Continuous release of mefloquine featured in electrospun fiber membranes alleviates epidural fibrosis and aids in sensory neurological function after lumbar laminectomy

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ABSTRACT

Recurrent low back pain after spinal surgeries, such as lumbar laminectomy, is a major complication of excessive epidural fibrosis. Although multiple preclinical and clinical methods have been aimed at ameliorating epidural fibrosis, their safety and efficacy remain largely unclear. Single implanted electrospun fibrous membranes provide physical barriers that can decrease tissue fibrosis after surgery; however, they also trigger local inflammation due to the implantation of a foreign body, thus subsequently attenuating their anti-fibrosis properties. Here, we designed a strategy that permits easy incorporation of mefloquine into polylactic acid membranes, and stable long-term mefloquine release, to potentially improve anti-fibrosis effects and relieve or prevent low back pain.

The electrospun fibrous membranes grafted with mefloquine showed a well-controlled early temporary peak release, and secondary drug release occurred smoothly over several weeks. Histopathological and histomorphometric results indicated that the drug-loaded membranes had excellent anti-fibrosis effects after laminectomy in rats. Inflammation and neovascularization at the surgical site indicated that the mefloquine-grafted electrospun fibrous membranes provided sustained anti-inflammatory outcomes while effectively alleviating associated neuropathic pain hypersensitivity. In summary, our study indicated that polylactic acid-mefloquine grafted electrospun fibrous membranes may be a potential local agent to mitigate epidural fibrosis and support sensory neurological function after laminectomy, thereby potentially improving patients' postoperative outcomes.

1. Introduction

Lumbar spine surgery is a common type of spinal surgery that has good effects in relieving spinal cord and nerve root compression. Despite great progress in operative treatments, the occurrence of failed back surgery syndrome (FBSS), whose typical clinical features include continued or recurring low back pain, remains a major concern after spinal surgery and has an incidence of approximately 8%–40% [1].

Recurrence of radicular pain in the postoperative period is not only a major personal and family issue but also a social problem: it makes patients feel desperate, increases their family's burden, and hinders their social adaptation.

Fibroblasts can migrate from the muscles around the vertebrae or past the surgical site through fluid circulation, thus resulting in the formation of epidural fibrosis (EF) in the process of repairing local vertebral lesions after activation by inflammatory cytokines, such as tumor necrosis factor...
Fig. 1. Schematic illustration of the study design. (a) Rat model of lumbar laminectomy. (b) Initial inflammation after operation plays a role in EF. (c) The only physical barrier of the polylactic acid (PLA) membrane cannot inhibit the secondary inflammation due to foreign body implantation, thus decreasing the efficacy of anti-adhesion. (d) With sustainable drug release, PLA-MFQ grafted membranes may solve the problem of initial and secondary inflammation after surgery. (e) Prevention of EF post-laminectomy by PLA-MFQ grafted membranes contributes to alleviating FBSS.

| Table 1 | Sequences of primers used. |
|---|---|
| Primers | Forward | Reverse |
| IL-1β | TGACCTGTCTTTGAGGCCGTAC | CATCAACCGAGTCACAGAG |
| TNF-α | CCAAGTCGTCTCAAGGACAA | GGTATGAAATGGCAAATCGGCT |
| NLRP3 | ATCAACCGGAGACCGCTG | GTCTGGTTGGCAGATTAG |
| GAPDH | GCCACAGTCAAGGCTGAAGT | ATGGTGGTGAAGGGCAGTA |

(TNF-α) and interleukin (IL-1β). The recruitment and development of macrophages also play critical roles in EF progression; however, the precise mechanisms regulating the progression of fibrosis during healing are poorly understood [2,3]. This scar tissue can migrate to the dura and grow into the nerve canal, extend into the vertebral canal, and adhere to the dura matter and nerve elements: this mechanism is considered the primary reason for the pain symptoms [4-6]. Many studies have confirmed that FBSS is directly associated with EF [7-9] (Fig. 1a and b). Recent studies on FBSS have also focused on perioperative inflammation management [10]. Although no direct evidence of a pathologic relationship between FBSS and inflammation has been reported, studies aimed at decreasing inflammation and anti-epidural scar adhesion have been widely performed to address the pain arising from unsatisfactory surgical results and reoperation [11,12].

Currently, the implantation of physical barriers, which are generally used in the form of membranes, is the most common anti-adhesion strategy in clinical settings, such as autologous tissues, natural polymers, and synthetic materials [13]. However, the anti-fibrosis membranes are usually cleared by macrophages during biodegradation, and the implantation of membranes, as foreign bodies, can provoke inflammatory responses, thus significantly decreasing the anti-adhesion efficacy [14-16] (Fig. 1c). Therefore, rigorously designed biomaterials are urgently needed for the effective treatment of excessive epidural scar formation after surgery [17].

Mefloquine (MFQ), a synthetic analog of quinine, is highly effective against drug-resistant P. falciparum [18]. MFQ has been described as a broad-spectrum pannexin-1 (PanX1) inhibitor [19]. Pannexins are a conserved family of transmembrane proteins in vertebrates that comprise three subtypes: PanX1, PanX2, and PanX3. Of the three pannexin members, PanX1 is a ubiquitous non-selective transmembrane channel that efficiently effluxes adenosine triphosphate into the extracellular environment. This extracellular adenosine triphosphate can act as a damage-associated molecular pattern (DAMP), thereby leading to downstream activation of NLRP3, enhancing the inflammatory response, and finally exacerbating tissue damage and promoting a tissue fibrotic phenotype [20]. Thus, PanX1 initiates the progression of inflammatory diseases and fibrogenesis [21]. Therefore, MFQ may be a promising treatment for postoperative EF by attenuating excessive inflammation. However, to ensure an effective MFQ concentration at the lesion site, high doses of oral or intravenous MFQ are required, which can elicit serious adverse events, such as hallucination, and liver and kidney function damage [22,23].

Electrospinning technology is a readily available, effective method for the acquisition of bio-inspired nanofibers, owing to its unique advantages of primary original porosity and heterogeneous distribution [24-27]. Given these advantages, this technology has been widely used in many fields, such as filtration, catalysis, optoelectronics, and particularly biomedical materials, in recent years [28-31]. Herein, sustained release of MFQ from electrospun fibrous membranes was found to maintain an effective drug concentration at the local site, effectively decrease EF and inflammation-associated pain, and alleviate sensory neuropathy, through inhibition of the PanX1/NLRP3 pathway (Fig. 1d and e).

In this study, we synthesized an MFQ-grafted PLA and prepared it into a functional nanofiber membrane via electrospinning. We showed that PLA-MFQ grafted membranes possess favorable anti-fibrosis effects after laminectomy as evidenced by a series of histopathological, histomorphometric, and functional experiments. We also demonstrated that PLA-MFQ grafted membranes can provide sustained anti-inflammatory effects and optimal alleviation of fibrosis-associated neuropathic pain. Overall, our study indicates that the PLA-MFQ grafted membranes may serve as a favorable translational therapeutic approach to treat epidural fibrosis and associated recurrent low back pain.

2. Materials and methods

2.1. Materials

Polyactic acid (PLA, Mw 80 kDa) was purchased from Rhawn Chemicals Co., Ltd. (Shanghai). MFQ hydrochloride, 1-(3-
Fig. 2. Characterization, hydrophilicity, mechanical properties, and drug release properties of different membranes. (a–c) SEM observation of PLA, PLA-MFQ, and PLA-MFQ grafted membranes by 10.0 k × magnification, respectively. (d–f) Frequency of fibrous diameter distribution of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (g–i) Images of water contact angles of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (j) Water contact angles for PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (k) Mechanical (strain %) curves of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (l) Moduli of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (m) Tensile stress of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. (n) Percentage MFQ release from PLA-MFQ and PLA-MFQ grafted membranes. (o) Mass residual percentages of PLA, PLA-MFQ, and PLA-MFQ grafted membranes (n = 3) (*) denotes p < 0.05.
dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), and dichloromethane were provided by Shanghai Aladdin Biochemical Technology Co., Ltd. HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) was obtained from Da-Rui Fine Chemical Co., Ltd. A Cell Counting Assay Kit-8 CCK8 was purchased from Dojindo China Co., Ltd. Phalloidin was purchased from Dojindo China Co., Ltd. (Shanghai). Fibronectin, anti-CD31 (catalog #: ab9498), and anti-CGRP (catalog #: ab36001) were purchased from Abcam. Anti-bodies to CD68 (catalog #: 97,778), NeuN (catalog #: 94403s), a-smooth muscle actin (a-SMA) (catalog #: 19245s), p-CREB (catalog #: 9197s), and TNF-α were obtained from Cell Signaling Technologies. Anti-IL-1β was purchased from Proteintech Group, Inc.

2.2. Synthesis of MFQ-grafted PLA

MFQ-grafted PLA was synthesized via EDCI/DMAP coupling method, by which the hydroxyl groups of MFQ and carboxyl groups of PLA were covalently conjugated through esterification reaction. Briefly, 3 g PLA and 0.3 g were uniformly mixed and dissolved in 50 mL dichloromethane in a 100 mL round bottom flask to obtain a uniform solution. Then 0.204 g EDCI and 0.086 g DMAP were added into the solution and gently stirred at room temperature for 6 h. After that, the mixture was precipitated in deionized water to give a white precipitate followed by extraction in dichloromethane and this procedure was repeated three times. The obtained product was dehydrated with anhydrous sodium sulfate in a rotary evaporator and stored in a desiccator for further use.

2.3. Fabrication of electrospun fibrous membranes

Electrospun fibrous membranes were prepared as described in our previous reports [32,33]. Briefly, polymers (PLA and MFQ-grafted PLA) were separately stirred in HFIP at a concentration of 10% (w/v) at room temperature until fully dissolved. The solution was fed at 1.5 mL/h through a blunt needle and electrospun with 15 kV high voltage to generate nanofibers. Electrospun nanofibers were collected with a slow-speed mandrel (6 cm diameter, 120 rpm) placed 10 cm from the needle. To obtain MFQ-blended nanofibers, we added 1% (w/v) MFQ to the PLA solution and performed electrospinning under the same conditions. Electrospun membranes obtained from pure PLA, MFQ-blended PLA, and MFQ-grafted PLA were referred to as PLA, PLA-MFQ, and PLA-MFQ grafted, respectively.

2.4. Characterisation of electrospun fibrous membranes

The micro-morphological features of the electrospun fibrous membranes were assessed with scanning electron microscopy (SEM, FEI Quanta 200, Netherlands) at an acceleration voltage of 21 kV, in at least five images per sample (10,000 × magnification). Final assessment was conducted with random views of each sample. The diameters of the nanofibers were calculated in ImageJ software (Bethesda, MD, USA). To investigate the tensile strength of fibrous membrane sections, we cut electrospun sections into similar dimensions of 1 cm × 1 cm (length × width). The tensile strength of the fabricated fibrous membranes was

Fig. 3. PLA-MFQ membranes inhibit fibroblast proliferation. (a) Immunofluorescence micrographs of chicken embryonic fibroblasts (UMNSAH/DF-1) incubated in control conditions or with different membranes for 1 and 4 days, respectively. The nuclei are stained blue, and the cytoskeleton was stained red. (b) Relative cell viability (OD) after 1 and 4 days by CCK8 assay, respectively: (n = 6) (*) denotes p < 0.05, (**) denotes p < 0.01, (***) denotes p < 0.001, vs control group. (i) denotes p < 0.05, (ii) denotes p < 0.01, (iii) denotes p < 0.001, vs PLA group. Scale bar, 100 μm.
evaluated with a mechanical machine (Instron 5567, Norwood, MA). In addition, stress-strain curves were established through documentation of load-deformation at a speed of 0.5 mm/s. A static contact angle measuring device with DSA 1.8 software (KRUSS, Hamburg, Germany) was used to determine the wettability of the electrospun membranes.

2.5. MFQ release of fibrous membranes

To test the MFQ release characteristics, we immersed exactly 100 mg of each membrane sample in 20 mL of phosphate-buffered saline (PBS, pH 7.4) with lipase (0.05 mg/mL). The release kinetics of MFQ was examined in a thermostatic shaking water bath (37°C–4°C, Tai chang Medical Apparatus Co., Jiangsu, China) at a frequency of 100 cycles per minute. Equal portions of release buffer (5.0 mL each) were collected and refreshed with 5.0 mL PBS at predetermined time points. The release of MFQ was determined by spectrophotometry (Shimadzu, Japan) at UV-2550.

2.6. In vitro degradation of electrospun fibrous membranes

The extent of membrane degradation was examined by monitoring the weight loss of the membranes. Drug-loaded PLA membranes (approximately 100 mg each) were soaked in PBS (50 mL) at 37°C. Next, membrane samples were harvested, washed with pure water, and dried under vacuum at each scheduled time point. By documenting the ratio of residual dry weight and the initial weight of the material, we calculated mass loss gravimetrically.

2.7. In vitro cell culture

Chicken embryonic fibroblasts (UMNSAH/DF-1) were cultured to investigate the cytocompatibility and cytotoxicity on the surfaces of PLA, PLA-MFQ, and PLA-MFQ grafted membranes. Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% antibiotic (10,000 units penicillin, 10,000 μg streptomycin) was used to culture the cells at 37°C and 5% CO2, and the medium was changed every 2–3 days. The cells were digested with 0.25% trypsin and washed with PBS for 90 min to achieve timely collection. Each well of a 24-well plate contained 100 mL per well of culture medium with a density of 1 × 10^5 cells/mL.

2.8. Cell proliferation and viability assays

A 100 μL volume of cell suspension (1 × 10^5 cells/mL) was inoculated into each well of a 96-well plate and incubated for 1 or 4 days (37°C, 5% carbon dioxide) to allow for cell adhesion and proliferation. At days 1 and 4, cells were cultured in medium mixed with 10% volume (10 μL) of cell counting kit-8 (CCK-8, Dojindo, Japan) reagent. The absorbance at 450 nm was measured with a microplate reader (Multiskan Mk3, Thermo Fisher, USA). The cell number was determined with the CCK-8 assay kit. Cells in each well were cultured at a density of 4 × 10^4/cm^2 for 1 day and 4 days in 24-well plates. The fibrotic cells were fixed in 4% formaldehyde and permeabilized with 0.5% (v/v) Triton X-100, then stained with rhodamine-phalloidin and 4′,6-diamidino-2-phenylindole dihydrochloride. Finally, the stained cells were observed under an immunofluorescence microscope (Olympus IX73). The cytoskeleton of cells was bright brown, thus revealing the morphology of living cells. All experiments were repeated three times.

2.9. Cell co-culture and treatment

RAW264.7 macrophages were treated with LPS (1 μg/mL) to simulate an inflammatory environment. MFQ, MDC950 (an NLRP3 inhibitor), or MFQ/MDC950 was added into the co-culture system. The control group did not receive any treatment. The co-culture of NIH-3T3 cells (lower chamber) and the RAW264.7 cells (upper chamber) was performed in a
Transwell plate with a permeable membrane separating the upper and lower media. The cells were cultured in high-glucose DMEM medium supplemented with penicillin-streptomycin and 10% FBS in a humid environment at 37 °C and 5% CO₂. Cells were incubated in a constant temperature incubator, and the drug treatment duration was 24 h.

2.10. Western blotting

Total protein from the cells was extracted via cell lysis in RIPA buffer (WB0101; Aowei) containing 1 mmol/L PMSF. The concentration was determined with a BCA assay. Equal amounts of protein were separated with 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 2.5% skim milk, the following primary antibodies were incubated at 4 °C overnight: anti-fibronectin (catalog #: ab268020; 1:1000), anti-NLRP3 (catalog #: ab263899; 1:1000), and anti-β-actin (catalog #: WB0196; 1:2000). The next day, the membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson; 1:2000) for 1 h at room temperature. The protein signals were detected with ECL luminescence reagent. The protein level indicated by the band intensity was analyzed in ImageJ image analysis software.

2.11. Animal study

The ethics and safety of animal experiments were approved and performed under the guidance of Chang Zheng Hospital of Naval Military Medical University. Sprague Dawley rats (200 ± 20 g) (provided by Shanghai SLAC Laboratory Co., Ltd.) were divided into four groups with ten rats per group at each time point. Anesthesia was induced by intramuscular ketamine injection (20 mg/kg). The second through fifth lumbar vertebral spinous processes were dissected under a microscope through a dorsal approach, by opening the lamina, exposing the spinal cord, and protecting the dura. PLA, PLA-MFQ, and PLA-MFQ-grafted electrostatically spun fiber membranes were placed on the surface of the spinal cord. The control group was not treated. After implantation, the incisions were sutured, and all rats were raised in captivity without restriction of activity. Animals with dura lesions, postoperative infection, and/or immediate spinal cord injury were excluded from the study. At predetermined time points, EF, neurological function, and inflammation were assessed. Finally, the rats were killed with a lethal dose of anesthesia. The intact vertebral columns at the modeling site were resected for histological and immune molecular examination.

2.12. Epidural fibrosis assessment

2.12.1. Magnetic resonance imaging (MRI) assessment of EF

MRI analysis was performed on animals at weeks 4 and 8 post-surgery to study EF. All MR images were collected with a Bruker 7.0 T Micro-MRI imaging system. After locating the image, we performed both sagittal T1/T2-weighted and transverse T1/T2-weighted imaging sequences (TR = 3000 ms, TE = 26.5 ms, slice thickness = 1 mm). The range of EF was assessed according to the methods established by Ross et al. [34–36]: grade 0 indicated no EF; grade 1 indicated more than 0% but less than 25% EF of the cross-sectional area of each quadrant; grade 2 indicated more than 25% but less than 50%; grade 3 indicated more than 50% but less than 75%; and grade 4 indicated more than 75% EF. The results were assessed independently by two senior diagnostic imaging technologists who were blinded to the treatments.
2.12.2. Assessment of the extent of fibrosis

Vertebral columns were collected at 4 and 8 weeks and fixed in 4% paraformaldehyde buffer (pH 7.4) for 5 days. Subsequently, decalcification was performed at 37 °C for 4 weeks at 10% EDTA (pH 7.4). Next, the samples were dehydrated with different concentrations of ethanol and embedded in paraffin. The section thickness was 4 μm. The cross-sections were observed with hematoxylin & eosin (H&E) and Masson’s trichrome staining for qualitative analysis.

The wound tissues were sliced into 4 μm sections and deparaffinized. Briefly, sections were blocked with 1% BSA and 0.3% Triton X-100 for 30 min at 37 °C. Then the slides were incubated overnight at 4 °C with specific primary antibodies against fibronectin. After flushing with PBS, the slides were incubated with rabbit anti-fibronectin for 2 h at room temperature. Finally, the nuclei were stained with DAPI. The immunofluorescence was observed with an Olympus IX73 fluorescence microscope.

The degree of EF was scored on the following scale [37]: grade 0, indicating no EF, no adhesion tissue invading the spinal canal, and no epidural adhesion tissue; grade 1, with part of the adhesive tissue invading the spinal canal edge without entry into the spinal canal, with only a small amount of fibrous bands on the dura; grade 2, with the corresponding fibrous tissue invading the spinal canal, and an adhesion rate less than 2/3 of the surgical defect; grade 3, with fibrous tissue infiltration and an adhesion rate greater than 2/3 of the surgical defect; grade 4, with complete invasion of the dural sac by fibrous tissue, involving the nonoperative side and/or nerve roots; and grade 5, with deformation and contraction of the entire sheath accompanied by extensive fibrous tissue.

2.12.3. Histological examination of fibroblast infiltration

Fibroblast infiltration was examined histologically to evaluate the anti-adhesion ability of the different membranes. Qualitative analysis was performed on H&E stained sections and scored with the following scale [38]: grade 1, <100 fibroblasts/field (400 × magnification); grade 2, 100–150 fibroblasts/field; and grade 3, >150 fibroblasts/field. The examination was conducted by two blinded independent investigators.

2.13. Nerve function assessment

2.13.1. Nerve function assessment of ultrastructural neurodegeneration

Transmission electron microscopy (TEM, HT7700 120 kV, HITACHI) was performed at 4 and 8 weeks after surgical membrane placement to observe the ultrastructure of neurodegeneration. Spinal nerves were immobilized at 4 °C in 2.5% glutaraldehyde buffer (pH 7.4) for 1 day, then immobilized with 1% osmium tetroxide, dehydrated with acetone, and embedded in epoxy resin. The spinal nerves were sliced into ultra-thin sections. After staining with uranyl acetate and lead citrate, the nerve function was examined through TEM. Immunofluorescence staining was performed with a standard protocol.

2.13.2. Molecular biological detection of dorsal root ganglia (DRG) of the spinal cord

Immunofluorescence staining was performed with a standard protocol. Briefly, at predetermined time points, the sections were incubated with primary antibodies to mouse NeuN (CST, 1:400), CGRP (Abcam, 1:200), and p-CREB (CST, 1:200) overnight at 4 °C. Secondary antibodies were used in immunofluorescence analysis to detect fluorescence signals after counterstaining with DAPI.

2.13.3. Nerve function assessment on the basis of pain

For pain assessment, hot plate tests were performed at predetermined time points [39]. The rats were held on the plate, and the time until a hind paw response (licking) was recorded as the pain threshold. The laboratory instrument consisted of a metal plate that was heated and maintained at a steady temperature (54 ± 0.5 °C) and monitored by...
Z. Yue et al. Materials Today Bio 17 (2022) 100469

an additional digital thermometer. The heated surface was fenced by a transparent cylinder (height 40 cm) to prevent the laboratory animals from jumping off the plate. Furthermore, mechanical allodynia was measured with von Frey filaments [40]. The left paw was vertically stimulated with a series of von Frey filaments with increasing stiffness (0.4–26 g). Stimuli were executed intermittently for several seconds, thus

Fig. 7. Nerve function assessment at both examination times postoperatively. (a) Representative triple-immunoﬂuorescence images of DRG neurons, in which neurogenic inﬂammation and stimulation are characterized by the presence of CGRP (pink) and p-CREB (red), and the neurons are indicated by NeuN (green). DAPI stained the nuclei blue. (b) Representative TEM images of DRG morphology, in which an abnormal structure of neurons is characterized by the stratiﬁcation of myelin. (c) The PLA-MFQ grafted group showed a signiﬁcant improvement in mechanical allodynia: (n = 6). (*) denotes p < 0.05, (**) denotes p < 0.01, (***) denotes p < 0.001. Scale bar, 20 μm.

Fig. 8. Immunoﬂuorescence assessment of NLRP3 and the density of macrophages in EF at both examination time points postoperatively. (a) Representative immunoﬂuorescence imaging showing highly decreased expression of NLRP3 (red) in the wound tissues with PLA-MFQ grafted membranes treatment. (b) Quantitative analysis of the relative ﬂuorescence intensity of NLRP3 (n = 10): (*) denotes p < 0.05, (**) denotes p < 0.01. (c) Representative IF imaging shows macrophages (CD68-positive) in green and cell nuclei in blue. (d) Quantitative analysis of macrophages (n = 10): (*) denotes p < 0.05, (**) denotes p < 0.01. Scale bar, 20 μm.
allowing for clear observation of any limb responses to previous stimuli. A positive response was noted if the paw was sharply withdrawn [41].

2.14. Inflammation assessment

2.14.1. Immunofluorescence of tissue around EF

The expression of inflammatory response and neovascularization markers (NLRP3, CD68, CD31, and α-SMA) was investigated with immunofluorescence histochemical staining in EF tissues. First, the slices were deparaffinized with xylene and dehydrated in a graded ethanol series. The slices were then immersed for 10 min in methanol containing 3% hydrogen peroxide. To extract antigens, we placed tissue slices in 10 mM citric acid buffer (pH 6.0) and treated them for 10 min at 100 °C for antigen recovery. Primary antibodies to CD68 (CST, 1:400), CD31 (Abcam, 1:500), α-SMA (CST, 1:400), and NLRP3 (NOVUS, 1:400) were incubated and stored overnight at 4 °C. After slices were washed with PBS three times, rabbit anti-CD68, anti-CD31, anti-α-SMA, and anti-NLRP3 were incubated for 2 h at room temperature. The peroxidation reaction was used for chromogenic detection, and DAPI was used for re-staining. For quantification, all results of molecular immunofluorescence expression were detected at the membrane implantation site with ImageJ software. The tissue area of each sample (n = 10) was normalized, and the degree of inflammation in each region was measured.

2.14.2. Tissue mRNA expression, determined by RT-PCR after different treatments

Inflammation was investigated with quantitative real-time PCR detection of inflammatory factors. The RT-PCR assays were performed with a PCR system (Applied Biosystems, ViiA™ 7 system) to evaluate the relative IL-1β, TNF-α, and NLRP3 mRNA levels in EF. A housekeeping gene (GAPDH) was used as an internal control. The sequences of primers for target genes are presented in Table 1.

2.15. Statistical analysis

In general, all data are presented as the mean ± standard deviation. Statistical analyses were evaluated in GraphPad Prism 8 with one-way analysis of variance between groups. A difference was considered statistically significant at p < 0.05. Analysis was performed in SPSS 24.0 statistical software (Chicago, IL, USA).
3. Results

3.1. Characterization of fibrous membranes

Electrospun membranes showed dense random nanofiber configurations (Fig. 2a–c), with fiber diameter sizes ranging from 100 to 400 nm, and average diameters of ~170–190 nm (Fig. 2d–f). Furthermore, these membranes were highly hydrophobic (Fig. 2g–i), as evidenced by contact angles of ~120°–130° (Fig. 2j). These results indicated that incorporation of MFQ into the electrospun membranes by either blending or covalent grafting did not affect the electrospinning processes. In contrast, incorporated MFQ compromised the mechanical properties (Fig. 2k) by decreasing the UTS and modulus from 25 MPa to 60 MPa for the PLA membrane to 12 MPa and 40 MPa for the PLA-MFQ grafted membrane (Fig. 2l and m), respectively. UV–vis spectrophotometry showed that the grafting rate of PLA-MFQ(G) was 88 ± 4% (Supplementary Fig. 1).

3.2. In vitro drug release and fibrous membrane degradation

The PLA-MFQ membranes showed short-term drug dissociation from PLA with a burst release of approximately 59% in the initial week and almost complete release at 5 weeks in PBS (Fig. 2n). In contrast, with the PLA-MFQ grafted membranes, MFQ was continuously released from PLA within at least 8 weeks, with an approximately 51% burst release in the initial week. By testing the ratio of degradation, we performed gravimetric analysis (Fig. 2o). The total mass loss was 67%, 74%, and 76% for PLA, PLA-MFQ, and PLA-MFQ grafted membranes, respectively.

3.3. Cell proliferation, viability, and migration

To examine the adhesion and proliferation of fibroblasts exposed to surfaces of PLA, PLA-MFQ, and PLA-MFQ grafted membranes, we cultured chicken embryonic fibroblasts (UMNSAH/DF-1) with different fibrous membranes and determined the cell viability at 1 day and 4 days after treatment. Cell viability decreased after treatment with PLA-MFQ and PLA-MFQ grafted membranes. The cell shapes were examined with rhodamine-phalloidin staining (Fig. 3a). No membranes caused clear morphological damage in fibroblasts (UMNSAH/DF-1). The cell viability and cytotoxicity were determined with CCK-8 assays and a TUNEL assay kit (Fig. 3a and b, Supplementary Fig. 2a and b and 2c). The PLA-MFQ grafted membranes showed greater inhibition of fibroblast proliferation at each successive time point. In addition, according to Transwell assays, the number of migrated fibroblasts was significantly lower in the PLA-MFQ and PLA-MFQ grafted membranes treated groups than that in the control groups, thus suggesting that released MFQ inhibits the migration capability of fibroblasts (Supplementary Figs. 2d and e).

3.4. Mefloquine inhibits expression of fibronectin in NIH-3T3 cells in the LPS-treated co-culture system

According to Transwell assays, the expression of fibronectin in the NIH-3T3 cells in the lower chamber was significantly lower in the MFQ/LPS-treated group than that in the LPS-treated group (Supplementary Figs. 5a and b). The MFQ/LPS-treated group showed a similar inhibitory level to the MCC950/LPS-treated group, thus suggesting that the inhibitory effect of MFQ on the fibronectin expression of fibroblast occurred through the PanX1/NLRP3 pathway.

3.5. In vivo assessment of EF

At both preset time points after laminectomy (4 and 8 weeks), all experimental rats were evaluated for post-operative EF with MRI. The MRI analysis showed severe hyperplastic fibrous tissues around the dural sac, and even compressing the spinal cord, after laminectomy in the control group, whereas only a slight peripheral flange was detected around the dural sac, with no direct adhesion on the dura in the PLA-MFQ grafted membranes treatment group in both T2-weighted sagittal MR and T1-weighted transversal MR images (Fig. 4a). Consistently, quantitative analysis of MRI-based grading scores revealed similar trends across groups [34,36] (Fig. 4b). After laminectomy at L2–L5, different membranes were precisely implanted on the dura mater (Fig. 4c).

To achieve clear and detailed visualization of the surgical site, we sacrificed the mice to dissect intact lesion tissue, which was processed for histological analysis (H&E and Masson staining) after MRI examination. The excessive proliferation of fibrous tissues clearly extended to the spinal cord and completely compressed the outside dural canal in the control groups. Only slight fibrous tissue was detected between the spinal cord and paravertebral tissues, and the subarachnoid space was relatively unpressed in the PLA-MFQ grafted group (Fig. 5a). At each examination time point, the control group had the highest EF grade (Fig. 5b). The PLA-MFQ group showed a lower average grade than the control (both time points) and PLA (only at latter time points) groups. Clear differences were observed between the PLA-MFQ grafted group and the control group at both time points, particularly at week 8. Furthermore, the degree of EF in the PLA-MFQ grafted group gradually indicated an advantage over the PLA-MFQ group at the latter examination time point. The fibrolamellar shape and thickness were examined with Masson staining. The fibrous tissues significantly decreased in thickness in the epidural space of the PLA-MFQ grafted group (Fig. 5c).

The density of fibroblast infiltration in epidural scar tissue, indicating the grade of EF [42,43], is shown in Fig. 6. Fibroblast density was highly correlated with the grade of EF (Fig. 6a). At both time points, no decrease in the fibroblast infiltration grades was observed in any groups, and the score for the PLA-MFQ grafted group was lowest (Fig. 6b). Possible reasons for the mild increase in fibroblast cell score between time points were the recruitment of inflammatory cells and cytokines, and fibroblast dispersion from posterior adjacent muscles and the fluid circulation [44, 45]. At both examination time points, the fibroblast infiltration grade of all membrane-treated groups was superior to that of the control, whereas the fibroblast density in the PLA-MFQ grafted group was the lowest among all groups, particularly at 8 weeks. Overall, a highly positive correlation between the grade of fibroblast infiltration and the extent of EF was indicated. In addition, fibronectin, a high molecular weight glycoprotein highly expressed in fibroblasts [46], was examined in the scar tissues. Compared with that in the control group, the expression of fibronectin in the PLA-MFQ grafted group was much lower in epidural tissue (Fig. 6c).

3.6. In vivo assessment of nerve function

Calcitonin gene-associated peptide (CGRP), a neuropeptide, is widely expressed in the nervous system, including both peripheral and central parts. The release of CGRP from sensory nerves has been found to be an effective indicator of neurogenic inflammation [47,48]. cAMP response element-binding protein (CREB) is a protein present in dimeric form in the nuclei of eukaryotic cells; the activated, phosphorylated form of CREB induces the expression of genes activating neuronal functions including cognition, long-term memory, the response to oxidative stress injury, and the response to neural ischemia and reperfusion [49,50]. We performed immunofluorescence staining of NeuN, CGRP, and p-CREB to assess nerve function.

After 4 weeks, the p-CREB in DRG in the PLA-MFQ grafted group was significantly more difficult to detect than that in the other three groups, particularly the control group, yet this distinction persisted by week 8. CGRP showed similar trends (Fig. 7a). The p-CREB and CGRP in the control group were significantly higher than those in the PLA, PLA-MFQ, and PLA-MFQ grafted groups at both examination times. The changes in immunofluorescence expression of p-CREB and CGRP in DRGs were associated with the mean grades of EF and the fibroblast density.

Furthermore, observation of the ultrastructure of myelin under TEM indicated possible ultrastructural pathomorphological changes in laboratory animals exposed to different treatments (Fig. 7b). Stratification of
myelin has been shown to be a possible indicator of neurodegeneration [51]. At predetermined time points, abnormal structure of myelin was detectable in the control and PLA groups, and the stratification of myelin was diminished and was rarely detected in the PLA-MFQ grafted groups.

In addition, von Frey and hot plate tests were conducted at 4 and 8 weeks after laminectomy as criteria for mechanical allodynia assessment [40,52,53] (Fig. 7c). Statistical differences were not found in the two tests between the control and PLA groups. In the PLA-MFQ and PLA-MFQ grafted groups, the paw response threshold was greater than that in the control group at each time point after implantation, whereas the PLA-MFQ grafted group showed a better effect of mitigation of hypersensitivity of postoperative neuralgia. These behavioral changes suggested that implantation of PLA-MFQ grafted membranes after lumbar laminectomy produced progressive development of neuropathic pain.

3.7. In vitro and in vivo evaluation of inflammation

We performed in vitro cell Transwell assays to examine the migration capability of inflammatory macrophages (RAW264.7). The numbers of migrating macrophages were significantly lower in the PLA-MFQ and PLA-MFQ grafted treated groups than that in the control groups, thus suggesting that released MFQ may attenuate the recruitment of macrophages (Supplementary Figs. 3a and b). Additionally, the protein level of NLRP3 in macrophages with LPS stimulation was significantly lower after MFQ treatment than LPS stimulation (Supplementary Figs. 4a and b). Simultaneously, the expression of inflammatory cytokines (IL-1β and TNF-α) was also decreased by MFQ treatment under LPS stimulation (Supplementary Figs. 4c and d).

Previous studies have revealed that neovascularization not only promotes persistent migration of inflammatory cells to lesion sites, but also is further affected by inflammation and oxidative stress [54,55], we tested markers of inflammation and angiogenesis. At each examined time point, the expression of NLRP3, CD68, α-SMA, and CD31 in EF tissues was detected by IF staining.

Compared with the control, the PLA-MFQ grafted membranes resulted in significantly lower expression of NLRP3 at the surgical sites. This trend was more clearly observed at week 8 (Fig. 8a and b). Moreover, the amounts of CD68-positive macrophages around PLA, PLA-MFQ, and PLA-MFQ grafted membranes gradually decreased and were clearly lower than those in the control group. The PLA-MFQ group showed a slight decline in the amounts of macrophages at 8 weeks, whereas that in the PLA-MFQ grafted group clearly decreased (Fig. 8c). Consistently, IF staining revealed the lowest macrophage count in the PLA-MFQ grafted group at week 8 postoperatively (Fig. 8d).

Moreover, IF analysis indicated significantly fewer α-SMA-positive and CD31-positive vessels around PLA-MFQ grafted membranes than in other groups at the 4- and 8-week examination time points, thus indicating that PLA-MFQ grafted membranes inhibited neovascularization at laminectomy sites. We also quantitatively evaluated the density of α-SMA-positive and CD31-positive vessels (Fig. 9a–d).

We additionally verified the expression of the mRNA levels of NLRP3, IL-1β, and TNF-α, to provide a more comprehensive understanding of the anti-inflammatory effects of the different fibrous membranes at predetermined time points (Fig. 10a and b and Supplementary Fig. 6). The mRNA expression of NLRP3, IL-1β, and TNF-α in the PLA-MFQ grafted group was the lowest among the four research groups, whereas the expression of IL-1β and TNF-α in the control group was highest among all groups. Distinct differences in the mRNA levels of NLRP3, IL-1β, and TNF-α at 4 and 8 weeks post-laminectomy were observed between the control and PLA-MFQ grafted groups.

4. Discussion

Laminectomy is the most commonly used technique during spinal surgery to explore the spinal canal and decompress the neural tissues. After lumbar laminectomy, EF, one of the most common complications, may occur during the repair response to the wound [56]. Furthermore, with excessive scar tissue formation around the epidural sac after surgery, extensive adhesion can compress or invade the epidural sac and nerve root and is an important cause of FBSS [6,57]. In scar formation, inflammatory cytokines and cell infiltration and proliferation play crucial roles. For example, IL-1β has been suggested to be a potent proinflammatory cytokine in fibrosis progression in many organs; it is synthesized as a precursor protein after stimulation of monocytes and/or macrophages [58–60]. The maturation and secretion of IL-1 are activated by NLRP3 in a protein-complex called the inflammasomes [61,62]. MFQ acts as not only a potent antimalarial drug but also as a broad-spectrum PanX1 inhibitor [19], which can decrease the downstream expression of NLRP3, alleviate the inflammatory response, and finally ameliorate tissue damage and decrease tissue fibrosis formation [20,63].

In recent years, with the extensive development of biomaterials, many strategies have been used to prevent epidural fibrotic adhesion. In our study, we successfully fabricated an MFQ-grafted membrane providing continuous drug release for fibroblast and fibrosis inhibition through electrospinning. Electrospinning is a traditional strategy to fabricate tissue engineering implants that provides many advantages, such as stable topology, porous structure, and excellent mechanical properties for maintaining effective local drug concentrations in vivo [24,25].

According to various reports, biomaterial membranes have been introduced to attenuate inflammation and EF [15,64–66]. In this study, we successfully fabricated PLA-MFQ grafted membrane through electrospinning [67]. In vitro drug release assays showed that the PLA-MFQ grafted membrane maintained nearly twice the drug release time of the PLA-MFQ membrane. This finding suggests that long-term fibroblast and fibrosis inhibition can be achieved by delivery of relatively effective and stable drug concentrations to local surgery sites in vivo.

Cell proliferation assays indicated that PLA-MFQ grafted and PLA-MFQ membranes inhibited fibroblast proliferation to a greater extent than observed in the PLA, and control groups. However, the inhibitory effects of macrophages (RAW264.7) disappeared (Supplementary Fig. 3c).

The process through which granulation gradually transforms into scar tissue lasts approximately 4 weeks. Furthermore, epidural scar maturation lasts approximately 2 months. Therefore, 4 weeks after surgery, the effect of antiadhesions after lumbar laminectomy could be observed, thus meeting the experimental requirements. We chose 4 and 8 weeks as the time points to collect imaging data and histological samples. In our in vivo study, we demonstrated that PLA-MFQ grafted membranes inhibited EF formation and fibroblast proliferation at the two time points, particularly at 8 weeks, with a lower EF grade, according to MRI, H&E staining, Masson staining, and fibronectin expression than those of the PLA-MFQ group, PLA group, and control group. We observed a better EF inhibitory effect at 8 weeks, owing to the continuous drug release; in an in vitro study, the PLA-MFQ grafted group, compared with the PLA-MFQ group, showed prolonged drug release after 5 weeks. Based on these changes, the physical barrier may decrease the recruitment of acute damage-associated molecular-pattern molecules triggered in response to surgery, and the sustained MFQ release may inhibit the secondarily local pathophysiological process of EF to some extent, thus indicating the relationships among inflammation and postoperative EF and neuropathic pain.

Robertson et al. have reported that excessive peridural scarring results in chemical and mechanical consequences for nerve tissue, thereby causing root-associated pain [68,69]. In addition, this pain might be aggravated by the presence of chronic inflammation at the lesion site. In this study, the PLA-MFQ grafted membranes decreased the abnormal activation of dorsal root ganglia by decreasing the expression of relevant markers such as CGRP and p-CREB, and mitigating stratification of the myelin, according to TEM imaging of neurons. All these data together demonstrated that long-term stable disassociation of MFQ from PLA-MFQ grafted membranes resulted in better amelioration of nerve deficits, and
alleviation of neuropathic pain.

Several studies have demonstrated the targeted transport of drugs to treatment sites by nanoparticles, which demonstrate better therapeutic effects than systemic administration [70,71]. We assumed that MFQ nanoparticles released from PLA-MFQ grafted membranes delivered MFQ to target therapeutic sites, decreased the local inflammatory response, and inhibited angiogenesis, thereby decreasing the expression of relevant markers such as NLRP3, CD 68, a-SMA, and CD 31. The decreased expression of NLRP3 in immunofluorescence analysis indicated that MFQ significantly inhibited NLRP3 activation through PANX1/NLRP3 signaling. In addition, the PLA-MFQ grafted membranes, with their effects on expression of CD 68, a-SMA, and CD 31, clearly decreased the recruitment of macrophages and inhibited the progression of neovascularization.

Here we demonstrated that the PLA-MFQ grafted membranes ameliorated epidural fibrosis after lumbar laminectomy. PLA-MFQ grafted membranes offered sustained drug release properties to achieve prolonged anti-inflammatory effects, and it also served as a physical barrier simultaneously. It could serve as a potential candidate for future clinical applications. However, our study has some limitations, first, here we showed that MFQ acted on macrophage PanX1/NLRP3 pathway to fulfill its anti-inflammatory function, however, NLRP3 may not be the only response element located downstream of PanX1. Therefore, we did not exclude the possibility that other molecular events could occur other that NLRP3 pathway activation downstream of PanX1 signaling. Moreover, concerning the potential of future clinical application of PLA-MFQ grafted membranes, more experimental evidence in larger animals such as non-human primates is needed. Collectively, our study revealed that PLA-MFQ grafted membranes may serve as a potential favorable candidate for the treatment of epidural fibrosis and associated recurrent low back pain.

5. Conclusions

FBSS remains a primary cause of recurrent discomfort in patients receiving spinal surgery, and innovative prevention strategies are urgently needed. Herein, MFQ-grafted copolymers were successfully synthesized to prepare biodegradable electrospinning fiber membranes that maintained stable release of MFQ for at least approximately 8 weeks in vitro. When measured as a drug-delivery bio-membrane with long-term stable release in a laminectomy rat model, the PLA-MFQ grafted membranes significantly improved adhesion resistance beyond that of the control (untreated) group, only membranes, and MFQ-loaded membranes, according to histological analysis. In addition, anti-inflammatory effects based on macrophages, neo-vascularization, and inflammatorymasomes, as well as gene expression (IL-1β and TNF-α), significantly decreased in laboratory animals with implantation of PLA-MFQ grafted membranes. Furthermore, neurological tests showed that the PLA-MFQ grafted membranes were also more effective in decreasing neuropathic pain and supporting sensory neurological function than the other treatments. Therefore, because of the continual anti-inflammatory activity mediated by MFQ, PLA-MFQ grafted membranes may decrease FBSS by effectively inhibiting EF.

Author contributions

Study conception and design: Huajian Zhong, Jinglei Wu, and Tao Zhang. Acquisition of data: Zhihao Yue, Bo Hu, Zhe Chen, Ganjiang Zheng, Peng Cao, and Xiaodong Wu. Analysis and interpretation of data: Zhihao Yue, Yunhao Wang, Chen Yang, Jing Li, Jianxi Wang, and Fazhi Zang, Lei Liang. All authors made substantial contributions in revising this manuscript for intellectual content and approved the final version to be published. Tao Zhang (zhangtao12345678@126.com), Jinglei Wu (jw@dhu.edu.cn), and Huajian Chen (spine_cenjb@163.com) had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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