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A Novel Polymeric Drug Delivery System for Localized and Sustained Release of Tacrolimus (FK506)

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ABSTRACT: Despite substantial improvement in microsurgical techniques for nerve repair, recovery after peripheral nerve injury is usually incomplete. FK506, an FDA approved immunosuppressant, improves functional recovery and reinnervation following peripheral nerve injury in animal models. However, systemically delivered FK506 causes undesirable global immunosuppression. We have, therefore, engineered a biodegradable local delivery system for FK506 using fibrin gel as a drug reservoir that could be placed at a site of nerve injury. FK506 was incorporated into fibrin gel in solubilized, particulated, and poly(lactic-co-glycolic) acid (PLGA) microspheres-encapsulated forms. A tunable release of FK506 in the fibrin gel from days to weeks was observed with the rate of release being most rapid for the solubilized form and then the particulate form. The most prolonged period of release was seen with the PLGA microsphere-encapsulated form. As analyzed by in vitro dorsal root ganglion (DRG) neurite extension assay, PLGA microsphere encapsulation of FK506 did not alter the drug's properties and the released FK506 maintained its bioactivity over the entire period of release. This study suggests that local delivery of FK506 with fibrin hydrogel could be used to enhance peripheral nerve regeneration.

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KEYWORDS: nerve injury; FK506; drug delivery; fibrinogen; poly(lactic-co-glycolic) acid; regenerative medicine

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Introduction

Peripheral nerve injuries are a common cause of severe disability, and they affect patients during their most productive years, resulting in devastating social and economic consequences (Ciaramitaro et al., 2010; Lundborg, 2000). Severe nerve injuries occur frequently following trauma and intraoperative nerve injuries are a common source of litigation accounting for 16% of all cases (Kroll et al., 1990). Presently, acute management following nerve transection injury is exclusively surgical focusing on restoring nerve continuity (Kouyoumdjian, 2006; Millesi, 1985). Despite optimal surgical management and prolonged rehabilitation programs, full recovery is rare (Intiso et al., 2010), particularly in cases where nerve repair is delayed (Fu and Gordon, 1995a,b). Novel and innovative pharmaceutical treatments that accelerate axonal regrowth and prevent prolonged end-organ denervation are needed to improve outcomes following nerve injury.

FK506 (Tacrolimus) was initially FDA-approved as an immunosuppressive agent for solid organ transplantation in humans (Starzl et al., 1989), and has recently demonstrated neurotrophic effects which appear to be independent of its immunosuppressive activity (Gold et al., 1994). In vitro, FK506 significantly increases neurite outgrowth from SH-SY5Y neuroblastoma cells (Gold et al., 1999) and from chick sensory ganglia (Steiner et al., 1997). Importantly, FK506 enhances nerve regeneration following rat sciatic nerve injury when administered systemically (Gold et al., 1994,1995). Moreover, sustained systemic administration of FK506 in combination with graft therapy or tube repair increases the rate of nerve regeneration and promotes functional recovery (Gold, 1997; Navarro et al., 2001).

However, systemically delivered FK506 may cause severe side effects including nephrotoxicity and global immunosuppression, potentially resulting in undesirable, debilitating consequences if used clinically following nerve repair (Udina et al., 2003; Varghese et al., 2014; Yamazoe et al., 2014). Thus, sustained localized delivery is required for a broad clinical use of FK506 to enhance nerve regeneration (Konofaos and Terziss, 2013). Current implantable local drug delivery methods that have been used in the peripheral nervous system, including silicone tubes attached to osmotic pumps, are not ideal as the implantable devices can fail, become infected and exposed, or induce a local, chronic inflammatory response leading to fibrosis and chronic nerve injury (Guilhem et al., 2009; Merle et al., 1989). Additionally, silicone tubes placed around nerves ultimately need to be removed because they are not biodegradable and may lead to chronic nerve compression secondary to capsular fibrosis (Lundborg, 2000). Therefore, other strategies, such as biodegradable biomaterials provide a more clinically practical alternative.

Local drug delivery systems must supply the agent for sufficient time to achieve optimized clinical outcomes; therefore, various release periods from about a week to even several months would be required (Boyd and Gordon, 2003). In order to achieve sustained drug release and eliminate the complications associated with current local delivery methods such as minipumps/catheter systems, natural biomaterials including fibrin gels can provide a clinically applicable solution (Wood et al., 2012, 2013). Due to their ease of placement (Jubran and Widenfalk, 2003) and lack of inhibitory effects on nerve regeneration (Sameem et al., 2011), fibrin gels are suitable as peripheral nerve drug delivery systems.

Soluble drugs have a rapid rate of release form the fibrin gel based drug delivery system (Wood et al., 2012, 2013). In order to achieve a more sustained release for over 7 days, hydrophobic drugs such as FK506 can be dispersed in the solid drug particulate form within the fibrin gel (Wang et al., 2009). In addition, by incorporating biomaterials such as polymeric microspheres with fibrin gel, various release profiles from weeks to months is possible (Baumann et al., 2009; Garbayo et al., 2008, 2009; Stanwick et al., 2012a,b; Wood et al., 2013). Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable polymer that has been previously used to encapsulate biologically active neurotrophic factors in polymeric microspheres (Garbayo et al., 2008, 2009; Kokai et al., 2010, 2011). These microspheres have been used for sustained release of neurotrophic factors, such as neurotrophin-3 and glial cell line-derived neurotrophic factor from implantable gels for central and peripheral nervous system injuries (Baumann et al., 2009; Wood et al., 2013a). Therefore, PLGA microspheres are ideal for developing a biocompatible FK506 delivery system with extended release.

The aim of the current in vitro study was to engineer several biocompatible and biodegradable FK506 delivery systems that are composed of fibrin gel as the drug reservoir. Our long-term objective is to obtain local and sustained FK506 release to an injured nerve in vivo for maximal effect simultaneous with minimal unwanted side effects. FK506 was incorporated in fibrin gel in solubilized, particulated, and PLGA microsphere encapsulated forms. We achieved tunable FK506 release ranging from 7 to 28 days by incorporating FK506 in the three forms within the gel. The PLGA-encapsulation method yielded the longest duration of release. Importantly, the bioactivity of the PGLA-encapsulated FK506 was equivalent to that of free drug as measured by a rat dorsal root ganglion neurite extension assay.

Materials and Methods

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

Microsphere Preparation

FK506 was encapsulated in PLGA microspheres (MSs). The encapsulation process was done using single emulsion solvent evaporation technique (Visscher et al., 1988). In summary, an organic phase consisting of 1 mL of dichloromethane (DCM)/acetone (75%/25%), 230 mg PLGA, 12.5 mg $MgCO_3$, and 12 mg FK506 was added to 25 mL of 2.5% polyvinyl alcohol (PVA) solution containing 10% NaCl. The emulsion was formed through homogenization (Kinematica, Bohemia, NY) at 6,000 rpm for 60 s. The entire mixture was poured into a 250 mL hardening bath of 0.25% PVA solution containing 10% NaCl under magnetic stirring at 125 rpm for 3 h. The hardened microspheres were collected and washed by centrifugation, lyophilized, and stored at −20°C until use.

Microsphere Characterization

Microsphere mean diameter and size distribution were measured via static light scattering using a Malvern Mastersizer 2000 laser diffraction particle sizer (Malvern Instruments Ltd., UK), using refractive indices of 1.33 and 1.59 for water and PLGA, respectively. In order to determine the encapsulation efficiency, 5 mg of microspheres were broken apart by acetonitrile as an organic solvent and the resulting solution was analyzed for FK506 content using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Caicco et al., 2013). Drug loading was determined as the FK506 mass per milligram of particles, while encapsulation efficiency was the measured drug loading of the particles divided by the theoretical maximum drug loading.

Drug Delivery Composite and In Vitro Release

Fibrin gel (80 μL total volume) was constructed by mixing equal parts of fibrinogen (75–115 mg/mL, 40 μL) and thrombin (5 IU/mL, 40 μL) obtained from a Tisseel® glue kit (Baxter Healthcare, IL), and then re-suspended according to the manufacturer's instructions. Fibrin gel was loaded with FK506 by incorporating 10 μL solubilized FK506 in acetonitrile (2 mg/mL), 80 μg solid FK506 particulates, and 10 mg of MSs into the thrombin solution before it was mixed with fibrinogen to form a gel at the bottom of a 2 mL Eppendorf tube. Release of FK506 from the drug delivery composite was accomplished by incubating the fibrin gels in 1 mL phosphate-buffered saline (PBS) at 37°C under constant gentle agitation by vortex. The PBS was removed and replaced at over 15 days and analyzed for FK506 content.

FK506 Detection

A Sciex API4000 triple quadrupole mass spectrometer (Ottawa, CA) fitted with an electrospray ionization interface was used for all analyses. The instrument was operated in electrospray positive ionization mode and coupled to an Agilent 1100 capillary LC system (Mississauga, CA). The separation of FK506 and internal standard (Cyclosporine A) was performed using a Spherisorb CN column (30×4.6 mm, 5 μm) (Waters, Milford) with a mobile phase composed of 65% aqueous acetonitrile containing 2 mM ammonium acetate and 0.1% (v/v) formic acid operating at a flow rate of 1 mL/min and a sample injection volume of 10 μL. Quantification was performed using multiple reactions monitoring of the ammonium-adduct transition masses of FK506 (m/z 822→768) and internal standard Cyclosporine A (m/z 1220→1202). Instrument parameters were optimized for the simultaneous detection of both the drug and internal standard.

Biological Activity Assay

Bioactivity of the FK506 released from the delivery system was evaluated using a dorsal root ganglia (DRG) neurite outgrowth assay. DRGs were dissected from 15-day-old rat embryos (E15) and placed in modified neurobasal media (NBM) with 2 vol.% B-27 serum free supplement, 1 vol% penicillin–streptomycin, and 1 vol% L-glutamine (Stanwick et al., 2012). Twelve millimeter diameter glass cover slips were placed in the wells of a 24-well plate. The cover slips were first pre-coated with poly-D-lysine (50 μg/mL) overnight and then laminin (5 μg/mL) for 2 h at 37°C. Three DRGs per well were then placed on the cover slips in the 24-well plate. The DRGs in each well were treated with 0.5 mL of modified NBM media and 0.5 mL of the samples from the in vitro release samples collected at days 7, 14, and 28 of the release period. For controls, 0.5 mL of FK506 concentrations of 0, 50, and 100 ng/mL were added to the wells. These concentrations were chosen based on previous studies demonstrating that rodent neurite outgrowth was maximal at a concentration range of 10–1 μg/mL of FK506 (Chang et al., 1995). This procedure was performed three separate times.

Neurite outgrowth was measured after 48 h. The DRGs were fixed by 4% paraformaldehyde (PFA) and stained for neurofilament (Kemp et al., 2009). Briefly, DRGs in each well were washed (1% Triton-X in PBS) and blocked for 1 h at room temperature (0.1% BSA/10% donkey serum in PBS). DRGs were then incubated with monoclonal mouse antibody neurofilament-160 (Sigma, 1:200) overnight at 4°C. The next day, each well was washed and incubated with donkey anti-mouse AlexaFluor 568 (Sigma, 1:500) for 2 h at room temperature. After washing the wells, images were taken under confocal fluorescence microscopy (Olympus IX81).

Average neurite lengths were calculated according to the methods published by Herbert et al., 1996), using the following formula:

$$L = \sqrt{\frac{1}{\pi}}\left[\sqrt{A_{\text{cell cluster}} + A_{\text{area of neurites}}} - \sqrt{A_{\text{cell cluster}}}\right] \qquad (1)$$

where L = average neurite length of a circular ganglion, which is the width of an annulus with an area equal to the area of the neurite zone (from the edge of the cluster of cell bodies to the wavefront of the growing neurites), $A_{\text{cell cluster}}$ that is the area of the cell cluster of the ganglion, and $A_{\text{area of neurites}}$, the area of the neurite zone. The average neurite outgrowth was normalized to the neurite extension measured for the control known FK506 concentrations which had consistent results for three consecutive experiments. The normalization was done by dividing the measured neurite lengths by the mean neurite length of the control.

Statistical Analysis

All data are presented as mean ± standard deviation. For pair-wise comparison, *t*-tests were carried out. For comparison of multiple groups, ANOVA comparisons were conducted and significance was assigned at $P < 0.05$.

Results and Discussion

In Vitro Microsphere Characterization and FK506 Release From Fibrin Gels

In order to temporally control drug release, FK506 was incorporated in the fibrin gel in solubilized (Fig. 1A), particulated (Fig. 1B), and PLGA microsphere (MS) encapsulated (Fig. 1C) forms. Figure 2A demonstrates the release of FK506 from the drug delivery system into phosphate buffered saline (PBS), where the data are normalized to the total amount of FK506 within the system. As shown in Figure 2A, solubilized FK506 was released from fibrin in 5 days, thus giving the most rapid rate of release from the drug delivery system. The rate of release was slower when FK506 was dispersed in fibrin gel in particulate form as previously described (Wang et al., 2009). This form of drug incorporation provided sustained release of FK506 for up to 15 days (Fig. 2A). The longer release period was likely due to the lesser amount of FK506 that is available to be released from the gel matrix in the dissolved form at any given time.

In another form, FK506 was encapsulated within PLGA MSs with diameter of 45 ± 5 μm, an encapsulation efficiency of $84.7 \pm 1.2\%$, and a loading of 40.85 ± 0.05 μg FK506 per milligram of microspheres. In this system, FK506 is released by sustained diffusion through the bulk-eroding surface of the PLGA MSs and then through the fibrin matrix. Such diffusion dramatically extended the drug release period to over 28 days as compared to 5 and 14 days for the solubilized and particulate forms of FK506. Moreover, the release occurred in two phases (Fig. 2A).

The microsphere and fibrin gel mixture began releasing FK506 from ~5 μg/mL per day, increasing to 20 μg/mL per day by day 9. This was followed by a decline in the rate of release from days 10 to 15 after which a surge of FK506 release was observed until all the FK506 was released from the PLGA MSs (Figure 2A and B). A possible mechanism to explain this release profile was that, during the first 9 days, FK506 was being released from the superficial layers of the PLGA microspheres. As these layers became depleted of FK506, the release rate decreased between

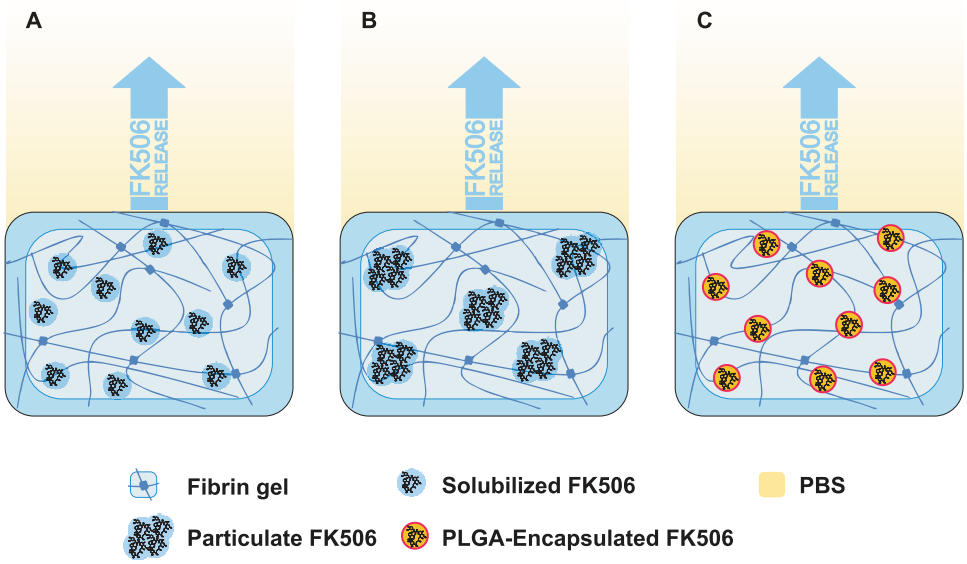

Figure 1. Three methods of incorporating FK506 in fibrin gel: (**A**) solubilized FK506, (**B**) particulate FK506, and (**C**) PLGA-encapsulated FK506. The arrows denote the release of FK506 from the fibrin gel.

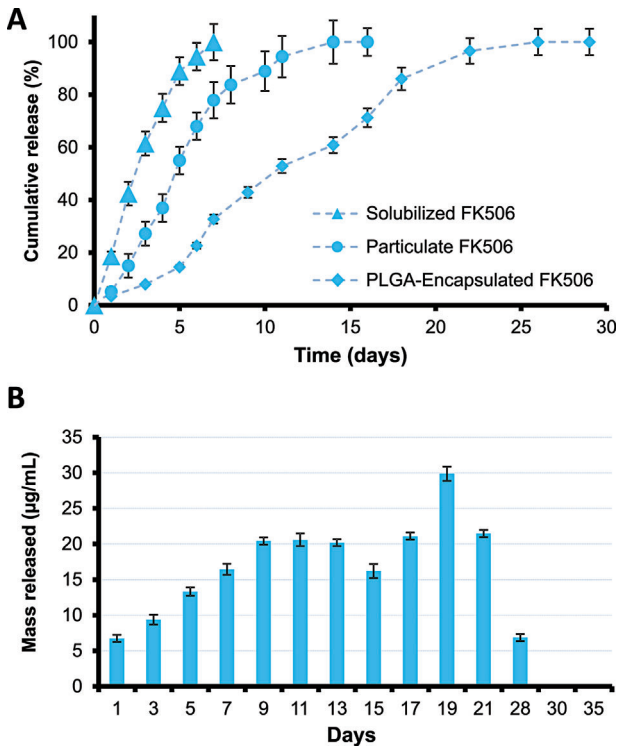

Figure 2. In vitro FK506 release profile from fibrin gel. (**A**) Cumulative mass release of FK506 in solubilized (▲), particulate (●), and PLGA encapsulated (◆) forms from fibrin gel shows incorporation of solubilized FK506 into the gel provides release for up to 5 days. The FK506 encapsulation in PLGA microspheres extends the sustained release to 28 days. (**B**) The daily mass release profile of FK506 from PLGA microspheres indicates a sustained drug release. FK506 content in the release samples was determined using LC-MS/MS. (Mean ± standard deviation, $n = 3$ per release study).

days 10 and 15. After degradation of the outer layers, FK506 began to be released from the core of the PLGA MSs resulting in an increase in the release rate until all FK506 had been released from the degrading PLGA MSs.

In Vitro Biological Activity of the Released FK506 From Fibrin Gel

Previous in vitro studies indicate that FK506 enhances neurite elongation of chick and rodent DRGs (Chang et al., 1995; Rosenstiel et al., 2003). The mechanism of the neurotrophic properties of FK506 is not fully understood but is thought to be a non-calcineurin dependent one. FK506 results in the dissociation of p23 from Hsp90 within a steroid receptor complex, which activates neurite outgrowth (Pratt, 1998). Beside its neurotrophic properties, FK506 can locally stimulate Schwann cell proliferation (Yin et al., 2015). FK506 can also promote peripheral nerve regeneration through reducing scar formation (Que et al., 2013); however, little is known about the mechanism of this action. Recently, Que et al. (2015) have shown FK506 has such an effect in sciatic nerve injured rat by inducing fibroblast apoptosis.

The bioactivity of the FK506 released from the PLGA MSs within fibrin gel was evaluated using neurite outgrowth from rat embryonic (E15) DRGs. The release media from the delivery system collected at days 7, 14, and 28 of the release duration was diluted to a 100 ng/mL concentration in the culture media of the rat E15 DRGs. After 48 h of culture, images of the DRGs under a red filter were obtained and the projected neurites were captured (Fig. 3). The length of the neurites was measured (Herbert et al., 1996) and normalized to the neurite extension of DRGs being cultured with 100 ng/mL of stock FK506 (Fig. 4).

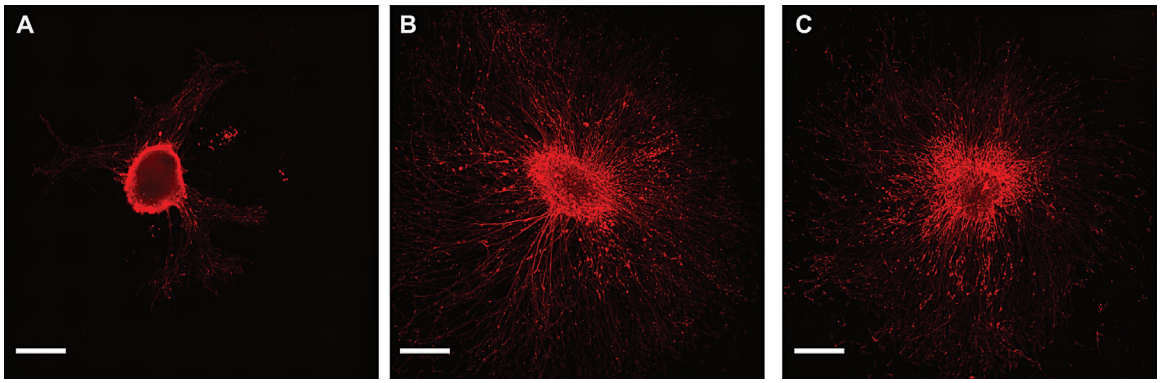

Figure 3. FK506 increased neurite extension. Neurite outgrowth of DRGs cultured with (**A**) media without any FK506, (**B**) media containing FK506 release sample from the delivery system, and (**C**) media with 100 ng/mL stock FK506 sloution. DRGs treated with FK506 from the drug delivery system and stock solution had significantly more neurite extension. The bars on each image indicate 330 μm.

Consistent with the previous studies, DRGs treated with FK506 demonstrated increased neurite extension compared with the cells cultured without FK506 (Fig. 3A–C). The DRGs cultured with the FK506 released samples at days 7, 14, and 28 despite the reduced mean release after 28 days, had statistically similar neurite extension lengths which were similar to that of the DRGs cultured with 100 ng/mL of stock FK506 (Fig. 4). Thus, FK506 remained bioactive during the entire period of release from the drug delivery system suggesting the encapsulation process did not alter the biological properties of the drug.

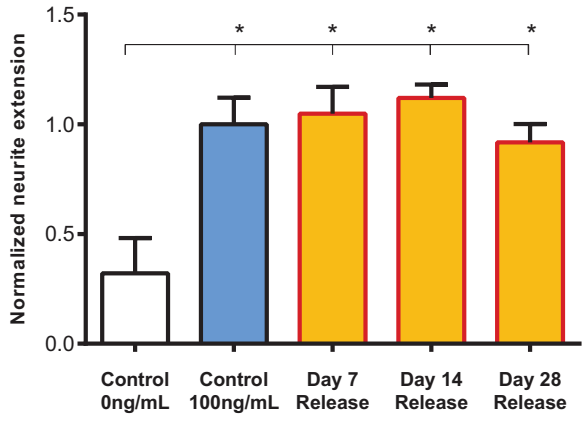

Figure 4. Encapsulation of FK506 in PLGA microspheres maintained the drug biological activity during the entire period of release. Released FK506 bioactivity was measured by the neurite extension assay. The released FK506 from the drug delivery system exerted substantial bioactivity during the entire period of release. FK506 release samples diluted to a concentration of 100 ng/mL had the equivalent bioactivity to a positive control group with 100 ng/mL of stock FK506. The neurite extension was significantly higher for the released samples and the positive control group as compared to the control group without FK506. The length of the neurites was normalized to the neurite extension of DRGs being cultured with 100 ng/mL of stock FK506. (Mean ± standard deviation; $n = 3$ per group; 3 wells per trial). *$P < 0.05$.

Conclusions

In this study, we engineered a novel drug delivery system for sustained and localized FK506 release to an injured peripheral nerve using fibrin gel. By changing the incorporation method of the drug into fibrin gel, a tunable release was achieved with the solubilized FK506 being released within 5 days. FK506 encapsulation in PLGA microspheres extended the sustained release to 28 days. Using the rat dorsal root ganglion neurite extension assay, FK506 was shown to maintain its bioactivity over 28 days of being released from the PLGA microspheres.

The novel system of FK506 local delivery may be utilized for enhancement of peripheral nerve regeneration. Such a system could be used at the time of nerve reconstruction. The drug loaded fibrin hydrogel placed around a nerve at the injury site, would allow a localized, sustained release of FK506. Because the treatment is localized to the injured nerve, the ability of the FK506 to enhance nerve regeneration would be achieved without the unwanted side effects of systemic immunosuppression and not requiring the high dosing of the drug when delivered systemically. Hence, the developed FK506 drug delivery system may be suitable as a clinically applicable treatment for peripheral nerve injury.

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