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Nanofibrous Patches for Spinal Cord Regeneration

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Abstract

The difficulty in spinal cord regeneration is related to the inhibitory factors for axon growth and the lack of appropriate axon guidance in the lesion region. Here we developed scaffolds with aligned nanofibers for nerve guidance and drug delivery in spinal cord. Blended polymers including Poly (l-lactic acid) (PLLA) and Poly (lactide-co-glycolide) (PLGA) were used to electrospin nanofibrous scaffolds with two-layer structure: aligned nanofibers in the inner layer and random nanofibers in the outer layer. Rolipram, a small molecule that can enhance cAMP activity in neurons and suppress inflammatory responses, was immobilized onto nanofibers. To test the therapeutic effects of nanofibrous scaffolds, the nanofibrous scaffolds loaded with rolipram were used to bridge the hemisection lesion in 8-week old athymic rats. The scaffolds with rolipram increased axon growth through the scaffolds and in the lesion, promoted angiogenesis through the scaffold, and decreased the population of astrocytes and chondroitin sulfate proteoglycans in the lesion. Locomotor scale rating analysis showed that the scaffolds with rolipram significantly improved hindlimb function after 3 weeks. This study demonstrated that nanofibrous scaffolds offered a valuable platform for drug delivery for spinal cord regeneration.

Keywords

Nanofibers; spinal cord; drug delivery; regeneration

INTRODUCTION

Spinal cord injury (SCI) and the resulting chronic paralysis affect more than a million individuals in United States. To date spinal cord regeneration remains as one of the most challenging problems in regenerative medicine, and there is no effective cure for spinal cord injury. The advance in nanotechnology and neuroscience provides new opportunities for us to meet this challenge. The difficulty in axon regeneration in spinal cord is largely due to glial scar formation and the release of inhibitory factors following spinal cord injury [1–5]. Following SCI, the lesion is invaded by fibroblasts, macrophages and glial cells, and a dense glial scar forms. The myelin sheath also degenerates. Both the glial scar and myelin breakdown products become barriers to axon regeneration [6–7]. To overcome this barrier, the stimulation and guidance of axon growth are needed.

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Previous studies have suggested that the combinations of strategies such as bridging the lesion, drug delivery and cell delivery could result in effective therapies for SCI [8–10]. There is evidence that the inhibitory effects of myelin-derived factors can be eliminated by elevating cAMP activity [11, 12]. For example, the inhibition of cAMP hydrolysis by the phosphodiesterase IV inhibitor rolipram (delivered by a mini pump) enhances axon growth in spinal cord [13]. Rolipram also has anti-inflammatory effects in central nervous system (CNS) [14]. However, how to combine axon guidance and rolipram delivery *in vivo* in a local and sustained manner needs to be addressed.

Several groups have used scaffolds to bridge the gap in the lesion area caused by the necrosis and apoptosis of cells after SCI [15–18]. These scaffolds are either porous solid materials or hydrogels. Recently, electrospinning has been used to fabricate nanofibrous scaffolds from both native matrix and synthetic polymers [19–22]. We have developed nanofibrous scaffolds for tissue regeneration by using electrospinning technology [23–25]. The aligned nanofibers not only guide axon growth, but also provide large surface area and can be functionalized by immobilizing bioactive factors [24]. For example, we have shown that aligned nanofibers immobilized with growth factor can synergistically promote axon growth from dorsal root ganglion (DRG) tissue. The prolonged release of bioactive factors by nanofibrous scaffolds can replace the injections or mini-pump administration of bioactive factors at the lesion site.

Here we developed nanofibrous scaffolds that not only guide axon growth and angiogenesis but also release drug locally to promote the regeneration of spinal cord tissue. Rolipram is a selective inhibitor of cyclic AMP phosphodiesterase IV and has antidepressant and anti-inflammatory effects in the central nervous system [26]. By using rolipram as a prototypical drug to augment the response to scaffold support, we showed that rolipram-loaded nanofibrous scaffolds can enhance axon growth and angiogenesis, suppress glial scar formation, and significantly improve the recovery of hindlimb function in the hemisection SCI model.

MATERIALS AND METHODS

Scaffold Fabrication and Characterization

We used electrospinning technology to fabricate biodegradable scaffolds with two-layer structure. Poly (L-lactide) PLLA (1.09 dl/g inherent viscosity) and Poly (D, L-lactide-co-glycolide) PLGA (50:50, 0.55~0.75 dl/g inherent viscosity) were from Lactel Absorbable Polymers Inc (Pelham, AL). To make the inner layer with aligned nanofibers, the jet stream of PLLA/PLGA (each 15% weight/volume) solution from the spinneret whipped between the two conductive ends of a plastic mandrel, resulting aligned nanofibers on the non-conductive portion in the middle of a slowly rotating mandrel. The outer layer with random nanofibers was generated by increasing the rotating speed of the mandrel to 800 rpm. The scaffold in half-cylindrical shape was made by cutting open the tubular scaffold in the longitudinal direction (Figure 1). The scaffolds were rinsed with phosphate buffer saline (PBS) and sterilized by UV irradiation before being used for rolipram immobilization or *in vivo* studies. The alignment of nanofibers and the structure of the nanofibrous scaffolds were examined by scanning electron microscopy (SEM). The diameter of nanofibers ranged from 500–800 nm.

Neural Stem cells (NSCs) interaction with Nanofibrous Scaffold

Human embryonic stem cells (hESC) line H9 (Wicell, Madison, WI) were cultured as previously described [27]. hESCs were detached with the treatment of 1 mg/mL collagenase, 0.5mg/mL dispase (Invitrogen, Carlsbad, CA) in Knock-out Dulbecco's Modified Eagle

Medium (KO-DMEM)/F12 for 20 minutes. The colonies were gently resuspended in B27 media (DMEM/F12 +2% B27 supplement, 1% L-glutamine, 1% non-essential amino acids (Invitrogen) and grown for 5 days in non-adhesive 6-well culture dishes (Nunc, Rochester, NY) to form embryoid bodies (EBs). EBs were then plated onto tissue culture dishes coated with laminin (1 mg/mL) (Invitrogen) and polyorithine (0.1 mg/mL) (Sigma, St. Louis, MO) (mixed in one solution for coating), and grown for 2–3 days in B27 media supplemented with 20ng/mL bFGF (Peprotech, Rocky Hill, NJ) to allow rosette formation. After rosettes formed, the rosettes were manually collected and cultured in suspension in B27 media with bFGF for one day and then seeded onto either aligned or random nanofiber membrane (pre-coated with laminin and polyorithine) in B27 media without bFGF for one week. Samples were fixed and stained with rabbit anti-rat β III tubulin (1:200 dilution, Sigma) to show the neurite extension (Figure 1D, 1E).

Immobilization of Rolipram and *In Vitro* Release Profile

To characterize the drug loading capacity of nanofibrous scaffolds *in vitro*, nanofibrous scaffolds were cut into circular membranes (6 mm in diameter and 600 μ m in thickness) with biopsy punches, and soaked in rolipram solutions with various concentrations for 12 hours. Then the nanofibrous scaffolds were washed twice with PBS (100 μ l each). The soaking solution and the washing solution were collected and combined, and the amount of rolipram was quantified by using high-performance liquid chromatography (HPLC) to deduce the amount of rolipram entrapped and adsorbed within the nanofibrous scaffolds. HPLC was performed by using a RP-18 column and eluent 30–42% acetonitrile/water. Rolipram was detected by UV absorbance at 280 nm. The standard curve of rolipram showed that the peak area obtained from HPLC analysis was linearly correlated with the amount of rolipram in a wide range of concentrations from 5 μ g/ml to 500 μ g/ml ($R=0.999$) (data not shown).

To obtain the release profile of rolipram, nanofibrous scaffolds were immersed into rolipram solution (500 μ g/ml) for 12 hours. The scaffolds were then rinsed twice with PBS, completely submerged in 3 ml PBS solution and incubated at 37°C for up to 20 days. At selected time points (0.5 hr, 4 hr, 1 day, 2 days, 4 days, 8 days, 12 days, 20 days), 0.3 ml solution was withdrawn and replaced by 0.3 ml of fresh PBS. The amount of rolipram in the withdrawn solution was determined by the HPLC, and the total amount of released rolipram was calculated to generate the release profile.

Spinal Cord Hemisection

All procedures in animal studies were approved by the Institutional Review Board Service and Institutional Animal Care and Use Committee at the University of California, Berkeley. Male adult athymic rats (250 $\pm$ 30 g; 8-week old) were anesthetized with 1.5% isoflurane in 70% N₂O/30% O₂. Body temperature was maintained at 37.0 $\pm$ 0.5°C. SCI was performed as described before^[11, 15, 28]. Briefly, the rat was set in the prone position, and the 9th to 11th thoracic vertebrae were exposed. After resecting the laminae, the spinal dura mater was incised to expose the spinal cord. A lateral hemisection at the T9–T11 level was made by creating a 4-mm long longitudinal cut along the midline of the cord, followed by lateral cuts at the rostral and caudal ends and the removal of the tissue by aspiration. Right after SCI, a scaffold (4-mm long, half of a tube with the diameter fitting in the cavity of the injured spinal cord and nanofibers aligned in longitudinal direction) with or without rolipram was used to cover the spinal cord surface and enclose the lesion site. The scaffold was thick enough (~600 μ m) to separate spinal cord from surrounding tissue. Fibrin glue was used to glue the outside of the scaffolds to the vertebra tissue as needed. The surgery site was closed in layers. After surgery, rats were kept on heating pads, and daily examination was performed. Abdomen massage was done to help evacuate urine from bladder as needed.

Immunohistochemistry

The frozen sections (10 μm in thickness) of spinal cord tissues were fan-dried for 1 hour at room temperature. Then the slides were fixed in 4% paraformaldehyde solution for 25 minutes at room temperature. Samples were incubated in 5% normal goat serum for 30 minutes to block the non-specific binding, and then incubated with primary antibody diluted in 5% normal goat serum overnight at 4°C. Primary antibodies include mouse anti-CD31 (1:100 dilution, BD Pharmingen) for endothelial cells (ECs), rabbit anti-neurofilament (1:200 dilution, Sigma) for axons, mouse anti-glial fibrillary acidic protein (GFAP) (1:2000 dilution, Sigma) for astrocytes, anti-chondroitin sulfate proteoglycans (CSPGs) (1:200 dilution, Millipore, Billerica, MA) for glial scar. Negative controls were included by omitting the primary antibody. The sections were incubated for one hour with either horseradish peroxidase-conjugated anti-mouse or rabbit IgG (1:1000, Alexa 594 for red and Alexa 488 for green, Invitrogen, Carlsbad, CA). Finally, slides were mounted and examined by using a fluorescence microscope (Zeiss Axioskop 2 MOT).

Hindlimb Behavioral Assessment

Three groups in this study were investigated to evaluate functional recovery after SCI by hindlimb behavioral assessment. Animals that had undergone SCI without any treatments were considered as the untreated group and experimental groups included scaffold only and scaffold plus rolipram groups. One day after SCI, and subsequently every other week, Basso-Beattie-Bresnahan (BBB) locomotor scale rating was performed to evaluate the functional recovery of disabled limb^[29]. The BBB locomotor rating scale is an open-field 21-point evaluation scale, with 21 point as the highest score (normal) and 0 as the lowest score (completely disabled). BBB scores were used to categorize the combinations of rat hindlimb movements, trunk stability, stepping, coordination, paw placement, toe drag and tail position. Briefly, BBB score rating was assessed by an observer blinded to the treatment every week. Animals were allowed to move freely in an open area and the movements of hindlimb were examined and recorded for at least five minutes. A digital camcorder was used to record the movement of rats and replayed in slow motions as needed.

Statistical Analysis

The data are presented as mean $\pm$ standard deviation (SD). Data were analyzed using analysis of variance (Statview 5.0) to examine BBB scores. Post hoc testing was performed by using Fisher's protected least significant difference (PLSD). A P value less than 0.05 was considered statistical significant.

RESULTS

Structure and Appearance of Nanofibrous Scaffold

We used electrospinning technology to make a tubular scaffold with longitudinally aligned nanofibers as inner layer. Layers of random nanofibers were generated outside of aligned nanofibers (Figure 1). The purpose of aligned fibers in the inner layer (~100 μm in thickness) was to provide guidance for the growth of axons in the longitudinal direction of spinal cord and facilitate axon growth into the three-dimensional (3D) scaffold, and the outer layer (~500 μm in thickness) with random nanofibers enhanced the mechanical property of the scaffolds.

Aligned Nanofibers Promotes Neurite Growth from NSCs

To characterize the roles of nanofibrous scaffold on neurite growth, we seeded NSCs onto scaffolds with either aligned or random nanofibers. As shown in Figure 1, aligned nanofibrous scaffold guide neurite growth from rosettes in the direction of nanofiber

alignment (Figure 1D), but neurites extended randomly on the surface of random nanofibers (Figure 1E).

Immobilization of Rolipram onto Nanofibrous Scaffold

As shown in Figure 2A, the amount of rolipram entrapped/adsorbed within the nanofibrous scaffolds increased with the input rolipram solution concentration, and reached a plateau beyond input concentration at 250 $\mu\text{g/ml}$. The *in vitro* rolipram release profile was shown in Figure 2B. The results showed that the drug release occurred in three phases: a first initial burst release, a fast release of the drug over the first 3 days, and a sustained and slow release of the drug over 20 days.

Nanofibrous Scaffolds with Rolipram Enhanced Axon Growth, Increased Angiogenesis and Decreased Glial Scar

To test the therapeutic effects of rolipram-loaded nanofibrous scaffolds, rats were subjected to T9–T11 spinal cord hemisection as SCI model. Right after the injury, the lesion was either untreated (control group), or bridged with nanofibrous scaffolds with or without immobilized rolipram (Figure 3A). After 12 weeks, three rats in each group were sacrificed, and the cross sections of the spinal cord tissues were used for histological analysis. The histological staining of samples from three groups (untreated control, scaffold and scaffold with rolipram) is shown in Figure 3 and 4. As an example, H&E staining of a cross section from the scaffold with rolipram group (Figure 3B) showed the intact white and grey matters on the left side of the spinal cord and the lesion area on the right side with some loose regenerated tissue. Black dashed lines sketched the boundary between the lesion and scaffold.

Neurofilaments are major elements of cytoskeleton in the axon cytoplasm, which were immunostained to show neurons and axon growth in the tissue. The scaffolds with or without rolipram increased axon growth through the scaffolds and in the lesion, with the most axon growth in the inner layer of the scaffolds with rolipram (Figure 3D–G). In addition, axon growth showed different directions in the longitudinal aligned and random nanofibrous layers, suggesting the primary role of nanofibers in the guidance of axon regeneration.

Moreover, positive CD31 staining for ECs was found in nanofibrous scaffolds. Angiogenesis through the scaffold was detected in the scaffold alone group (Figure 4B), suggesting that the aligned nanofibers guided EC migration. Interestingly, the combination of nanofibrous scaffold and rolipram further promoted angiogenesis through the scaffold (Figure 4C).

Furthermore, the least GFAP staining was found in the lesion treated with scaffolds plus rolipram, which was correlated well with the lowest CSPGs expression (Figure 4F and Figure 4I), suggesting that rolipram could reduce the number of reactive astrocytes and suppress inflammatory responses and glial scar formation.

Release of Rolipram from Nanofibrous Scaffolds Promoted the Functional Recovery

To determine whether the insertion of nanofibrous scaffold with or without rolipram promoted the recovery of hindlimb motor function, behavioral analysis was performed by using BBB locomotor scale rating. As shown in Figure 5, scaffold alone only had modest effect on functional recovery after 6 weeks. Rolipram plus scaffold significantly improved hindlimb function after 3 weeks. During 3–8 weeks, BBB score of hindlimb movement increased at every observation point in the group with scaffold plus rolipram. After 8 weeks, the hindlimb function had no further improvement in all three groups.

DISCUSSION

The difficulty in spinal cord regeneration can be attributed to multiple factors, including the limited regeneration capability of neurons in CNS, the lack of appropriate axon guidance in the lesion, and the inhibitory factors such as myelin breakdown products and CSPGs in glial scar. Most efforts to restore function after spinal injury have been directed toward enhancing damaged axons to regenerate through injury site, and then reconnecting the proper targets to restore motor and sensor function. Here we developed the biodegradable nanofibrous scaffold with immobilized rolipram to facilitate spinal cord tissue regeneration.

Nanostructured materials have tremendous potential for tissue engineering. Our results showed that aligned nanofibers guided neurite growth *in vitro* (Figure 1). In addition, axon growth in the inner and outer layers of the scaffolds show different directions following nanofibers (Figure 3D–3G), indicating that aligned nanofibers can guide axon growth *in vivo*. Furthermore, aligned nanofibers could also promote axon growth and cell infiltration [25] into the 3D scaffolds. The release of rolipram from the nanofibers significantly increased axon growth through the inner layer of the scaffolds (Figure 3F–3G), suggesting a synergistic effect of aligned nanofibers and drug release, which is well correlated with the behavior test results (Figure 5). The modest functional recovery in scaffold group suggests that axon guidance alone is not sufficient, but it is likely that aligned nanofibers are necessary to achieve the functional recovery—otherwise axon growth may be disorganized and cannot reach the target area effectively. Whether random nanofibrous scaffolds with rolipram could promote functional recovery awaits further study.

In addition to promoting axon growth, the therapeutic effects of rolipram could also be attributed to the suppression of inflammatory responses and glial scar formation, as shown by the decrease of CSPGs and glial cells in the lesion area (Figure 4D–4I). CSPGs are a major component of glial scar synthesized by glial cells and inflammatory cells, which forms a physical and biochemical barrier to axon growth [30–31]. Rolipram can elevate cAMP activity, thus suppressing inflammatory effects and inhibiting reactive astrocytes related astrogliosis [32–33]. Generally, rolipram at high dose can be used as an inflammatory drug, which prevents secondary cell loss in the acute stage [26, 34]. Acute rolipram treatment retards the injury-induced increase of IL-1 β and TNF- α , which are prominently elevated at the lesion and cause the secondary tissue degeneration [35]. Moreover, recent evidence suggests that rolipram modulated pro-apoptotic caspase-3 activity to provide neuroprotection against several apoptotic insults [36].

Our rolipram release profile is well suited to address the needs to suppress inflammatory responses at the early stage and promote axon growth at the later stage. Rolipram is a small hydrophobic molecule with three-ring structure. Here rolipram was adsorbed onto hydrophobic PLLA/PLGA nanofibers, potentially through hydrophobic interactions through multiple sites on the molecule. The exact binding sites have not been mapped out. We found that most rolipram was released in the first day, and then nanofibrous scaffold continuously released lower level of rolipram into the solution continuously for 20 days. The instant release of 90% rolipram from nanofibrous scaffold may play a major role in attenuating early inflammation. Following the initial burst of rolipram release, low dose of rolipram release might be effective in stimulating axon growth. Previous investigation showed that increasing cAMP levels in neurons enhanced the responsiveness to diffusible growth factor and overcame myelin-associated inhibitory molecules to promote axonal growth and neurites [37]. Further investigations will be conducted to engineer the drug loading capacity and release profile and to determine the differential effects of rolipram at the early and late stages of the spinal cord repair. It should also be noted that the athymic rats used in this

study have T-cell deficiency, and possible immunological responses involving T cells are not included in this animal model.

Interestingly, in addition to axon growth into the scaffolds, extensive angiogenesis was detected in the scaffold, which was further enhanced by rolipram treatment. The mechanisms are not clear and need further investigation. Recent investigation reported that axon regrowth was simulated by new blood vessel formation in the damaged tissue after spinal cord injury [38] and that vascular endothelial growth factor (VEGF) induced posttraumatic angiogenesis to improve functional recovery of injured rodents [39]. Whether and how angiogenesis in the scaffold contributes to spinal cord regeneration need further investigation.

Overall, this study has demonstrated that nanofibrous scaffold offered a valuable platform of drug delivery for spinal cord regeneration in addition to the guidance of axon growth and angiogenesis, and that scaffold-mediated rolipram release not only promotes axon growth and angiogenesis but also suppresses glial scar formation.

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Figure 1.

Characterization of nanofibrous scaffolds with both aligned and random layers. (A) The scaffold in half-cylindrical shape was made from the tubular scaffold and cut open in the longitudinal direction. SEM images showed the inner layer (B) with longitudinal aligned fibers and outer layer (C) with random fibers of the scaffold. Scale bar = 20 μ m. Human ESC derived neural cells cultured on aligned (D) and random (E) nanofiber surface *in vitro* for one week in B27 media. Neurites were stained by β III-tubulin antibody. Scale bar = 100 μ m.

Figure 2.

In vitro immobilization and release profile of rolipram with nanofibrous scaffolds by performing the HPLC test. (A) The amount of rolipram immobilized within the nanofibrous scaffolds with different rolipram loading concentrations. (B) The release profiles of rolipram in PBS at 37°C.

Figure 3.

(A) Schematic illustration of our approach to promote spinal cord regeneration. Scaffolds with nanofibers (in the inner layer) aligned in the longitudinal direction were used to bridge the lesion, guide axons and deliver rolipram locally. (B) H&E staining showed injury lesion after 12 weeks of SCI in the group with scaffolds plus rolipram. Black dashed lines showed the boundary between the lesion and scaffold. Scale bar = 500 μ m. (C–G) Immunofluorescent staining of cross sections showed neurofilament staining of axons in the area of damaged white matter and scaffold in the untreated (C), scaffold (D, E) and scaffold with rolipram (F, G) groups. White dashed lines showed the boundary either between the lesion and inner layer of scaffold (D, F) or between inner layer and outer layer of scaffold (E, G). Scale bar = 20 μ m. Abbreviations are: WM, white matter; GM, grey matter; L, lesion; S, scaffold; S-IL, scaffold-inner layer; S-OL, scaffold-outer layer.

Figure 4.

Histological analysis of CD31, GFAP and CSPG staining after 12 weeks of spinal cord hemisection surgery. Immunofluorescent staining of cross sections (in the area of damaged white matter around the longitudinal middle section of the lesion and near the scaffolds) from untreated lesions (A, D, G), lesions treated with scaffold alone (B, E, H) and lesions treated with scaffold plus rolipram (C, F, I). ECs in capillaries were stained by CD31 antibody (A–C). White dashed lines showed the boundary between the lesion (L) and scaffold (S). Astrocytes in the lesion were stained for GFAP antibody (D–F). Glial scars were stained for CSPG antibody (G–I). Scale bar = 20 μm .

Figure 5.

Functional recovery of hindlimb locomotor as indicated by BBB score. After spinal cord hemisection, the hindlimb locomotor function was analyzed every week throughout 12-week survival time periods. * indicates significant difference ($P < 0.05$) compared to the control group at the respective time points. Three animals were used in each group.