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Micropatterned Schwann Cell–Seeded Biodegradable Polymer Substrates Significantly Enhance Neurite Alignment and Outgrowth

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ABSTRACT

Biomimetic strategies were employed to promote directional outgrowth of neurites in vitro by using a synergistic combination of physical, chemical, and cellular cues. Compression molded and solvent cast biodegradable polymer substrates made of poly(D,L-lactic acid) were micropatterned to form grooves on the substrate surfaces. Laminin was localized in the grooves, and rat sciatic Schwann cells were seeded on the substrates. Whole as well as dissociated rat dorsal root ganglia were seeded on the substrates along with Schwann cells, and neurite outgrowth and alignment were measured. The micropatterns provide physical guidance, laminin provides chemical cues, and the Schwann cells provide biological cues to the axons. The presence of Schwann cells in the grooves was found to promote neurite alignment as well as outgrowth and help the neurites orient even on shallower grooves and exhibit continued alignment even as the grooves degrade. The synergistic combination of physical, chemical, and cellular guidance enabled greater than 98% alignment of neurites and accelerated outgrowth of neurites in the direction of the microgrooves.

INTRODUCTION

The most common repair technique for peripheral nerve injuries and transections is autografting. This procedure suffers from disadvantages such as deinnervation at the donor site and limited tissue availability. Entubulization methods are being explored to connect transected nerve endings by guiding the axons towards the distal side of the injury. However, these have had limited success because they provide guidance to the regenerating axons at a macroscopic level.1

When a peripheral nerve is transected, Wallerian degeneration occurs. The proximal side of the transection degenerates near the wound site, and the debris is removed by macrophages. On the distal side, the axons degenerate completely as they are separated from the cell body. When a nerve transection or injury occurs, glial cells or Schwann cells of the peripheral nervous system respond by helping to remove cellular debris, facilitating remyelination, and forming columns of Schwann cells to promote nerve fiber regenera-

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tion. Schwann cells modify their surroundings by synthesizing and secreting extracellular matrix (ECM) components. Recent work has also demonstrated that glial cells, once thought to play merely a supporting role, are vital in enabling the neurons to form synapses. Laminin, a noncollagenous multidomain glycoprotein, is one ECM component that is continuously synthesized by Schwann cells to mediate interactions between ECM and other cells.

Previous studies have investigated the effect of physical topography on the growth of neurons. Most of these studies have been conducted on glass or other nondegradable substrates. Some efforts have also been made to adsorb chemical cues in patterns on glass substrates to investigate the effect of the patterns on cell behavior. The effects of Schwann cells seeded in conduits on nerve regeneration rates have been investigated. Entubulation methods involving conduits have been modified using guidance cues such as oriented collagen in the tubes. However, some studies have demonstrated that filling the lumen of a conduit with additional materials reduces the amount of space available for neurons to grow and can actually lead to a reduction in the outgrowth of neurites and slower nerve regeneration.

Our approach tries to mimic natural regeneration processes in vitro by using a combination of microgrooves with adsorbed laminin and Schwann cells seeded in the grooves as cues to guide and enhance axonal outgrowth at the cellular level. Our previous work has focused on the development of patterning techniques to create microgrooves on biodegradable polymer substrates with adhesive proteins selectively attached to the grooves. These micropatterned biodegradable films can then be rolled up and used as conduits, and they provide guidance at the periphery without filling up the lumen of the conduit and reducing the space available for neuronal outgrowth. The techniques used in this work to pattern biodegradable polymer films involve both indirect transfer of patterns from micropatterned quartz/silicon substrates followed by a surface tension–based method to adsorb laminin selectively in the grooves. These novel indirect methods were developed because none of the conventional lithographic techniques are suitable for etching biodegradable polymer substrates. Other techniques like microstamping are too compliant to be able to produce microgrooves on biodegradable polymer substrates. We have developed biodegradable polymer substrates that present physical and chemical cues that help Schwann cells align along the grooves. We have shown that neurites align along the microgrooves and that the presence of laminin in the grooves enhances neurite outgrowth. However, the use of synergistic effects of these various stimuli on biodegradable polymers to obtain accelerated directional neuronal outgrowth has not been explored in the past. In this paper, we demonstrate that an integrated approach with physical, chemical, and biological cues in the form of microgrooves, laminin, and Schwann cells, respectively, helps neurons to undergo accelerated oriented growth in a direction that is crucial for nerve regeneration processes.

MATERIALS AND METHODS

Micropatterned substrate fabrication

Poly(d,l-lactide) (PDLA; Birmingham Polymers, Birmingham, AL) was used for fabricating the substrates. PDLA was chosen because it is a biocompatible polymer that degrades by hydrolysis. The use of biocompatible polymers ensures that this work can be extended to in vivo studies. Microgrooves were produced on flat substrates made of PDLA by transferring the patterns from quartz or silicon wafers by compression molding or solvent-casting. Photolithographic techniques and reactive ion etching were used to fabricate the micropatterned quartz and silicon wafers. These quartz dies were placed on top of flat PDLA substrates in a Carver press and compressed at 500 psi pressure at 50°C for 5 min to transfer the grooves onto the PDLA substrates. Alternatively, solutions of PDLA in chloroform were spun on top of the patterned silicon wafers and floated off the surface to obtain very thin micropatterned PDLA films.

The depth of the grooves fabricated varied from 1 to 4 μm; the width used was 10 μm, and the spacing between the grooves was either 10 or 20 μm. These dimensions were based on previous studies involving Schwann cell or neurite guidance on these substrates. The dimensions of the microgrooves are indicated as groove width/groove spacing/groove depth in microns. The compression molded films were found to retain their microgrooves for only up to a week, while the microgrooves on the solvent cast films show only about 10% degradation after 4 weeks. Laminin (using a 200 μg/mL solution in phosphate-buffered
saline [PBS]) was adsorbed into the microgrooves by surface tension effects. The concentration used was the optimum laminin concentration for neurite outgrowth based on previous studies. These studies showed that this method concentrated the laminin in the grooves and not on the spacing between the grooves.\textsuperscript{11}

**Cell culture and seeding**

Primary cultures of Schwann cells obtained from 16–20-day-old Sprague-Dawley rats using the method developed by Morrissey and co-workers\textsuperscript{14} were used for the studies. Schwann cell cultures were purified using a method adapted from the work of Rutkowski and co-workers.\textsuperscript{15} Cells were not passaged more than two times because subculturing the cells more than twice leads to immortalization of Schwann cells. Schwann cell numbers were determined by trypan blue exclusion using a hemacytometer. Counts were done in triplicate and averages for total cell numbers and viability were calculated.

The neurons were collected from the dorsal root ganglia (DRG) of 1–3-day-old Sprague-Dawley rats using a procedure described earlier.\textsuperscript{13} Both whole and dissociated DRG were used for seeding the substrates. Studies with whole DRG better represent neurite behavior after transsections, while the dissociated DRG allow an investigation of individual cell behavior.

Schwann cells were seeded onto the etched laminin-coated patterned polymer surfaces at an initial density of 50,000 cells/cm\textsuperscript{2} and incubated at 37°C for a few minutes before seeding the neurons. This cell density was used as it was found to be optimal for Schwann cell alignment from our previous studies.\textsuperscript{13} Lower cell densities did not promote significant cell alignment, while cell densities higher than 50,000 cells/cm\textsuperscript{2} did not improve alignment. Whole ganglia were dipped into chicken plasma and then into thrombin and placed on the substrate for attachment. They were cultured in media consisting of 50% basal medium Eagle (BME), 25% Earle’s balanced salt solution (EBSS), 25% horse serum (HS), 5 μL of gentamicin per mL of media, and 10 ng of nerve growth factor–β (NGF-β) per mL of media. The cultures were placed on a slowly oscillating tilt table in the incubator at 37°C with 5% CO\textsubscript{2}. Dissociated neurons were also seeded onto the substrates shortly after seeding the Schwann cells. One ganglion was dissociated and seeded on two 1 cm\textsuperscript{2} micropatterned polymer substrates. Both neurons and Schwann cells were cultured in a medium consisting of 90% Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS), 5 μL of gentamicin per mL of medium and 10 ng of NGF-β per mL of medium. After 24 h, the culture was supplemented with 5 mM cytosine-β-arabinocid to prevent the proliferation of fibroblasts.

**Cell staining**

Live staining techniques were used to observe Schwann cells and neurons on the micropatterned substrates after seeding using light microscopy. An Olympus IMT-2 bright field/phase contrast microscope with an attached digital camera was used. DiI (Molecular Probes, Eugene, OR), a retrograde neuronal tracer, labels both neurons and Schwann cells. In order to distinguish between neurons and Schwann cells, the neurons were stained with DiI and the Schwann cells were labeled with SYTO23. SYTO23 (Molecular Probes, Eugene, OR) is a green fluorescent nucleic acid stain with an absorption of 499 nm and emission of 520 nm.\textsuperscript{16} Prior to seeding, the neurons were labeled with 3.7 μL Dil/mL DMEM and incubated at 37°C for 30 min. The cells were viewed with the light microscope using a 590-nm barrier filter. SYTO23 was dissolved in 5 mM dimethylsulfoxide (DMSO) and 3.7 μL/mL DMEM to label the Schwann cells. SYTO23 is very light sensitive and the fluorescent intensity decreases with exposure to all light. For simultaneous seeding of Schwann cells and neurons, the SYTO23 labeled Schwann cells were mixed with DiI labeled neurons and then seeded.

Fixed neurons were labeled using a primary antibody (neurofilament protein) with a modified staining protocol. This procedure stained neurons but not Schwann cells so that the differences between elongated Schwann cell processes and neurites could be observed. This was also done to verify that neurons were indeed seeded on the surface of the substrates. The cells were fixed with 4% paraformaldehyde for 10 min and then rinsed with Tris buffered saline (TBS) three times for 1 min each. Using a DAKO APAAP Kit (System 40, K670), the primary antibody (neurofilament protein) was applied to the films and incubated for 30 min at 37°C. The films were rinsed with TBS two times for 1 min each. The link (rabbit anti-mouse immunoglobulins) was applied and incubated for 30 min at 37°C. The films were rinsed and alkaline phos-
phate antialkaline phosphatase (APAAP) was added and incubated at 37°C for 30 min. By repeating the link and APAAP steps, the stain intensity was increased. The substrates were viewed using light microscopy. The neurites were labeled red and Schwann cells may appear pink from background labeling. The Schwann cells were counter stained with working hematoxylin and Scott’s tap water for 1 min. The hematoxylin was monitored during staining so that the Schwann cells were not overdeveloped.

**Alignment and outgrowth measurements**

The stained neurons and Schwann cells were imaged by light microscopy. Fixed staining was used to verify that the live staining techniques with double staining accurately distinguished between Schwann cells and neurites. Photomicrographs were taken daily and outgrowth and alignment of the stained neurites along the grooves was ascertained by microscopy 48 h after seeding. Extension of the neurites was measured at the rate of μm/day. Alignment was determined by whether the neurites grew in the direction of the grooves without crossing from one groove to another. 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 using 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 microgroove dimensions, concentration of laminin in the grooves, and the presence of Schwann cells, on the adhesion, outgrowth and alignment of neurites was ascertained.

**RESULTS**

Since Schwann cell alignment is controlled primarily by groove width and spacing, and neurite alignment by groove depth, the groove dimensions were independently varied to optimize both Schwann cell and neurite alignment on PDLA microgrooved substrates with adsorbed laminin. Figure 1 shows neurites growing randomly on the smooth part of a PDLA substrate while they are aligned on the microgrooved part of the substrate with laminin adsorbed in the grooves. Interactions between neurons disrupt neurite alignment on shallow grooves, but these interactions are very advantageous *in vivo* because chemotaxis allows regenerating axons to be guided by Schwann cells in the neurilemma for reinnervation. A combination of aligned Schwann cells in the grooves in addition to chemical and physical cues was used to investigate neurite behavior.

![FIG. 1. Half smooth (top) and half 10/20/3 μm patterned laminin coated (200 μg/mL) PDLA solvent-cast substrate with DiI-labeled neurons and Schwann cells showing aligned and nonaligned cells, respectively. Bar = 50 μm.](image-url)
Live staining of the Schwann cells with SYTO23 and the neurons with DiI helped to differentiate between cell types. Figure 2 shows a same-location image of Schwann cell nuclei fluorescing green and neurons fluorescing red (in gray scale). Figure 1 shows the synergistic effects of the grooves and the Schwann cells on neurite alignment. Labeling the neurons using immunohistochemistry required that the cells be structurally reinforced. The cells were fixed with 4% paraformaldehyde and then labeled. Labeling the fixed neurons with neurofilament protein definitively differentiates between Schwann cells and neurons (Fig. 3). This technique was used to verify that the cells being labeled by DiI were indeed neurons and not other cell types.

**Effect of Schwann cells on neurite alignment**

On 10/10/4 μm PDLA solvent-cast substrates within laminin coatings (200 μg/mL), 95 ± 1% of neurites aligned on Schwann cell-seeded substrates while 92 ± 1% of neurons seeded without Schwann cells aligned. On 10/10/3 μm PDLA solvent-cast substrates with laminin in the grooves (200 μg/mL), 98 ± 1%

![Image A](image1.png)

![Image B](image2.png)

**FIG. 2.** Laminin (200 μg/mL) coated 10/10/4 μm PDLA solvent-cast substrates with (A) SYTO23 labeled Schwann cells and (B) DiI-labeled neurons. Bar = 50 μm.
neurites aligned in the presence of Schwann cells, as opposed to $77 \pm 1\%$ in the absence of Schwann cells (Fig. 4). Therefore, preseeded Schwann cells promote neurite alignment even on shallower grooves and reduce the effect of groove depth on neurite alignment.

Similar trends were observed on substrates with groove spacings of $20 \mu\text{m}$. On 10/20/3 $\mu\text{m}$ PDLA solvent-cast substrates coated with 200 $\mu\text{g/mL}$ laminin, Schwann cells--seeding improved neurite alignment from $70 \pm 1\%$ to $97 \pm 1\%$. On 10/20/4 $\mu\text{m}$ substrates, Schwann-cell seeding improved alignment from $88 \pm 1\%$ to $95 \pm 1\%$. Preseeding Schwann cells helps to reduce the effect of groove spacing on neurite alignment.

**FIG. 4.** Effect of preseeded Schwann cells on neurite alignment. □, substrates without Schwann cells; ■, substrates preseeded with Schwann cells. The cells were seeded on PDLA solvent-cast substrates. The means and SEs are shown for 18 substrates. *Significant difference at a 95% confidence level.
Effect of Schwann cells on neurite outgrowth

The neurites on 10/10/3 and 10/20/3 μm solvent-cast PDLA solvent-cast substrates coated with laminin and preseeded with Schwann cells experienced elongation at the rates of $82 \pm 15$ and $81 \pm 17 \mu m/day$ after 2 days. The neurites experienced elongation rates similar to rates previously documented ($77 \mu m/day$ during initial growth) on smooth substrates due to biological cues expressed by the Schwann cells. The neurites on 10/10/4 and 10/20/4 μm PDLA substrates coated with laminin and preseeded with Schwann cells experienced elongation rates of $108 \pm 43$ and $106 \pm 31 \mu m/day$ as opposed to neurites on substrates without preseeded Schwann cells that elongated at $56 \pm 5$ and $64 \pm 7 \mu m/day$ (Fig. 5). Large variations in the neurite outgrowth were observed in the presence of preseeded Schwann cells. The groove depth or spacing did not seem to affect the neurite outgrowth.

Continued effects of Schwann cells on neurite alignment

Preseeding of Schwann cells followed by neurons on micropatterned compression molded substrates were examined to monitor cell behavior during accelerated degradation of the substrate. The compression molded substrates are subjected to higher temperatures and pressures during the fabrication process and degrade much faster than the solvent-cast films. As observed in earlier studies, the topography of a 3-μm-deep pattern on compression molded films was almost completely eliminated after 7 days in media at 37°C. Therefore, cells were seeded on these substrates to see if they continue growing in the same direction and maintain alignment even as the grooves degrade. Figure 6 shows a fluorescent microscopy image of Dil-labeled Schwann cells and neurons showing aligned cells after 2 days. After 5 days in medium, fluorescent microscopy shows that the cells have not detached because of erosion of the polymer (Fig. 7) and the cells continue to align in the direction of the grooves even after the grooves have almost disappeared.

This effect is also seen in the case of whole ganglia. Whole ganglion shed Schwann cells which help to promote neurite outgrowth. The Schwann cells migrate out of the ganglion along with the neurites and align with the grooves. But the Schwann cells and neurites were randomly organized in the smooth substrate region (Fig. 8) as soon as they exit from the grooves. This demonstrates the importance of preseeding the substrate with Schwann cells in order to maintain the continued alignment of neurites along the substrates.

![Graph showing neurite elongation rates](image)
Although some Schwann cells were present in the dissociated DRG cells, the Schwann cells preseeded from primary cultures were labeled so that the Schwann cell nuclei fluoresced green and the neurons fluoresced red. Double labeling of the cells allowed for the collection of more accurate neurite alignment and elongation data. Immunocytochemistry was used to verify that the DiI-labeled cells were indeed neurons.

Seeding neurons on the laminin coated Schwann cell preseeded films significantly improved neurite alignment at a 95% confidence level compared to the coated films without Schwann cells. Preseeding Schwann cells on substrates with shallower grooves of 3 μm improved neurite alignment considerably (Fig. 4). This

**FIG. 6.** Laminin (200 μg/mL) coated 10/10/1.9 μm compression-molded PDLA substrates with DiI labeled neurons and Schwann cells 50 h after seeding. Bar = 100 μm.

**DISCUSSION**

**FIG. 7.** Laminin (200 μg/mL) coated 10/10/1.9 μm compression-molded PDLA substrates with DiI labeled neurons and Schwann cells 120 h after seeding. Bar = 100 μm.
improvement in alignment is important because as the groove depth decreases due to degradation, the Schwann cells provided guidance cues that continued to maintain neurite alignment. We hypothesize that the interactions between the Schwann cells and neurites on these shallow microgrooved substrates is stronger than the interactions between the neurites themselves, maybe due to proximity of Schwann cells and neurites, leading to better alignment in the presence of Schwann cells even on shallower grooves.

Without Schwann cell preseeding, substrates with 10 μm groove spacings were found to align the neurites better than substrates with 20 μm spacings. This is attributed to the fact that in the absence of pre-seeded Schwann cells, some neurites tend to grow on the spacing between the grooves, and for larger spacings, the alignment of neurites decreases.\(^\text{13}\) However, this effect is not significant when Schwann cells are pre-seeded on the substrates. Therefore, the presence of Schwann cells in the grooves seems to considerably reduce the influence of groove depth and groove spacing on neurite alignment. This is because the Schwann cells tend to align along the grooves\(^\text{11}\) and the interactions between the Schwann cells and neurites cause the neurites to preferentially grow in the grooves rather than on the spacings between the grooves, thereby improving alignment even on widely spaced grooves. However, completely eliminating the microgrooves causes the neurites to grow in random directions and display no orientation along any particular direction as seen in Figure 1. In all cases when Schwann cells and neurons were seeded together on grooved substrates, excellent alignment was observed along the grooves.

The aligned preseeded Schwann cells improve neurite outgrowth at a 90% confidence level (Fig. 5). Although neurite growth cone sensitivity increases with increases in guidance cues,\(^\text{19}\) the neurite elongation was not slowed in the presence of preseeded Schwann cells. This was contrary to what was observed with just physical and chemical guidance cues only in the absence of preseeded Schwann cells.\(^\text{13}\) In this case, neurite outgrowth was significantly slowed due to the presence of additional guidance cues. Schwann cells secrete NGF and other growth factors that may have contributed to the increased neurite outgrowth even in the presence of additional cues. These findings reinforce the idea that Schwann cells act synergistically with the physical and chemical guidance cues and enhance the environment that promotes the best neurite regeneration and also improve alignment in conjunction with the microgrooves.

The preseeded Schwann cells help maintain alignment of the neurites even after the grooves degrade. Otherwise, without the preseeded Schwann cells, the neurites do not maintain their alignment as the groove depth decreases or disappears due to degradation, as seen in the case of whole ganglia also. Therefore, in contrast to the preseeded Schwann cells, the Schwann cells shed later by the whole ganglia do not seem to
help the neurites maintain alignment after the grooves disappear. This might be due to the inherent tendency of Schwann cells to form columns and orient themselves in particular directions.\textsuperscript{11} The key to alignment seems to be to have the Schwann cells align first along the grooves when the grooves are still present, and they, in turn, help maintain the alignment of neurites even after the grooves disappear.

These findings suggest that axons in vivo will have continued physical influence promoting neurite alignment as a result of preseeding Schwann cells even as the substrate degrades. The preseeded Schwann cells form columns and appear not to migrate once they are in the grooves and promote orientation even after the grooves disappear. In contrast, as seen in Figure 8, the Schwann cells migrating out of the whole ganglion, and thereby the neurites, do not continue to align in the absence of grooves when no Schwann cells were preseeded. Therefore, it is not just the individual cues but a combination of different cues that are required for enhanced guided growth of the regenerating neurons.

The synergistic effects of physical, chemical, and biological cues were successfully optimized to enhance neurite alignment and outgrowth on polymer films in vitro. Excellent prolonged spatial control, with over 98\% of aligned neurites, was obtained using this technique. Since biodegradable polymers have been used in these studies, the modifications can be implemented into an in vivo experiment to facilitate peripheral nerve regeneration.

CONCLUSIONS

Preseeding of Schwann cells on laminin-coated and micropatterned biodegradable films incorporates physical, chemical, and cellular guidance cues to enhance neurite alignment and outgrowth, promoting faster and more direct axonal regeneration. This cooperative interaction between Schwann cells and neurons is mediated by the physical effects of the grooves and the chemical cues from the laminin. Preseeding Schwann cells with the neurons significantly increases neurite alignment. Effect of Schwann cells on neurite alignment was greater on the shallower grooves. Schwann cells minimized the influence of groove depth and groove spacing on neurite alignment. Therefore, Schwann cells maintained alignment of neurons over time even though the physical effects of the grooves were diminished by degradation. Preseeding Schwann cells did notably improve neurite elongation compared with cultures with only neurons. Therefore, by combining directed neurite alignment with faster extension rates, neurites can be prompted to regenerate faster. These results show that the production of uniform patterned substrates coated with laminin and preseeded with Schwann cells is key to promoting fast and the most direct nerve regeneration in vitro. By utilizing physical, chemical, and cellular cues, the ability to manipulate neurite outgrowth at the cellular level does enhance guided peripheral nerve regeneration in vitro. Using this technique, excellent prolonged spatial control was obtained with over 98\% of neurites, aligning in the direction of the microgrooves. It is the combination of these cues, rather than individual cues themselves, that seems to have a maximum synergistic effect on guided accelerated outgrowth of neurites.

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