

Full length article

## Local delivery of FK506 to injured peripheral nerve enhances axon regeneration after surgical nerve repair in rats



Kasra Tajdaran<sup>a,b,\*</sup>, Katelyn Chan<sup>a,b</sup>, Molly S. Shoichet<sup>b,c</sup>, Tessa Gordon<sup>a,d,e</sup>, Gregory H. Borschel<sup>a,b,d,e</sup>

<sup>a</sup> Division of Plastic and Reconstructive Surgery, The Hospital for Sick Children, 555 University Ave, Toronto, Ontario M5G1X8, Canada

<sup>b</sup> Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

<sup>c</sup> Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada

<sup>d</sup> Division of Plastic and Reconstructive Surgery, Department of Surgery, University of Toronto, Toronto, Ontario, Canada

<sup>e</sup> Program in Neuroscience, The Hospital for Sick Children Research Institute, Toronto, Ontario, Canada

### ARTICLE INFO

#### Article history:

Received 2 January 2019

Received in revised form 23 May 2019

Accepted 23 May 2019

Available online 26 May 2019

#### Keywords:

Nerve injury

Nerve regeneration

FK506

Local drug delivery

Fibrinogen

Controlled release

Biomaterial

Rat model

Regenerative medicine

### ABSTRACT

Administration of FK506, an FDA approved immunosuppressant, has been shown to enhance nerve regeneration following peripheral nerve injuries. However, the severe side effects of the systemically delivered FK506 has prevented clinicians from the routine use of the drug. In this study, we analyzed the effectiveness of our fibrin gel-based FK506 delivery system to promote axon regeneration in a rat peripheral nerve transection and immediate surgical repair model. In addition, biodistribution of FK506 from the local delivery system to the surrounding tissues was analyzed *in vivo*. Rats in the negative control groups either did not receive any delivery system treatment or received fibrin gel with empty microspheres. The experimental groups included rats treated with fibrin gel loaded with solubilized, particulate, and poly(lactic-co-glycolic) acid microspheres-encapsulated FK506. Rats in experimental groups receiving FK506 microspheres and the particulate FK506 regenerated the highest number of motor and sensory neurons. Histo-morphometric analysis also demonstrated greater numbers of myelinated axons following particulate FK506 and FK506 microspheres treatment compared to the negative control groups. In biodistribution studies, FK506 was found at the nerve repair site, the sciatic nerve, and spinal cord, with little to no drug detection in other vital organs. Hence, the local application of FK506 via our delivery systems enhanced axon regeneration whilst avoiding the toxicity of systemic FK506. This local delivery strategy represents a new opportunity for clinicians to use for cases of peripheral nerve injuries.

### Statement of Significance

This work for the first time investigated the influence of locally administered FK506 to the site of nerve injury and immediate repair directly on the number of motor and sensory neurons that regenerated their axons. Furthermore, using the immediate nerve repair model, we obtained valuable information about the biodistribution of FK506 within the nervous system following its release from the delivery system implanted at the site of nerve injury and repair. The strategy of local FK506 delivery holds a great promise in the clinical translation, as the localized delivery circumvents the main limitation of the systemic delivery of FK506, that of immunosuppression and toxicity.

© 2019 Published by Elsevier Ltd on behalf of Acta Materialia Inc.

## 1. Introduction

Peripheral nerve injuries, with more than 800,000 cases per year in the US only [1], result in substantial social and personal costs. Patients with nerve injuries suffer from paralysis, loss of sensation, and frequently suffer from neuropathic pain. Addressing the

deleterious effects of peripheral nerve injury is still a considerable challenge, particularly when there is a delay in nerve repair or when axons are required to reestablish connections with peripheral targets over large nerve defects or long distances [2,3]. Currently, only 10% of patients with peripheral nerve injury recover full function, despite the improvements in surgical interventions over the past decades [4,5]. Therefore, it is essential to develop clinically applicable techniques for treating nerve injuries and restore sensory and functional outcomes after nerve injuries.

\* Corresponding author.

E-mail address: [kasratajdaran@gmail.com](mailto:kasratajdaran@gmail.com) (K. Tajdaran).

Early work of Gold and colleagues (1994) showed FK506 (Tacrolimus), an immunosuppressant drug used extensively for organ transplantation, has neuroregenerative effects via a calcineurin-independent pathway distinct from the FK506 immunosuppression pathway. The neuroregenerative effect of FK506 is thought to be mediated by its interaction with the FK506 binding protein-52 (FKBP52) that initiates downstream intracellular signaling cascades [6], the details of which presently remain unknown. *In vitro*, FK506 significantly increases neurite outgrowth from SH-SY5Y neuroblastoma cells [6], chick sensory ganglia [7,8], and rat dorsal root ganglia [9]. Importantly, sustained systemic administration of FK506 improves the rate of nerve regeneration and functional recovery after peripheral nerve transection [10–12], crush [12], chronic axotomy [13], ischemic denervation [14], and in combination with nerve graft therapy or tube repair [8,15,16]. Clinically, FK506 has been recently implemented in a growing number of cases of reconstructive nerve allotransplantation, as an effective agent capable of preventing organ rejection, while also improving axonal regeneration [17,18].

While there was an interest in utilizing FK506 for enhancing nerve regeneration over the previous decades [19–22], the significant toxicity of systemic FK506 deterred further investigations. The drug is toxic to the kidney and there is an elevated risk of development of both skin cancers and opportunistic infections due to the systemic delivery of FK506 [23–25]. Systemic FK506 increases risks of stroke or heart attack due to chronic hypertension. The systemic immunosuppression can also lead to nephrotoxicity, and metabolic derangement such as diabetes mellitus [24,26]. These effects potentially result in undesirable, debilitating consequences if the FK506 is delivered systemically in patients following nerve repair. Thus, a sustained localized delivery system which avoids the above-noted deleterious side effects is essential for the broad clinical use of FK506 to enhance nerve regeneration after nerve injuries and surgical repair [27].

In order to address the limitations of the current local delivery systems, such as catheters and osmotic pumps, which can cause nerve tissue damage due to compression or infection [28], we have engineered a biocompatible and biodegradable FK506 delivery system that is composed of a fibrin gel as the drug reservoir [9]. This drug delivery system was designed to provide local and sustained FK506 release to an injured nerve *in vivo* for maximal effect simultaneous with minimal unwanted side effects. FK506 was incorporated in the fibrin gel in solubilized, particulate, and poly(lactico-glycolic) acid (PLGA) microsphere (MS) encapsulated forms, providing a wide range of drug release periods from 7 to 28 days. In this study, first, we asked whether the local delivery of FK506 using our engineered drug delivery system is effective in enhancing nerve regeneration in a rat model of immediate nerve transection and repair. Second, we asked where and what quantity of FK506 is transported following its release from the drug delivery system implanted at the site of nerve transection and surgical repair.

## 2. Materials and methods

All chemicals were obtained from Sigma-Aldrich (MO, USA) unless otherwise specified.

### 2.1. Microsphere preparation and characterization

The encapsulation process of FK506 within the poly(lactico-glycolic) acid (PLGA) microspheres (MS) was done using the single emulsion solvent evaporation technique [9,29]. Briefly, an organic phase consisting of 1 mL of dichloromethane (DCM)/acetone (75%/25%), 230 mg PLGA, 12.5 mg MgCO<sub>3</sub>, and 12 mg FK506 powder

(LC Laboratories, Woburn, MA) 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. Empty PLGA MSs were similarly prepared without the addition of FK506 in the organic phase. The mean ( $\pm$ SE) MS size was  $45 \pm 5 \mu\text{m}$ , as measured by laser diffraction (Malvern Mastersizer 2000, Malvern, UK). In order to determine the encapsulation efficiency, 5 mg of microspheres were broken apart by the organic solvent, acetonitrile, and the resulting solution was analyzed for FK506 content using liquid chromatography tandem mass spectrometry (LC-MS/MS) [30]. Drug loading was determined as the FK506 mass per milligram of particles, while encapsulation efficiency was reported as the ratio of drug loading of the particles and the theoretical maximum drug loading. An average encapsulation efficiency of  $84.7 \pm 1.2\%$ , and a loading of  $40.85 \pm 0.05 \mu\text{g}$  FK506 per milligram of microspheres was achieved.

### 2.2. Drug delivery composite and *in vitro* release

Fibrin gel (80  $\mu\text{L}$  total volume) was constructed by mixing equal parts fibrinogen (75–115 mg/mL, 40  $\mu\text{L}$ ) and thrombin (5 IU/mL, 40  $\mu\text{L}$ ) obtained from a Tisseel<sup>®</sup> glue kit (Baxter Healthcare, IL, USA), and then re-suspended according to the manufacturer's instructions. Fibrin gel was loaded with FK506 by incorporating 10  $\mu\text{L}$  solubilized FK506 powder (LC Laboratories, Woburn, MA) in acetonitrile (2 mg/mL), 200  $\mu\text{g}$  solid FK506 particulates, or 5 mg of FK506 MSs into the thrombin solution before it was mixed with fibrinogen to form a gel at the bottom of a 2 mL Eppendorf tube. The incorporation of particulate FK506 in fibrin gel was achieved by physically mixing FK506 powder (LC Laboratories, Woburn, MA) in fibrin gel. The concentration of FK506 was selected based on the local dose of Cyclosporin-A that was used previously in the published studies [30,31].

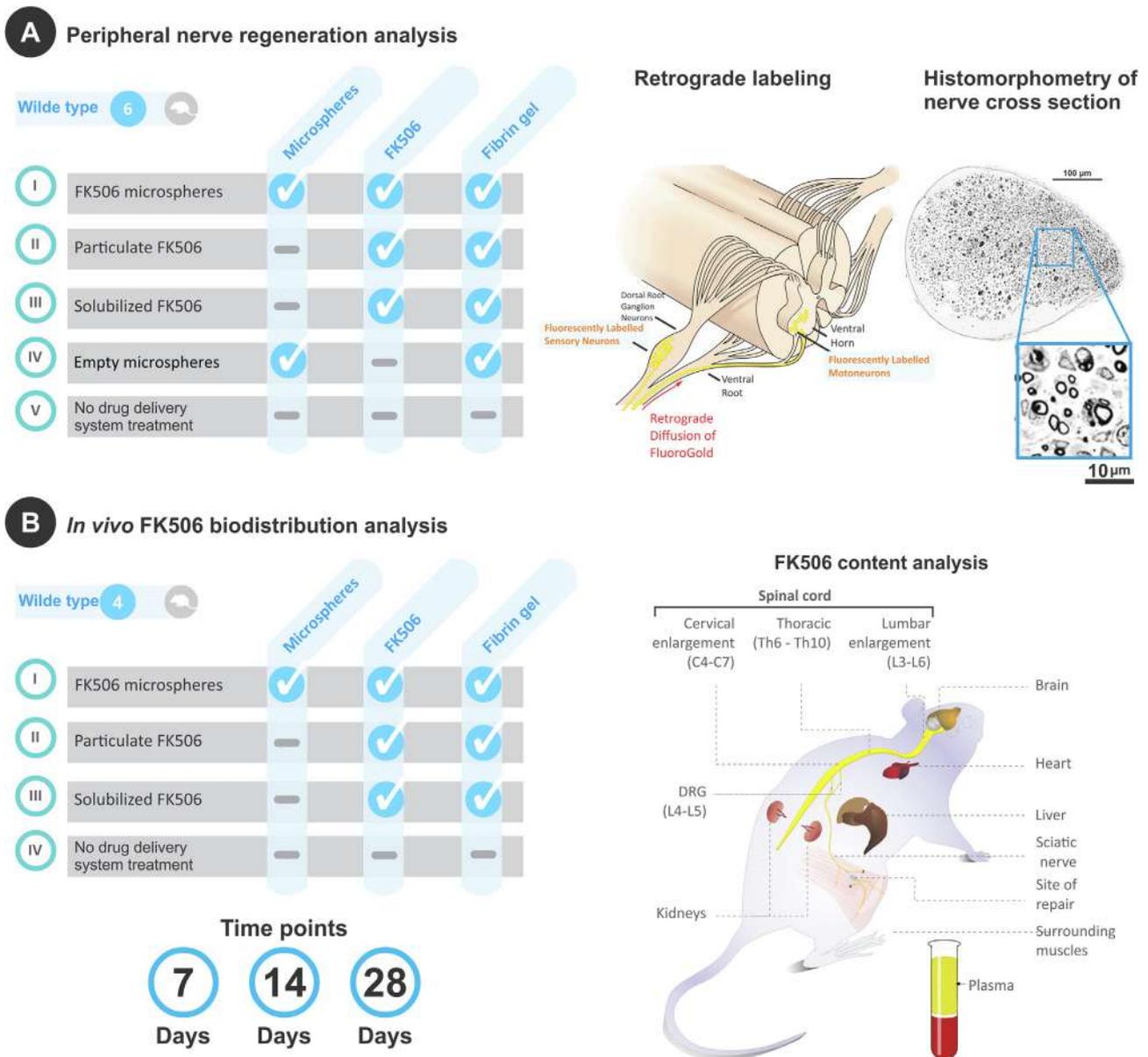
### 2.3. Experimental animals

In this study, 78 adult female Sprague–Dawley rats (Harlan, Indianapolis, USA), each weighing 250–300 g, were used. Currently, there have been no reports on whether there are sex differences in the effectiveness of FK506 in enhancing nerve regeneration. Female rats were used to ensure consistent dosing and avoid issues associated with weight change during the experimental period. Female rats gain less weight over time as compared to male rats. All surgical procedures and perioperative care measures were performed in strict accordance with the National Institutes of Health guidelines, the Canadian Council on Animal Care (CCAC) and were approved by the Hospital for Sick Children's Laboratory Animal Services Committee.

### 2.4. Experimental design

#### 2.4.1. Peripheral nerve regeneration analysis

We analyzed the efficacy of the FK506 delivery systems in enhancing peripheral nerve regeneration using a nerve transection and immediate surgical repair model in 30 randomized rats ( $n = 6$  per group, Fig. 1A). In this model, the tibial (TIB) nerve was transected and its proximal nerve stump was cross-sutured to the distal stump of a freshly cut common peroneal (CP) nerve (see Section 2.5). In addition to analyzing the neuroregenerative effect of local FK506 on immediate nerve repair, the TIB to CP nerve transfer model was used to compare the results of this study to



**Fig. 1.** Experimental design. A) The experimental groups in the peripheral nerve regeneration analysis consisted of rats receiving fibrin gels loaded with FK506 microsphere (MS) (I), particulate FK506 (II), solubilized FK506 (III). Control groups of rats received fibrin gels with empty MS (IV), and no drug delivery system treatment after nerve repair (V). Three weeks following nerve repair and drug delivery system implantation, nerve regeneration was analyzed using retrograde labeling and histological analysis of the distal CP nerve. B) The experimental groups in the *in vivo* FK506 biodistribution analysis received delivery systems loaded with FK506 MS (I), particulate FK506 (II), solubilized FK506 (III). The control group did not receive any drug delivery system treatment. Rats were sacrificed 7, 14, 28 days post repair. The FK506 content was analyzed at the site of nerve repair, in the gluteal muscles surrounding the nerve repair site, entire sciatic nerve, dorsal root ganglia (L4, L5), spinal cord (lumbar, thoracic and sacral sections), brain, heart, liver, kidneys, plasma.

an ongoing chronic axotomy study with local FK506 delivery system administration. In three experimental groups, fibrin gel loaded with FK506 MS, particulate FK506, or solubilized FK506 was placed at the suture site. Rats in the control groups either did not receive any delivery system or received fibrin gel containing empty MS (without any FK506). Nerve regeneration was evaluated using retrograde labeling of motor and sensory neurons that regenerated their axons (see Section 2.6) and histomorphometric analysis of regenerated nerve fibers (see Section 2.7) three weeks post nerve transfer at 7 mm distal from the nerve transfer site.

#### 2.4.2. *In vivo* FK506 biodistribution analysis

To characterize the local delivery of FK506 to peripheral nerve tissues and FK506 biodistribution *in vivo*, the TIB to CP nerve

immediate transfer model was used in 48 randomized rats (Fig. 1B). Following nerve transfer, three experimental groups received the three forms of the FK506 delivery system containing solubilized FK506, particulate FK506, or FK506 MS (see Section 2.5).

A total of 80 µL fibrin gels that contained applicable components for each group were placed at the site of the nerve transfer (see Sections 2.2 and 2.5). Rats in a negative control group did not receive any delivery system treatment. The treated animals in each group were sacrificed at 7, 14, and 28 days post nerve repair and drug delivery system implantation (n = 4 per time point for each group). Using liquid chromatography–tandem mass spectrometry (LC–MS/MS), FK506 tissue concentrations were analyzed at 1) the site of nerve repair, 2) gluteal muscles surrounding the

nerve repair site (lateral and contralateral sides), 3) entire sciatic nerve (lateral and contralateral sides), 4) L4, and L5 dorsal root ganglia (DRG) (lateral and contralateral sides), 5) spinal cord (lumbar, thoracic and sacral sections), 6) brain, 7) heart, 9) liver, 10) kidneys, and 11) plasma (see Sections 2.8 and 2.9).

### 2.5. Operative procedure

All surgical procedures were performed using aseptic techniques. Isoflurane (3%) gas anesthesia was used for animal induction followed by 2.5% isoflurane gas for maintenance. The hind leg of the rat was surgically cleaned with a betadine/alcohol rub. The sciatic nerve was exposed through a dorsolateral-gluteal muscle-splitting incision. Wounds were irrigated with saline, dried and closed in two layers, utilizing 4–0 Vicryl™ (Ethicon, OH, USA) sutures to close the muscle layers, and 5–0 Vicryl™ sutures to close the skin. Experimental animals were recovered in a warm environment prior to returning to the housing facility.

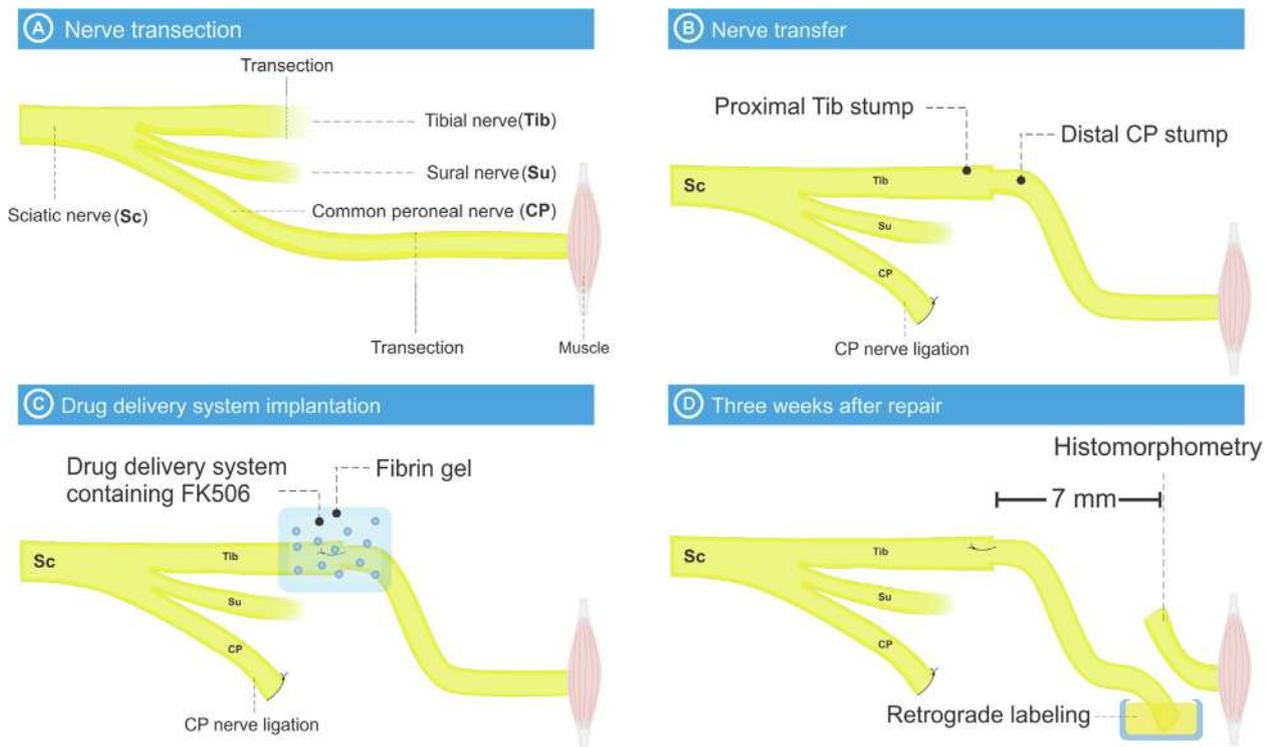
During the procedure, the rat sciatic nerve was exposed and the main branches including TIB and CP nerves were dissected (Fig. 2A). The TIB nerve was transected 5 mm distal to its trifurcation from the CP nerve and sural nerves with fine microsurgical scissors. The proximal TIB nerve stump was cross-sutured to the distal stump of a cut CP nerve using 9–0 Nylon sutures (Fig. 2B). Proximal CP nerve and distal TIB nerve were ligated to the surrounding muscles to prevent regeneration using 6–0 silk sutures. In the groups receiving the fibrin gel-based drug delivery system (Fig. 2C), the nerve suture site was surrounded by two 40  $\mu$ L fibrin gels (containing applicable components for each group) formed by pipetting the fluid fibrin mixture, before setting as a gel, onto Parafilm as semispherical drops ( $\sim 5\text{mm} \times 5\text{mm}$ ). The gel drops were then placed centered above and below the repair sites and secured

by gently opposing the gel drops on one another. The fibrin gel drops instantly adhered to each other around the nerve suture site and the fibrin gel mixture did not show any signs of migration upon implantation, as shown previously by our research group [32,33].

### 2.6. Retrograde labeling of motoneurons (in ventral horn) & sensory neurons (in dorsal root ganglia)

To assess nerve regeneration three weeks after nerve repair, the surgical site of the rats allocated for analysis of peripheral nerve regeneration was reopened under general anesthesia. The CP nerve was transected 7 mm distally from the repair site and the proximal nerve stump was immediately placed in a silicone well containing 4% Fluoro-Gold™ in sterile saline for 1 h (Fig. 2D). At the same time, the distal stump was harvested for histology (described below, Section 2.7). The silicone wells and Fluoro-Gold solutions were removed, incisions were closed, and rats were allowed to recover as described previously. The 7 mm distance distal from the repair site was chosen to enable us to analyze axon regeneration three weeks following anastomosis. Such distance takes into account axon regeneration through the sutured nerve transection site and within the denervated distal nerve stump.

Seven days following the procedure, the rats were euthanized and perfused with 0.9% NaCl saline and cold 4% paraformaldehyde in phosphate buffer saline (PBS). The lumbar region (L3–L6) of the spinal cord and L4–L5 of the dorsal root ganglia (DRG) were dissected free for frozen sectioning. Axial sections of the lumbar spinal cord (50  $\mu$ m) or the DRGs (20  $\mu$ m) were obtained on a cryostat (Leica, ON, Canada). The number of labeled neuronal cell bodies within the ventral horn of each spinal cord section or within every fifth DRG section was counted using a fluorescent micro-



**Fig. 2.** Surgical procedures performed on rats. Following exposure of the sciatic nerve, TIB and CP branches were transected 5 mm distal to the sciatic nerve trifurcation (A). A nerve transfer from the proximal TIB nerve to distal CP nerve was performed with an epineural suture (B). Proximal CP nerve stump and distal TIB nerve stump were ligated to the surrounding muscles to prevent regeneration. Following nerve transfer, the experimental groups received drug delivery systems containing solubilized FK506, particulate FK506, and FK506 microspheres at the nerve repair site (C). Three weeks following nerve repair, the distal CP nerve was harvested and labeled with retrograde dye 7 mm distally from the repair site (D). TIB: Tibial nerve, CP: common peroneal nerve.

scope with a 10× objective (100× overall magnification; Leica). Spinal cord counts were adjusted to account for split nuclei using the methods of Abercrombie [34].

### 2.7. Histology & morphometric evaluation of nerves

At the time of the retrograde labeling surgery, the nerve tissue taken 7 mm distal to the nerve repair site was collected (Fig. 2D), fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, ethanol dehydrated and embedded in Araldite® 502 (Polyscience, Inc., PA, USA). Thin (0.6 μm) sections were made from the tissue using an LKB II ultramicrotome (LKB-Producter AB, Sweden) and then stained with 1% toluidine blue for examination by light microscopy. The slides were evaluated for overall nerve architecture and quality of regenerated fibers. At 1000× overall magnification, the entire nerve cross-section was captured to count regenerated axons, measure myelin thickness and fiber diameter, and calculate G-ratio using a semi-automated MATLAB program [35].

### 2.8. Tissue harvest for FK506 content analysis

Rats allocated for the *in vivo* FK506 biodistribution analysis were sacrificed 7, 14 and 28 days after CP nerve repair and the drug delivery system implantation. At each time point, the entire sciatic nerve from the site of nerve injury to the DRG spinal roots was harvested from both the ipsilateral and the contralateral sides of the rats. At the site of nerve injury and repair, a 5 mm segment of the CP nerve together with the delivery system placed around the site of nerve repair was harvested for analysis of the FK506 content. Each 5 mm segment of the sciatic nerve was analyzed for the FK506 content using LC–MS/MS. The gluteal muscles surrounding the nerve repair site were harvested on both the lateral and the contralateral hind legs. Muscle volumes of 5 mm × 5 mm × 5 mm were collected and analyzed for FK506 content using LC–MS/MS. The lateral and the contralateral L4 and L5 DRGs were collected as well. In addition, brain and the entire spinal cord divided into the three sections of lumbar, thoracic, and sacral spinal cord were harvested. Sections of the liver, heart, kidneys with the volume of 5 mm × 5 mm × 5 mm, and 1 mL blood samples were collected. Plasma was extracted by collecting the supernatant following centrifuging the collected blood samples at 4 °C for 10 min at 20,000g. One hundred microliters of the collected plasma per rat were used to analyze the FK506 content.

### 2.9. Analysis of FK506 tissue content

The collected tissues were placed in 1.5 mL microcentrifuge tubes, which were weighed before and after sample collection to determine the mass of tissue contained therein. Prior to tissue homogenization, 300 μL lysis buffer (50 mM Tris (Fisher), 150 mM NaCl, 2 mM EDTA (Ambion), 0.1% Sodium Dodecyl Sulfate (Ambion), 1% NP40, 0.5% DOC, 1% Protease inhibitor, 0.05% Cyclosporine A (as the internal standard) was added to each tube containing the tissues. In each tube, the tissues were homogenized by sonication on ice for 30 s (45% amplitude), cooled on ice for 15 min, and homogenized again for 30 s (30% amplitude). FK506 was extracted by adding 900 μL of acetonitrile (ACN, HPLC grade, Caledon Labs) to 100 μL of the processed tissues, homogenizing for 1 min, centrifuging at 4 °C for 5 min at 16,000g, and removing the supernatant for analysis. FK506 was detected by LC–MS/MS, as described previously [9].

### 2.10. Body mass monitoring

All rats were weighed weekly by means of a digital scale (Sartorius 1413 MP 8/8-1, USA). The normalized body mass for each rat

was calculated by dividing the weekly body mass by the mass of the animal prior to surgery.

### 2.11. Statistical analysis

The *in vivo* retrograde labeling data in Section 3.1 and the quantitative histology data in Section 3.2 were reported as mean ± standard error. The *in vivo* dynamic drug release data in Section 3.3, the FK506 tissue concentration in Section 3.4, and the normalized rat body mass in Section 3.5 were reported as mean ± standard deviation. Statistical testing was performed in GraphPad Prism 6 to confirm the normality of the data, and then differences between groups were assessed by analysis of variance (ANOVA) with Bonferroni's post-hoc at 95% confidence intervals.

## 3. Results

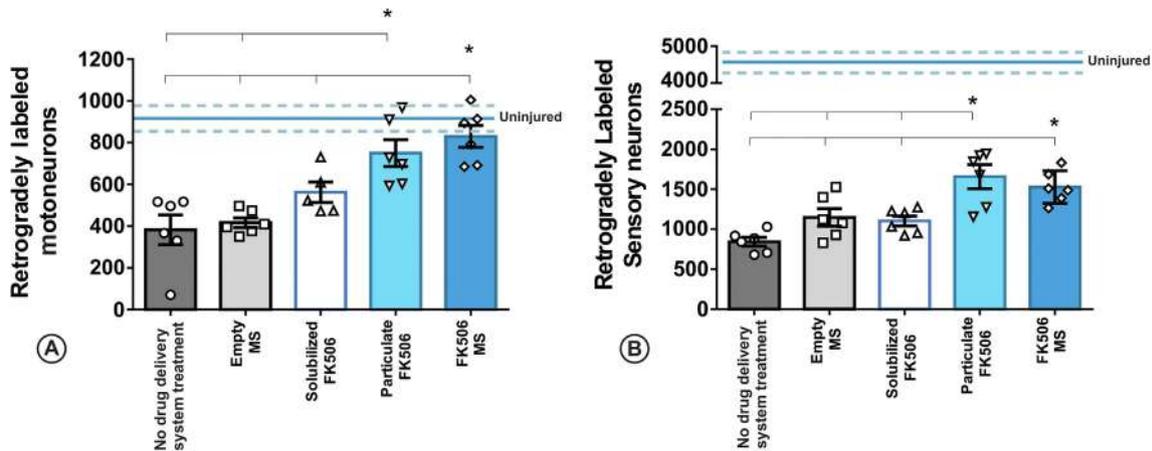
### 3.1. *In vivo* retrograde labeling of neurons following nerve repair

In order to quantify the number of the neurons regenerating their axons through the nerve repair site and to compare the extent of motor versus sensory axon regeneration, retrograde labeling was performed 3 weeks after nerve repair. Fluoro-Gold was applied to the regenerated tibial (TIB) axons within the CP distal nerve stump 7 mm distal to the site of the cross-suture of TIB and common peroneal (CP) nerves (Fig. 2D). Similar to our previous investigations [32,36], the number of motoneurons that regenerated their axons across the repair site was the same when the empty microsphere (MS) was placed at the repair site and when there was no drug delivery system treatment ( $p = 0.6544$ , Fig. 3A), indicating the fibrin gel-based delivery system with MS did not negatively affect nerve regeneration. Treating the nerve repair site with the solubilized FK506 containing delivery system did not increase the number of motoneurons regenerating their axons compared to the control groups with empty drug delivery system treatment or no drug delivery system treatment ( $p = 0.5936$ , Fig. 3A). When FK506 MS or the particulate FK506 was loaded in the delivery system, double the number of motoneurons regenerated their axons, resulting in regeneration of all TIB motoneurons (represented by the dashed line in Fig. 3A). The numbers of motoneurons that regenerated their axons in both the FK506 MS treated rats and the particulate FK506 treated rats were significantly higher than that of the control groups, including the no drug delivery system treated and the empty MS treated groups ( $p < 0.0001$ , Fig. 3A), where approximately 50% of the TIB motoneurons regenerated their axons.

Sensory neuron regeneration pattern mirrored that of the motoneurons. The control groups with no drug delivery system treatment and empty MS loaded delivery system had a similar number of sensory neurons that regenerated their axons 3 weeks post repair ( $p = 0.6619$ , Fig. 3B). Treating the site of nerve transection and surgical repair with the drug delivery system containing solubilized FK506 did not improve sensory neuron regeneration compared to the control groups ( $p = 0.5644$ , Fig. 3B). Sensory neuron regeneration was likewise significantly higher than the controls only in the FK506 MS treated rats and the particulate FK506 treated rats ( $p < 0.0001$ , Fig. 3B).

### 3.2. *In vivo* nerve histology and morphometric measures of regeneration

Three weeks following nerve repair, nerve samples were harvested 7 mm distal from the repair site for histological analysis by light microscopy. Qualitative analysis of nerve samples revealed a low number of myelinated axons and similar nerve morphology



**Fig. 3.** Local delivery of FK506 *in vivo* doubled tibial (TIB) neuron numbers regenerating their axons across a suture site into the distal common peroneal (CP) nerve stump. Retrograde labeling of neurons regenerating their axons was performed 7 mm distal from the repair site 3 weeks following TIB-CP nerve transfer and drug delivery system implantation. Motor ventral horn neurons in the spinal cord (A) and DRG sensory neurons (B) were counted to assess motor and sensory neuron regeneration, respectively. FK506, delivered in either microsphere or particulate form, greatly enhanced neuron regeneration compared with the negative control groups. Data ( $n = 6$ ) represent the mean  $\pm$  standard error of the mean.  $p < 0.05$ . The dashed line indicates the number of neurons in the uninjured TIB.

for the groups receiving no drug delivery system treatment or solubilized FK506 treatment (Fig. 4A–B). Drug delivery system loaded with FK506 MS or the particulate form of FK506 (Fig. 4C–D) promoted regeneration of a higher number of myelinated axons within the nerve cross-sections compared to the negative control groups (Fig. 4A–B) and uninjured nerve (Fig. 4E).

Quantitative histomorphometric analysis of the entire nerve cross-sections confirmed the significant increase in the number of myelinated axons following particulate FK506 and FK506 MS treatments as compared to the solubilized FK506 treatment and the control groups ( $p < 0.0001$ , Fig. 4F). The number of the myelinated axons following FK506 MS treatment was statistically higher compared to that of the particulate FK506 treatment ( $p < 0.0270$ , Fig. 4F). As a measure of nerve maturity, nerve fiber diameter, and myelin thickness was determined. There were no significant differences in myelin thickness and fiber diameter among all the groups. The G-ratio, calculated as the ratio of the axon diameter to the total fiber diameter, was statistically equivalent for all groups (data not shown), and all were below the values of normal uninjured nerves (Fig. 4E).

### 3.3. *In vivo* detection of FK506 at the site of nerve repair

The mass of detected drug within each delivery system containing solubilized FK506, particulate FK506, or FK506 MS was the same following their implantation at the site of TIB-CP nerve cross-suture in rats (Fig. 5). Seven days after implanting the solubilized FK506 containing delivery system, the detected mass of FK506 at the site of nerve repair fell to  $8.2 \pm 1.4 \mu\text{g}$  and was undetectable by 14 days (Fig. 5). Incorporation of particulate FK506 within the drug delivery system extended FK506 release to 14 days when  $2.65 \pm 1.2 \mu\text{g}$  of the drug was still detectable, but no drug release was detected by 28 days at the site of nerve repair (Fig. 5). The release of FK506 was clearly much longer from the drug delivery system loaded with FK506 MS. After 28 days,  $15.1 \pm 1.5 \mu\text{g}$  was still detectable at the site of nerve repair (Fig. 5). At all the time points after the implantation of the drug delivery systems, the highest amount of FK506 at the site of nerve injury was detected in rats treated with FK506 MS.

### 3.4. *In vivo* FK506 bio-distribution within rat organs

In this study, we analyzed the bio-distribution of FK506 following its release from the drug delivery system implanted at the site

of nerve transection and surgical repair. Fig. 6 shows the detected concentration and mass of FK506 within the rat organs over 28 days after the drug delivery system implantation. During the first week (Fig. 6A&B), FK506 was detected in the proximal injured sciatic nerve, the gluteal muscles surrounding the nerve, and at the highest level, in the entire spinal cord. The concentration of FK506 was similar in the lumbar, thoracic, and cervical segments of the spinal cord. The particulate FK506 treated group had a significantly higher amount of FK506 within the entire spinal cord by 7 days ( $p < 0.05$ , Fig. 6A&B). Similar and lower amounts of FK506 was detected within the spinal cord after the first week. Kidney tissues also had low FK506 levels only during the first week in the solubilized FK506 treated rats. The detected FK506 levels during the first week declined significantly to very low but detectable levels at 14 days (Fig. 6C&D) and undetectable levels by 28 days (Fig. 6E&F).

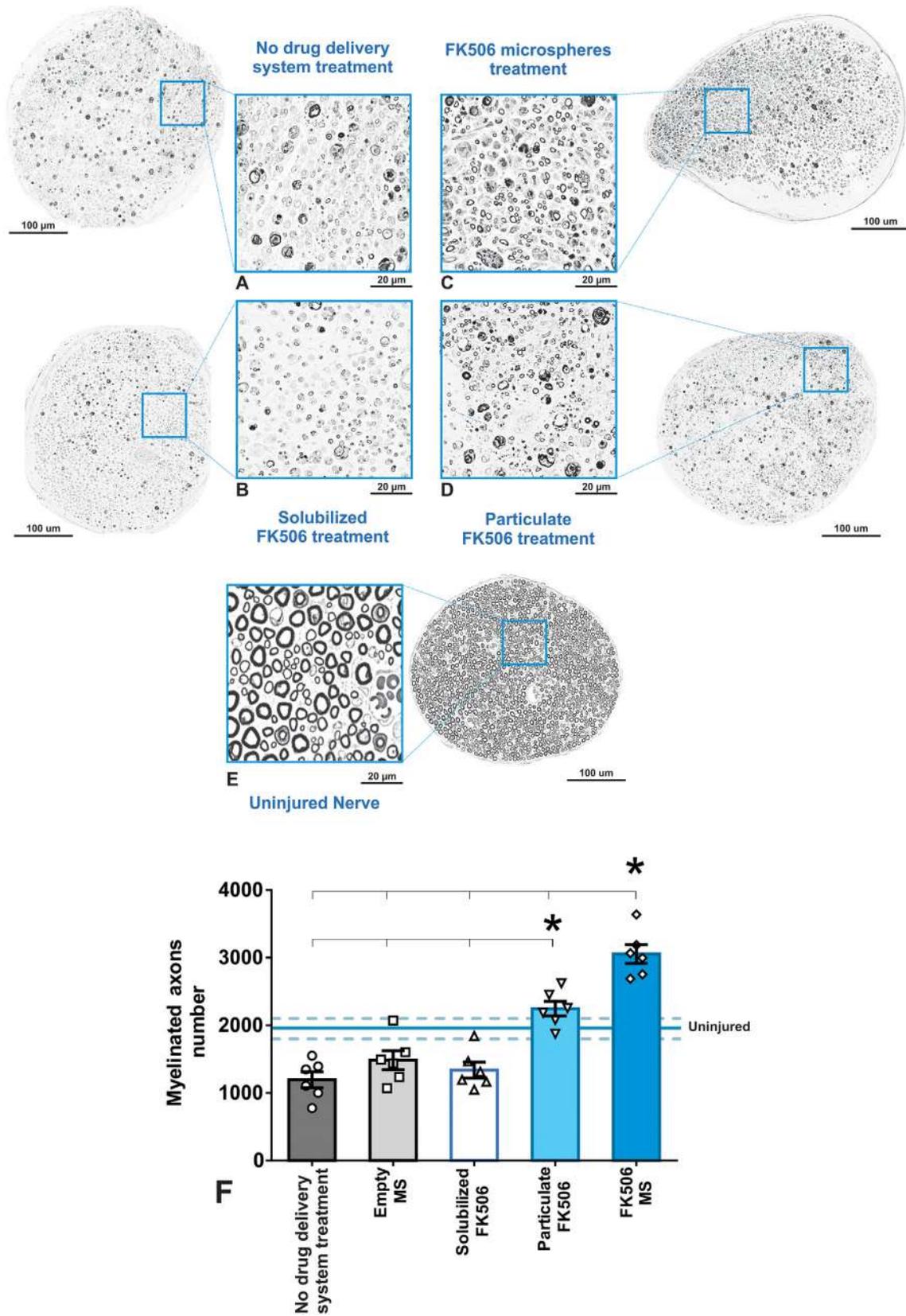
Within the DRGs (L4, L5) of the particulate FK506 and FK506 MS treated groups, there was an accumulation of FK506 at 14 days, which was undetectable at 28 days. Detectable FK506 levels remained in the ipsilateral gluteal muscles by 28 days (Fig. 6E&F). The contralateral sciatic nerve, contralateral gluteal muscles, and contralateral L4–L5 DRGs, plasma, brain, liver, or heart did not contain any detectable levels of FK506 during the entire period of the treatment. Overall, the *in vivo* FK506 biodistribution analysis, in this study, shows the locally released FK506 at the nerve repair site is mainly transported and distributed within the nervous tissues, specifically sciatic nerve and spinal cord.

### 3.5. Body mass analysis following local FK506 administration

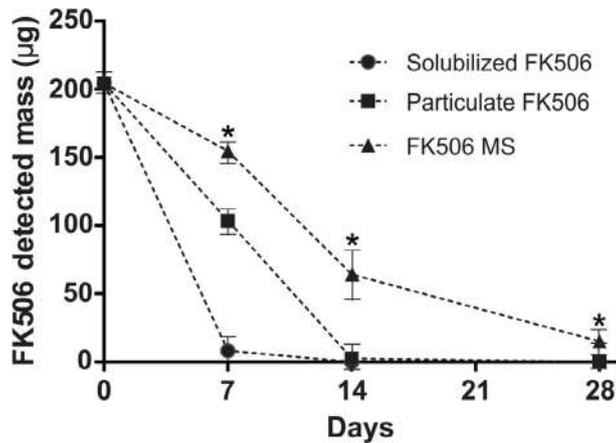
Most importantly, FK506 local delivery in rats did not cause discernable side effects typically associated with the FK506 systemic administration such as body mass loss, malaise, lethargy, coarsening of the coat, and hair loss [23–25]. As shown in Fig. 7, following local FK506 administration at the nerve repair site, body weight of the rats in any group did not change throughout the 28 days of treatment. In addition, the absence of any detectable FK506 within the vital organs including brain, liver, heart, and plasma during the entire study period, supports our observation in the prevention of systemic adverse effects following the local FK506 administration.

## 4. Discussion

We sought to determine whether the engineered fibrin gel-based delivery system for FK506 could improve nerve regeneration



**Fig. 4.** Treatment of nerve injury site with FK506 delivery system containing particulate FK506 or FK506 microsphere (MS) increased myelinated axon regeneration. Light micrographs of nerve cross sections were analyzed in (A) no drug delivery system treated group, (B) solubilized FK506 treated group, (C) FK506 MS treated group, (D) Particulate FK506 treated group, and (E) uninjured common peroneal nerve. (F) Histo-morphometric analysis of the nerve cross-sections demonstrated a significantly higher number of myelinated axons in particulate FK506 and FK506 MS treated groups as compared with all other groups. Data represent the mean  $\pm$  standard error of the mean for 6 animals per group.  $p < 0.05$ . Sections A–D are representative light micrographs of nerve cross sections for each group.



**Fig. 5.** In vivo FK506 release at the site of nerve repair. FK506 daily mass release at the site of nerve repair from the drug delivery systems containing solubilized FK506 (●), particulate FK506 (■), and FK506 microspheres (MS) (▲) showed that incorporation of FK506 in the solubilized form provided the fastest release period up to a week. The FK506 encapsulation in MS resulted in drug release extension to 28 days. After the implantation of drug delivery system, the FK506 MS delivery system maintained significantly higher amounts of FK506 for up to 28 days as compared to the solubilized FK506 and particulate FK506 delivery systems ( $p < 0.05$ ). FK506 content within the tissues at the site of nerve repair was determined using LC-MS/MS. (Mean  $\pm$  standard deviation,  $n = 4$ ).

following immediate nerve transection and surgical repair. In addition, we analyzed the biodistribution of FK506 following its release from the implanted drug delivery system at the site of nerve repair. Our findings, in this study, demonstrated the effective local release of FK506 from the drug delivery system containing FK506 microspheres (MS) or particulate FK506. The drug delivery system resulted in *all* the motoneurons regenerating their axons across a transection site, 3 weeks after immediate cross-suture of the tibial (TIB) and the common peroneal (CP) nerves. The numbers of motor and sensory TIB neurons that regenerated their axons into the CP nerve stump 3 weeks after nerve repair was doubled in the rats receiving particulate FK506 or FK506 MS treatments (Fig. 3). Histo-morphometric analysis of the regenerated TIB axons distal to the cross-suture site revealed parallel elevated numbers of myelinated axons 3 weeks after repair and local FK506 administration from the delivery systems containing particulate FK506 and FK506 MSs as compared to the control group in which FK506 was not delivered to the cross-suture site and the group received the solubilized FK506 treatment (Fig. 4).

Comparing the numbers of the regenerated myelinated axons (Fig. 4) with the numbers of neurons that regenerated their axons (Fig. 3) indicates more myelinated axons ( $\sim 3000$  axons) than the neurons regenerated their axons ( $\sim 2300$  motor and sensory neurons) following FK506 MS and particulate FK506 administration. This increased number of regenerated nerve fibers in the distal nerve stump reflects the well-known emission of more than one regenerating daughter axon from each parent nerve in the proximal nerve stump [3]. The enhanced nerve outgrowth and population of the distal nerve stump within a short period of time, reflects the ability of FK506 to accelerate axon outgrowth as well as to increase the rate of nerve regeneration within the distal nerve stump [11].

Biodistribution analysis of FK506 *in vivo* after implanting the local drug delivery system at the site of TIB-CP cross-suture demonstrated that our local delivery system, specifically the particulate FK506 and FK506 MS forms, provide a sustained FK506 release to the site of nerve injury (Fig. 5). The *in vivo* drug release profile confirmed the tunable release of FK506 from the fibrin gel as observed *in vitro* [9] from  $\sim 7$  to 28 days. The rate of *in vivo* drug

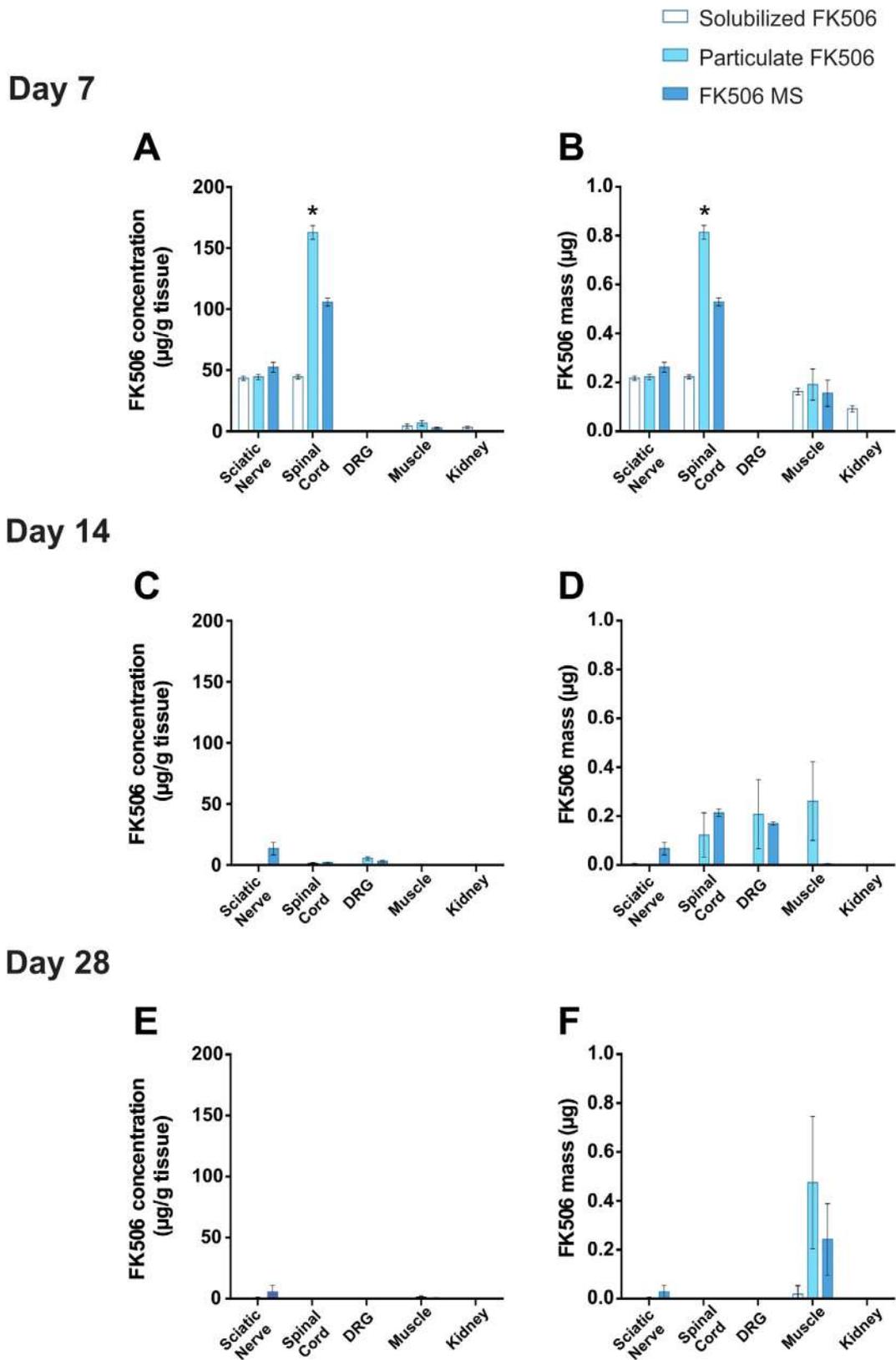
release was similar to the rate of *in vitro* [9] release with the release in  $\sim 7$  days from the solubilized form being the most rapid followed by the release from the particulate form in  $\sim 14$  days. The most prolonged period of release of  $\sim 28$  days was found with the FK506 MS form in both *in vivo* and *in vitro* [9] studies.

These findings provide a proof of concept that the engineered delivery system functioned as designed [9]. The *in vivo* drug release rate at the site of nerve injury was very similar, however, slightly slower than that observed *in vitro* [9]. The slightly faster release rate *in vitro* can be due to the idealized case where the sink conditions are maintained, maximizing the driving force for diffusion. Whereas, *in vivo*, the ideal sink conditions are not always provided due to various factors including the potentially slower rate of FK506 transport within the peripheral nerve, and hindered diffusion due to scar tissue formation at the site of nerve injury and surgical repair.

Based on the *in vivo* (Fig. 5) and *in vitro* [9] release profiles, the particulate and the FK506 MS forms of the delivery system had a sustained drug release without a burst release. The solubilized form of the delivery system had a burst release of the drug. The burst release of FK506 was not detrimental to nerve regeneration: the number of motor and sensory neurons that regenerated their axons after the nerve injury and repair and the number of myelinated axons in the solubilized FK506 treated group were the same as those of the no drug delivery system and the empty drug delivery system treated groups. In addition, the rats that were treated with the solubilized FK506 treated group maintained their body mass during the entire period of study as did the rats in the remaining groups. Hence, by comparing the outcomes of the three forms of the delivery system, we can conclude that the duration of FK506 sustained release locally to the site of nerve injury and repair impacts nerve regeneration, such that the optimal nerve regeneration following immediate nerve repair can be achieved with release periods longer than 14 days. Such prolonged release period is achieved with the particulate FK506 and FK506 microspheres containing delivery systems. However shorter period of drug release of about 1 week, which is achieved using the solubilized FK506 delivery system, does not improve nerve regeneration in this model.

FK506 released from the delivery system was transported retrogradely to the spinal cord where it accumulated during the first 14 days following the drug delivery system implantation. On the other hand, FK506 accumulation within the DRGs (L4, L5) did not occur during the first 7 days (Fig. 6C-D). Low concentrations of the drug were detected within the DRGs 14 days post delivery system implantation in the groups receiving the particulate FK506 or FK506 MS treatments. This differential course of FK506 detection in motor and sensory neurons indicates that the transport systems differ in the motor and sensory axons. The faster and higher accumulation of FK506 within the spinal cord, as compared to the DRGs, can be a confounding factor to promoting all motoneurons to regenerate their axons (Fig. 3A) compared to only  $\sim 50\%$  of the sensory neurons that regenerated their axons (Fig. 3B). The detected concentration of FK506 was the same within the entire spinal cord including the lumbar, thoracic, and cervical segments. The transport of FK506 into the spinal cord and DRGs is not considered an off-target effect because these tissues contain the neural cell bodies that regulate the regeneration of the axons at the site of nerve injury. No FK506 detection was observed within the brain. Such finding suggests a potential mechanism of FK506 transport within the spinal cord neurons, which requires further investigation.

Whilst FK506 is considered an immunosuppressant, the total of amount of FK506 used in this model was not enough to induce discernible immunosuppression, which could mitigate the required immune response for nerve regeneration [37]. In all drug delivery



**Fig. 6.** Concentration and mass of FK506 in rat organs (A–B) 7 days (C–D) 14 days, and (E–F) 28 days post drug delivery system implantation. FK506 was detectable within the proximal injured sciatic nerve, DRGs (L4, L5), the entire spinal cord, and the muscles surrounding the nerve repair site, decreasing in concentration over time. The particulate FK506 treated group had a significantly higher amount of FK506 within the entire spinal cord at 7 days ( $p < 0.05$ ). FK506 content within the tissues was determined using LC–MS/MS. (Mean  $\pm$  standard deviation,  $n = 4$ ).

system treated groups, including the solubilized FK506 treatment, no diminished nerve regeneration was observed. Following the local FK506 delivery system implantation, there was no long-

term vital organ exposure to FK506, including within the brain, liver, heart, kidneys, and plasma. Thus, our findings strongly suggest the engineered delivery system can deliver FK506 to the ner-

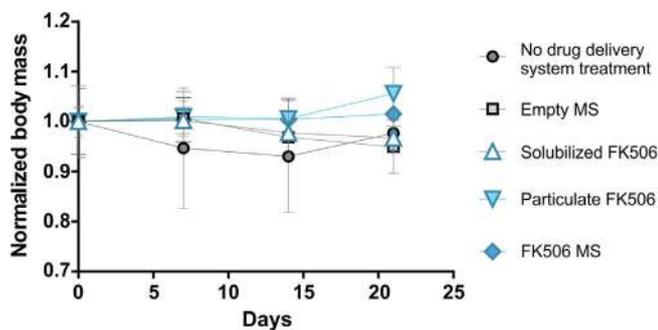


Fig. 7. FK506 local delivery did not affect the animal mass over the entire period of treatment. The presented body mass is normalized by the mass of the animals in each group on the day of surgery. (Mean  $\pm$  standard deviation,  $n = 4$ ).

vous tissues including sciatic nerve, and spinal cord; with minimal drug penetration into the surrounding muscles. No visual signs of toxicity were observed within the gluteal muscles surrounding the delivery system. Future histological studies are required to investigate any potential local muscle toxicity due to the delivery system implantation. It is important to note that the lack of discernable side effects and the maintenance of the body mass in rats receiving the local administration of FK506 was very encouraging (Fig. 7), which paralleled our finding in lack of systemic FK506 detection within the plasma and the vital organs. However, one of the limitations of this study is the lack of detailed toxicological evaluation to ensure no systemic toxicity could occur following the local administration of FK506 using our delivery system.

The strategy of local FK506 delivery holds a great promise in the clinical translation, as the localized delivery circumvents the main limitation of the systemic delivery of FK506, that of immunosuppression. The FK506 delivery systems tested in this study were engineered by focusing on the ease of clinical use. The drug delivery system, specifically the FK506 particulate form, contains readily available components to surgeons: fibrin gel [38] and FK506 [24,27], which have been used in the surgical field for decades. Thus, the application of the delivery system at the surgical site will not require extensive learning process for the surgeons. Fabricating the drug delivery system from biodegradable and biocompatible materials had eliminated the need for a secondary surgery for the removal of the delivery system. The delivery system alone without any FK506 did not diminish nerve regeneration following implantation at the site of nerve injury. Following three weeks of implantation, the delivery system was mainly resorbed. No foreign body formation or nerve compression was observed, confirming the system's clinical value for nerve repair.

The fibrin gel-based delivery systems containing the FK506 MS or particulate FK506 provide a tunable and controlled release of FK506 without any initial drug burst release at the site of nerve injury and surgical repair. Preventing the initial drug burst release has been a challenge and shortcoming in the recently developed delivery systems for FK506 [8,39]. Compared to the currently developed conduit-based FK506 delivery systems [8,39], the fibrin gel-based delivery system has the versatility to be used in various clinical cases of nerve injury and surgical repair. The modular property of the fibrin gel-based delivery system allows its implantation at different anatomical locations and around the injured nerve with any diameter.

For the first time, in this study, we showed the beneficial effects of the local FK506 released from our delivery system on axon regeneration following peripheral nerve transection and immediate surgical repair. In addition, biodistribution analysis of FK506 following local release at the site of nerve repair showed minimal vital organ exposure to FK506 and accumulation of the drug

mainly within spinal cord. This study provides the first biodistribution analysis of FK506 following the release of the drug from the site of peripheral nerve injury and repair. Based on our promising results in this study, we are testing our engineered drug delivery system in the more clinically relevant model of delayed nerve repair. Our preliminary results demonstrate that the effects of the drug delivery system of the FK506 MSs and particulate forms of FK506 are effective in promoting nerve regeneration and functional recovery after delayed surgery following a 2 month period of chronic axotomy, i.e. transecting the TIB nerve and preventing regeneration for 2 months prior to the cross-suture of the proximal nerve stump to the freshly denervated CP distal nerve stump [40]. Furthermore, functional assessments, such as muscle force analysis, and behavioral studies will be performed, which will allow us to assess the functional benefits of local FK506 administration to the site of peripheral nerve injury and surgical repair.

## 5. Conclusions

In this study, a fibrin gel-based drug delivery system for sustained and localized FK506 release at the site of peripheral nerve transection and surgical repair was utilized for enhancement of axon regeneration following immediate nerve transection and surgical repair. By having FK506 incorporated within the fibrin gel in solubilized, particulate or poly(lactic-co-glycolic) acid (PLGA) microsphere (MS) encapsulated forms, a tunable *in vivo* FK506 release from days to weeks was achieved. The local application of FK506 in the particulate form or MS encapsulated form resulted in a superior axon regeneration while preventing the toxicity of systemic FK506 that has prevented clinicians from using FK506 routinely for treating severe cases of peripheral nerve injuries. We have demonstrated effective release of FK506 from the fibrin gel-based delivery system *in vivo*. For the first time, this study showed the *in vivo* transport of the locally released FK506 to the nervous tissues, specifically to the sciatic nerve and spinal cord, with undetectable vital organ exposure to FK506. The findings in this study provided compelling evidence that the tested FK506 delivery system can be a new clinical solution for treatment of peripheral nerve injury.

## Funding

The authors have no financial disclosures to reveal.

## Declaration of Competing Interest

The authors have no conflict of interest relevant to the subject of the manuscript.

## Acknowledgements

We are grateful to Jennifer Zhang for their help on spinal cord tissue analysis. We wish to thank the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding Kasra Tajdaran and Katelyn Chan.

## References

- [1] K. Brattain, *Analysis of Peripheral Nerve Repair Market in the United States*, Magellan Medical Technology Consultants, Inc., 2013.
- [2] O. Sulaiman, R. Midha, T. Gordon, *Pathophysiology of Surgical Nerve Disorders*, 6th ed., Saunders, 2011.
- [3] S.Y. Fu, T. Gordon, The cellular and molecular basis of peripheral nerve regeneration, *Mol. Neurobiol.* 14 (1–2) (1997) 67–116, <https://doi.org/10.1007/BF02740621>.
- [4] A.W. English, J.C. Wilhelm, P.J. Ward, Exercise, neurotrophins, and axon regeneration in the PNS, *Physiology* (Bethesda) 29 (6) (2014) 437–445, <https://doi.org/10.1152/physiol.00028.2014>.

- [5] T. Scholz, A. Krichevsky, A. Sumarto, D. Jaffurs, G. Wirth, K. Paydar, G. Evan, Peripheral nerve injuries: an international survey of current treatments and future perspectives, *J. Reconstr. Microsurg.* 25 (6) (2009) 339–344, <https://doi.org/10.1055/s-0029-1215529>.
- [6] B.G. Gold, V. Densmore, W. Shou, M.M. Matzuk, H.S. Gordon, Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506, *J. Pharmacol. Exp. Ther.* 289 (3) (1999) 1202–1210. <http://www.ncbi.nlm.nih.gov/pubmed/10336507>.
- [7] P. Labroo, J. Shea, H. Sant, B. Gale, J. Agarwal, Effect of combining FK506 and neurotrophins on neurite branching and elongation, *Muscle Nerve*. 55 (4) (2017) 570–581, <https://doi.org/10.1002/mus.25370>.
- [8] P. Labroo, S. Ho, H. Sant, J. Shea, B.K. Gale, J. Agarwal, Controlled delivery of FK506 to improve nerve regeneration, *Shock* 46 (3 Suppl 1) (2016) 154–159, <https://doi.org/10.1097/SHK.0000000000000628>.
- [9] K. Tajdaran, M.S. Shoichet, T. Gordon, G.H. Borschel, A novel polymeric drug delivery system for localized and sustained release of tacrolimus (FK506), *Biotechnol. Bioeng.* 112 (9) (2015) 1948–1953, <https://doi.org/10.1002/bit.25598>.
- [10] B. Gold, T. Storm-Dickerson, D. Austin, The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury, *Restor. Neurol. Neurosci.* 6 (1994) 287–296.
- [11] B.G. Gold, K. Katoh, T. Storm-Dickerson, The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve, *J. Neurosci.* 15 (11) (1995) 7509–7516. <http://www.ncbi.nlm.nih.gov/pubmed/7472502>.
- [12] S.C. Jost, V.B. Doolabh, S.E. Mackinnon, M. Lee, D. Hunter, Acceleration of peripheral nerve regeneration following FK506 administration, *Restor. Neurol. Neurosci.* 17 (1) (2000) 39–44. <http://www.ncbi.nlm.nih.gov/pubmed/11490076>.
- [13] O.A. Sulaiman, J. Voda, B.G. Gold, T. Gordon, FK506 increases peripheral nerve regeneration after chronic axotomy but not after chronic schwann cell denervation, *Exp. Neurol.* 175 (1) (2002) 127–137, <https://doi.org/10.1006/exnr.2002.7878>.
- [14] M. Kihara, M. Kamijo, Y. Nakasaka, Y. Mitsui, M. Takahashi, J.D. Schmelzer, A small dose of the immunosuppressive agent FK506 (tacrolimus) protects peripheral nerve from ischemic fiber degeneration, *Muscle Nerve* 24 (12) (2001) 1601–1606. <http://www.ncbi.nlm.nih.gov/pubmed/11745968>.
- [15] B.G. Gold, FK506 and the role of immunophilins in nerve regeneration, *Mol. Neurobiol.* 15 (3) (1997) 285–306, <https://doi.org/10.1007/BF02740664>.
- [16] X. Navarro, E. Udina, D. Ceballos, B.G. Gold, Effects of FK506 on nerve regeneration and reinnervation after graft or tube repair of long nerve gaps, *Muscle Nerve* 24 (7) (2001) 905–915. <http://www.ncbi.nlm.nih.gov/pubmed/114100918>.
- [17] S.E. Mackinnon, V.B. Doolabh, C.B. Novak, E.P. Trulock, Clinical outcome following nerve allograft transplantation, *Plast. Reconstr. Surg.* 107 (6) (2001) 1419–1429. <http://www.ncbi.nlm.nih.gov/pubmed/11335811>.
- [18] S.W. Glaus, P.J. Johnson, S.E. Mackinnon, Clinical strategies to enhance nerve regeneration in composite tissue allotransplantation, *Hand Clin.* 27 (4) (2012) 495–509, <https://doi.org/10.1016/j.hcl.2011.07.002>. *Clinical*.
- [19] L.M. Diaz, M.H. Steele, A.B. Guerra, F. Aubert, G. Sloop, H. Diaz, R. Metzinger, D. Blake, C. Delaune, S. Metzinger, The role of topically administered FK506 (Tacrolimus) at the time of facial nerve repair using entubulation neurotrophin in a rabbit model, *Ann. Plast. Surg.* 52 (4) (2004) 407–413, <https://doi.org/10.1097/01.sap.0000107780.37285.6d>.
- [20] J. Steiner, M. Connolly, H. Valentine, Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporin A, *Nat. Med.* 3 (4) (1997) 421–428.
- [21] E.C. Toll, A.M. Seifalian, M.A. Birchall, The role of immunophilin ligands in nerve regeneration, *Regen. Med.* 6 (5) (2011) 635–652, <https://doi.org/10.2217/rme.11.43>.
- [22] E. Udina, E. Verdú, X. Navarro, Effects of the immunophilin ligand FK506 on nerve regeneration in collagen guides seeded with Schwann cells in rats, *Neurosci. Lett.* 357 (2) (2004) 99–102, <https://doi.org/10.1016/j.neulet.2003.11.070>.
- [23] K. Yamazoe, K. Yamazoe, T. Yamaguchi, M. Omoto, J. Shimazaki, Efficacy and safety of systemic tacrolimus in high-risk penetrating keratoplasty after graft failure with systemic cyclosporine, *Cornea* 33 (2014) 1157–1163.
- [24] T.H. Tung, Tacrolimus (FK506): safety and applications in reconstructive surgery, *Hand (N Y)* 5 (1) (2010) 1–8, <https://doi.org/10.1007/s11552-009-9193-8>.
- [25] P.E. Wallemacq, R. Reding, FK506 (tacrolimus), a novel immunosuppressant in organ transplantation: clinical, biomedical, and analytical aspects, *Clin. Chem.* 39 (11 Pt 1) (1993) 2219–2228. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2978067&tool=pmcentrez&rendertype=abstract>.
- [26] M.J. Brenner, T.H. Tung, J.N. Jensen, S.E. Mackinnon, The spectrum of complications of immunosuppression: is the time right for hand transplantation?, *J. Bone Joint Surg. Am.* 84-A (10) (2002) 1861–1870.
- [27] P. Konofaos, J.K. Terziss, FK506 and nerve regeneration: past, present, and future, *J. Reconstr. Microsurg.* 29 (2013) 141–148.
- [28] I. Guilhem, B. Balkau, F. Lecordier, J.M. Malecot, S. LA Elbadi, A. Leguerrier, Insulin pump failures are still frequent: a prospective study over 6 years from 2001 to 2007, *Diabetologia* 52 (12) (2009) 2662–2664.
- [29] G.E. Visscher, J.E. Pearson, J.W. Fong, G.J. Argenti, R.L.M.H. Robison, Effect of particle size on the in vitro and in vivo degradation rates of poly(DL-lactide-co-glycolide) microcapsules, *J. Biomed. Mater. Res.* 22 (1988) 733–746.
- [30] M.J. Caicco, M.J. Cooke, Y. Wang, A. Tuladhar, C.M. Morshead, M.S. Shoichet, A hydrogel composite system for sustained epi-cortical delivery of Cyclosporin A to the brain for treatment of stroke, *J. Control. Release* 166 (3) (2013) 197–202, <https://doi.org/10.1016/j.jconrel.2013.01.002>.
- [31] A. Tuladhar, C.M. Morshead, M.S. Shoichet, Circumventing the blood-brain barrier: local delivery of cyclosporin A stimulates stem cells in stroke-injured rat brain, *J. Control. Release* 215 (2015) 1–11, <https://doi.org/10.1016/j.jconrel.2015.07.023>.
- [32] K. Tajdaran, T. Gordon, M.D. Wood, M.S. Shoichet, G.H. Borschel, A glial cell line-derived neurotrophic factor delivery system enhances nerve regeneration across acellular nerve allografts, *Acta Biomater.* 29 (2016) 62–70, <https://doi.org/10.1016/j.actbio.2015.10.001>.
- [33] M.D. Wood, T. Gordon, H. Kim, M. Szyrakur, P. Phua, C. Lafontaine, S. Kemp, M. Shoichet, G. Borschel, Fibrin gels containing GDNF microspheres increase axonal regeneration after delayed peripheral nerve repair, *Regen. Med.* 8 (1) (2013) 27–37, <https://doi.org/10.2217/rme.12.105>.
- [34] M. Abercrombie, Estimation of nuclear population from microtome sections, *Anat. Rec.* 94 (1946) 239–247.
- [35] H.L. More, J. Chen, E. Gibson, J.M. Donelan, M.F. Beg, A semi-automated method for identifying and measuring myelinated nerve fibers in scanning electron microscope images, *J. Neurosci. Methods* 201 (1) (2011) 149–158, <https://doi.org/10.1016/j.jneumeth.2011.07.026>.
- [36] K. Tajdaran, T. Gordon, M.D. Wood, M.S. Shoichet, G.H. Borschel, An engineered biocompatible drug delivery system enhances nerve regeneration after delayed repair, *J. Biomed. Mater. Res. – Part A* 104 (2) (2016) 367–376, <https://doi.org/10.1002/jbm.a.35572>.
- [37] P. Dubový, R. Jančálek, T. Kubek, Role of inflammation and cytokines in peripheral nerve regeneration, *Int. Rev. Neurobiol.* 108 (2013) 173–206, <https://doi.org/10.1016/B978-0-12-410499-0.00007-1>.
- [38] M. Sameem, T. Wood, J. Bain, A systematic review on the use of fibrin glue for peripheral nerve repair, *Plast. Reconstr. Surg.* 127 (6) (2011) 2381–2390.
- [39] B. Davis, S. Wojtalewicz, P. Labroo, J. Shea, H. Sant, B. Gale, J. Agarwal, Controlled release of FK506 from micropatterned PLGA films: potential for application in peripheral nerve repair, *Neural Regen. Res.* 13 (7) (2018) 1247–1252, <https://doi.org/10.4103/1673-5374.235063>.
- [40] K. Tajdaran, K. Chan, M. Shoichet, T. Gordon, G.H. Borschel, Local FK506 delivery enhances nerve regeneration and functional recovery following chronic axotomy, *Am. Soc. Peripher. Nerve* (2019). PN20.