

Oriented inner fabrication of bi-layer biomimetic tendon sheath for anti-adhesion and tendon healing

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Abstract

Introduction: Synthetic fibrous membranes unveil a promising field in anti-adhesion of tendons. Meanwhile, oriented nanofiber structures have been widely studied and used in the application of biomedical engineering, particularly in repairing and strengthening effects.

Methods: In this study, a bi-layer poly(L-lactic acid) (PLLA) electrospun membrane was fabricated, in which the inner oriented fibrous layer was designed to promote tendon healing while outer random aligned layer was designed to prevent peritendinous adhesion.

Results: It was found that fibroblasts were aligned along the oriented fiber of membranes *in vitro* and in a Leghorn chicken model. In biomechanical tests of repaired tendons, no significant difference was found between oriented fibrous membrane and blank control in maximum tensile strength; both oriented fibrous membranes and random fibrous membranes showed lower work of flexion than blank control, which was consistent with gross assessment.

Conclusion: It was practicable to promote tendon healing while preventing adhesion *via* bi-layer PLLA membranes with an inner-oriented-fiber fabricated structure.

Keywords: anti-adhesion, oriented fiber, tendon adhesion, tendon healing

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Introduction

Tendon is vulnerable to adhesion after hand surgery, which could lead to malfunction of the extremities.¹ Quality of life of patients is impaired and medical expenses accrued along with unsatisfactory clinical outcomes. Nowadays, synthetic fibrous membranes unveil a promising field in anti-adhesion of tendon.²

An ideal fabrication of anti-adhesion membrane should fulfill several demands, including obstruction of peritendinous adhesion, promotion of tendon healing, provision of initial gliding space, and inherent properties of materials such as biodegradability, biocompatibility, and sufficient mechanical strength. In previous studies, biomimetic membranes with functional drugs were fabricated

for anti-adhesion and repair of tendon.^{3,4} However, the ambiguous effects and mechanism impeded the progress of clinical transformation. Recently, many studies suggested that the characterizations of biomedical materials could regulate cell behavior such as proliferation and differentiation *via* remodeling of cytoskeleton.^{5–8} Meanwhile, oriented structured nanofibers have been applied extensively in certain areas of cardiology,⁹ urology,¹⁰ and ophthalmology,¹¹ as well as orthopedics (annulus fibrous implant,¹² peripheral nerve defect,¹³ rotator cuff tendon defect,¹⁴ cartilage degeneration,¹⁵ and peritoneal adhesion.¹⁶)

Thus, we designed a bi-layer tendon membrane, where the inner layer was fabricated with oriented poly (L-lactic acid) (PLLA) electrospun nanofiber

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to promote tendon healing and the outer layer was fabricated in a random pattern to obstruct peritendinous adhesion. This novel multi-functional membrane was synthesized successfully and its effect in preventing tendon adhesion and promoting tendon healing was further investigated *in vitro* and *in vivo*.

Materials and methods

Materials and electrospun fabrication of fibrous membrane

PLLA (Mw=50,000 Da, Mw/Mn=1.61) was purchased from Jinan Daigang Co., Jinan, China. N,N-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Sigma. To prepare electrospinning solution, 10 g PLLA was dissolved in the mixture of 30 g DMF and 70 g DMC and placed in a 10 ml plastic syringe. The diameter of the steel needle was 0.7 mm. The electrospinning procedure was conducted with parameters of 15 kV and 1 ml/h solution flow rate. The randomly oriented electrospun membrane was collected on the collector placed 15 cm away from the needle. The bi-layer membrane was fabricated first with a randomly oriented layer using a grounded collector, then an oriented layer with a rotating drum collector at a velocity of 1500 rpm.

In vitro fibroblasts culture

Chicken embryonic fibroblasts (UMNSAH/DF-1) were used to assess the adhesion and proliferation on the random and aligned PLLA scaffold surfaces. The cells were incubated in DMEM (37°C, 5% CO₂, 10% fetal bovine serum and added antibiotics with 100 U/ml penicillin and 100 mg/ml streptomycin). Culture liquid was changed three times per week. Cells were collected using 0.25% trypsin by post-trypsinization methods on confluence. We used phosphate-buffered saline (PBS) to wash ethanol remnant away after a 90-min immersion. In the end, samples were transferred to a 24-well plate (1 × 10⁵ cells/ml, 100 ml/well).

Immunofluorescence assay

Fluorescence microscopy was used to observe UMNSAH/DF-1 cells on the surface of random and aligned PLLA fibrous membranes. Cells (initial density 10⁵/ml) were cultured on each group for 24 h. After 30 min staining with 4',6-diamidino-2-phenylindole (DAPI) and phalloidin (Sigma),

fluorescence microscopy (LEICA, USA) was used to observe the cell morphology. The blue and red staining on the surface of anti-adhesion scaffold indicated nuclei and cytoskeleton, respectively. In quantification of cell orientation, 70 cells were randomly selected from each sample, the orientation based on the standard reference was analyzed by MetaMorph image processor.

In vivo assays

The animal experimental ethics was approved by Shanghai Six People's Hospital (IRB number: 2020-0507), following the guidelines of Shanghai Jiao Tong University. As in a previous study,¹⁷ Leghorn chickens (*n* = 30, around 1.5 kg each) were anesthetized by intramuscular injection of ketamine hydrochloride at a dose of 50 mg/kg. An elastic tourniquet was used after surgical area preparation. The flexor digitorum profundus tendon was exposed at the lateral side of the phalanges from the third toe, with an incision that was about 1.5 cm. Then, the tendon sheath was incised, the FDP was separated and cut in a transverse direction. The 6-0 prolene (Ethicon Ltd.) was used to repair tendon using a modified Kessler technique.¹⁸ The chickens were separated randomly into blank control group, aligned fibrous membrane group, and random fibrous membrane group. A 1.0 × 1.0 cm² sheet was cut from each fibrous membrane. After tendon repair, it was enclosed with or without sheet. Finally, we sutured the skin and immobilized the operated leg with a customized splint.³ Animals were raised in separated cages.

Tissue orientation characteristics

The tissue surrounding repaired tendon was obtained after 3 weeks. After dehydrating, drying, and spraying, samples were detected in the view of scanning electron microscopy (FEI Quanta 200). To quantify the cell orientation, 70 cell lines were randomly selected from each sample, the orientation based on the standard reference was analyzed by MetaMorph image processor.

Macroscopic evaluation

The tendon surrounding adhesion was evaluated according to the macro-scale grading system by two independent investigators. In detail: level 1 indicates adhesion free; level 2 indicates slightly

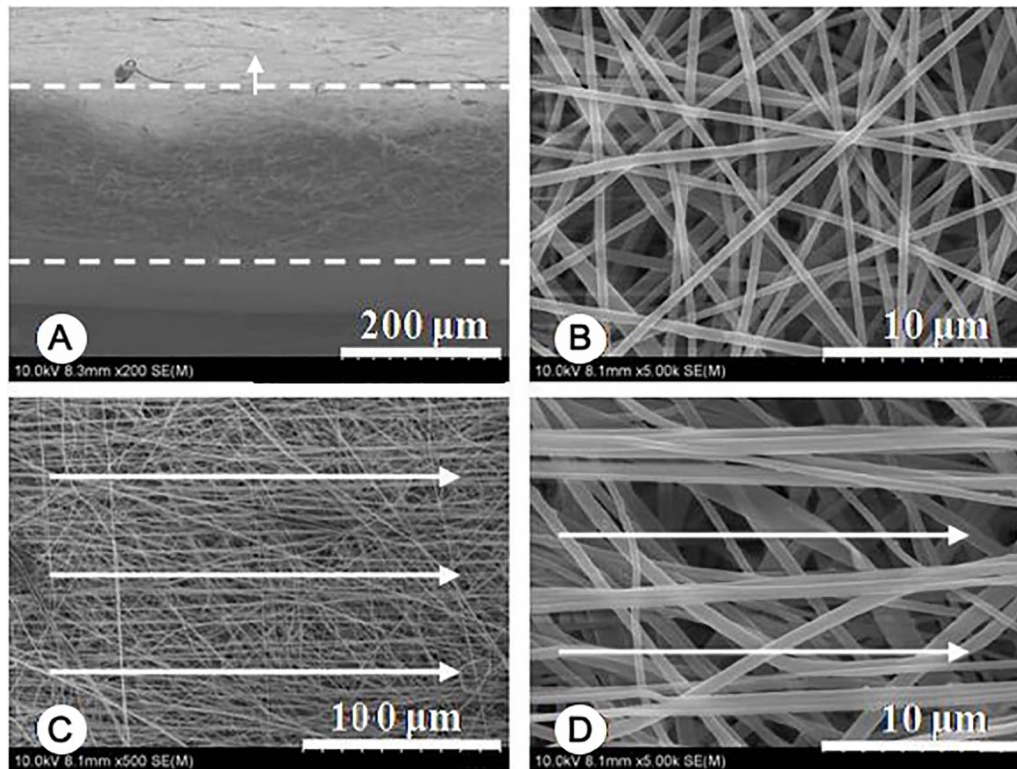


Figure 1. SEM photograph of bi-layer PLLA fibrous membranes: (A) sectional view of bi-layer fibrous membranes with inner aligned fibers; (B) surface view of the randomly aligned layer; (C, D) surface views of the aligned fibrous layer. The white arrow indicates the inner aligned fibrous layer.

separable adhesion; level 3 indicates mildly unseparable adhesion; level 4 indicates moderate adhesion (affecting 35–60% of adhesion area); level 5 indicates severe adhesion (affecting over 60% in total adhesion tissues).

Biomechanical assay

First, the specimens were prepared by exposing repaired tendon at the level of the ankle joint. The peak tensile strength was measured by a tensile tester (Instron 5548), as well as the work of flexion. The proximal side of each sample was closely set on a dynamometer, while the other side was fixed in a customized clamp. In work of flexion test, stress (N) and displacement (mm) data were collected before proximal interdigital joint angle reached 40°, while the repaired tendon was pulled at the rate of 10 mm per minute. The area under the curve of stress and displacement represented the work of flexion. Meanwhile, the stress showed maximum tensile strength when FDP tendon completely separated from its sheath.

Statistics

Mean \pm SD was calculated to present the parameters. One-way analysis of variance (ANOVA) was used (SPSS 11.0). A significant difference was considered when $p < 0.05$.

Results

Morphology of PLLA fibrous membrane

Two types of bi-layer anti-adhesion membranes were fabricated. One was a bi-layer membrane with randomly aligned fiber in both inner and outer layers, while the other was fabricated with inner aligned fibers and outer randomly aligned fibers. The morphology of both layers was detected by a scanning electronic microscope (SEM) and presented in Figure 1. The aligned fibers of inner layer were densely arranged (Figure 1A), and the fibers presented more oriented pattern compared with the randomly aligned layer (Figure 1C and D). The thickness of each layer in random and aligned fibrous membranes were

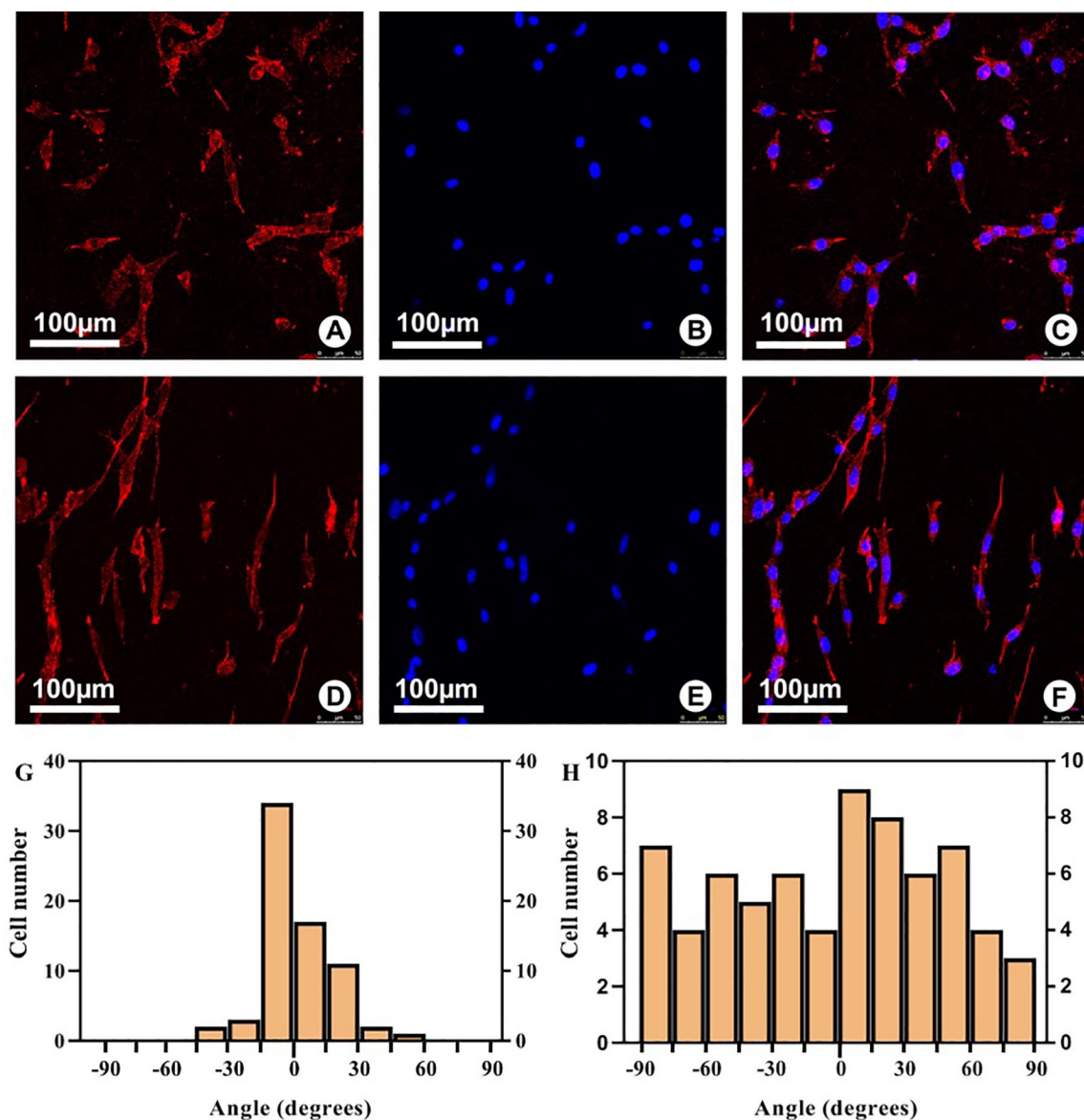


Figure 2. Fluorescent micrographs of chicken embryonic fibroblasts (UMNSAH/DF-1) after 24 h of incubation. The nuclei appeared blue and the cytoskeleton appeared red on the surface of the fibrous membranes: [A–C] randomly aligned fibrous membrane; [D–F] aligned fibrous membrane. Cell orientation angle distributions on aligned fibrous membrane (G) and random fibrous membranes (H).

near 200 μm . The porosities were $79.23 \pm 5.05\%$ and $76.16 \pm 4.46\%$ in random and aligned fibrous groups, respectively.

In vitro adhesion and proliferation of fibroblasts

The confocal fluorescent images present the adhesion and morphology of fibroblasts on the random fibrous layer and aligned fibrous layer after a 24-hour incubation (Figure 2). The morphology of fibroblasts of oriented fibrous membrane showed significant arrangement along with the

aligned fibers (Figure 2D–F) and cell orientation angle distributions were illustrated. On aligned fibrous layer, the orientation angle was concentrated between 0 and 30°; meanwhile (Figure 2G), it had an unordered distribution on randomly aligned fibers (Figure 2H).

Animal model

The repaired tendon of each group was obtained postoperatively after 3 weeks. The surgical sites were unveiled to assess peritendinous adhesion of

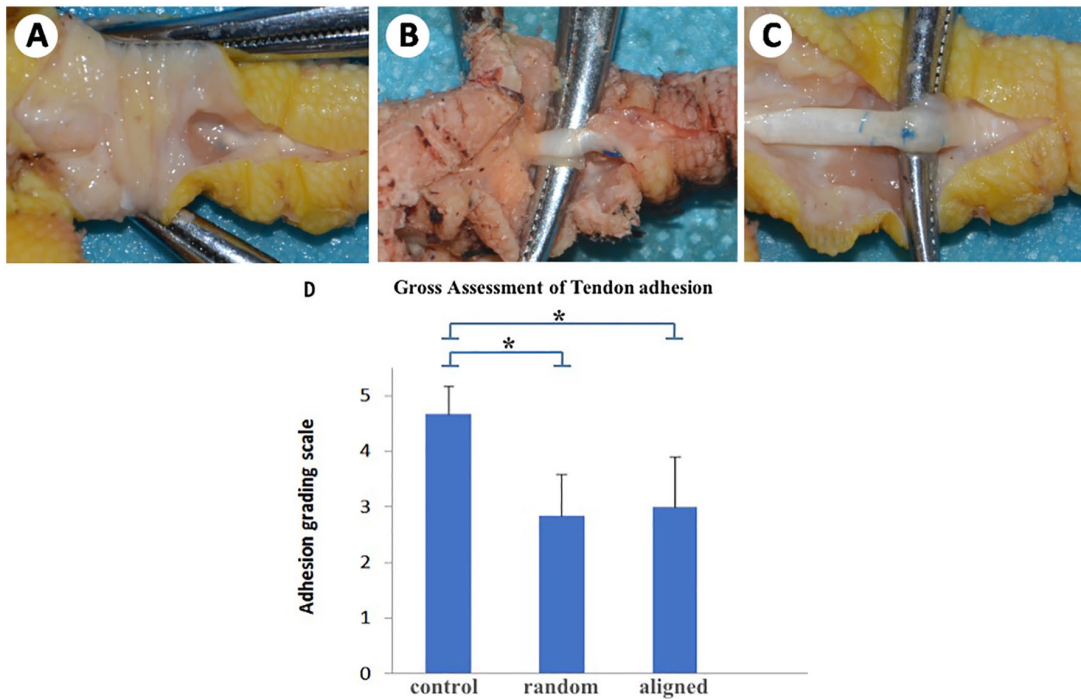


Figure 3. The adhesion evaluation of repaired flexor digitorum profundus (FDP) in chicken models after 3 weeks: (A) blank control group; (B) random fibrous membranes group; (C) aligned fibrous membranes group; (D) adhesion grading scale of the three groups. * $p < 0.05$.

tendon, as well as the healing situation. In the blank control group, extensive peritendinous adhesion was observed in the repaired sites, thus it was difficult to separate tendon from surrounding tissues (Figure 3A). Both in random and aligned fibrous membranes, the surrounding tissue could be easily isolated from repaired areas (Figure 3B, C). Adhesion grading scale (as detailed in the Materials and methods section) of aligned and random fibrous membranes were significantly lower than those of the untreated group; meanwhile, no significant difference was found between aligned and random fibrous membranes (Figure 3D). Figure 4 showed the SEM imaging of cells at the surgical site *in vivo* with random and aligned layers. Cells showed no specific alignment in the random layer, while they were significantly aligned along the aligned fibers (Figure 4B and D). Cell orientation angle distributions unveiled the same pattern (Figure 2E and F).

Biomechanical test

The peak tensile strength and work of flexion were measured to detect the tendon healing strength and extent of adhesions by using a tensile tester. The maximum tensile strength of blank

control group and oriented fiber group (group B) were similar, relatively higher than that of the random fiber group (Figure 5A). Meanwhile, the parameters showed that the work of flexion of both the oriented fiber group and randomly aligned fiber group were significantly lower than in untreated control group (Figure 5B).

Discussion

We have fabricated a biomimetic tendon sheath scaffold, in which the fibers of the inner layer were fabricated in an oriented array aiming to promote tendon healing, and the fibers of the outer layer were in a random pattern acting as a physical barrier to secondary adhesion after intervention. The bi-layer structure of the PLLA membrane provided a gliding space for early motion after surgery, which increased the anti-adhesion effect of the scaffold. The results suggested that this innovation could prevent adhesion as well as promoting the quality of tendon healing.

Without the encumbrances such as complexity of the production process, uncertainty of mechanism involving drug delivery, and disturbance of tendon regeneration, the inherent plain structure is one of

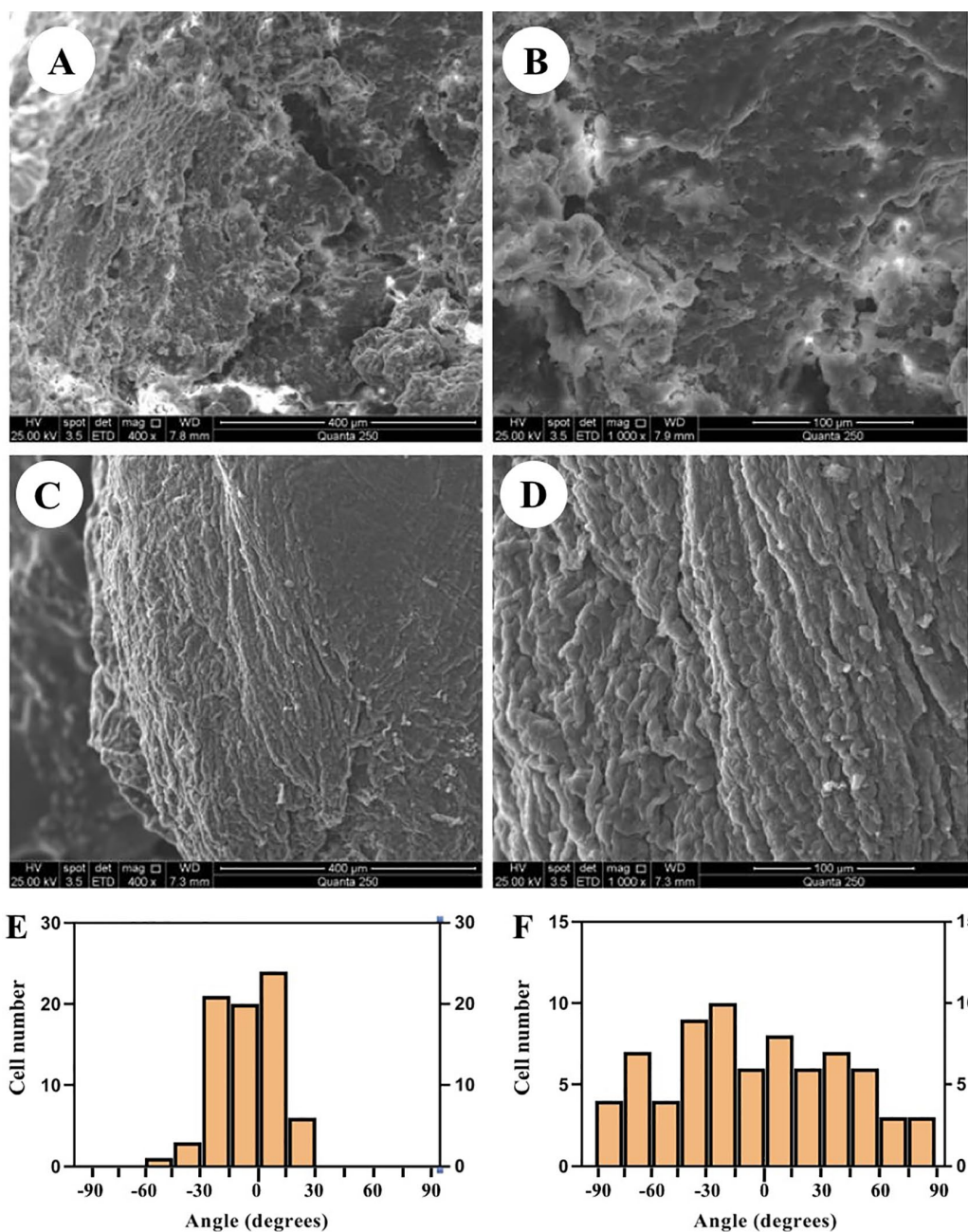


Figure 4. SEM photograph (400× and 1000×) of the surface of membranes at the surgical site after 3 weeks: (A, C) surfaces of random fibrous membranes *in vivo*; (B, D) surfaces of oriented fibrous membranes *in vivo*. Cell orientation angle distributions *in vivo* on oriented fibrous membrane (E) and random fibrous membranes (F).

the advantages of this scaffold,^{4,19} making both manufacturing and application in clinical situations easier.

Recently, oriented materials and their applications in orthopedic tissue engineering have been studied intensively, including peritoneal adhesion,¹⁶ cartilage degeneration,¹⁵ peripheral nerve defect,¹³

annulus fibrosus implant,¹² and rotator cuff tendon defect.¹⁴ However, there is still no application in the field of biomaterials for tendon adhesion. Thus, we have introduced oriented fibrous membrane to prevent tendon adhesion while promoting tendon healing. The tendon cells can secrete type I collagen and lead traction force to recombine and arrange collagen molecules along cell shape, and

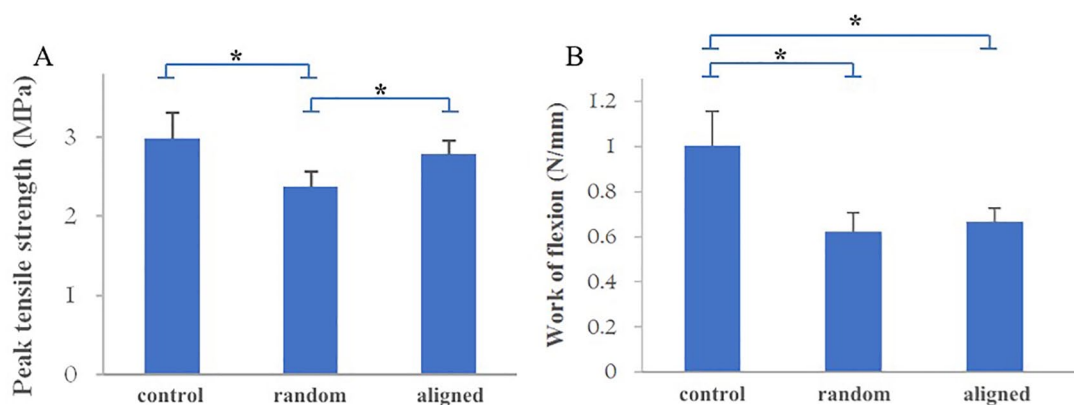


Figure 5. Biomechanical test of FDP samples: (A) peak tensile strength; (B) work of flexion. * $p < 0.05$.

the aligned collagen can further promote tenogenic differentiation and tendon regeneration, Therefore, the mechanical strength of healed tendon in aligned fibrous PLLA was higher than that of random fiber membrane due to cell morphological structure as well as the regulation of cell behavior.²⁰

There are only a few papers on the tendon repair of oriented bi-layer or multi-layer electrospun nanofibrous scaffolds to date. Rothrauff *et al.*²¹ found that aligned multi-layer PCL and PLLA nanofibrous scaffold could support the expression of tenogenic markers and enhanced cell numbers of hMSCs, as well as total collagen content, and total sulfated glycosaminoglycan content. In another study, Yang *et al.*²² built up multilayered polycaprolactone/gelatin fiber-hydrogel composite for tendon tissue engineering and revealed that cells impregnated into the constructs remained responsive to topographical cues and exogenous tenogenic factors, such as TGF- β 3. However, they focused on tendon graft regeneration and did not investigate the influence on the adhesion of aligned nanofibrous scaffold. Further investigations should be carried out to reveal the effects of aligned nanofibrous scaffolds on tendon regeneration while preventing adhesion.

Owing to its biodegradability, biocompatibility, and US FDA approval, this structured aligned PLLA fibrous membrane is very close to clinical application and further regulatory mechanisms have been verified in cell compressive stress, remodeling of cytoskeleton, and cross-link signaling pathway. Lack of degradation study *in vivo* was one of the limitations of this paper. Inflammation and its effect on peritendinous adhesion caused by

PLLA degradation will be investigated in further study. Since the pathological situations are different between human and chicken, further pre-clinical research is necessary to reveal the insightful relationship and mechanisms between tendon regeneration and inflammation.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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