RESEARCH ARTICLE

Controlled release of FK506 from micropatterned PLGA films: potential for application in peripheral nerve repair

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Abstract

After decades of research, peripheral nerve injury and repair still frequently results in paralysis, chronic pain and neuropathies leading to severe disability in patients. Current clinically available nerve conduits only provide crude guidance of regenerating axons across nerve gap without additional functionality. FK506 (Tacrolimus), an FDA approved immunosuppressant, has been shown to enhance peripheral nerve regeneration but carries harsh side-effects when delivered systemically. The objective of this study was to develop and evaluate a bioresorbable drug delivery system capable of local extended delivery of FK506 that also provides topological guidance cues to guide axon growth *via* microgrooves. Photolithography was used to create micropatterned poly(lactide-co-glycolic acid) (PLGA) films embedded with FK506. Non-patterned, 10/10 μm (ridge/groove width), and 30/30 μm patterned films loaded with 0, 1, and 3 μg/cm^2 FK506 were manufactured and characterized. *In vitro* FK506 rate of release testing indicated that the films are capable of an extended (at least 56 days), controlled, and scalable release of FK506. Neurite extension bioactivity assay indicated that FK506 released from the films (concentration of samples tested ranged between 8.46–19.7 ng/mL) maintained its neural bioactivity and promoted neurite extension similar to control FK506 dosages (10 ng/mL FK506). The multi-functional FK506 embedded, micropatterned poly(lactide-co-glycolic acid) films developed in this study have potential to be used in the construction of peripheral nerve repair devices.

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doi: *10.4103/1673-5374.235063*

Accepted: *2018-05-06*

Key Words: *nerve regeneration; FK506; controlled release; micropatterns; topological cues; PLGA*

Introduction

Peripheral nerve injuries of varying degrees of severity often lead to permanent loss of sensory and motor function (Navarro et al., 2007). Treatment of peripheral nerve injuries frequently result in inadequate functional recovery, especially for nerve gap-injuries when a tension-free direct neurorrhaphy cannot be performed (Grinsell and Keating, 2014). Autologous nerve grafts are the gold standard treatment for nerve gap-injury repair, but even this treatment often leads to insufficient functional recovery and causes loss of function at the donor site (Lee and Wolfe, 2000). Alternatives to the autograft are bioresorbable hollow guidance conduits and decellularized allogenic nerve grafts. Clinically available nerve conduits provide gross approximation of the two severed nerve ends for crude guidance of regenerating axons, while decellularized allogenic nerve grafts provide the topological and chemical guidance cues of native nerve extracellular matrix (Cho et al., 2012). Decellularized allogenic nerve grafts can be considered the greatest advancement of clinically available nerve repair devices, but these still are not as efficacious as the autograft in long-gap nerve repair (> 3 cm). A clinical need still exists to improve patient outcomes after peripheral nerve injury of all severities.

The majority of clinically available nerve repair devices lack added functionality. An intriguing small-molecule that has been widely studied in peripheral nerve regeneration applications is FK506 (Tacrolimus). FK506 is an FDA approved immunosuppressant drug used to prevent allograft organ rejection. FK506 can improve functional outcomes *in vivo* after peripheral nerve inury *via* multiple mechanisms of action including neurotrophic effects and reduction of scar formation (Gold et al., 1994, 1995; Que et al., 2012, 2013; Yan et al., 2012). Clinical adoption of FK506 has not occurred because the benefits are not perceived to outweigh the harsh systemic toxicity and immunosuppresive effects associated with long-term systemic use (*e.g.*, kidney toxicity, liver toxicity, increased risk of infection) (Felldin et al., 1997; Naesens et al., 2009; Konofaos and Terzis, 2013). Localized delivery of FK506 to a nerve injury/repair site could provide the peripheral nerve regeneration enhancing effects without the injurious side-effects associated with systemic use. Additionally, topological guidance cues are a critical component for sustaining nerve regeneration across large gaps (Ide et al., 1983; Hoffman-Kim et al., 2010). Previous studies by us and others have shown that longitudinally aligned surface micropatterns can increase neurite extension and neural cell alightment *in vitro* (Vaidya et al., 1998; Yao et al., 2009; Su et al., 2013; Li et al., 2014, 2018). Previously, we conducted micropattern optimization studies to investigate the effect pattern size had on neuronal cell extension, orientation, and viability. Results indicated that 10 μm ridges spaced by 10 μm grooves and 30 μm ridges spaced by 30 μm grooves were the optimal micropattern dimensions (**Figure 1**).

For this study, we set out to investigate these previously developed micropatterned poly(lactide-co-glycolic acid) (PLGA) films as a local, controlled FK506 delivery system.

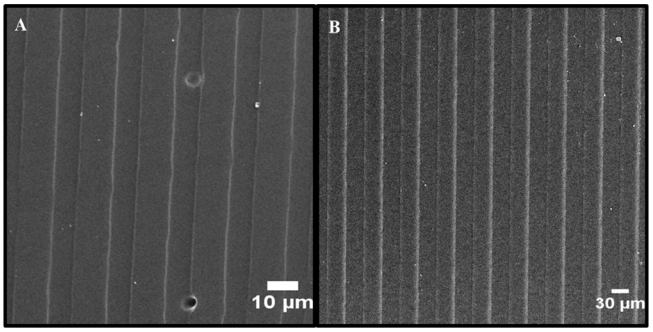

Figure 1 Scanning electron microspcopy images of micropatterned films.
(A) 10/10 µm film, scale bar: 10 µm, 1000× magnification. (B) 30/30 µm film, scale bar: 30 µm, 200× magnification.

Polyesters (PLGA, poly(Lactic acid) (PLA), poly(ε-caprolactone) (PCL), *etc.*) have been widely studied for the development of drug delivery systems for the delivery of small molecules in various clinical applications, therefore making our previously developed PLGA micropatterned films a prime candidate for the creation of a combination technology encompassing topological guidance cues and local, controlled delivery of FK506 (Pillai and Panchagnula, 2001).

We hypothesized that the micropatterned PLGA film sembedded with FK506 could release FK506 in a therapeutic range over an extended period of time (**Figure 1**). Characterization testing of the FK506 embedded micropatterned PLGA films was performed in this study. These micropatterned PLGA films capable of extended, localized delivery of FK506 have the potential to be used in the construction of nerve repair devices.

Materials and Methods

Flat and micropatterned mold fabrication

Flat and micropatterned PLGA films were manufactured by solvent casting onto polydimethylsiloxane (PDMS) molds. PDMS molds were created using a previously developed photolithography technique (Li et al., 2018). The photolithography process was performed to produce both 10/10 µm (groove width/ridge width) and 30/30 µm micro-patterns on a silicon substrate which PDMS was cast on to create the inverse mold for film production. AZnLOF2020 (Microchemicals, Ulm, Germany) negative photoresist was coated on the surface by spin coating with a series of rotating speeds (30 r/min 5 seconds, 500 r/min 5 seconds and 1400 r/min 45 seconds). The silicone substrates were soft baked at 110°C using a hotplate for 1 minute. Silicon substrates with photoresist were exposed to ultraviolet light by OAI 200 mask aligner (OAI, San Jose, CA, USA) for 8.5 seconds. AZ 1:1 developer solution (Microchemicals) was applied for 45 seconds, and then rinsed with deionized water for 1 minute. Silicon substrates were dried with N_2 gas and the silicon substrate mold was obtained. To create the inverse micro-patterned PDMS molds, 20 mL PDMS (10:1, v/v) was poured onto the silicone mask and degassed in a vacuum for 30 minutes. The PDMS molds were left to cure at room temperature for 24 hours on a leveling table and then at 80°C for 2 hours. A PDMS mold for the flat, non-patterned films was made by pouring 30 mL of PDMS (10:1, v/v) in a 60 mm glass petri dish and left to cure at room temperature for 24 hours on a leveling table and then at 80°C for 2 hours. A 60 mm diameter PDMS (10:1, v/v) ring was made and bonded to each PDMS mold to create a flange around the mold to contain the PLGA/FK506 solutions.

FK506/PLGA solutions and film casting

To make the FK506 (Astellas Pharma, Northbrook, IL, USA) containing PLGA solutions, 10 g of 75/25 poly-lactic-glycolic-acid (Evonik Industries, Essen, Germany) was dissolved in 20 mL acetone and stirred at 60 r/min at room temperature for 2 hours. 6 mL of 100% ethanol was added to the solution, and stirring continued at room temperature for 2 days for complete dissolution of the PLGA.Two different concentrations of FK506/PLGA solutions were made to produce films with 1 and 3 µg/cm^2 FK506 of the final casted film. A solution without drug was made with 0 µg/cm^2 FK506. FK506 was dissolved in 100% dimethyl sulfoxide (DMSO) and used for dilutions. Each solution was mixed at a ratio of 90/10% PLGA/DMSO. Micropatterned films were made with either ridge/groove ratios of 10/10 µm widths or 30/30 µm widths and flat non-micropatterned films were made for each FK506 concentration. Each type of FK506/PLGA solution was poured into a PDMS mold and left at room temperature for 48 hours to cure and then in a vacuum for 12 hours.

Micropattern design verification *via* scanning electron microscopy

FEI Quanta 600 FEG scanning electron microscope (SEM) (Thermofisher, Waltham, MA, USA) was used to verify the micropattern design specifications after the PLGA film fabrication was complete. The surface was coated with a 5 nm gold film (Thermofisher) by sputter for conductivity. The FEI Quanta 600 FEG (Thermofisher) at the Utah Nanofab of the University of Utah. Using ImageJ (ImageJ 1.31v, National Institutes of Health, Bethesda, MD, USA) the ridge and groove widths were measured (n = 10 measurements of ridge and groove each).

Weight loss

A weight loss study was conducted to evaluate the degradation rate of the various films (flat, 10/10 and 30/30 µm groove/ridge widths with 0, 1, and 3 µg/cm^2 FK506) loaded into the film. 1 cm^2 films were cut out of each of the cast films. Each film was weighed prior to the start of the study to determine a starting weight and was placed into a 5 mL conical tube containing 5 mL of PBS. The conical tubes were kept at 37°C and 5% CO_2 for 8 weeks. Every 7 days the PBS was aspirated and replaced with 5 mL of fresh PBS. At weeks 2, 4, 6, and 8, n = 4 films of each film type was removed, rinsed in DI water and left in a vacuum for 48 hours to dry for weighing. Material degradation rate was calculated by the following equation (W_o is original dry weight prior to test and W_n is dry weight at time point n):

$$\text{Material degradation rate (\%)} = \frac{W_0 - W_n}{W_0} \times 100\%$$

In vitro FK506 release characterization

An *in vitro* drug release test was conducted to determine the release rate of FK506 from the PLGA films. 1 cm^2 film

devices were cut out of each casted film (flat, 10/10, 30/30 with 0, 1, and 3 μg/cm^2 FK506 concentrations), disinfected by washing in 70% ethanol, and placed in a 24-well plate with 2 mL of culture media (Gibco™ DMEM F12 + 10% FBS + 1% Pen-Strep) in each well (n = 8 for each group). The films were incubated at 37°C and 5% CO_2 for 56 days. The 2 mL media samples were collected and replaced with fresh media at 24 hours and then every 72 hours for 28 days. The concentration of FK506 within the samples was determined using an ELISA (Abnova, Taiwan, China) and then the mass of released FK506 calculated.

FK506 bioactivity evaluation

The collected media samples from the *in vitro* FK506 rate of release testing were used for the bioactivity study. The 3 μg/cm^2 films were used for the bioactivity study, 3 collected media samples were chosen per group per time point for evaluation (n = 3 per sample and day 1, 16, 35, and 49 were evaluated). Control groups of 0 ng/mL and 10 ng/mL FK506 were used for comparison (n = 8 per control group, dorsal root ganglions (DRGs) were tested) and for the experimental groups a total of 12 DRGs for each timepoint and group were cultured. Fertilized chicken eggs (Merrills Poultry, ID, USA) were incubated at ~39°C under 100% relative humidity for 12 days. The eggs were cleaned with 70% ethanol and then opened to extract the embryos. DRGs were dissected from the embryos under a microscope using a previously published method (Labroo et al., 2017b). 24-well plates were coated with laminin (1 μg/mL), then 500 μL from each media sample was placed into 3 wells. Chick DRGs were separated carefully from connective tissue for culturing and a single DRG was placed into each well with the 500 μL of media sample (n = 3/group). The plate was incubated for 72 hours at 37°C and 5% CO_2 to evaluate the released drug's bioactivity. After 72 hours the media was aspirated from the wells, the DRG's were fixed with methanol and rinsed with deionized water. Each DRG was imaged using a wide field light microscope at 4× magnification. Images of DRGs were used to analyze neurite extension. Neurite extension measurements were done using a previously described method

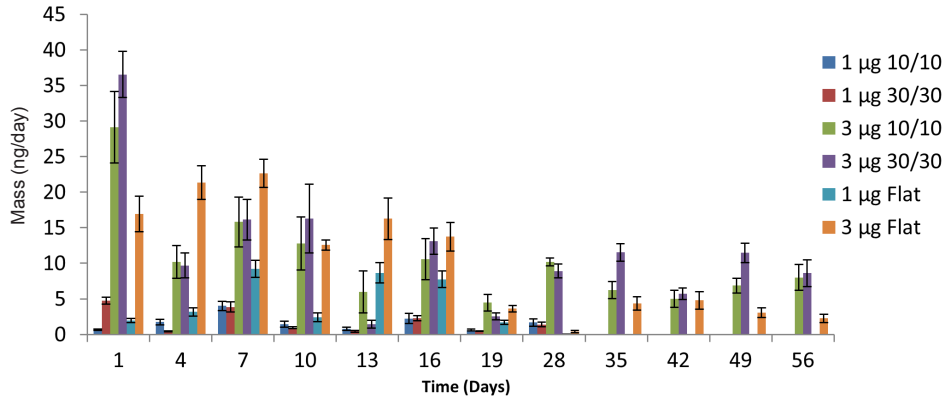

Figure 2 FK506 release profiles of the various PLGA films.
In vitro drug release testing was performed to determine the FK506 (Tacrolimus) release profiles from the various PLGA films. PLGA films with 1 μg/cm^2 and 3 μg/cm^2 initial loading concentrations were used for this study with either flat, 10/10 μm, or 30/30 μm micropatterns. The rate is presented in ng/day ± SEM, n = 8 per group for this experiment. 1 μg/cm^2 films were discontinued after day 28 due to the fact that they were releasing drug at such a low rate. PLGA: Poly(lactide-co-glycolic acid).

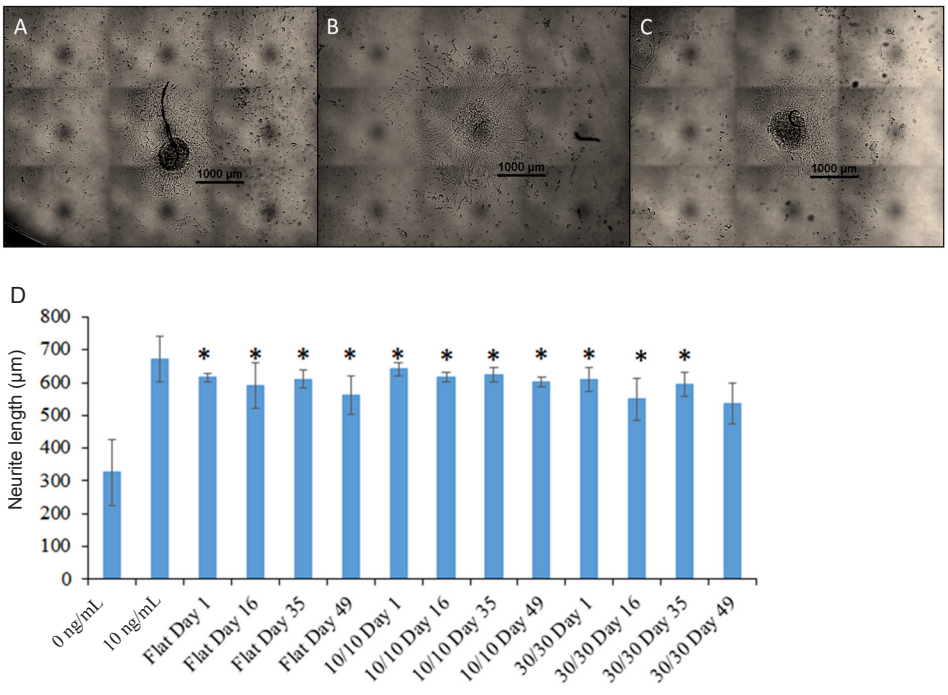

Figure 3 Dorsal root ganglion neurite extension assay for FK506 (Tacrolimus) bioactivity assessment.
(Top) Phase-contrast images of the dorsal root ganglions (DRGs) cultured for 72 hours which were used for average neurite extension assessment. (A) DRG grown in 0 ng/mL FK506 control media (B) DRG grown in 10 ng/mL control media (C) DRG grown in collected media from 3 μg/cm^2 film drug release. (D) Neurite extension values of DRGs cultured in media collected during the drug release test. Neurite extension was evaluated and compared to known FK506 concentrations 0 and 10 ng/mL to verify that drug retains its bioactivity after being released from the films (n = 8 per 0 and 10 ng/mL control groups and for experimental groups n = 3 per time point for a total of n = 12 per flat, 10/10, and 30/30). ∗Indicates groups that are significantly different (P < 0.05) from the 0 ng/mL control and are not significantly different (P > 0.05) from the 10 ng/mL control.

Table 1 Results of the weight loss study

	Flat (%)	10/10 (%)	30/30 (%)
Week 2	22.1±0.46	19.9±0.92	22.2±0.86
Week 4	21.5±1.11	22.5±1.25	23.0±0.92
Week 6	20.1±2.30	23.2±6.19	20.1±1.76
Week 8	17.5±1.51	17.4±0.28	21.2±0.88

Relative change values are presented in % change of initial weight of the poly(lactide-co-glycolic acid) film. At each time point $n = 4$ films per group were dried and weighed. Values are presented as % weight loss ± SD relative to the initial weight.

(Labroo et al., 2017a). Briefly, the area of the ganglion body (A_{DRG}) and the total area of the DRG with the growing axons (A_{tot}) were measured using ImageJ (ImageJ 1.31v, National Institutes of Health, Bethesda, MD, USA). The average neurite length (lavg) was calculated by: $l_{avg} = (A_{tot}/\pi)^{1/2} - (A_{DRG}/\pi)^{1/2}$.

Statistical analysis

One-way analysis of variance with Tukey's *post-hoc* test was performed on the DRG neurite extension bioactivity assay. A Student's *t*-test was performed on the drug release data. Values of $P < 0.05$ were considered significant. Statistical analysis was performed in Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

Results

Flat and micropatterned PLGA film characterization

Pictures of micropattern ridge/groove topography can be seen in **Figure 1A** and **B**. The 10/10 films had an average ridge and groove width of 10.7 ± 0.419 μm and 7.76 ± 0.596 μm, respectively. The 30/30 films had an average ridge and groove width of 28.9 ± 0 .788 μm and 27.4 ± 0.682 μm, respectively. Additionally, a weight loss test was conducted to determine the degradation rate of the PLGA films. Four time-points (2, 4, 6 and 8 weeks) were used to determine the weight loss profile of the PLGA films. Weight loss values are shown in **Table 1**.

Flat and micropatterned PLGA film *in vitro* FK506 release profile

An *in vitro* drug release assay was used to determine the release kinetics of FK506 from the embedded PLGA films over a 56 day period. The samples were collected every 72 hours but the data is reported in average mass release per day. For the first 28 days, the 1 and 3 μg/cm^2 FK506 flat films released an average of 3.58 ± 2.95 ng and 12.8 ± 7.79 ng per day, respectively. For the first 28 days the 10/10 1 and 3 μg/cm^2 FK506 films released an average of 1.65 ± 1.04 ng and 12.4 ± 7.14 ng per day, respectively. For the first 28 days, the 30/30 1 and 3 μg/cm^2 FK506 films released an average of 1.83 ± 1.57 ng and 13.1 ± 10.3 ng per day, respectively. **Figure 2** shows a more detailed depiction of the release profile, displaying the average mass/day values for every time point. Statistical analysis found no significant distances between the release the 10/10 and 30/30 micropatterned groups, indicating that the size of the micropatterns did not have an effect on the drug release rate. After day 28, the 1 μg films were discontinued from the study because they were releasing at undesirably low levels, and the 3 μg films were continued on for an additional 28 days. From days 29–56, the flat, 10/10, and 30/30 3 μg/cm^2 films released an average of 3.61 ± 1.01, 6.53 ± 1.09, and 9.33 ± 2.39 ng per day, respectively.

In vitro FK506 biological activity

Cell culture media samples that were collected from the drug release study were used to culture whole DRGs to evaluate the bioactivity of the FK506 released from the films. DRG neurite extension was measured to evaluate FK506 bioactivity. 10 ng/mL of freshly prepared FK506 was used as the positive control and a 0 ng/mL FK506 solution was used as a negative control. DRGs were cultured in media collected from the 3 μg/cm^2 films (flat, 10/10, and 30/30) at days 1, 16, 35, and 49. The average DRG neurite extension for all the time points and groups was 596 ± 30.3 μm treated with FK506 containing samples collected from the drug release test and the average DRG neurite extension for the 0 and 10 ng/mL control groups were 333 ± 82.04 μm and 671 ± 69.0 μm, respectively. Data for individual groups and timepoints can be seen in **Figure 3**. All groups were found to be significantly higher ($P < 0.05$) than the 0 ng/mL control group except 30/30 Day 49. No differences were found from all experimental groups compared with the 10 ng/mL ($P > 0.05$).

Discussion

Previous studies conducted by others have given systemic delivery of FK506 to investigate the neurotrophic effects of FK506, but less is known about localized delivery of FK506 to nerve injuries (Gold et al., 1994, 1995, 1997; Doolabh and Mackinnon, 1999; Lee et al., 2000; Navarro et al., 2001; Udina et al., 2003, 2004; Yan et al., 2012; Konofaos and Terzis, 2013; Que et al., 2013). Recently, local application of FK506 to peripheral nerve injuries has been studied with positive results indicating local delivery of FK506 to peripheral nerve injuries can improve nerve regeneration outcomes (Azizi et al., 2012; Mekaj et al., 2017). Both of these studies did not incorporate a drug-carrier to control the release of the drug, a physical barrier to prevent tissue adhesion, or the ability to provide structural support to bridge nerve gaps. Additionally, Tadjaran et al. (2015) demonstrated that PLGA microspheres loaded with FK506 can provide controlled release of FK506, but these microspheres would need to be used in conjunction with other technologies such as nerve conduits and wraps in order to be useful in many nerve repair scenarios (Tajdaran et al., 2015). The technology developed in this study differs from the other local FK506 delivery strategies mentioned in that it can offer controlled release, added functionality in the form of topological guidance cues for regenerating axons, and the ability to be used to construct nerve conduits and wraps combined into one technology, whereas others are limited in their functionality and use.

The goal of this project was to assess the drug delivery capabilities of previously developed micropatterned PLGA films (Li et al., 2018). These films are an ideal candidate to be

used as a drug delivery system because PLGA has been extensively studied for drug delivery applications over the last few decades. PLGA is ideal because of its biocompatibility, bioresorbability, and highly tunable properties such as mechanical properties, degradation rate and drug release rates (Makadia and Siegel, 2011). PLGA and other polyesters have obtained FDA approval for various clinical applications, including nerve conduits and wraps (Kehoe et al., 2012). FK506 has shown to increase nerve regeneration outcomes by multiple mechanisms including increasing axon regeneration rates, increasing Schwann cell proliferation, and by reducing scarring at the repair site (Gold et al., 1995; Fansa et al., 2000; Udina et al., 2003; Atkins et al., 2006). FK506 is an ideal candidate drug for a PLGA based drug delivery system because both FK506 and PLGA are hydrophobic materials. The hydrophobic nature of FK506 allows it to readily disperse in the hydrophobic PLGA polymer network which allows for higher control and duration of the release. It was hypothesized that micropatterned PLGA films homogenously loaded with FK506 would be capable of an extended, localized, and scalable release of FK506.

SEM was used to observe the surface patterns of the FK506 containing micropatterned PLGA films, and analysis of the SEM images verified that our fabrication technique could produce micropatterned films with longitudinally aligned ridges and grooves of varying dimensions. The 10/10 ridge width was slightly larger than the designed 10 μm, and the 30/30 ridge width was slightly smaller than designed 30 μm. These variations can be attributed to the nature of molding and can be accounted for in the future production of molds by scaling. Qualitatively, the micropatterns on the PLGA films appeared crisp and complete across the whole film. Data from the weight loss study showed an increase in weight of about 20% in the first two weeks and then the weight did not change for the remainder of the time points (weeks 4, 6, and 8). The observed weight gain was contradictory to what would be expected to occur during an *in vitro* PLGA degradation study (Lu et al., 1999). The increase in weight could have been caused by swelling and potentially trapping of water molecules and salts that could not escape during the drying process (Tang and Hunt, 2006).

The drug release study verified that FK506 could be released over a period of two months and is characterized by a byphasic release. Statistical analysis found no differences in release rates of the 10/10 and 30/30 drug release rates, this result was expected because the release of a hydrophobic drug from 75:25 PLGA is primarily dependent on bulk diffusion and bulk erosion. Bulk eroding polymer drug delivery systems, such as many polyesters, release drug based on the bulk erosion rate of the specific material used, while surface area has a diminished effect (Uhrich et al., 1999; Makadia and Siegel, 2011). PLGA-based drug delivery systems have been shown to have a biphasic release curve, characterized by an initial phase based on bulk diffusion and then as bulk erosion of the polymer frees the entrapped drug molecules for release (Faisant et al., 2002). An initial higher concentration burst occurred in the first 16-19 days and then a slower steadier release occurred from day 20 on. This phenomenon could be explained by FK506 molecules which are weakly interacting with the polymer matrix initially diffusing out of the film when the film is exposed to the aqueous solution, and then as the polymer matrix erodes, the more entrapped FK506 is freed to diffuse out of the films (Makadia and Siegel, 2011). Use of a small fraction of DMSO in the polymer-drug solution may have formed a semi-porous film which would allow an initial uptake of water into the film causing the initial burst phase of the drug release. It was found that the release is scalable and can be tuned for specific applications which may require differing dosages and sizes of films to be used. Bioactivity testing found that the FK506 released from the films retains its bioactivity and that neurite extension was found to be similar to the 10 ng/mL control group (previous studies found 10 ng/mL to be an optimal dosage for chick DRG neurite extension (Labroo et al., 2017a)).

This study was limited to *in vitro* analysis of the drug-releasing capabilities of the micropatterned films and *in vitro* bioactivity assessment. Future studies will investigate the efficacy of these films *in vivo*. The micropatterned PLGA film drug delivery system investigated in this study has the potential to be used in various peripheral nerve injury models by incorporation into different devices (nerve conduits and nerve wraps). The micropatterned films could be used to line the inner lumen of nerve conduits to provide surface topological guidance, drug delivery, and a physical barrier simultaneously. Bioresorbable nerve wraps are used in the clinic to prevent immune cell infiltration, reduce scarring, and limit nerve adhesion to surrounding tissues (Masear, 2011; Kokkalis et al., 2015). FK506 embedded PLGA films developed in this study can be wrapped around crush, stretch, direct-suture repair, autograft and allograft repairs to provide a physical barrier to impede infiltration of immune cells and prevent undesirable nerve adhesion to surrounding tissue while simultaneously delivering FK506 to the peripheral nerve injury site. *In vivo* investigation in various injury and repair models is needed to assess the nerve regeneration efficacy of these multi-functional micropatterned PLGA with added drug delivery capabilities.

Conclusion

We have successfully developed a bioresorbable micropatterned PLGA film drug delivery system capable of extended, controlled, and localized FK506 delivery for use in the construction of peripheral nerve repair devices. The FK506 embedded PLGA films were fabricated with a surface ridge/groove micropattern using a photolithographic technique to promote neural cell alignment and growth (Li et al., 2018). Results of this study exhibited that bioactive FK506 can be released over a period of 56 days within a therapeutic range. Additionally, the amount of FK506 released per day can be tailored by altering the initial loading concentration of the FK506/PLGA films. These FK506-containing PLGA films were developed for potential use in various peripheral nerve repair scenarios including nerve wrapping of non-transection injuries or direct suture repaired transection injuries, lining the inner lumen of nerve conduits for gap-injury repair, and wrapping around autograft/allograft repairs to increase func-

tional outcomes. This technology allows for extended, localized delivery of FK506 to the repair site while avoiding the side-effects associated with systemic delivery of FK506.

Author contributions: *Concept, design, definition of intellectual content, literature search, experimental studies, data acquisition, data analysis, manuscript preparation: BD. Manuscript preparation, experimental studies, data acquisition: SW. Experimental studies, data acquisition: PL. Concept, design, definition of intellectual content, manuscript review: JS. Concept, design, definition of intellectual content: HS. Concept, design, definition of intellectual content, manuscript review: BG. Guarantor, concept, design, definition of intellectual content, manuscript editing and manuscript review: JA. All authors approved the final version of this paper.*
Conflicts of interest: *The authors have no conflicts of interest to declare.*
Financial support: *None.*
Institutional review board statement: *IACUC approval was not required for the experiments performed in this study in accordance with the University of Utah's IACUC Guidelines.*
Biostatistics statement: *The statistical analysis was developed and performed by the study investigators with assistance from the University of Utah biostatistics core as needed.*

Open peer reviewer: *Kirsten Haastert-Talini, Hannover Medical School, Institute for Neuroanatomy and Cell Biology, Germany.*
Additional file: *Open peer review report 1.*

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