Preparation and Evaluation of Decellularized Epineurium as an Anti-Adhesive Biofilm in Peripheral Nerve Repair

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Abstract

Following peripheral nerve anastomosis, the anastomotic site is prone to adhesions with surrounding tissues, consequently impacting the effectiveness of nerve repair. This study explores the development and efficacy of a decellularized epineurium as an anti-adhesive biofilm in peripheral nerve repair. Firstly, the entire epineurium was extracted from fresh porcine sciatic nerves, followed by a decellularization process. The decellularization efficiency was then thoroughly assessed. Subsequently, the decellularized epineurium underwent proteomic analysis to determine the remaining bioactive components. To ensure biosafety, the decellularized epineurium underwent cytotoxicity assays, hemolysis tests, cell affinity assays, and assessments of immune response following subcutaneous implantation. Finally, the functionality of the biofilm was determined using a sciatic nerve transection and anastomosis model in rat. The result indicated that the decellularization process effectively removed cellular components from the epineurium while preserving a number of bioactive molecules, and this decellularized epineurium was effective in preventing adhesion while promoting nerve repairment and functional recovery. In conclusion, the decellularized epineurium represents a novel and promising anti-adhesion biofilm for enhancing surgical outcomes of peripheral nerve repair.

Key Words: Peripheral nerve; Anastomatic adhesions; Epineurium;
Decellularization; Proteomics; Anti-adhesion

Graphical abstract

1. Introduction

Peripheral nerve injury (PNI) is a typical disease leading to motor, sensory, and trophic impairment in innervated region [1]. Annually, over 1 million individuals globally are afflicted with PNI, with approximately 300,000–500,000 cases reported in China, constituting 2.8% of all trauma cases [2], which imposes a significant medical and economic burden on society. Recently, due to the fast development of microsurgical techniques, the quality of nerve anastomosis has been greatly improved [3, 4]. Nevertheless, a significant challenge associated with neuroanastomosis is the development of fibroblast scars at the neuroanastomosis site [5]. Even in adequately repaired nerves, roughly 50% of the regenerated axons might extend into tissue scarring, potentially resulting in a localized neuroma and impeding axon regeneration toward its intended target [6]. Consequently, regenerative nerve function often remains unsatisfactory. Therefore, preventing nerve adhesion and providing a good
anastomosing microenvironment is of great significance in promoting nerve regeneration and repair.

In recent years, a series of materials have been developed to avoid site adherence following nerve anastomosis, which are mainly polymer films [7-9]. While these materials offer some resistance to adhesion, they exhibits low biocompatibility due to the lack of bioactive components. This has led to an increasing shift towards natural materials like chitosan [10-12] and collagen [13, 14]. Their enhanced biocompatibility is conducive to adhesion of cells and proliferation, crucial for tissue engineering applications. However, natural materials are not without their drawbacks. One significant issue is the fast degradation rate, which cannot match the rate of nerve repair. Additionally, the variability and inconsistency in their physical and chemical properties, which can arise from differences in their biological sources, can lead to challenges in standardizing and replicating results. Research indicates that when it comes to tissue rebuilding, extracellular matrix (ECM) components—especially those sourced from tissues specific to a certain site—work better than synthetic or non-specific tissue-derived materials [15-17]. Extracellular matrix (ECM) is a complex, multidimensional structural network present in a variety of tissues. It is essential for tissue remodeling, organization, and control over cellular functions [18]. Collagen, laminin, proteoglycans (including glycosaminoglycans), elastic fibers and elastin, fibronectin, and other glycoproteins are some of its primary constituents. The ECM serves as a communication network between cells within organs and tissues, transmitting
biochemical and mechanical signals. During cellular migration, it acts as a scaffold, providing structural support and potential attachment points for cells [19-21]. Decellularized extracellular matrix (dECM) refers to a biological material derived from tissues or organs of humans or animals through decellularization techniques, which eliminate immunogenic cellular components, mostly retaining the extracellular matrix as their primary component [22, 23]. Therefore, dECM is under the spotlight. Ting Li et al [24] prepared the nerve anti-adhesion membrane after the decellularization of the porcine sciatic nerve and carried out studies in vitro and in vivo respectively, which confirmed this anti-adhesion membrane could effectively avoid the invasion of tissues adjacent into the site of anastomosis, lessen adherence of tissues while promoting regeneration of nerves. However, this kind of membrane has a low mechanical property and rapid degradation in vivo, which makes it difficult to match the speed of nerve repair and has certain limitations. The epineurium is a natural biological scaffold extracted from nerve tissue that has been specially processed to remove cellular components but retain the intact structure of the extracellular matrix. It has good biocompatibility and bioactivity, and a suitable degradation rate in vivo, which will minimize the risk of postoperative adhesion as well as offer the best possible support structure for nerve regeneration.

The present study explores the development and efficacy of decellularized epineurium as an anti-adhesive biofilm, aiming to address the challenges of peripheral nerve repair with an innovative, biomimetic approach.
2. Materials and methods

2.1 Fabrication process of epineurium

Epineurium was prepared followed the guidelines outlined in the ISO 22442-2 standard.

Fresh nerves of the lower limbs of crossbred porcine gained from the market were
removed from external fibrous tissue, blood, and fatty tissue, then cut into 5 cm
segments. Following freezing at -80°C for 2 hours, the sciatic nerves were lyophilized
in a freeze drier for six hours. The dried nerves were temporarily submerged in PBS for
30s for sufficient moisturization of the epineurium. Subsequently, microscopic scissors
and forceps were used to delicately separate the epineurium from the nerves. The
isolated epineurium was then sliced into small pieces of 2 cm by 2 cm and repeatedly
cleaned in PBS adding 1% antibiotics. Two sections of materials were separated: half
lyophilized and kept for further program; the other was put in PBS for additional
decellularized treatment.

2.2 Process of the epineurium decellularization

The epineurium was first immersed for six hours, with three fluid changes. Following
this, submerged in 3% Triton X-100 solution (Sigma, USA) about 12 hours. Subsequent
to this immersion, three ten-minute washes with deionized water were performed on
the samples. They were then placed in sodium deoxycholate solution (4% (w/v)) (Sigma,
USA) about 24 hours, after which there were three further 10-minute rinses with
deionized water. These treatments were all carried out in a shaking incubator with a 120 rpm setting at ambient temperature. Ultimately, the decellularized epineurium was placed at 4°C for later use after being moved to a PBS solution containing 1% antibiotics.

2.3 Assessing the efficacy of decellularized epineurium

To evaluate the effectiveness of decellularized epineurium, both decellularized epineurium (DEP) and fresh epineurium (FEP) samples underwent H&E staining, along with DNA analysis. Following fixation in a 4% (w/v) paraformaldehyde solution for 24 hours, after being dehydrated in ethanol, FEP and DEP were embedded in paraffin and sectioned into slices that were 4 μm. H&E staining was used for observation of nuclei. Furthermore, a genomic DNA extraction kit (Tiangen, China) was used to extract DNA in accordance with the instructions provided by the manufacturer. In order to determine the quantity of DNA was still present in the DEP, the extracted DNA was measured using an absorbance spectrophotometer (Bio Drop Duo, UK). Utilizing 1% agarose gel electrophoresis, DNA fragments were isolated in order to conduct a qualitative analysis.

2.4 Glycosaminoglycans Analysis

By employing the dimethylmethylene blue method (GenMed Scientific INC, USA), the glycosaminoglycans (GAGs) content was evaluated and determined in accordance with the instructions provided by the manufacturer.

2.5 Proteomic analysis

Following the iFASP method's preparation of protein samples and determination of
their protein concentration, the samples underwent enzymatic digestion. The results of enzymatic digestion were subjected to RP-ESI-MS/MS analysis, employing an Orbitrap Fusion Lumos mass spectrometry equipment in conjunction with an Easy-nano LC 1200 liquid phase apparatus. Positive ion scanning mode was used for mass spectrometry, and data-dependent mode was used for the acquisition of secondary mass spectrometry. Utilizing software called Xcalibur 1.4 (Thermo Fisher, USA), system control and data capture were handled. Retrieval of cellular proteome mass spectrometry data for label-free quantitative samples was achieved through Maxquant (version 1.6.5.0), with the database sourced from the UniProt website (Sus Scrofa domestic’s whole proteome of domestic porcine origin). The LFQ algorithm was employed for protein quantification, with the "match between runs" function enabled. The target-decoy approach was utilized to set the false discovery rate (FDR) at 1% for both protein and peptide levels.

Bioinformatics analysis encompassed protein enrichment and the Gene Ontology (GO) analysis, covering cellular composition, biological processes, and molecular functions based on the protein data.

**2.6 SEM observation of the decellularized epineurium**

Utilizing scanning electron microscopy (SEM), the microscopic structure of the decellularized epineurium was observed. After fixing the material for six hours at 4°C with 2.5% glutaraldehyde (Sigma, USA), they were rinsed three times for five minutes each with PBS. Then utilizing an ethanol gradient to do successive dehydration, three rinses with deionized water were conducted. Samples were then dried in a vacuum freeze dryer for an entire night. A 10 kV accelerating voltage was used for observing the microstructure with a tungsten scanning electron microscope (TESCAN, Czech).
2.7 Water absorption analysis

To assess the water uptake of decellularized epineurium, studies with water absorption were conducted. In order to achieve a consistent dry mass, the materials (n = 5) were lyophilized for 12 hours after being submerged in PBS for 24 hours. After lyophilization, the materials were immersed in PBS for a whole day at 4°C in order to attain a stable weight upon absorption. The weight after absorption was subtracted from the dry weight to calculate the water weight. By dividing the water weight by the dry weight, the water absorption rate was determined.

2.8 Evaluation of the decellularized epineurium's biosafety

2.8.1 Cytotoxicity assays

The Cell Counting Kit-8 (CCK-8) assay was used to assess the cytotoxicity of decellularized epineurium (DEP). The DEP was sterilized with 75% alcohol about two hours, and washed three times with sterile PBS, and then submerged in sterile PBS to be used. Extracts were produced by incubating the sterilized DEP for 24 hours at 37°C at a concentration of 6 cm³/ml in a medium adding serum. Positive controls (DMSO), negative controls (polyethylene), and blank controls (complete medium) were prepared accordingly. In 96-well plates, RSC-96 cells were planted with a density of 3×10³ each well. Upon completion of the incubation period of 24 hours, the culture medium was changed and 100 μL of either the positive, negative, or blank control extract (DEP) was added into every well. Afterwards further incubation of 1, 3, and 5 days, 10 μL of the CCK-8 solution was put into every well, and then the incubation time was extended to 1 hour. After that, an enzyme-linked measuring device was employed to ascertain absorbance at 450 nm. The OD value was then used to compute cell viability, which
was used to assess DEP cytotoxicity.

2.8.2 Hemolysis test

Blood was extracted from Sprague Dawley rats in order to get cleaned erythrocytes. The plasma was extracted from the blood by centrifuging it for ten minutes at 4°C at 1000 rpm. Erythrocytes, usually leftover red blood cells, were suspended in saline solution. Three to five times was the washing procedure repeated, up to when the supernatant turned translucent and colorless. Following this, the blood cells were suspended in 2% (v/v) saline.

To obtain DEP extracts, the DEP was sterilized with 75% alcohol about two hours, and washed three times with sterile PBS, and then immersed into saline with a concentration of 6 cm²/ml and incubated over 24 hours at 37°C. The supernatant that resulted was gathered. Three centrifuge tubes were then filled with 0.5 mL of a 2% erythrocyte suspension, 1 mL of distilled water, DEP extract, and saline (as a positive control and negative control, respectively). The supernatant was removed after hemolysis was visually inspected, and an enzyme-linked measuring device was employed to ascertain the absorbance at 545 nm. The hemolysis rate (%) was used to indicate the extent of hemolysis, calculated using the formula provided below:

\[ HR(\%) = \frac{(OD_{test} - OD_{negative})}{(OD_{positive} - OD_{negative})} \times 100\% \]  

2.8.3 Cell affinity assay

The cell affinity of RSC 96 cells on decellularized epineurium was observed by SEM.

(1) Sample preparation: The materials were sliced into 1 cm by 1 cm pieces, sterilized with 75% alcohol about two hours, three times with sterile PBS washed, and then submerged in sterile PBS to be used. (2) The cell suspension was added at a cell count
of $1 \times 10^5$ per well, then cultured in incubator after the materials were progressively put into the 24-well plate. (3) After 3 d of incubation, the materials were taken out and repeated three times for five to ten minutes each time, followed by an overnight soak in 2.5% glutaraldehyde. (4) Then washed three times for five to ten minutes each time with PBS solution, and placed in an ultra-clean bench at room temperature to allow them to dry. (5) Observe the changes in cell morphology on the surface by scanning electron microscopy.

In addition, cell suspensions containing $1 \times 10^5$ RSC 96 were cultured in DEP extract. After 3 days, after fixation with 4% paraformaldehyde, the samples were stained with AbFluor 488 Reagent (BMD0082, Abbkine, USA) and then viewed by confocal microscopy.

### 2.8.4 Immune reaction to implanting subcutaneously

Experiments on animals adhered strictly to the approved regulations by the Experimental Animal Ethics Committee of China Medical University (CMU2019192). Animals were divided into 2 groups: decellularized epineurium (DEP) and decellularized nerve repair membrane (DNRM), respectively. The decellularized epineurium and decellularized nerve repair membrane were cut to the size of 1 cm × 0.5 cm, sterilized with 75% alcohol about two hours, three times with sterile PBS washed, and then submerged in sterile PBS to be used. SD rats were anesthetized with sodium pentobarbital (using a ratio of 30 mg/kg), and then dorsal skin preparation and disinfection were done. A 1.5 centimeter longitudinal incision was created along the rat's back's midline. The material was inserted into a subcutaneous capsule that was created by blunt dissection and was situated 10 mm to the left of the midline. In the same way, a subcutaneous capsule was prepared 10 mm to the right of the midline, but
no material was implanted as a control group (Fig. 5D). The incision was sutured. After
the rats had awakened from anesthesia, they were returned to the cage for normal
rearing.

Samples were collected at 1, 4, and 6 weeks post-implantation. Following humane
euthanasia, the implanted material with surrounding tissue was obtained. To assess the
immunological response to the implanted materials, immunofluorescence and H&E
staining were used. Anti-CD11b antibody (diluted 1:100; Abcam, USA) was used to
identify neutrophils, while anti-CD68 antibody (diluted 1:100; Abcam, USA) was used
to identify macrophages. Images of stained sections were viewed by light microscope,
and for analysis, the fields with top, bottom, left, right, and center of vision were
selected. The CD68 and CD11b positive area ratios were calculated using ImageJ
software

2.9 Functionalities evaluated in vivo

2.9.1 Surgical protocols
Rats were fasted and dehydrated for 8 hours. Animal experimentation adhered strictly
to the approved regulations by the Experimental Animal Ethics Committee of China
Medical University (CMU2019192). All materials were sterilized with alcohol. In brief,
materials were immersed in 75% alcohol for 2 hours, then washed three times with
sterile PBS, and finally stored in sterile PBS for later use. All surgical operations were
performed on the right leg. The right leg was weighed and given an intraperitoneal
injection of sodium pentobarbital (1% w/v) at a dose of 40 mg/kg to induce anesthesia,
after which the limbs were fixed on surgical plates. The surgical area was subjected to
a series of operations such as hair removal, skin preparation, disinfection, and toweling.
A 20 mm incision was made by longitudinal dissection in the posterior lateral femur, and blunt separation was performed along the course of the muscle fibers till the sciatic nerve is exposed. Then the nerve was cut. 8-0 nylon microsutures were used to close the epineurium both distally and proximally to the nerve terminal. The DEP and DNRM were wrapped around the anastomosis, respectively. The sciatic nerve was cut for end-to-end anastomosis without the use of materials wrapping in the control group. Afterwards, a 4-0 nylon suture was used to suture together the muscle and skin, and finally, a little penicillin powder was applied to the wound. After awakening, the rats were put back in the cage to be raised norm.

2.9.2 Claw-spread reflex examination

At 4th and 8th weeks following surgery, the claw-spread reflex was tested to assess the functionality of regenerating nerves. Using the following standards, nerve function recovery was categorized into grades A, B, or C: Grade A indicates that the rat reacts to acupuncture and spreads the claws; Grade B indicates a response to acupuncture with no claw spreading; Grade C indicates no response to acupuncture. A double-blind method was used for the statistics.

2.9.3 Observation of tissue adhesion at nerve anastomosis

At four and eight weeks following surgery, the adhesion with adjacent tissues was assessed at 4th and 8th weeks post-surgery using a grading system comprising three levels: Grade 0 indicates no adhesion; Grade 1 indicates slight adhesion, where the anastomosis site can be separated with ease; Grade 2 indicates tight adhesion, making separation difficult. A double-blind method was used for the statistics.

2.9.4 Sciatic nerve function index
The CatWalk XT gait analysis system was purchased from Noldus Corporation. Briefly, the instrument has a single-channel system, when the rat passes through the channel, there is a camera at the bottom of the channel that can record each step of the rat and transfer the data into the system software, after the CatWalk XT Version 10.5 software measured the left side of the footprints, i.e., the normal side (N), and the right side, i.e., the surgical side (E), the parameters: the furthest distance between the heel and the tip of the foot (Print length, PL), with EPL representing the length of the footprint on the surgical side and NPL representing the length of the footprint on the normal side; the distance of the opening from the first toe to the fifth toe (Toe spread, TS), with ETS representing the width of the toe spread on the surgical side and NTS representing the width of the toe spread on the normal side; and the width of the intermediate toe spread from the second toe to the fourth toe (ITS). Intermediary toe spread, ITS), with EITS being the width of the intermediate toe on the operative side and NITS being the width of the intermediate toe on the normal side (Fig. 7A). SFI was calculated according to the Bain sciatic functional index (SFI) score[25]:

\[
SFI = -38.3 \frac{(EPL-NPL)}{NPL} + 109.5 \frac{(ETS-NTS)}{NTS} + 13.3 \frac{(EITS-NITS)}{NITS} - 8.8
\]  

(2)

The given formula was used to calculate the value of SFI. The normal sciatic nerve function is indicated by SFI=0, while full loss of sciatic nerve function is SFI=-100.

2.9.5 Electrophysiological evaluation

A multichannel electrophysiologic signal acquisition system was used to obtain experimental electrophysiologic images to evaluate postoperative sciatic nerve recovery in rats. Compound muscle action potentials (CMAP) were detected by electrophysiological instruments. Bipolar stimulating electrodes from a multichannel electrophysiology system were used to stimulate the proximal and distal nerve trunks.
of the regenerated sciatic nerve after the proximal and distal ends of the anastomosis were exposed. The electrodes were then inserted into the anterior tibial muscle. Then stimulated the nerve with continuously intensified electrical pulses until a response was produced, then the distance between the distal and proximal positions of the stimulating electrodes and the receiving electrodes, the latency, and the CMAP were recorded, and the motor nerve conduction velocity (MCV) was calculated, to assess the recovery of the sciatic nerve of the rats after surgery.

2.9.6 Toluidine blue staining of regenerating nerve

The distal nerve sections next to the anastomosis location were removed after electrophysiological testing (Fig.7E). Axonal regeneration and myelin growth were assessed for each group through toluidine blue staining of the nerves. The specific steps were as follows: (1) After removing the distal nerve, it was placed in 2.5% (v/v) glutaraldehyde and soaked about 2 hour at 4°C. Then fixed for roughly 30 minutes using 1% (v/v) osmium tetroxide; (2) After gradient alcohol dehydration, the embedding was carried out by Epon 812 epoxy resin, and the embedded samples were semi-thinly sliced by ultra-thin slicer, with the thickness of 0.5 μm; (3) Section staining was performed using a 1% toluidine blue staining solution; (4) The results were observed with light microscope and images were captured; (5) The myelinated axons’ density (measured as the number of axons/mm²) was quantified utilizing ImageJ software.

2.9.7 Detection of specific markers for regenerating nerves

The connective tissue of the distal nerve was removed, immersed in paraformaldehyde solution, and fixed about 12 hours. Following a series of 20% and 30% sucrose
solutions dehydration steps, OCT-embedded frozen sections measuring 8 μm in thickness were created. Fluorescence staining was performed. In short, a mixed antibody dilution of Tuj1 (dilution ratio of 1:100) and S-100 (dilution ratio of 1:200) was added dropwise to the slices, then which were kept at 4°C for the whole night. The fluorescently labeled secondary antibody dilution (dilution ratio 1:500) was dropped and incubated at 37°C for 1 h; and finally blocked with an anti-fluorescence quencher. The staining results were observed by confocal microscopy, the graphs were captured, and for analysis, the top, bottom, left, right, and center fields of vision were selected. The Tuj1 positive ratio of area was determined by ImageJ software.

2.10 Statistical analyses

SPSS 25.0 statistical software was used to handle and analyze the data. The data obtained were expressed as mean ± standard deviation. Comparisons between two groups were analyzed by $t$-test, and comparisons between multiple groups were analyzed by two-way ANOVA. ns indicates that the difference is not statistically significant, and $p < 0.05$ means that the difference is statistically significant.

3 Results

3.1 Decellularization efficiency analysis

The effectiveness of decellularization was evaluated using tissue staining and biochemical analysis. When compared to fresh epineurium (FEP), decellularized epineurium (DEP) had lower number of nuclei, as demonstrated by H&E staining. In DEP, agarose gel electrophoresis did not reveal any discernible DNA bands. In addition,
analysis of residual DNA content showed a significant reduction in DEP compared with FEP ($p < 0.05$), with DEP's DNA level falling short of the 50 ng/mg international norm (Fig. 1). These findings validate the effectiveness of decellularization by showing that it satisfies the established standards [26].

Fig. 1 Assessing the epineurium's decellularization effectiveness. FEP: Fresh epineurium; DEP:
Decellularized epineurium. Decellularized epineurium (A and B). H&E staining of fresh epineurium (C). H&E staining of decellularized epineurium (D). Quantitative analysis of dsDNA (E). DNA agarose gel electrophoresis (F). Data are expressed as the mean ± SD (n = 5). *p < 0.05.

3.2 GAGs content in decellularized epineurium and fresh epineurium

Glycosaminoglycans are essential for nerve regeneration. In fresh epineurium and decellularized epineurium, the GAGs content was 9.46 ± 0.36 and 3.39 ± 0.48 μg/mg of dry tissue, respectively, which revealed a notable reduction in the DEP group compared to the FEP group (p < 0.05) (Supplementary figure 1).

3.3 Proteomics analysis

3.3.1 Coexisting proteins in FEP and DEP

The GO analysis of proteins commonly present in both FEP and DEP reveals that the majority of these proteins are concentrated in the extracellular matrix components (Fig.2A). Specifically, from the perspective of biological processes, they predominantly participate in the organization of collagen fibrils and promote the migration and adherence of cells. The relevant terms include collagen fibril organization (GO: 0030199), elastic fiber assembly (GO: 0048251), cell-matrix adhesion (GO: 0007160), collagen biosynthetic process (GO: 0032964), cell adhesion (GO: 0007155), cell migration (GO: 0016477), and axon extension involved in regeneration (GO: 0048677). In terms of cellular components, they are primarily associated with the extracellular matrix, such as collagen and basement membranes, with relevant terms including extracellular space (GO: 0005615), basement membrane...
(GO: 0005604), collagen trimer (GO: 0005581), extracellular region (GO: 0005576), extracellular matrix (GO: 0031012), and collagen type I trimer (GO: 0005584). Furthermore, from a molecular function perspective, most are related to protein binding, as indicated by terms such as extracellular matrix structural constituent (GO: 0005201), collagen binding (GO: 0005518), protein binding, bridging (GO: 0030674), cell adhesion molecule binding (GO: 0050839), and laminin-binding (GO: 0043236).

Enrichment analysis was performed on the protein constituents detected in both FEP and DEP samples (Fig. 2B). The analysis revealed a substantial presence of collagen and basement membrane proteins, such as types I, II, IV, V, and VI collagens (COL I, COL II, COL IV, COL V, COL VI), along with laminins (LAMA, LAMB), and fibronectin (FN), in the epineurium both before and after decellularization. Additionally, there was an observed increase in the relative abundance of certain collagen proteins. The epineurium also contained a variety of glycoproteins, including but not limited to alpha-2-HS-glycoprotein (AHSG), decorin (DCN), heparan sulfate proteoglycan 2 (HSPG2), and alpha-1β-glycoprotein (AIBG). Furthermore, a minor complement of cytokines, such as transforming growth factor-beta 1 (TGFB1), was also present in the epineurium.
**Fig. 2** Coexisting protein analysis. Protein gene ontology enrichment analysis (A). BP: Biological Process; CC: Cellular Components; MF: Molecular Functions. The analysis of protein abundance for certain identified proteins. The bluer color, the lower the in relation content of protein (B). FEP: Fresh epineurium; DEP: Decellularized epineurium. COL1A2, collagen type I alpha 2 chain; COL2A1, collagen type II alpha 1 chain; COL4A1, collagen type IV alpha 1 chain; COL4A2, collagen type IV alpha 2 chain; COL5A1, collagen type V alpha 1 chain; COL5A2, collagen type V alpha 2 chain; COL6A1, collagen type VI alpha 1 chain; COL6A2, collagen type VI alpha 2 chain; COL6A3, collagen type VI alpha 3 chain; COL12A1, collagen type XII alpha 1 chain; COL14A1, collagen type XIV alpha 1 chain; COL24A1, collagen type XIV alpha 1 chain; COL28A1, collagen type XXVII alpha 1 chain; LMNA, lamin filament; LAMA1, laminin subunit alpha 1; LAMA2, laminin subunit alpha 2; LAMA5, laminin subunit alpha 5; LAMB1, laminin subunit beta 1; LAMB2, laminin subunit beta 2; LAMC1, laminin subunit gamma 1; FN1, fibronectin; FBN1, fibrillin-1; FGG, Fibrinogen gamma; AHSG, α2-HS glycoprotein; DCN, decorin; APOH, beta 2 glycoprotein I; HSPG2, heparan sulfate proteoglycan 2; AIBG, α1-beta glycoprotein; TXNB, tenascin; MPZ, myelin protein; NEFL, neurofilament; ANXA7, annexin; GFAP, glial fibrillary acidic protein; PEA15, phosphoprotein enriched in astrocytes 15; TGFB1, transforming growth factor, beta 1
3.3.1 Comparisons of distinct proteins in FEP and DEP

Differential analysis was conducted on the identified proteins, with differential proteins (DEPs) being selected based on a threshold of $p < 0.05$ and an absolute log2 fold change $|\log2\text{FoldChange}|$ of at least 1. This analysis resulted in the identification of 116 DEPs, consisting of 100 proteins with decreased relative abundance and 16 proteins with increased relative abundance. The GO analysis was utilized to elucidate the biological function disparities among these DEPs.

The GO analysis's findings indicate that the proteins with increased relative abundance primarily play a crucial part in the structure and function of the extracellular matrix (Fig. 3). A significant number of these genes contribute to collagen-related structures and functions and their interactions with other molecules. For the category of Biological Processes, the relevant terms include collagen fibril organization (GO: 0030199), blood vessel development (GO: 0001568), collagen biosynthetic process (GO: 0032964), and cell adhesion (GO: 0007155). Regarding the Cellular Component category, implicated terms are collagen trimer (GO: 0005581), extracellular space (GO: 0005615), extracellular matrix (GO: 0031012), and basement membrane (GO: 0005604). As for Molecular Function, some of the associated terms are platelet-derived growth factor binding (GO: 0048407), extracellular matrix structural constituent (GO: 0005201), protease binding (GO: 0002020), and collagen binding (GO: 0005518).
Fig. 3 GO analysis of up-regulated proteins. The horizontal bars are categorized by color to indicate three distinct GO terms: Biological process (red), Cellular component (blue), and Molecular function (green). The x-axis shows two scales: on the left, there's the negative logarithm to the base 10 of the p-value (-log10(p-value)), which indicates the statistical significance of each term; on the right, the Gene count, which denotes the number of genes associated with each term.

The proteins exhibiting decreased relative abundance are predominantly associated with cellular structure and activity (Fig. 4). Within the Biological Process category, the processes implicated include intermediate filament cytoskeleton organization (GO: 0045104), positive regulation of protein processing in phagocytic vesicle (GO: 1903923), actin cytoskeleton organization (GO: 0030036), and regulation of establishment of T cell polarity (GO: 1903903). In terms of Cellular Components, the relevant terms are intermediate filament (GO: 0005882), cytoplasm (GO: 0005737),
cytosol (GO: 0005829), and intracellular membrane-bounded organelle (GO: 0043231).

For Molecular Function, some pertinent terms include calcium ion binding (GO: 0005509), actin filament binding (GO: 0051015), actin binding (GO: 0003779), and superoxide dismutase activity (GO: 0004784).

**Fig. 4** GO analysis of Down-regulated proteins. The horizontal bars are categorized by color to indicate three distinct GO terms: Biological process (red), Cellular component (blue), and Molecular function (green). The x-axis shows two scales: on the left, there's the negative logarithm to the base 10 of the p-value (-log10(p-value)), which indicates the statistical significance of each term; on the right, the Gene count, which denotes the number of genes associated with each term.
3.4 Morphology and structure of the decellularized epineurium

The microstructure of the decellularized epineurium was observed using via SEM, and it was noted that the surface of DEP is relatively flat with no obvious pores. (Supplementary figure 2).

3.5 Water absorption

In order to promote the interchange of nutrients and metabolites and support the nerve regeneration, effective water absorption is essential. In this study, the water absorption rate of fresh epineurium was 5.729±0.319, while the decellularization epineurium was 7.771±1.528, indicating that decellularization treatment could increase the water absorption of epineurium.

3.6 Cytotoxicity

The CCK-8 assay is a colorimetric method that evaluates metabolic activity of cells as an indicator for cytotoxicity and cell growth (Fig. 5A). The results showed that at all-time points (1, 3, and 5 days), the negative control group exhibits a constant increase in proliferation of cells. The DEP group also shows an increase in proliferation of cells, similar to the negative control, which suggests that the DEP does not exhibit cytotoxic effects on the cells. On the other hand, the positive control group displays a significantly decreased cell proliferation rate. In summary, the tested material (DEP) does not appear to have a cytotoxic effect on the cell culture within the observed time frame of the experiment, as the proliferation rates are comparable to those of the negative control and significantly higher than the positive control ($p < 0.05$). This suggests that DEP is potentially biocompatible and safe for the cells.
Fig. 5 Characterization and biocompatibility of decellularized epineurium (DEP). CCK-8 test of the extract medium was used to evaluate the cytotoxicity of DEP. (A). The test's qualitative hemolysis results (B). The negative control of normal saline exhibited no hemolysis, and the DEP extract showed no signs of causing hemolysis. In contrast, the positive control of distilled water demonstrated hemolysis. The adherence of Schwann cells on the surface of DEP was examined via SEM at ×1000 magnifications on the third day (C). Schematic diagram of experimental subcutaneous implantation on the back of rats (D), the circle on the left represents the control group, and the box on the right represents the material group. H&E staining at various times (E). DEP: Decellularized epineurium; DNRM: Decellularized nerve repair membrane; Control: no material. (a1, b1, c1, d1, e1, f1, g1, h1, i1, scale bar = 100 μm). The dotted red box is observed at a higher magnification (a2, b2, c2, d2, e2, f2, g2, h2, i2, scale bar = 50 μm). Data are expressed as the mean ± SD (n = 5). *p < 0.05, compared with positive control group.
3.7 Hemolysis test

The hemocompatibility of the DEP was assessed via a hemolysis assay. In the negative control, physiological saline was used, resulting in erythrocyte sedimentation with a clear and colorless supernatant, indicating no hemolysis. Conversely, distilled water served as the positive control, leaving no residual erythrocytes at the bottom of the tube, indicative of complete hemolysis. DEP extract supernatant retained its clear, colorless look, exhibiting not significantly change from the negative control. It suggests that contact with the tubular material did not induce hemolysis (Fig. 5B). The hemolysis rate (HR) quantifies the extent of erythrocyte rupture and dissolution resulting from material contact. In standardized hemolysis assessments, substances with an HR below 2% are categorized as non-hemolytic, while those with HR between 2% and 5%, or higher, is classified as little hemolytic or hemolytic, correspondingly. As Table 1 illustrates, the HR for DEP was 1.11%, classifying it as a non-hemolytic material, demonstrating favorable hemocompatibility.

<table>
<thead>
<tr>
<th>Group</th>
<th>OD&lt;sub&gt;545&lt;/sub&gt;</th>
<th>HR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEP extract</td>
<td>0.0504±0.0132</td>
<td>1.11</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.259±0.002</td>
<td>100</td>
</tr>
<tr>
<td>Normal saline</td>
<td>0.0475±0.0005</td>
<td>0</td>
</tr>
</tbody>
</table>

3.8 Cell affinity assay

In order to evaluate the affinity between DEP and Schwann cells, SEM was employed to analyze cell morphology and adhesion in cultivated together cells and materials. (Fig. 5C). From the image, we can observe that the Schwann cells have attached to and spread across the surface of the material, and the three-dimensional sense is strong, and the
long and slender protrusions are interwoven into a network. This indicates that the
DEP has a good affinity for cells and can promote cell growth and adhesion, which is
an indicator of good biocompatibility.

Following three days of Schwann cell culture in DEP extract, confocal microscopy
images showed stretched morphology of Schwann cells (Supplementary Figure 3).

3.9 H&E staining of subcutaneous implants in rats

In the subcutaneous implantation experiments, the immunological inflammatory
response induced by the material after implantation was analyzed through H&E staining
and immunohistochemical staining. Without any issues, every rat lived until the
scheduled time intervals. H&E staining indicated that from the first to the fourth week,
there was significant inflammatory cell infiltration in both the decellularized nerve
repair membrane (DNRM) group and the decellularized epineurium (DEP) group in
contrast to the control group. However, by the sixth week, the infiltration of
inflammatory cells was markedly reduced (Fig. 5E).

3.10 Immunohistochemical observation of subcutaneous implants
in rats

Figure 6 presents the results of immunohistochemical staining for CD11b and CD68 on
the dorsal subcutaneous implants from rats. CD68 is used to label macrophages, while
CD11b marks neutrophils. Positive staining is indicated by cells that exhibit brownish
or light brown cytoplasm.

The staining results for neutrophils labeled with CD11b indicate that there was no
significant inflammatory response from the first to the sixth week in the Control group.
From the results of the DNRM group, infiltration of neutrophils began in the first week, but there was no cellular invasion into the membrane. The DEP group, however, showed a small amount of cell infiltration, with some positive expression found within the membrane. By the fourth week, both material groups exhibited a large amount of positive expression and began to progressively infiltrate into the interior of the materials.

By the sixth week, there was a notable decrease in CD11b expression (Fig. 6A). In addition, according to the CD11b positive area ratio, the positive expression of neutrophils was the highest in the first week, and the DNRM group had the most severe infiltration ($p < 0.05$). The positive expression rate of each group gradually decreased over time, and by the sixth week, the three groups' neutrophil positive area ratios did not significantly differ from one another ($p > 0.05$) (Supplementary Fig. 4A).

The immunohistochemical results for the macrophage marker CD68 showed that, compared to the control group (Control), both material groups exhibited varying degrees of CD68 positive expression, which gradually diminished by the sixth week. Among them, the positive expression rate of the DEP was slightly lower than that of the DNRM (Fig. 6B). According to the CD68 positive area ratio, in contrast to the control group, the DNRM group had the highest positive expression rate of macrophages ($p < 0.05$) until the sixth week, which was not significantly distinct with the control group, while the DEP group was closer to the control group ($p > 0.05$) (Supplementary Figure 4B).
**Fig. 6** Samples stained with immunohistochemicals at different times, where CD11b signifies neutrophils (A) and CD68 indicates macrophages (B). DEP: Decellularized epineurium; DNRM: Decellularized nerve repair membrane; Control: no material. (a1, b1, c1, d1, e1, f1, g1, h1, i1, scale bar = 100 μm). The red box with dots is seen at a higher magnification (a2, b2, c2, d2, e2, f2, g2, h2, i2, scale bar = 50 μm).

### 3.11 In vivo functional study

#### 3.11.1 Claw-spread reflex analysis

Assessment of the claw-spread reflex provides insights into the recovery status of nerve
function post-sciatic nerve injury, graded from Grade A (normal function) to Grade C (complete functional impairment) (Table 2). At 4 weeks post-neurorrhaphy, the majority of rats in the DEP group were graded as B (responsive to needle prick but without toe extension reflex), with a minority assessed at Grade C (no response to needle prick); the DNRM group displayed an equal distribution of rats at Grades B and C; while the majority of the control group were at Grade C. Eight weeks after the procedure, 50% of rats in the DEP group were at Grade A (responsive to needle prick with toe extension reflex) and 50% at Grade B; in the DNRM group, the majority were Grade B, accounting for 66.67%, with Grades A and C each representing 16.67%; in the control group, Grade B rats were the most prevalent at 50%, while Grade A was the least common, at 16.67%.

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade A, n (%)</th>
<th>Grade B, n (%)</th>
<th>Grade C, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEP</td>
<td>0</td>
<td>4(66.67%)</td>
<td>2(33.33%)</td>
</tr>
<tr>
<td>4 W</td>
<td>DNRM</td>
<td>0</td>
<td>3(50%)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2(33.33%)</td>
<td>4(66.67%)</td>
</tr>
<tr>
<td>DEP</td>
<td>3(50%)</td>
<td>3(50%)</td>
<td>0</td>
</tr>
<tr>
<td>8 W</td>
<td>DNRM</td>
<td>1(16.67%)</td>
<td>4(66.67%)</td>
</tr>
<tr>
<td>Control</td>
<td>1(16.67%)</td>
<td>3(50%)</td>
<td>2(33.33%)</td>
</tr>
</tbody>
</table>

3.11.2 Assessment of tissue adhesion at the nerve anastomosis site

Adhesions at the neurorrhaphy site was assessed at 4th and 8th weeks postoperatively. At 4th weeks, extensive degradation was observed in DNRM, while DEP exhibited only partial degradation. By the 8th week post-surgery, DEP had completely degraded.
As shown in Table 3, in the fourth week, the number of rats graded level 1, wrapped with DNRM and DEP, was higher than that in control group. In the eighth week, both groups had more rats with a score of 0 in contrast to the control group. The DEP group had higher scores at both points compared to the DNRM group, demonstrating DEP’s superior anti-adhesion effects.

Table 3 Number of rats at each grade according to the adhesion assessment at anastomosis sites

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEP</td>
<td>0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>4 W</td>
<td>DNRM</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>DEP</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>8 W</td>
<td>DNRM</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

3.11.3 SFI evaluation

SFI in the DEP, DNRM, and Control groups all showed a trend of increase over time. In the 4th week post-surgery, there was no significant difference in the SFI between the DEP and DNRM groups in contrast to the control group ($p > 0.05$). However, at the 8th week post-surgery, the SFI of the DEP and DNRM groups was higher than that of the control group, exhibiting a difference that is statistically significant ($p < 0.05$) (Fig. 7B).
Fig. 7 Assessment of the in vivo nerve regeneration's reparative impact in rats. DEP: Decellularized epineurium; DNRM: Decellularized nerve repair membrane; Control: Without materials wrapping. Postoperative weeks 4 and 8 footprint patterns of rats in each group (LH: left hind healthy side, RH: right hind operative side) (A). Sciatic function index (SFI) values of each group at 4 and 8 weeks post-surgery (B). Motor conduction velocity (MCV) (C) and Compound action muscle potential (CAMP) amplitude ratio (D) for each group at 4 and 8 weeks post-surgery. Schematic diagram of postoperative tissue sampling and staining (E). Analysis of toluidine blue staining results for regenerated myelinated nerves at 4 and 8 weeks post-surgery (F). Analysis results of myelinated nerve fiber density (G). Data are represented as mean ± standard deviation (n=6), with comparisons between multiple groups made using two-way ANOVA. *p < 0.05, compared with the control group.

1. 3.11.4 Electrophysiological analysis

At the 4th and 8th weeks post-surgery, through electrophysiological experiments, the
regenerated nerves' functional recovery was evaluated. After surgery, about the fourth
week, the MCV in the DEP group was significantly higher than in the Control group ($p < 0.05$), but there was no significant difference in the CMAP compared to the Control
group ($p > 0.05$). The MCV and CMAP amplitude ratio in the DNRM group did not
show a significant difference in contrast to the Control group ($p > 0.05$). At the 8th
week post-surgery, both the MCV and CMAP amplitude ratio in the DEP group were
significantly higher than those in the Control group ($p < 0.05$). The MCV in the DNRM
group was also significantly higher than in the Control group ($p < 0.05$), but the CAMP
showed no difference in contrast to the Control group ($p > 0.05$) (Fig. 7C and 7D).

3.11.5 Analysis of toluidine blue staining

The density of myelinated fibers in regenerated nerves was evaluated using toluidine
blue staining. It was observed that at the 4th week post-surgery, the density and number
of myelinated sheaths in the DEP, DNRM, and Control groups were significantly
reduced compared to normal nerve myelin (Fig. 7F). Among these, the number of
regenerated myelin sheaths in the DEP group was significantly higher compared to the
Control group ($p < 0.05$), while the number in the DNRM group did not show a
significant difference compared to the Control group ($p > 0.05$). At the 8th week post-
surgery, the number of myelin sheaths in all three groups was significantly higher than
at the 4th week, with thicker myelin diameters. Compared to the Control group, both
the DEP and DNRM groups revealed a difference in the number of regenerated myelin
sheaths that was statistically significant ($p < 0.05$) (Fig. 7G).
Fig. 8 Immunofluorescence staining results of Tuj1 and S-100 in nerve samples. Normal: Normal nerve; DEP: Decellularized epineurium group; DNRM: Decellularized nerve repair membrane group; Control: Without materials wrapping group. Immunofluorescence staining results of regenerated nerves at the 4th-week post-surgery (A) and the positive area ratio of Tuj1 in regenerated nerves (B). Immunofluorescence staining results of regenerated nerves at the 8th-week post-surgery (C) and the positive area ratio of Tuj1 in regenerated nerves (D). Scale = 20 μm. Data are represented as mean ± standard deviation (n=6), with comparisons between multiple groups made using two-way ANOVA. *p <0.05, compared with the control group.

3.11.6 Immunofluorescence analysis of regenerating nerve fibers

At the 4th and 8th weeks after transplantation, slices of the renewed nerve were subjected to immunofluorescence staining with two distinct markers, S-100 and Tuj1, to observe nerve regeneration. S-100, a protein that nourishes neurons, is typically
secreted by Schwann cells in the peripheral nervous system. Hence, immunofluorescence staining for the S-100 marker could observe the proliferation and migration of Schwann cell throughout the whole process of nerve repairment and regeneration. Tuj1 is a microtubule protein considered to be involved in neuron-specific differentiation and can serve as an early neuronal characteristic immunomarker. In the confocal microscope images, green represents S-100 protein, red indicates Tuj1 protein, and blue is for cell nuclei.

From Figure 8A, it can be observed that at the 4th week after surgery, in normal nerves, the intensity of both red and green fluorescence is higher compared to the other surgical groups. The green and red fluorescence intensities of the three surgical groups are not significantly different, indicating that no obvious nerve regeneration had occurred by the 4th week.

At the 8th week post-surgery, the intensity of both green and red fluorescence in all three groups was significantly stronger than at the 4th week. Among these, the fluorescence intensity in the DEP and DNRM groups was noticeably stronger than in the Control group, with the regenerated myelin sheaths in the DEP group being closest to that of normal nerves (Fig. 8C).

Through immunofluorescence staining, the positive area ratio of Tuj1 was calculated for assessing the quality of the regenerated nerve (Fig. 8B and 8D). From 4th to 8th week post-surgery, the positive area ratio of Tuj1 gradually increased. In the 4th week post-surgery, compared with the Control group, DEP showed a significant increase in the positive area ratio of Tuj1 fluorescence ($p < 0.05$), while there was no significant difference between the DNRM and the Control group ($p > 0.05$) (Fig. 8B). At the 8th week post-surgery, both the DEP and DNRM groups exhibited a significantly higher
positive area ratio of Tuj1 fluorescence compared with the Control group ($p < 0.05$) (Fig. 8D).

### 4 Discussions

The conventional method for repairing severed peripheral nerves often entails directly suturing the nerve ends. Nonetheless, this approach may result in adhesion of the nerve anastomosis to adjacent tissues or the development of neuromas, impeding the restoration of nerve function [3, 27]. Thus, we have devised an innovative xenogeneic decellularized epineurium as an anti-adhesion membrane, aimed at averting adhesions and fostering nerve repairment and regeneration.

This study utilized the epineurium of the sciatic nerve derived from pigs, which was decellularized and then used as an anti-adhesion membrane at the site of nerve anastomosis. Compared to complete nerve tissue, the epineurium is thinner and contains fewer cells. Therefore, this study improved the Sondell method for decellularization. Based on the Sondell method [28], we performed only a single round of decellularization. The results showed that after the decellularization process, H&E staining did not reveal any visible cell nuclei, and the DNA content measured by quantitative DNA detection was significantly less than the standard of 50 ng/mg. In addition, the decellularized epineurium did not exhibit any discernible DNA bands in the DNA gel electrophoresis results. Therefore, that could be shown that the modified Sondell decellularization technique employed in this work produced the decellularized epineurium in an effective way, eliminating the cellular components and satisfying the criteria put forth by Crapo [26] and other scholars.

By modifying the activity of the corresponding tissue cells, the physiologically active
chemicals found in the extracellular matrix (ECM) are essential for tissue regeneration [29-31]. This study analyzed the compositional structure of the epineurium both prior to and following decellularization using proteomics. The findings indicated that the epineurium contains an abundance of extracellular matrix components, like collagen, fibronectin, laminin, and proteoglycans, which are preserved after the decellularization process. Studies have indicated that collagen type IV (COL IV) is a key signaling molecule that activates axon fasciculation and promotes axonal growth [32, 33]. Laminin (LN) can enhance the differentiation, migration, and adhesion of Schwann cells and, following peripheral nerve injury can bind to integrin receptors to facilitate nerve regeneration[1, 34, 35]. The presence of proteoglycans aids axonal regeneration after peripheral nerve injury [36, 37]. Additionally, certain cytokines such as TGF-β were identified, which can influence cell growth, differentiation, apoptosis, and immunomodulation through various signaling pathways [38-40]. However, the mechanisms by which the components of the decellularized epineurium participate in cellular signaling pathways to regulate the repair of injured nerves remain to be further explored.

Through biocompatibility experiments, it has been observed that in addition to being non-toxic to Schwann cells, the decellularized epineurium promotes their adherence and growth on its surface. This is attributed to the existence of proteins like laminin (LN) and fibronectin (FN) [35, 41] of decellularized epineurium, which promotes cell adhesion and proliferation. Additionally, through subcutaneous implantation experiments in rats’ backs, it has been demonstrated that the material does not cause severe immune rejection reactions upon contact with tissue. This is due to the substantial removal of cellular antigenic components from the epineurium. Therefore, the decellularized epineurium could be considered a safe and effective material in the
Furthermore, in this research, we made a model of sciatic nerve transection in rats and optimized the regrowth of transected peripheral nerves by using the decellularized epineurium. The study employed the claw spread reflex test to assess the rat sciatic nerve's functional recovery. This test can show that both motor and sensory nerve function have recovered at the same time. From the experimental results, at the 4-week mark, the claw spread reflex test showed poor functional recovery in rats, with both material groups showing no significant advantage over the untreated group. This could be because, in the short period after surgery, effective nerve innervation had not yet been established, and the regenerating nerves had not completely reached the target muscles, so the sensory and motor functions of the rats had not been restored. By the 8th week, the groups using the decellularized epineurium and the decellularized nerve repair membrane showed a significant advantage in promoting nerve regeneration compared to the untreated group. This demonstrates the effectiveness of using anti-adhesion membranes after end-to-end nerve anastomosis surgery. Additionally, when evaluating the recovery of motor function in tissues innervated by the sciatic nerve, one often used indicator is the Sciatic Functional Index [25]. Early on the deficiency of the sciatic nerve, SFI value is very low. As the treatment time increases, this value changes accordingly. An increase in the SFI indicates that the material has a certain reparative effect on the deficient nerve. From the experimental results, there was a significant upward trend in the SFI from the 4th to the 8th week. Notably, in the 8th week, the group with the decellularized epineurium exhibited the highest SFI value, demonstrating that the decellularized epineurium promotes the recovery of impairments in the sciatic nerve.
Electrophysiological examination results indicate that both the decellularized epineurium group and the decellularized nerve repair membrane exhibited significantly better nerve conduction velocities compared to the untreated group. Notably, at the 4-week mark, as the nerve anastomosis location was exposed again, the decellularized nerve repair membrane had almost completely degraded, whereas the decellularized epineurium group had only partially degraded. This partial degradation provided a physical barrier that avoided fibroblasts invading the anastomosis site. Therefore, during the nerve repairment and regeneration, the degradation rate of decellularized epineurium is more closely matched with the timeline of nerve regeneration, demonstrating a more suitable effect in preventing adhesions.

Toluidine blue staining can be employed to further observe the number of axons, myelin sheath thickness, and diameter in the cross-section of the regenerated nerve. It is observable that the diameters and thicknesses of the regenerated nerve axons vary, with a sparse and irregular arrangement. However, in contrast to the other two groups, the group with the decellularized epineurium showed a more orderly arrangement of regenerated nerves and higher myelin sheath maturity. This was more evident in the 8th week. These morphological changes are consistent with the trends observed in the SFI and electrophysiological results.

The assessment of nerve-associated marker expression through Tuj1 and S-100 immunofluorescence staining on the regenerated nerves provides insights into the degree of nerve regeneration. The level of expression of these nerve-associated markers can reflect the effectiveness of nerve regeneration to some degree. At the 4-week mark, the expression levels of both Tuj1 and S-100 were relatively low, indicating poor nerve regeneration at this stage. By the 8th week, however, there was a significant increase in
the expression of TuJ1 and S-100, with the highest expression levels observed in the
decellularized epineurium group. This suggests that the decellularized epineurium can
enhance the expression levels of nerve-associated markers, thereby promoting nerve
regeneration.

5 Conclusion

The decellularized epineurium prepared in this study has effectively removed cell-
derived antigenic components while preserving a substantial amount of extracellular
matrix proteins and wrapping the nerve anastomosis site with decellularized epineurium
to achieve the perfect biomimicry. This is the first instance of employing
decellularized epineurium as a novel promising anti-adhesion bio-film for enhancing
surgical outcomes of peripheral nerve repair.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or
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