Chinese Journal of Polymer Science Vol. 33, No. 4, (2015), 587–596

Chinese Journal of Polymer Science
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Institute of Chemistry, CAS
Springer-Verlag Berlin Heidelberg 2015

Prevention of Postoperative Tendon Adhesion by Biodegradable Electrospun Membrane of Poly(lactide-*co*-glycolide)*

Zhi-ming Song[a], Bo Shi[b, c], Jian-xun Ding[b, d**], Xiu-li Zhuang[b, d], Xiao-nan Zhang[a], Chang-feng Fu[c**] and Xue-si Chen[b, d]

[a] *Department of Sports Medicine, First Hospital of Jilin University, Changchun 130021, China*
[b] *Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China*
[c] *Department of Spine Surgery, First Hospital of Jilin University, Changchun 130021, China*
[d] *Changchun SinoBiomaterials Co., Ltd., Changchun 130103, China*

Abstract An electrospun poly(lactide-*co*-glycolide) (PLGA) membrane was prepared and used to perform the anti-adhesion of Achilles tendon. Throughout the experiments, the membrane showed an appropriate degradation rate, and the pH values of degradation media were maintained at around 7.4. Simultaneously, the excellent biocompatibility of the membrane *in vitro* and *in vivo* was confirmed by live/dead and histopathological analyses. Meanwhile, the membrane can reduce tendon adhesion significantly and promote functional recovery effectively. The encouraging results were further demonstrated by hematoxylin and eosin (H&E), and Masson's trichrome stainings, and type I collagen immunohistochemical analysis. It was concluded that the model treated with the electrospun PLGA membrane was significantly better with respect to the adhesion prevention and tissue repair than that without treatment. Considering the results of degradation and adhesion prevention efficacy, the electrospun PLGA membrane would be a great candidate for the prevention of postoperative tendon adhesion.

Keywords: Anti-adhesion; Achilles tendon repair; Biodegradability; Electrospun membrane; Poly(lactide-*co*-glycolide).

INTRODUCTION

Tendon adhesion is one of the severe and widespread complications after tendon injury and tendon repair surgery, which is not conducive to tendon activity[1, 2]. For effective prevention of tendon adhesion after surgery, different approaches are exploited, such as inhibition of inflammation[3] and physical barrier with biomaterials[4, 5]. For the latter one, the injured area and surrounding tissue are isolated by a physical barrier, which is a promising technique and arouses extensive interests[6]. So far, more and more bioabsorbable materials are applied to prevent tendon adhesion, such as carboxymethyl chitosan and hyaluronic acid[7, 8]. However, the above systems have not been ideal for effective clinical validation[9]. For example, the degradation rate of carboxymethyl chitosan is too slow to result in the formation of bladder that increases tissue adhesion[10]. On the other hand, hyaluronic acid disappears from the damage location fast, which will largely affect the anti-adhesion efficacy[11].

* This work was financially supported by the National Natural Science Foundation of China (Nos. 51303174, 51321062, 51233004, 51390484, 51273196, and 51203153) and the Scientific Development Program of Jilin Province (No. 20140520050JH).
** Corresponding authors: Jian-xun Ding (丁建勋), E-mail: jxding@ciac.ac.cn
Chang-feng Fu (付长峰), E-mail: 13804312088@163.com
Received July 25, 2014; Revised October 29, 2014; Accepted November 1, 2014
doi: 10.1007/s10118-015-1611-5

To overcome the shortcomings of the above anti-adhesion materials, the biodegradable and biocompatible aliphatic polyester films have been extensively studied in recent years, which are prepared by three-dimensional braiding, spin coating, membrane casting, electrospinning, *etc.*[12–14]. Among them, electrospinning exhibits obvious advantages: large surface area, variable aperture, and high anti-adhesion efficiency[15, 16]. The researchers reveal that these electrospun fibrous membranes of biodegradable polyesters can not only provide a comfortable texture and flexibility, but also be discharged by the body's physiological metabolism[17, 18]. In addition, it is suggested that the membranes from electrospinning can effectively reduce the inflammatory response and lesion tissue adhesion after surgery[19].

Poly(lactide-*co*-glycolide) (PLGA) as a member of biodegradable polyesters has been widely studied and benefited from its high efficiency and fewer complications. Moreover, it has long been used in surgical sutures, tissue repair, controlled release, and other biomedical fields because of its reliable mechanical properties, suitable biodegradation, and good biocompatibility[20–24]. Through researching the nanofiber scaffold of PLGA, it was discovered that the structure of PLGA scaffold was similar to the extracellular matrix, and it would facilitate the exchange of nutrition and metabolism between PLGA scaffold and environment[25]. In the traditional understanding, the anti-adhesion membrane should be designed to possess appropriate mechanical properties, rate of degradation, and stability after *in vivo* implantation[19]. Based on the above background, the PLGA membrane was prepared by electrospinning for the prevention of postoperative Achilles tendon adhesion in this work (Fig. 1). The morphology, degradation rate, and biocompatibility *in vitro* and *in vivo* were systemically revealed. In addition, the anti-adhesion assay revealed that the electrospun PLGA membrane was a promising candidate for the prevention of surgical adhesion and reduction of inflammation.

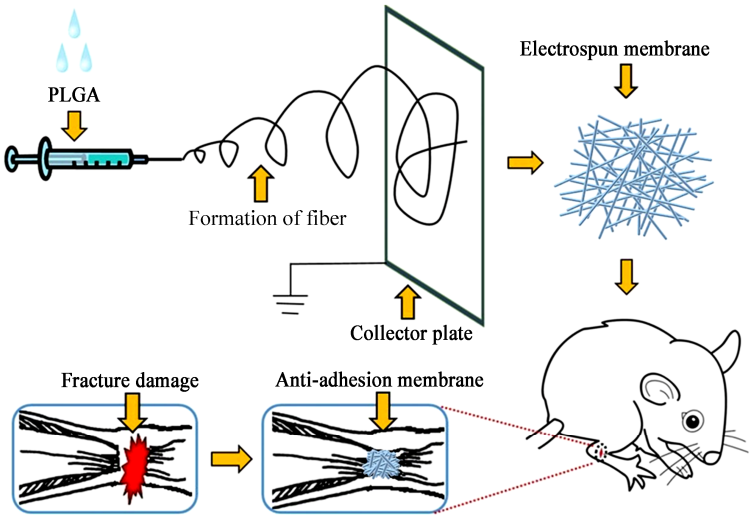


Fig. 1 Schematic diagram for preparation of electrospun PLGA membrane and application in prevention of postoperative Achilles tendon adhesion of a rat

EXPERIMENTAL

Materials

PLGA (viscosity (η) = 1.03 Pa·s, viscosity-average molecular weight (M_η) = ~ 40 kD) was obtained from Changchun SinoBiomaterials Co., Ltd. (Changchun, China). α-Chymotrypsin and elastase were bought from Aladdin (Shanghai, China). Acetoxymethyl ester (calcein AM) and propidium iodider (PI) were purchased from Sigma-Aldrich (Shanghai, China).

Preparation of Electrospun PLGA Membrane

As shown in Fig. 1, the electrospun PLGA membrane was manufactured through the following steps[16]. First, PLGA was dissolved in chloroform at a concentration of 6% (*W*/*W*). Second, the PLGA solution was loaded in a 10 mL syringe, which was fixed at approximately 10° from horizontal in order to minimize the falling drop at the end of capillary tip. An electrical field of 20 kV was applied by a high voltage power supply, and the injection rate of solution was set as 0.1 $mm \cdot min^{-1}$. The electrospun PLGA membrane with a thickness of 0.1 mm was collected on a grounded aluminum sheet kept at a distance of 15 cm from the needle tip.

Characterizations of Electrospun PLGA Membrane

Morphology assay

The surface morphology of electrospun PLGA membrane was observed using a field emission scanning electron microscope (SEM, Inspect-F, FEI, Finland) under high vacuum and with an acceleration voltage of 20 kV. The average diameter of electrospun PLGA fibers was obtained from the SEM microimage through the image analysis software of Nano Measurer 1.2.

In vitro degradation evaluation

The electrospun PLGA membrane had diverse degradation rates in different hydrolysis solutions. 3.0 mg of electrospun PLGA membrane was placed in a vial, and then 2.0 mL of phosphate-buffered saline (PBS, 0.1 $mol \cdot L^{-1}$, pH 7.4), or PBS with α-chymotrypsin (1.0 $IU \cdot mL^{-1}$, pH 7.4) or elastase (1.0 $IU \cdot mL^{-1}$, pH 7.4) was added. The vial was incubated with continuous reciprocal oscillation (75 $r \cdot min^{-1}$) at 37 °C. The whole degradation process was lasted for a cycle of 42 days in PBS or 21 days in PBS with α-chymotrypsin or elastase. The incubation medium was replaced daily to maintain the activity of enzyme. When it reached a predetermined time, the medium was carefully removed, and the sample was rinsed with distilled water, freeze-dried, and subsequently weighed. In order to clarify the weight loss of experimental sample during the degradation process, the real-time weight (W_r) was normalized in contrast to the initial one (W_0), that is, normalized weight (%) = $W_r/W_0 \times 100\%$. Meanwhile, the pH value of residual medium after the degradation, which was replaced daily, was also detected carefully to assess the impact of degradation on the body fluids.

Biocompatibility of electrospun PLGA membrane

The biocompatibility of electrospun PLGA membrane was characterized *in vitro* and *in vivo*. A live/dead test was applied as a method to evaluate the cytocompatibility of electrospun PLGA membrane. After the sample was sterilized by ultraviolet-irradiation on each side for 0.5 h, the electrospun PLGA membrane was placed on the bottom of a 48-well tissue culture plate (TCP). Subsequently, the L929 cells, a mouse fibroblast cell line, were seeded in Dulbecco's modified Eagle's medium (Invitrogen corporation, Gibco, USA) with a density of 1.0×10^4 cells each well. The sample was incubated for 1, 4 or 7 days, and then the medium was removed, and the cells were washed for three times with PBS. Subsequently, 10.0 μL of PBS containing calcein AM (2.0 $g \cdot mL^{-1}$) and PI (3.0 $g \cdot mL^{-1}$) was added to each well, and incubated for 30 min at 37 °C. Finally, the stained cells were detected by a Nikon fluorescence microscope (TE 2000, Nikon Co., Japan).

To evaluate the *in vivo* inflammatory response, the electrospun PLGA membrane (3.0 mg) was subcutaneously implanted to three healthy male Sprague-Dawley (SD) rats weighing 250–300 g. All animals were handled under the protocol approved by the Institutional Animal Care and Use Committee of Jilin University, and all efforts were made to minimize suffering. After the operation for 3, 6, and 9 weeks, one rat was randomly sacrificed by cervical amputation. And then, the skin tissue close to the material was isolated, sliced and stained by hematoxylin-eosin (H&E), and observed by an optical microscope (Nikon Eclipse Ti, Optical Apparatus Co., Ardmore, PA).

In vivo Evaluation of Electrospun PLGA Membrane

For this assay, a total of 20 male SD rats weighing 250–300 g were selected. The rats were randomly divided into two groups: Group I without treatment was set as control group; Group II was wrapped with 1 cm × 2 cm electrospun PLGA membrane for anti-adhesion in the site of Achilles tendon suture. After the intramuscular

injection of chloral hydrate with a dosage of 30.0 mg·kg^{-1}, the rat fur was shaved, and the skin was disinfected with alcohol. A transverse incision distancing from calcaneal bone for about 5 mm was made in the middle position of right Achilles tendon skin. The tissues were isolated, and then the tenotomy was implemented and sutured with 4-0 silk suture (PGA Resorba®, Resorba, Nürnberg, Germany). The wound was sutured directly in Group I after completing the above steps. In Group II, the tendon was surrounded with electrospun PLGA membrane before the wound closure.

Macroscopic evaluation

Before the animals were sacrificed, the apparent inflammation and ulcer in surgical sites were rigorous recorded. The breadth and severity of Achilles tendon adhesion were scored. According to the adhesion scoring system of previous studies, the degree of tendon adhesion based on surgical findings was evaluated into 1–5 grade: score 1 meant no adhesion; score 2 referred to mild adhesion, which could be separated by blunt dissection; score 3 meant that less than or equal to 50% of the adhesion scopes needed to be separated by sharp dissection; score 4 showed that 51%–97.5% of the adhesion area needed to be separated by sharp dissection; and score 5 meant no less than 97.5% of the adhesion scopes needed to be separated by sharp dissection[26, 27]. The rate of adhesion was used to quantify the degree of tendon adhesion[28]. The entire region of tendon adhesion which must be separated by sharp dissection was detected by caliper, and two parameters were determined by two observers under double-blind conditions to assess each experimental group.

Histological analysis

The tissue was separated and rinsed with PBS, and then fixed in 4% (*W*/*V*) PBS-buffered paraformaldehyde and embedded in paraffin after the animals were sacrificed. 5 μm thick longitudinal slices were observed after H&E or Masson's trichrome staining. In Masson's trichrome staining, the collagen was stained green, nucleus was stained blue or brown, and cytoplasm, blood cells, and muscle fibers were stained red.

The expression and localization of type I collagen were analyzed in Achilles tendon tissue by immunohistochemistry. Typically, the slice was dewaxed in xylene and hydrated in a graded array of alcohol. For the recovery of antigen, sections were placed in 10.0 mL of citrate buffer (pH 6.0) and then heated in the microwave twice. Respectively, the goat serum (diluted 1:100) and 0.3% (*V*/*V*) hydrogen peroxide were used to block the nonspecific binding and the activity of endogenous peroxidase. Subsequently, the antibody of type I collagen (Abcam company, Cambridge, USA) was used to incubate overnight at 4 °C. The section was washed for three times with PBS and nurtured with anti-mouse rabbit secondary antibody at 37 °C for 1 h. Finally, it was completed with staining in dia-minobenzidine solution (Dako primary antibody incubation developed, Hamburg, Germany) and contrast staining by hematoxylin.

Statistical Analysis

All experiments were performed for at least three times, and the results were represented as means ± standard deviation (SD). Statistical significances were analyzed using SPSS (Version 13.0, Chicago, IL, USA). $p < 0.001$ were considered highly statistically significant.

RESULTS AND DISCUSSION

Property Characterizations of Electrospun PLGA Membranes

The diameters and distributions of electrospinning fibers are the key parameters, which can influence the performance of PLGA. The electrospun PLGA membrane showed a clear microstructure. According to the SEM micrograph in Fig. 2(a), the electrospun PLGA fibers with the diameters in a range of 1–10 μm were distributed randomly and had a smooth surface. Figure 2(b) shows the semiquantitative diameters of electrospun PLGA fibers and their normal distribution. It revealed that the average diameter of electrospun PLGA fibers was 5.27 μm.

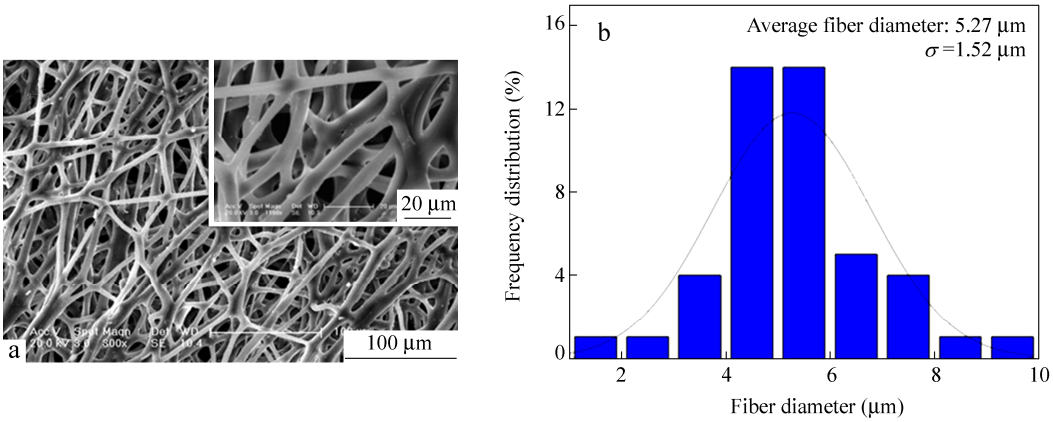


Fig. 2 Typical SEM micrograph of electrospun PLGA membrane (a), and statistical diameter and frequency distribution of electrospun PLGA fiber (b)

The degradations of biodegradable polyesters are decided by the intrinsic properties of polymer and the impact of external environment. The properties of polymer include internal structure, molecular weight of monomer, linkage, ratio of copolymer, crystallinity, surface area, *etc*. Simultaneously, the external environment contains enzymes, temperature, pH, *etc*.[29–35]. The *in vitro* degradation of electrospun PLGA membrane in terms of mass consumption was performed by three kinds of biodegradable solutions during 21 days or even longer. As shown in Fig. 3, the degradation rate of electrospun PLGA membrane was affected significantly by different types of media. Figure 3(a) shows that the PLGA sample in the medium with elastase exhibited the fastest degradation rate during 21 days of incubation. As a result, the remaining mass of electrospun PLGA membrane was 67.97%. In clear comparison, the degradation kinetics of α-chymotrypsin and PBS groups were relatively slow. Even the mass loss of PBS group was lower than that of elastase group for over 30% of initial weight in the same duration. It was explained that the electrospun PLGA membrane was more sensitive to elastase than any others, which may be due to the enzymatic hydrolysis characteristic of PLGA[36]. Throughout the degradation of electrospun PLGA membrane, less pre-mass loss of specimen was observed. When the degradation reached a certain level, the rate began to accelerate. The relevant literatures showed that the stability of substrate could be determined by the destruction of ester bond, and which led to the significant loss of mass,

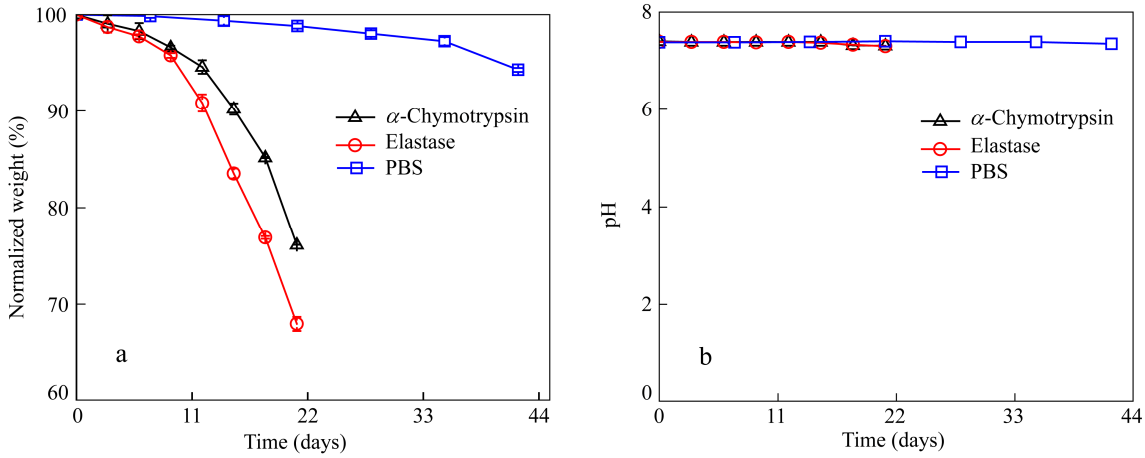


Fig. 3 Degradation profiles of electrospun PLGA membrane in PBS and PBS with α-chymotrypsin or elastase (a), and pH values of degradation media at different degradation times (b)
Data were presented as mean ± SD (n = 3).

when the molecular weight loss reached a certain stage in the process of polyester degradation[37, 38]. The degradation trend of electrospun PLGA membrane was well consistent with the above theory, and the *in vitro* biodegradation rate of electrospun PLGA membrane could be adjusted by different hydrolysis solutions. Meanwhile, at the whole detected degradation time, the pH values of degradation media were not significantly fluctuant in all three groups (Fig. 3b), indicating that the degradation did not greatly affect the surrounding medium, on the contrary, the pH values of surrounding environment less affected the degradation process of sample in the current study. This above adjustable degradable characteristic was extremely useful in the prevention of adhesion after surgery, because it can meet more specific biological treatment applications and the needs of body's internal homeostasis.

In vitro and *in vivo* compatibility of electrospun PLGA membrane were assessed by live/dead staining and histopathological analysis. First, the cytocompatibility of electrospun PLGA membrane toward the proliferation of L929 cells was detected by a live/dead assay. As shown in Fig. 4, L929 cells were cultured on electrospun PLGA membrane during 1, 4, and 7 days, at which the live and dead cells were fluorescently labeled to green and red, respectively[39]. Blank TCP was used as control. The fluorescence microimages showed that the cell viabilities on the electrospun PLGA membrane were close to those of control, that was, almost all L929 cells were alive and no significant dead cells were tracked. It meant that the electrospun PLGA membrane had excellent cytocompatibility and did not show toxicity to fibroblasts. Even more interesting was that, compared with control group, the electrospun PLGA membrane presented a slightly lower cell density probably due to its sufficient mechanical property and not uniform porous structure. Observed directly after PLGA implantation, the subcutaneous profile was performed as no significant congestion, degeneration or necrosis. Material was wrapped around the fibrous connective tissue. In addition, H&E staining showed no infiltration of neutrophils, multinucleated giant cells and other inflammatory cells after the operation for all 3, 6, and 9 weeks. The number of inflammatory cells had no significant difference in the site of implantation by H&E staining at the above three time points. It was found that the biodegradable membrane had no serious inflammatory response (Fig. 5), which once again demonstrated that the PLGA film exhibited good biocompatibility.

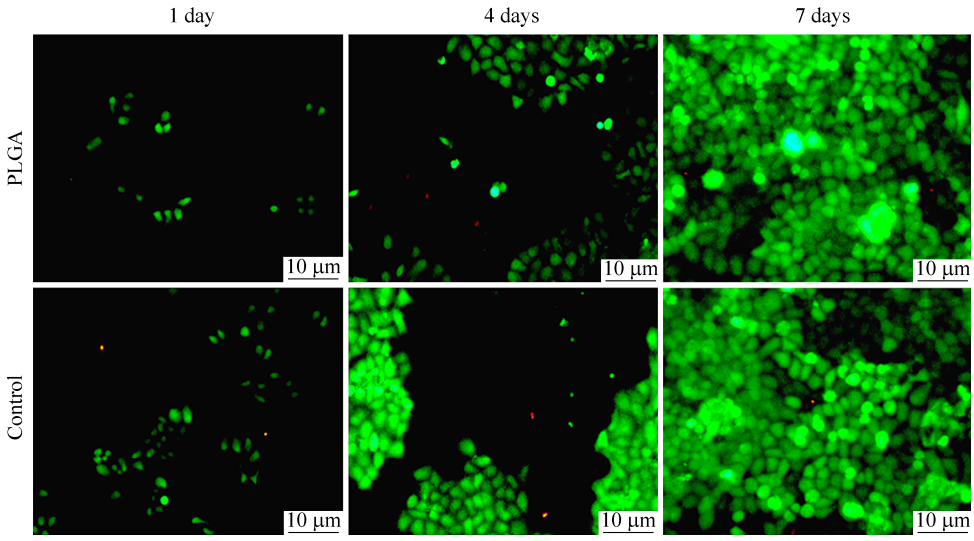


Fig. 4 Typical fluorescence micrographs of L929 cells on electrospun PLGA membrane and TCP after *in vitro* culture for 1, 4 and 7 days
Live cells were stained green by calcein AM, and dead cells are stained red with PI.

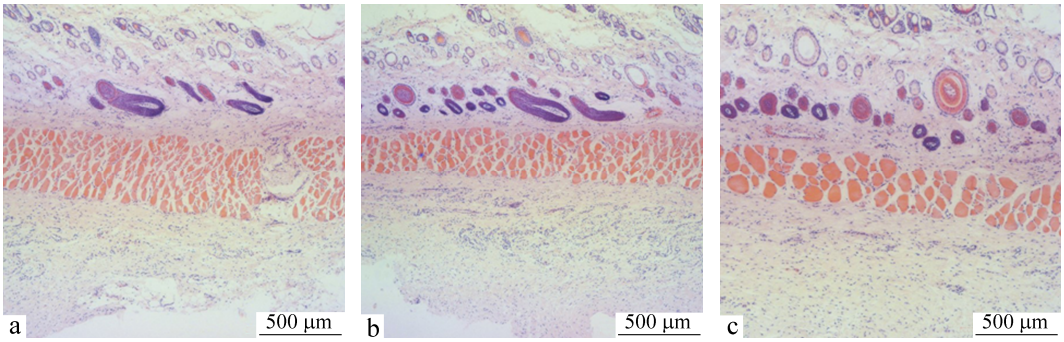


Fig. 5 H&E staining of skin tissue sections after 3 weeks (a), 6 weeks (b), and 9 weeks (c) of subcutaneous implantation of electrospun PLGA membrane

In Vivo Anti-adhesion Assessment for Achilles Tendon Repair

In order to evaluate the anti-adhesion effect of electrospun PLGA membrane toward damaged Achilles tendon, the animal experiment was conducted. All animals remained healthy and no inflammation of wound throughout the experiment. Figure 6 shows the photographs of preoperative Achilles tendon and the injury sites, when the SD rats were sacrificed at 21 days postoperation. Compared with the normal one (Fig. 6a), there was almost no residual membrane observed in the electrospun PLGA membrane group. Although a small amount of tissue adhesion was observed between tendon and tendon sheath tissue, the adhesion region could be easily split by blunt dissection (Fig. 6b). However, Achilles tendon in control group without treatment showed the presence of a broad layer of fibrous tissue adhesion between tendon and surrounding tissue (Fig. 6c). Compared to the electrospun PLGA membrane group, a significant thickening of Achilles tendon was detected for control group. It is because the boundary between tendon and surrounding tissue could not be observed clearly, which made difficult to be completely separated in control group.

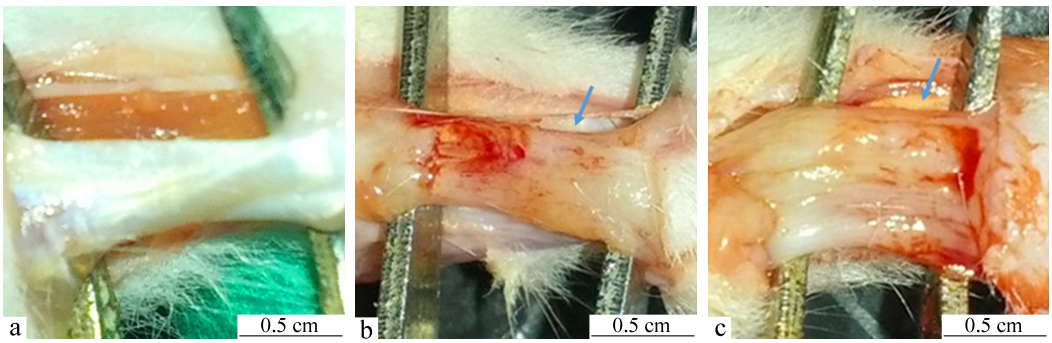


Fig. 6 Postoperative adhesion photographs of experimental animals: gross appearance of preoperative Achilles tendon tissue (a), Achilles tendon injury groups with (b) and without the treatment of electrospun PLGA membrane (c)
The arrows indicate the Achilles tendon adhesions at 3 weeks after repair surgery.

In order to measure the extent of adhesion, the distributions of adhesion fraction (Fig. 7a) and bonding areas (Fig. 7b) of rats were evaluated carefully in two groups. Achilles tendons without anti-adhesion disposal showed a high degree of adhesion (4 or 5 points) in injury site, and the average adhesion area was 1.82 cm^2. In comparison, the electrospun PLGA membrane group showed a slight adhesion (1 or 2 points), the proportion of adhesion was less than 30%, and the average adhesion area was only 0.15 cm^2. The excellent anti-adhesion efficacy endowed the electrospun PLGA membrane with great prospect for clinical application.

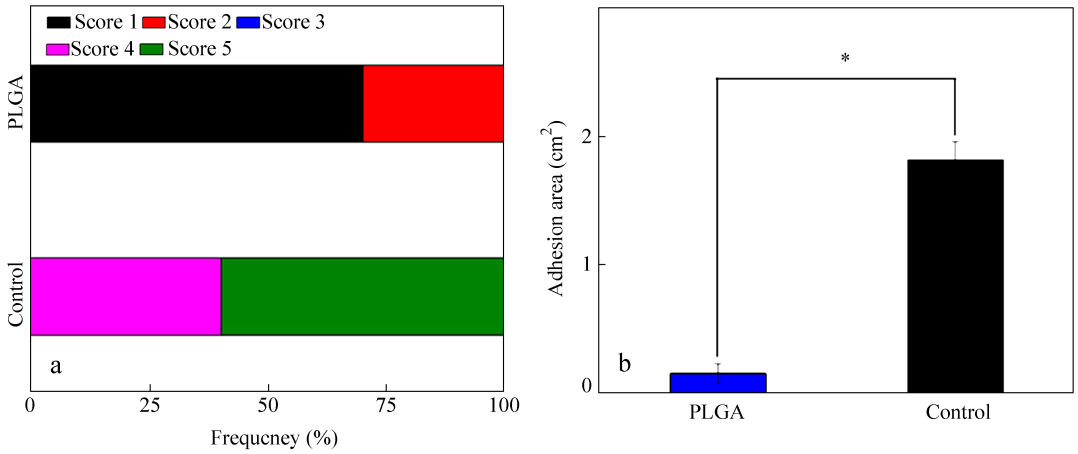


Fig. 7 Distribution of adhesion scores of Achilles tendon after treatment with or without electrospun PLGA membrane for 3 weeks after Achilles tendon repair surgery (a), and statistical adhesion areas of Achilles tendon (b) Data were presented as mean ± SD (n = 10, * p < 0.001).

As we all know, the regenerated collagen may be deposited in the wound and caused adhesion between tendon and surrounding tissue. To explore the degrees of adhesion and recovery of tendon, which could be detected through the distribution and density of collagen regeneration after repair surgery, Achilles tendons were observed by H&E and Masson's stainings. The results showed that less collagen was discovered between tendon and surrounding tissue indicating almost no adhesion formation in the electrospun PLGA membrane group (Fig. 8a and 8b). In addition, white cavities appeared in the site of material due to the biodegradation of electrospun PLGA membrane. In stark contrast, in control group, a large amount of collagen fibers were

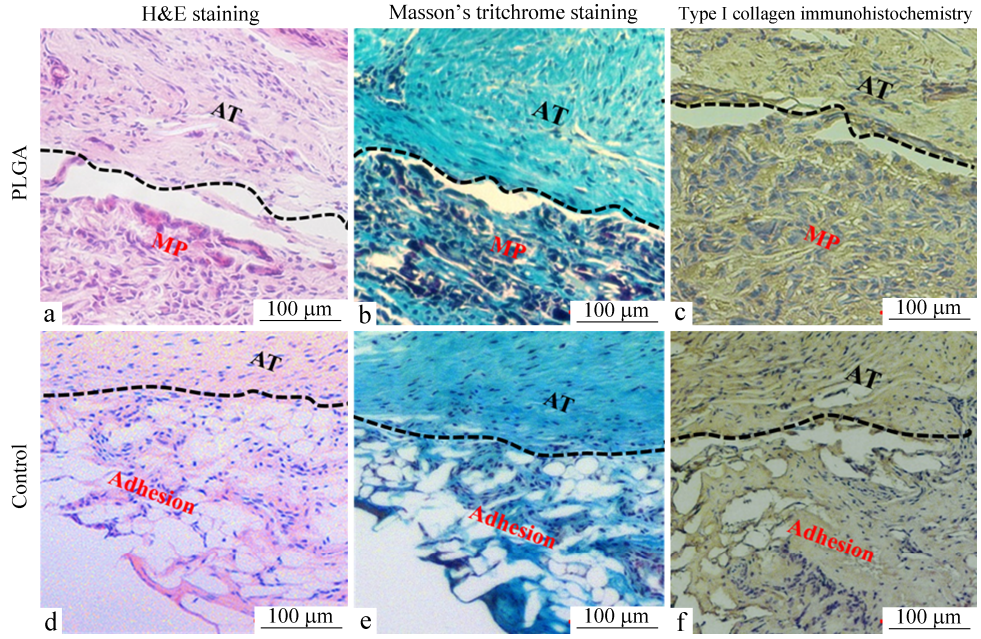


Fig. 8 Typical microdiagrams of H&E staining (a and d), Masson's tritchrome staining (b and e) and type I collagen immunohistochemistry (c and f) for injure site of Achilles tendon at 3 weeks after repair surgery, treated with (a–c) or without electrospun PLGA membrane (d–f)
AT: Achilles tendon, MP: material position.

generated and connected with each other between tendon and surrounding tissue (Fig. 8d and 8e). It demonstrated the existence of a wide range of tissue adhesion, and further validated that the PLGA films have significant anti-adhesion ability. Similarly as control group, collagen fibers and tendon cells were arranged densely and neatly in the electrospun PLGA membrane-treated group, and the staining area exhibited no visual difference in the normal and repaired positions. It revealed that the anti-adhesion electrospun PLGA membrane did not produce unfavorable effects on Achilles tendon.

The relevant literatures suggested that the extracellular matrix of tendon is predominantly composed of type I collagen that is also participate in many of the metabolic and inflammatory peritendinous connective tissues[40, 41]. In this work, immunohistochemistry was used to further confirm the degree of adhesion and recovery of tendon. In the assay, type I collagen in all groups rendered as brown (Fig. 8c and 8f). Compared to that of the electrospun PLGA membrane group, the boundary of type I collagen was not clear between the tendon and surrounding tissue in control group. It was directly shown that the tissue adhesion was lighter in the electrospun PLGA membrane group. The arrangement and direction of collagen fibers exhibited no visual difference in the electrospun PLGA membrane and control group, while the positive staining area of treated group was slightly larger than that of control group. It demonstrated that the electrospun PLGA membrane presented positive effect on the repair of Achilles tendon. Through comprehensive comparison of all above results, it meant that the Achilles tendon could be effectively separated from the surrounding tissue without significant damage to the surrounding tissue by the electrospun PLGA membrane, which simultaneously promoted the repair of Achilles tendon.

As well known, the degradation rate of anti-adhesion membrane is very important. Too fast degradation will cause serious tissue adhesion, while too slow degradation may adversely affect the normal function of tissue and lead to the prolonged postoperative recovery time. To overcome this situation, the electrospun PLGA membrane is chosen in this work, because it exhibits an appropriate degradation rate among a variety of polyester materials. In addition, the degradation rate of PLGA can be regulated in different approaches, such as changing the molecular weight or composition ratio. In order to regulate these parameters, more experimental research will be conducted in the future. Considering the above outcome and analysis, the electrospun PLGA membrane can be speculated to exhibit potential application in reducing the postoperative adhesion after tendon repair, which is mainly due to the porous microstructure and appropriate rate of degradation. Therefore, the overall anti-adhesion effect of PLGA material is worthy of recognition.

CONCLUSIONS

Adhesion is an inevitable consequence of trauma surgery. Herein, the electrospun PLGA membrane was prepared by electrospinning and used to prevent or reduce the adhesion after tendon surgery. The morphology, degradation kinetics, compatibility, anti-adhesion efficacy, histopathological, and immunohistochemical analyses were focused on. The electrospun PLGA membrane was revealed to exhibit an excellent microstructure and an appropriate degradation kinetics. More importantly, it was found to significantly reduce the adhesion between tendon and surrounding tissue, and improve tendon healing, which might be regarded as a good candidate barrier for the prevention of postoperative tendon adhesion. Furthermore, many other anti-adhesion therapeutics by the electrospun PLGA membrane is expected to be performed in the future, such as the anti-adhesion of flexor tendon and abdominal wall.

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