the microgrooves. The Schwann cell alignment was found to be sensitive to groove widths but not to groove depths; therefore, the groove depth was varied to optimize neurite alignment. Because of constraints imposed by the fabrication techniques, the groove depths were varied from 1 to 4  $\mu\text{m}$ . On the basis of previous Schwann cell studies, groove widths were maintained at 10  $\mu\text{m}$  and the groove spacings were either 10 or 20  $\mu\text{m}$ . Measurements of the degradation rates of these polymers have shown that the microgrooves on solvent-cast films remain intact for up to 4 weeks whereas the microgrooves on the compression-molded films disappear in 1 week.<sup>8</sup>

#### *Laminin adsorption*

A solution of laminin (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) (100  $\mu\text{g}$  of laminin per milliliter of PBS) was adsorbed selectively into the microgrooves on the polymer substrates, using surface tension effects. The laminin distribution on the surface was observed by using an assay involving fluorescein isothiocyanate (FITC)–anti-laminin conjugate solution, AlexaFluor 488 rabbit anti-FITC IgG antibody solution, and AlexaFluor 488 goat anti-rabbit IgG conjugate solutions. The fluorescence of the substrate was examined under a microscope with a 490-nm cutoff filter and laminin was found to adsorb selectively to the edges of the grooves when this technique was used.<sup>8</sup>

#### *Isolation of whole dorsal root ganglia*

Neurons were collected from the dorsal root ganglia (DRG) of 1- to 3-day-old Sprague-Dawley rats. The rats were anesthetized with halothane in a closed chamber system. The spinal cords were gently removed with forceps. The ganglia were removed by carefully inserting the forceps behind the ganglia and lifting them out by the distal processes. The processes were cut with iris scissors and the ganglia were placed in Gey's balanced salt solution (GBSS). The sheaths were removed from the whole DRG after chilling them on ice for about 1 h.

#### *Isolation of dissociated neurons*

The DRG obtained by the above-cited procedure were dissociated by placing the ganglia in a centrifuge tube and removing the GBSS. The DRG were incubated at 37°C for 45 min on 5 mL of filtered (0.22- $\mu\text{m}$  pore size) 0.1% trypsin and 0.1% collagenase in Hanks' balanced salt solution (HBSS). The cells were centrifuged at 1000 rpm for 10 min and the supernatant was removed. The DRG were resuspended in 5 mL of 0.25% trypsin in HBSS and incubated for 10 min at 37°C. The cells were centrifuged again for 10 min at 1000 rpm and the trypsin solution was removed. Five milliliters of medium (90% Dulbecco's modified Eagle's medium [DMEM], 10% fetal bovine serum [FBS], 5  $\mu\text{l}$  of gentamicin per milliliter of medium, and 10 ng of nerve growth factor [NGF- $\beta$ ] per milliliter of medium) was added to inhibit the enzymatic reaction of the trypsin. The cells were dissociated mechanically with flame-narrowed Pasteur pipettes.

The whole DRG provides controlled placement of cells that dissociated DRG cells do not provide. Dissociated DRG cells provide insight into individual cell behavior with respect to chemical and physical guidance cues and enable elongation measurements, whereas whole DRG better represent neurite behavior after a nerve has been transected. Therefore, both whole and dissociated DRG were used for these studies.

### *Cell seeding on the substrates*

Whole ganglia were dipped into chicken plasma (5 mg/mL H<sub>2</sub>O) and then into thrombin (10 units/mL) and placed on the substrate. This combination of plasma and thrombin attaches the cells to the substrate. For a 60 × 15 mm culture dish, 1 mL of medium (50% basal medium eagle [BME], 25% Earle's balanced salt solution [EBSS], 25% horse serum [HS], 5 μl of gentamicin per milliliter of medium, and 10 ng of NGF-β per milliliter of medium) was added. After 24 h, the culture was supplemented with 5 mM cytosine-β-arabincoside to prevent the proliferation of fibroblasts and Schwann cells. The cultures were placed on a slowly oscillating tilt table in an incubator at 37°C with 5% CO<sub>2</sub>. Dissociated DRG were seeded at the rate of one ganglion on two 1-cm<sup>2</sup> micropatterned polymer substrates in 5 mL of the medium used earlier to inhibit the trypsinization reaction. This cell density was adequate for studying individual neurite behavior on laminin-coated patterned substrates. The cell suspension in medium was placed on the laminin-coated patterned substrates.

### *Cell staining*

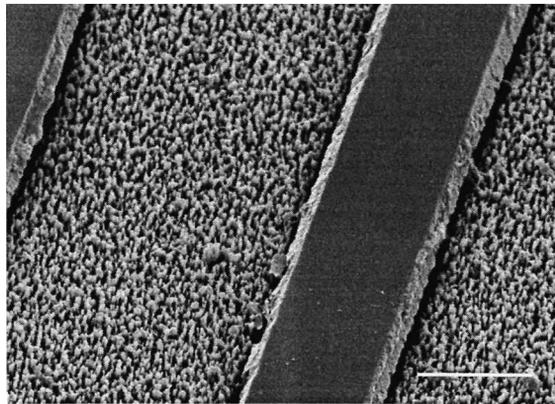
Live cell staining was used to improve cell visibility and to monitor the cells as they grew. The lipophilic tracer DiI<sub>C18(3)</sub> (DiI) (Molecular Probes, Eugene, OR) is a retrograde neuronal tracer in living and fixed cells. The neurons were labeled with 3.7 μL of DiI per milliliter of DMEM and incubated at 37°C for 30 min. The cells were viewed under a light microscope, using a 590-nm barrier filter. The dye uniformly labeled neurons through lateral diffusion in the plasma membrane at a rate of 0.2–0.6 mm/day and can remain detectable for up to 4 weeks in culture.<sup>18</sup> If the neurites extended farther than 0.2–0.6 mm/day, the cells were redyed to label the fine processes of the neurons.

### *Alignment and outgrowth measurements*

The neurons were imaged by light microscopy. An Olympus (Melville, NY) IMT-2 bright-field/phase-contrast microscope was used to take digital images (Olympus DP10 digital camera) and color slide images. Photomicrographs were taken daily and outgrowth and alignment of the stained neurites along the grooves were ascertained by microscopy 48 h after seeding. Previous work has demonstrated that neurite outgrowth occurs in two phases: the first 3 days, during which the axons grow slower (77 μm/day), and from day 4 onward, during which growth rates are much faster (283 μm/day).<sup>19</sup> In this study, initial extension of the neurites was measured 48 h after seeding and expressed as micrometers per day in order to obtain the initial growth rate of the neurites and compare them with the rates obtained in other studies on smooth substrates. Quantitatively, alignment was defined as a neurite aligned parallel to the grooves and maintaining its original growth position without crossing the border between a groove and a groove spacing. It was reported as a percentage of the total number of neurites aligned in the direction of the grooves. The alignment of the neurites was evaluated by an *F* test with two-sample variance (neurites aligned and neurites not aligned) with  $\alpha = 0.05$ . The confidence interval was calculated for independent samples. Mean values and standard errors are reported for 18 samples. The effect of polymer type, microgroove dimensions, and the concentration of laminin in the grooves on the adhesion, outgrowth, and alignment of neurites was ascertained.

## RESULTS

The quartz die produced by reactive ion etching that acts as a template for indirect pattern transfer to biodegradable polymer films is shown in Fig. 1. The microgrooves were successfully transferred to biodegradable PLGA and PDLA substrates by compression molding and solvent casting. Here we report the efficacy of these micropatterned biodegradable laminin-coated substrates developed by these novel techniques in promoting outgrowth and alignment of neurites.



bar = 10  $\mu\text{m}$

**FIG. 1.** Reactive ion-etched region of a 10/20/3.3 (groove width/groove spacing/groove depth)  $\mu\text{m}$  quartz die.

### *Effect of physical guidance*

Initially, visual inspection of dissociated ganglia on compression-molded PDLA substrates that provide only physical guidance showed that groove depth had a significant influence on neurite alignment. Groove dimensions are listed as groove width/groove spacing/groove depth, in micrometers. Groove depths of less than 2  $\mu\text{m}$  had little influence on the physical guidance of the neurites. Interactions between neurons were found to diminish the influence of physical topography, as the neurites exhibited a tendency to grow toward other neurites (Fig. 2A). Groove depths greater than 2  $\mu\text{m}$  acted as physical barriers and provided better alignment of neurites (Fig. 2B).

These trends were also observed in the case of whole ganglia. By removing the sheath, the regenerating neurite grew away from the mass of cells and aligned with the grooves on compression-molded PDLA substrates (Fig. 2C) when the groove depth was greater than 2  $\mu\text{m}$ . This indicates that the deeper grooves provide physical barriers to the neurites, thereby promoting alignment. The width of the grooves did not have a significant effect on neurite alignment, but because Schwann cells were found to align along grooves with widths of 10  $\mu\text{m}$ ,<sup>8</sup> those microgroove dimensions were chosen for the studies. Alignment was determined by whether the neurites grew in the direction of the groove without crossing from one groove to another.

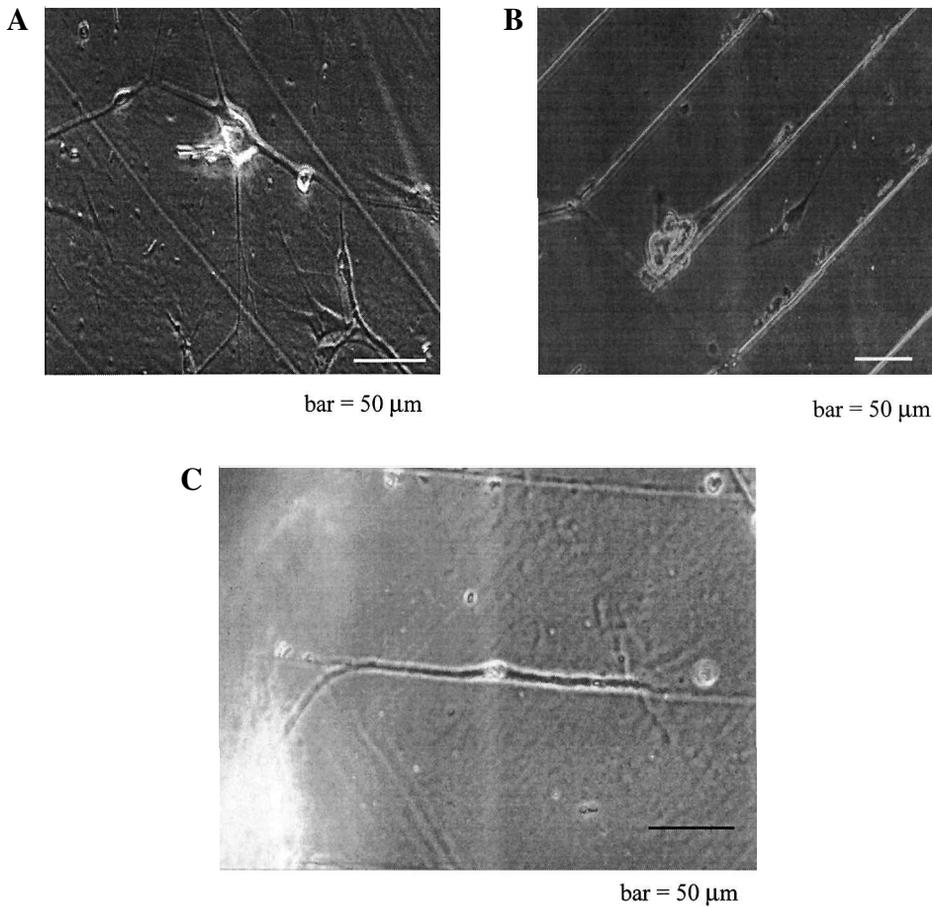
### *Effect of laminin on cell adhesion*

Laminin improves adhesion of neurons to biodegradable polymer substrates; 82.5  $\pm$  2.3% more neurons attached to smooth solvent-cast PDLA substrates with laminin (200  $\mu\text{g}/\text{mL}$ ) as opposed to substrates with no laminin, 48 h after seeding. Laminin has the added benefit of promoting neurite outgrowth. Neurites on smooth solvent-cast PDLA laminin-coated substrates (200  $\mu\text{g}/\text{mL}$ ) grew three times longer than neurites on smooth noncoated PDLA substrates, 48 h after seeding (Fig. 3). Therefore laminin was used in conjunction with the microgrooved substrates to promote neurite alignment.

### *Effect of groove dimensions on neurite alignment*

Solvent casting was used to produce substrates with deeper grooves and all the subsequent studies were conducted on solvent-cast films for this reason. On laminin (200  $\mu\text{g}/\text{mL}$  PBS-coated) solvent-cast PDLA films, 92  $\pm$  1% of neurites aligned on 10/10/4  $\mu\text{m}$  films as opposed to only 77  $\pm$  1% on 10/10/3  $\mu\text{m}$  substrates (Fig. 4), reinforcing the fact that deeper grooves are crucial for neurite alignment even in the presence of additional chemical cues. Statistically significant differences with confidence levels greater than 95% are indicated by an asterisk in Fig. 4 as well as in subsequent figures.

Groove spacing also seems to play a role: 70  $\pm$  1% of neurites aligned on 10/20/3  $\mu\text{m}$  substrates coated with laminin at 200  $\mu\text{g}/\text{mL}$  compared with 77  $\pm$  1% for 10/10/3  $\mu\text{m}$  substrates; and 84  $\pm$  1% of neurites



**FIG. 2.** (A) Neurons growing toward each other on a 4/100/1  $\mu\text{m}$  compression-molded PDLA substrate. (B) Clustered neuron cell bodies with their neurites following a groove on a 4/100/2.3  $\mu\text{m}$  compression-molded PDLA substrate. (C) Neurite exiting a whole ganglion and turning to grow along a groove on a 4/100/2.3  $\mu\text{m}$  PDLA substrate. The images were taken 48 h after seeding.

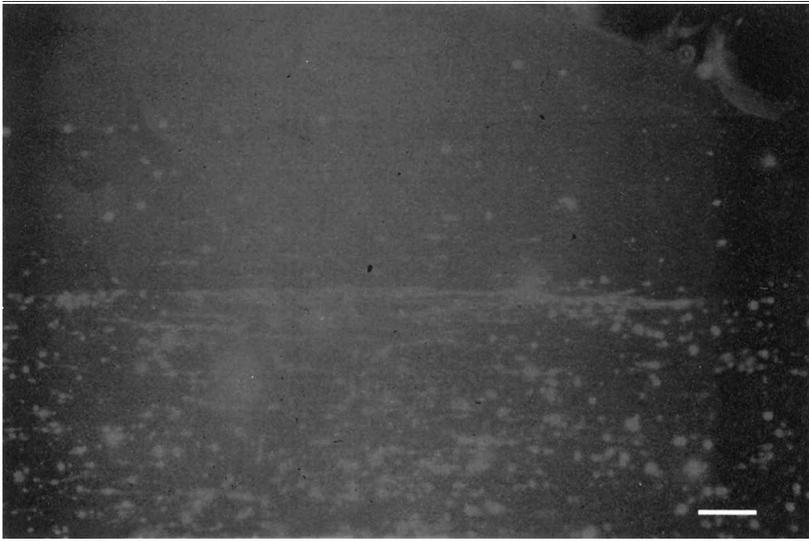
aligned along 10/20/4  $\mu\text{m}$  PDLA substrates with laminin at 200  $\mu\text{g}/\text{mL}$  compared with  $92 \pm 1\%$  on 10/10/4  $\mu\text{m}$  PDLA substrates. Spacings of 10  $\mu\text{m}$  between grooves seem to significantly promote (95% confidence level) neurite alignment compared with 20- $\mu\text{m}$  spacings.

#### *Effect of laminin on neurite alignment*

The neurites on 10/10/4  $\mu\text{m}$  solvent-cast PDLA substrates with no laminin in the grooves showed an alignment of  $86 \pm 1\%$ , whereas those with laminin at 200 and 1000  $\mu\text{g}/\text{mL}$  showed neurite alignments of  $92 \pm 1$  and  $93 \pm 1\%$ , respectively. On 10/10/4  $\mu\text{m}$  solvent-cast PDLA substrates, increasing laminin concentration improved neurite alignment at a 95% confidence level for laminin at 0 to 200  $\mu\text{g}/\text{mL}$  but not for laminin at from 200 to 1000  $\mu\text{g}/\text{mL}$  (Fig. 5). In contrast, changing the laminin concentration from 200 to 1000  $\mu\text{g}/\text{mL}$  on 10/20/4  $\mu\text{m}$  PDLA substrates caused a significant increase (at a 95% confidence level) in neurite alignment from  $84 \pm 1$  to  $90 \pm 1\%$ . Similar trends were observed with the 10/20/4  $\mu\text{m}$  PLGA substrates, with better neurite alignments on changing the laminin concentration from 200 to 1000  $\mu\text{g}/\text{mL}$ .

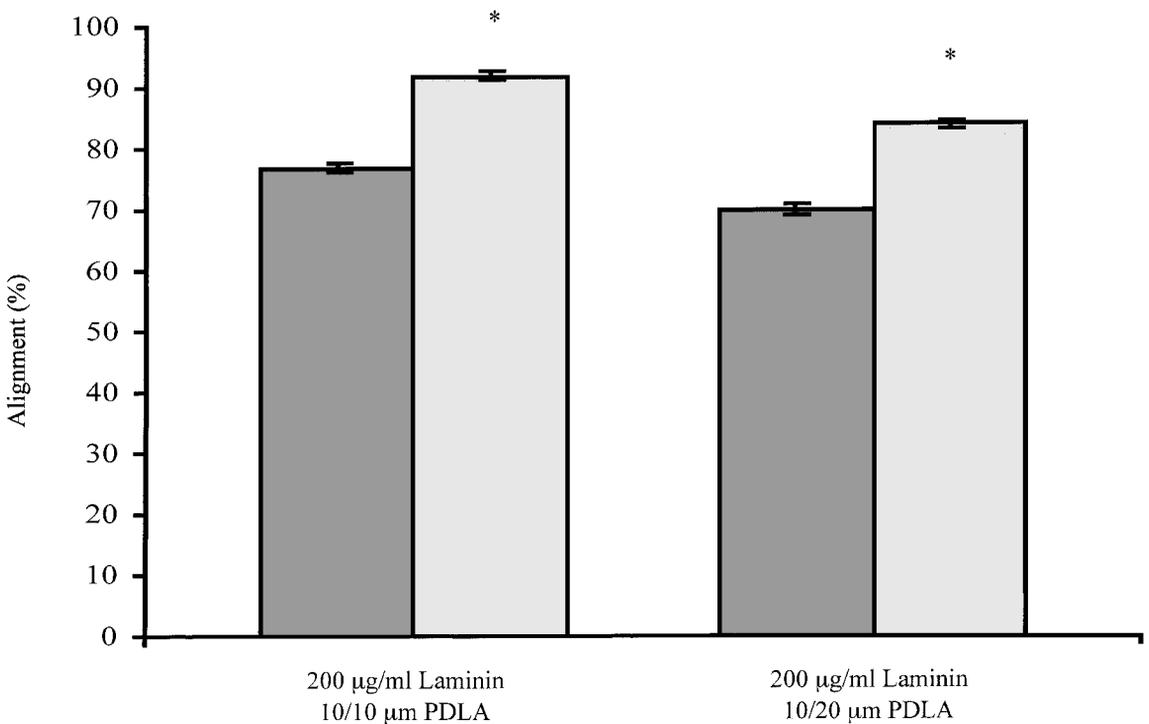
Studies were also conducted to measure alignment of neurites along a preferred direction on smooth substrates. The direction selected was that along which a majority of neurites aligned. The alignment along smooth PDLA substrates without laminin was  $28 \pm 1\%$  as opposed to  $23 \pm 1\%$  on smooth substrates with laminin at 100  $\mu\text{g}/\text{mL}$ . Therefore, the presence of microgrooves significantly improved neurite alignment

## SYNERGISTIC EFFECTS ON NEURITE OUTGROWTH

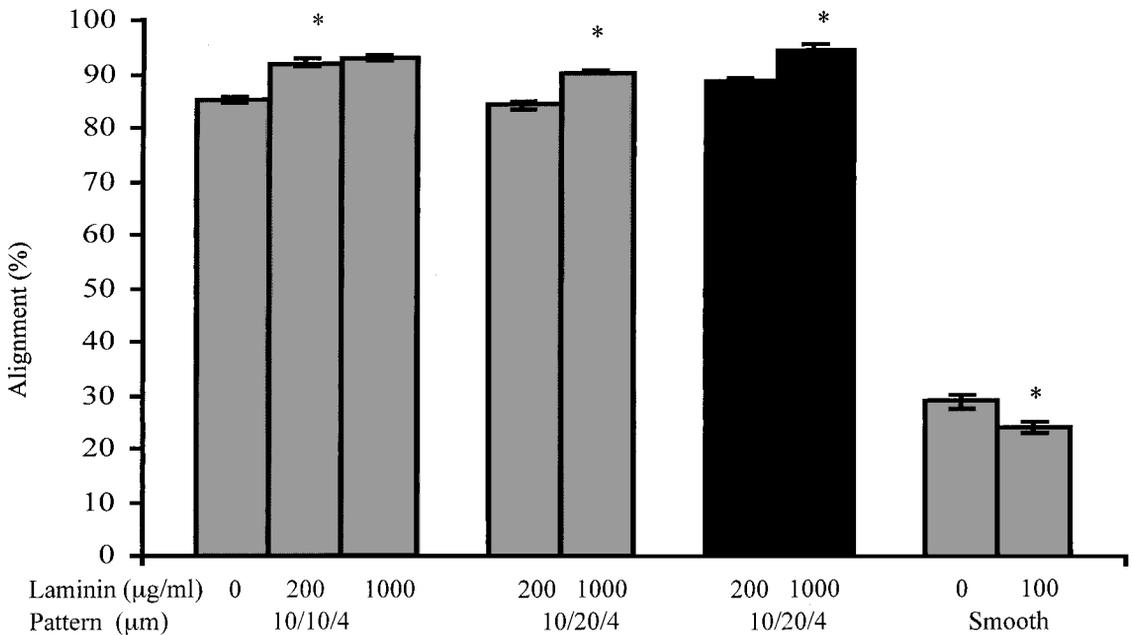


bar = 50  $\mu\text{m}$

**FIG. 3.** The bottom half of this laminin-coated (200  $\mu\text{g}/\text{ml}$ ) 10/20/4  $\mu\text{m}$  PDLA solvent-cast substrate has a higher population of neurons with greater outgrowth and better adhesion than the top half of the substrate, which is not coated with laminin, 48 h after seeding.



**FIG. 4.** Effect of groove depth and spacing on neurite alignment 48 h after seeding. Groove depth on PDLA solvent-cast substrates coated with laminin: 3  $\mu\text{m}$  (dark gray columns); 4  $\mu\text{m}$  (light gray columns). Values are reported as means and standard errors for 18 samples. The asterisks indicate statistically significant differences with confidence levels greater than 95%.



**FIG. 5.** Effect of laminin concentration on neurite alignment on solvent-cast PDLA substrate (gray columns) and solvent-cast PLGA substrate (solid columns) 48 h after seeding. Values are reported as means and standard errors for 18 samples. The asterisks indicate statistically significant differences with confidence levels greater than 95%.

(almost 3- to 4-fold increase in number of neurites aligning) and the presence of laminin on smooth surfaces actually decreased neurite alignment slightly.

#### *Effect of polymer type on neurite alignment and outgrowth*

Polymer type significantly affected neurite alignment at a 95% confidence level (Fig. 6). On 10/20/4 μm solvent-cast PLGA substrates with laminin at 200 μg/mL, 88 ± 1% neurites aligned as opposed to 84 ± 1% on PDLA substrates. This trend was observed even on 10/20/4 μm PLGA substrates with laminin at 1000 μg/mL, where 94 ± 1% alignment was observed as opposed to 90 ± 1% on PDLA substrates. Therefore the PLGA substrates were found to display slightly better neurite alignments compared with the PDLA substrates.

Polymer type did not significantly affect neurite elongation at a 95% confidence level, even at various laminin concentrations. On 10/20/4 μm solvent-cast PLGA substrates with laminin at 200 μg/mL, neurite elongations of 85 ± 16 μm/day were observed as opposed to 43 ± 8 μm/day on PDLA. Increasing laminin concentration to 1000 μg/mL on the 10/20/4 μm increased the elongation on solvent-cast PDLA substrates to 64 ± 7 μm/day as opposed to 91 ± 20 μm/day on PLGA substrates.

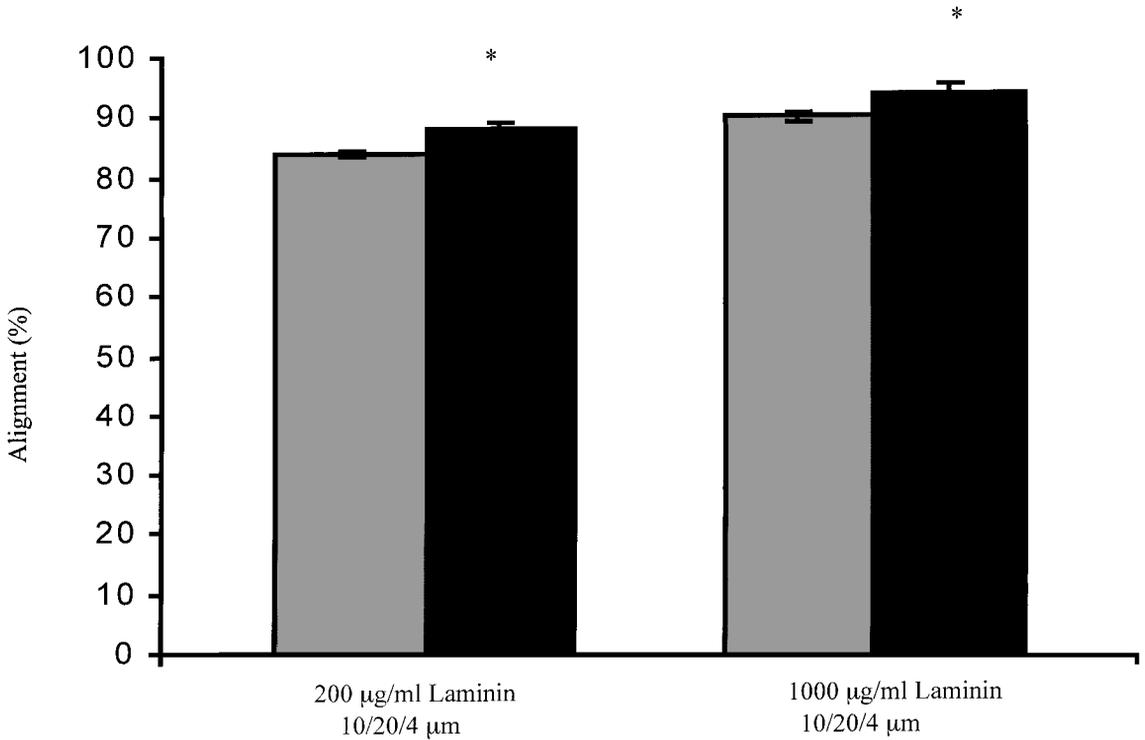
#### *Effect of laminin on neurite outgrowth*

On 10/10/4 μm PDLA substrates, neurite outgrowth improved significantly (95% confidence level) with an increase in laminin concentration from 0 to 200 μg/mL but not from 200 to 1000 μg/mL (Fig. 7). The neurites elongated at 21 ± 3 μm/day for PDLA without laminin and at 56 ± 5 μm/day for PDLA with laminin at 200 μg/mL and at 58 ± 7 μm/day with laminin at 1000 μg/mL. Similar trends were observed on 10/20/4 μm PDLA substrates as well as PLGA substrates. The presence of laminin improved neurite outgrowth tremendously, but increasing the laminin concentration from 200 to 1000 μg/mL did not.

#### *Effect of groove dimensions on neurite elongation*

No statistically significant differences in neurite elongation were observed with groove depth or groove

## SYNERGISTIC EFFECTS ON NEURITE OUTGROWTH



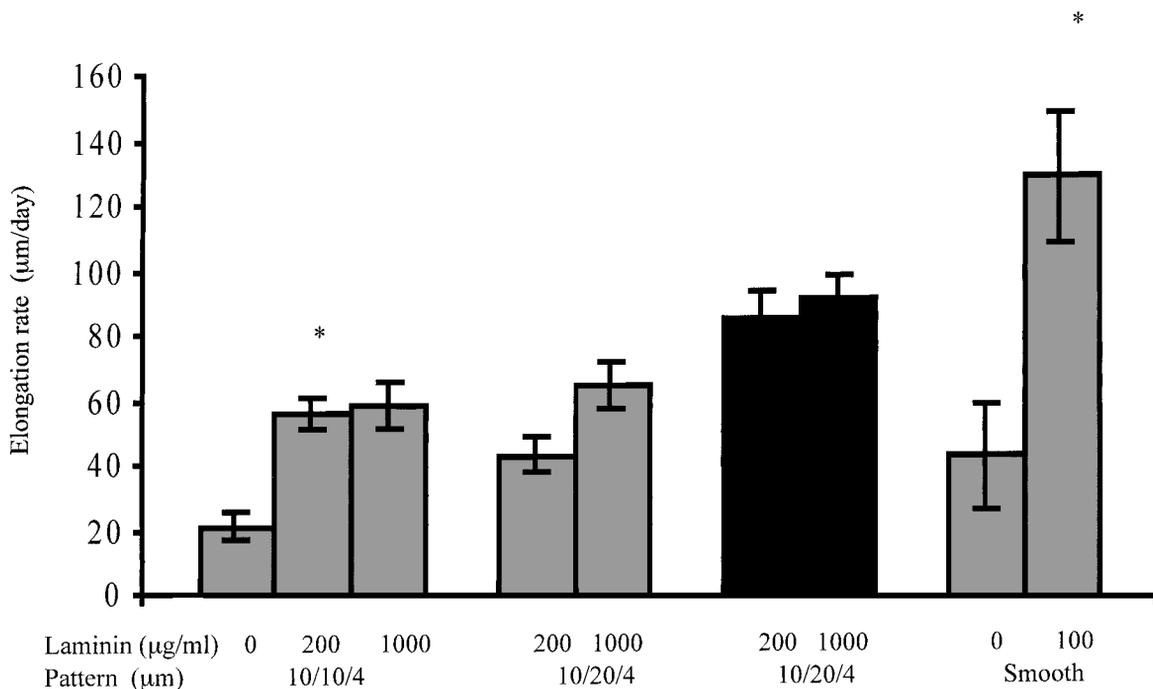
**FIG. 6.** Effect of polymer type on neurite alignment on solvent cast: PDLA substrate (gray columns) and PLGA substrate (solid columns) 48 h after seeding. Values are reported as means and standard errors for 18 samples. The asterisks indicate statistically significant differences with confidence levels greater than 95%.

width. On 10/10/3 and 10/10/4 µm PDLA substrates with no laminin, the elongation rates were  $21 \pm 4$  and  $21 \pm 3$  µm/day. Even in the presence of laminin (200 µg/mL) the same trends were observed on 10/10/4 and 10/20/4 µm PDLA substrates, where the elongation rates were  $56 \pm 5$  and  $43 \pm 8$  µm/day. On smooth films with no laminin, elongations of  $42 \pm 12$  µm/day were observed, compared with  $128 \pm 27$  µm/day with laminin at 100 µg/mL (Fig. 7). Therefore, the neurites on smooth films showed significantly higher elongation rates compared with neurites on the microgrooved substrates.

## DISCUSSION

Axons exhibit a high degree of selectivity in the recognition of guidance cues. Developing axons use adhesive cell contacts between the growth cone and cell adhesion molecules mediating interactions between the cell surface and extracellular matrix molecules.<sup>20</sup> Laminin acts as a cell adhesion molecule and also provides chemotropism by binding chemical factors such as nerve growth factor to the substrate surface, orienting neurite outgrowth. By combining laminin as a chemical cue with the patterned substrates for physical guidance, disruption in alignment due to interactions between cells on microgrooved substrates presenting only physical cues were found to be overcome to a great extent. Laminin was also found to greatly enhance neurite adhesion and outgrowth on the biodegradable polymer substrates.

Groove depth plays an important role in controlling neurite alignment, with or without the presence of laminin. Significant differences in neurite alignment were observed with groove depths because deeper grooves provide physical barriers that make it difficult for the neurites to cross over. Additional chemical cues in the grooves further encourage the neurites to grow along the grooves. Groove spacing also seems to play an important role (Fig. 4) in neurite alignment. A groove spacing of 10 µm proved better than a spacing of 20 µm for neurite alignment, at a 95% confidence level, because of the “flow effect.” The flow



**FIG. 7.** Effect of laminin concentration and pattern dimensions on neurite elongation: PDLA solvent-cast substrate (gray columns) and PLGA solvent-cast substrate (solid columns) 48 h after seeding. Values are reported as means and standard errors for 18 samples. The asterisks indicate statistically significant differences with confidence levels greater than 95%.

effect is presumed to occur because of the few neurites that are located not within the grooves but in the spacing between the grooves. On the narrower, 10- $\mu\text{m}$  grooves, the neurites that are located in the groove spacings do not have much space between the grooves to grow in different directions and they tend to “flow” in the direction of the grooves. The deep grooves prevent the neurites from crossing over to other groove spacings. On the other hand, substrates with 20- $\mu\text{m}$  spacings have enough space for the neurites on the groove spacings to grow in other directions and not just parallel to the grooves. Therefore the narrower spacings between the grooves promote better alignment of neurites by contributing to the flow effect.

On 10/10/4  $\mu\text{m}$  PDLA substrates, the presence of laminin (200  $\mu\text{g}/\text{mL}$ ) in the grooves improved alignment significantly, but increasing the laminin concentration to 1000  $\mu\text{g}/\text{mL}$  did not seem to further affect the alignment (Fig. 5). In contrast, changing the laminin concentration from 200 to 1000  $\mu\text{g}/\text{mL}$  significantly enhanced neurite alignment on 10/20/4  $\mu\text{m}$  PDLA and PLGA substrates. The difference is attributed to groove spacing, in which the flow effect of the neurites with the grooves was stronger on the narrowly spaced 10- $\mu\text{m}$  pattern than on the wider 20- $\mu\text{m}$  spacing. Therefore, increasing the laminin concentration from 200 to 1000  $\mu\text{g}/\text{mL}$  in the grooves significantly improved alignment as it may have encouraged more neurites, even in the wider spacings, to grow along the grooves and not in the groove spacings. The same trends were observed on 10/20/4  $\mu\text{m}$  PLGA substrates as well. On smooth solvent-cast PDLA substrates, alignment of neurites along a preferred axis decreased when the laminin concentration was increased from 0 to 100  $\mu\text{g}/\text{mL}$  because of greater neurite outgrowth in random directions. The preferred direction of alignment is random, and hence the smooth substrates are not useful in promoting oriented nerve growth along desired directions, especially in the presence of laminin.

In the presence of laminin, PLGA showed slightly improved neurite alignment over PDLA (Fig. 6), but the mechanism for the difference was not clear. One possibility could be the differences in laminin unfolding on the two polymer surfaces, leading to the observed differences in neurite alignment. Another factor, however, is the larger variation in alignment of neurites on PLGA substrates compared with that on PDLA substrates, because PLGA degrades faster than PDLA,<sup>8</sup> thereby contributing to heterogeneities on

the surface. This faster degradation of PLGA might have negated the influence of differences in laminin unfolding between the two polymers, thereby leading to no significant differences in neurite outgrowth on PDLA compared with PLGA.

The trend in the effect of laminin concentration on neurite outgrowth is somewhat similar to the effect of laminin on neurite alignment. The presence of laminin on the substrates improved neurite elongation significantly (Fig. 7), but increasing the laminin concentration from 200 to 1000  $\mu\text{g}/\text{mL}$  did not significantly improve elongation either on the PDLA or PLGA substrate. This trend is similar to that seen by others<sup>15</sup> using strips of laminin patterns adsorbed on glass substrates. Changes in groove dimensions also did not have an effect on neurite outgrowth, as expected.

But the presence of the grooves themselves slowed neurite outgrowth: the smooth PDLA films with laminin displayed greater neurite outgrowth than the grooved films. This was most likely due to a light decrease in growth cone locomotion owing to the physical guidance. The proposed mechanisms that cells use to detect topography involves the cytoskeleton, which causes the growth cone to become more sensitive as the density of cues increases.<sup>13</sup> According to this postulate, the neurite elongation on smooth films would be greater than on grooved films with chemical cues. However, because neurite alignment on microgrooved films was much better, neurite outgrowth along a desired direction was faster on microgrooved substrates than on smooth substrates.

## CONCLUSIONS

Biomimetic strategies were employed to promote and accelerate directional nerve growth *in vitro* at the cellular level with a view to aiding peripheral nerve regeneration *in vivo*. A novel microfabrication technique developed specifically for biodegradable polymers was used to provide neurites a combination of physical and chemical cues involving micropatterns with adsorbed laminin. This approach mimics the processes occurring *in vivo* during peripheral nerve regeneration, where endoneurial tubes provide physical guidance and laminin provides chemical cues.

The neurons required groove depths greater than 3  $\mu\text{m}$  for the best alignment. However, physical guidance alone does not assure good alignment of neurites because of interference with alignment caused by interactions between neurons. The presence of laminin in the grooves helped to overcome that effect and significantly improved neurite alignment and outgrowth. Neurite adhesion was significantly improved on the patterned substrates by adsorbing laminin selectively into the grooves, thereby providing an area of better adhesion for the neurite growth cone to advance. On the basis of previous Schwann cell studies,<sup>8</sup> 10- $\mu\text{m}$  groove spacings were found to promote better neurite alignment than the 20- $\mu\text{m}$  grooves, because the closeness of the grooves promoted a flow effect for neurite orientation. PLGA substrates displayed slightly better neurite alignment compared with PDLA substrates, but did not show any differences in neurite elongation rates.

Neurites did experience greater elongation on smooth substrates than on grooved substrates. A possible explanation is that neurite outgrowth is slowed while the growth cone investigates the various physical and chemical guidance cues. However, outgrowth along a preferred direction was greater in the case of neurites seeded on microgrooved substrates with adsorbed laminin. On the basis of these studies, the biodegradable substrates were successfully optimized to ensure best alignment and outgrowth of the neurites. The synergistic effect of chemical and physical cues was utilized to obtain much better neurite alignments than those obtained with just individual cues. Excellent spatial control of neurites, with alignments of up to 95%, was obtained by optimizing physical and chemical guidance cues. Therefore a combination of physical and chemical cues on biodegradable polymer substrates can be used to guide regenerating neurites at an accelerated pace along the direction of the grooves.

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