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Antibacterial and anti-adhesion effects of the silver nanoparticles-loaded poly(L-lactide) fibrous membrane

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ABSTRACT

The complications of tendon injury are frequently compromised by peritendinous adhesions and tendon sheath infection. Physical barriers for anti-adhesion may increase the incidence of postoperative infection. This study was designed to evaluate the potential of silver nanoparticles (AgNPs)-loaded poly(L-lactide) (PLLA) electrospun fibrous membranes to prevent adhesion formation and infection. Results of an in vitro drug release study showed that a burst release was followed by sustained release from electrospun fibrous membranes with a high initial silver content. Fewer fibroblasts adhered to and proliferated on the AgNP-loaded PLLA electrospun fibrous membranes compared with pure PLLA electrospun fibrous membrane. In the antibacterial test, the AgNP-loaded PLLA electrospun fibrous membranes can prevent the adhesion of Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* and Gram-negative *Pseudomonas aeruginosa*. Taken together, these results demonstrate that AgNP-loaded PLLA electrospun fibrous membranes have the convenient practical medical potential of reduction of infection and adhesion formation after tendon injury.

1. Introduction

The complications of tendon injury are frequently compromised by peritendinous adhesions and tendon sheath infection; an untreated tendon sheath infection is further recognized as crucial etiology of adhesion formation underscoring the importance of early prevention of infection [1,2]. Recently, aiming to prevent infection and adhesion formation, biochemical pharmacology and physical barriers have been used respectively [3,4]. However, antibiotics have limited effects on anti-adhesion while physical barriers may increase the incidence of postoperative infection. Although NSAIDs-loaded physical barriers have been used to prevent adhesion formation and inflammatory response of biomaterials [5,6], these drugs only focus on the anti-adhesion without taking prevention of infection caused by bacteria into consideration.

Silver ions are famous for their powerful antibacterial ability [7]. However, cytotoxicity of silver ions may lead to a decrease in cell viability and proliferative activity [8–10]. Recently, silver nanoparticles (AgNPs) have been widely used in sol–gel method and implantable materials to reduce the incidence of postoperative infection [11,12] but these various applications refer to the potential risk related to their toxicity. For example, in NIH3T3 fibroblasts, AgNPs were found to own cytotoxic effects and induce apoptosis [13]. Nevertheless, NIH3T3 fibroblast was a widely-used cell model for anti-adhesion formation, which makes AgNPs just suitable for anti-adhesion formation and simultaneously early prevention of infection. Previously, Ag ions were introduced onto the material surfaces by sol–gel method, ion implantation, ion exchange and sputtering, or into the electrospun fibers by adsorbing silver ions with subsequent hydrogen reduction [7,11,14–17]. The antimicrobial agents on nanofiber surfaces resulted in initial burst releasing of Ag ions and unmatched Ag ions releasing with material degradation, and the complex manufactory process limits their practical medical application. Recently, no antibacterial membrane has been invited to distribute AgNPs throughout the materials for long-lasting release as the material degradation with attempt to reduce peritendinous adhesion and infection.

Electrospun fibrous membranes are attractive barriers for tissue separation and drug delivery to get drug-loaded materials with lengthened releasing time because of their large surface area and controlled porous structure [4,18]. With the porous structure, when fibrous membranes are used to prevent peritendinous adhesion, the passage of nutrients from outside the tendon sheath to promote

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intrinsic healing is allowed [18]. Therefore, it can be envisaged that, if these admirable features of an electrospun membrane are combined with the proper properties arising from silver ions, a type of excellent antibacterial and anti-adhesion membrane can be gained.

Nowadays, biodegradable poly(L-lactide) (PLLA) has been widely used in clinic because it can be used in important biosafety applications approved by FDA. Therefore, in this study, the AgNPs are directly electrospun into biodegradable PLLA fibrous membrane. Ag release study was performed. It is expected that the antibacterial and anti-adhesion effects of the AgNP-loaded PLLA fibrous membrane can be combined together.

2. Materials and methods

2.1. Materials

Poly(L-lactide) (PLLA, Mw = 50 kDa, Mw/Mn = 1.6) was prepared by bulk ring-opening polymerization of L-lactide using stannous chloride as an initiator (Jinan Daigang Co., Jinan, China). Silver nanoparticles with 99.9% purity and a typical size of 60–100 nm were used as an anti-bacterial additive in this study, and these were purchased from the Aladdin Regents Company (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were supplied by Gibco (Grand Island, NY). All other chemicals and solvents were of reagent grade or better and purchased from GuoYao Regents Company (Shanghai, China), unless otherwise indicated.

2.2. Electrospinning and characterization of AgNP-loaded fibrous membranes

1.0 g PLLA was completely dissolved in 3.5 g dichloromethane to concentration of 25% (w/w). 0.04, 0.08 and 0.12 g AgNPs were completely dispersion in 1.5 g N,N-dimethlformamide (DMF), respectively. Later, AgNP suspension were then added to the PLLA solution and dispersed using a vortex mixer. The AgNP/PLLA blend solutions with different weight ratios from 100:4, 100:8 to 100:12 were prepared for electrospinning and the name are PLLA/Ag4%, PLLA/Ag8% and PLLA/Ag12% respectively.

The electrospinning processes were performed as described previously [19]. Briefly, the electrospinning apparatus was equipped with a high-voltage statitron (Tianjing High Voltage Power Supply Co., Tianjing, China) whose maximal voltage is 30 kV. Flow rate of the polymer solution was controlled by a precision pump (Zhejiang University Medical Instrument Co., Hangzhou, China) to maintain a steady flow from the capillary outlet. The AgNP-loaded PLLA electrospun fibrous membranes were collected on the surface of grounded aluminum foil and vacuum dried at room temperature for 24 h.

The morphology of fibrous scaffolds was observed by scanning electron microscopy (SEM, FEI Quanta 200, Netherlands). At least five images were taken for each scaffold sample and fiber diameter of scaffolds was measured from SEM images with 10,000× magnification. From each image, at least 20 different fibers and 200 different segments were randomly selected to generate an average fiber diameter using Photoshop [20].

The structure of Ag in the PLLA electrospun fibers were observed with a transmission electron microscope (TEM, JEM-2100F, Japan).

2.3. Ag ions release study

For evaluating the in vitro release behavior of Ag ions, the AgNP-loaded PLLA fibrous membranes were first punched into small squares with a total mass of ca. 100 mg, which were immersed in 20 ml of 154 mM phosphate buffered saline (PBS, pH 7.4), containing 0.02% sodium azide as a bacteriostatic agent. The suspension was kept in a thermostated shaking water bath (Taichang Medical Apparatus Co., Jiangsu, China) with a shake speed of 50 cycles per minute at 37 °C. At predetermined time intervals, 1.0 ml of the release buffer was removed for analysis and 1.0 ml of fresh PBS was added back for continuing incubation. The amount of released Ag ions in the collected medium was determined by UV–VIS spectroscopy at 411 nm. A standard calibration plot of Ag ions in the concentration range of 0–0.1 mg/ml (411 nm absorbance) was used to determine the concentration of the Ag ions released, and a linear correlation ($\gamma^2 = 0.9948$) was determined between the absorption strength and Ag ion concentration. The percentage of the released Ag ions were then calculated based on the initial weight of AgNPs incorporated in the electrospun scaffold.

This converter calculates the measured value in units of "mg/ml" into units of "ppm" and vice versa. The solution volume was changed to 500 ml to mimic the amount of body solution.

2.4. Proliferation assay

C3h 10 1/2 mouse fibroblasts were used to evaluate proliferation on PLLA electrospun fibrous membrane surfaces with or without AgNPs. The cells were cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Tauranga, New Zealand) and antibiotics (penicillin 100 U/ml, streptomycin 100 g/ml; Hyclone, Logan, UT, USA) at 37 °C in a humidified atmosphere with 5% CO_2. After sterilized by immersing in 75% ethanol for 1.5 h in a 24-well plate, the specimens were washed repeatedly with PBS to remove residual ethanol. Cells (2×10^5 cells/ml) were seeded into each well (100 μl/well) and analyzed for proliferation on days 1 and 4. The number of cells grown on different specimens was observed by scanning electron microscopy (SEM, FEI Quanta 200, Netherlands) after fixation with 2.5% glutaraldehyde (Gibco Laboratories) and dehydration in ethanol graded series. For the cell number counting, the cells were detached from the surfaces of each specimen by trypsinization and subsequently counted with a hemacytometer (Reichert Co., USA). The results were expressed as the average number of cells attached per cm^2 of surface.

2.5. Cytotoxicity

Murine fibroblast line L929 was used to determine the cytotoxicity of PLLA electrospun fibrous membranes with or without AgNPs by following the instructions of ISO standards (1992) [21]. L929 cells were provided by the Chinese Academy of Sciences (Shanghai) and grown in DMEM supplemented with 10% FBS and antibiotics (penicillin 100 U/ml, streptomycin 100 g/ml; Hyclone, Logan, UT, USA) at 37 °C with 5% CO_2. The contents of PLLA electrospun fibrous membranes with or without AgNPs were extracted from specimens in DMEM medium for 24 h at 37 °C. According to the instructions, L929 cells were plated at a density of 1.5×10^3 cells (96-well plate) and incubated in the DMEM medium, 100 U/ml penicillin, and 100 μg/ml streptomycin containing extracts of PLLA electrospun fibrous membranes with or without AgNPs for 24 h. Then, 20 μl of a 5 mg/ml MTT (Sigma; Saint Louis, MO) was added into each well. After 4 h of culture at 37 °C, the medium was removed. The MTT formazan crystals were solubilized in 200 μl dimethyl sulfoxide on a shaking platform for 10 min. Absorbance at 490 nm was determined using a spectrophotometer (Synergy 2; BioTek, Winooski, VT). Cell toxicity of extracted contents from specimens was rated as follows: severe (<30%), moderate (30%–60%), slight (60–90%), or non-cytotoxic (>90%) of MTT activity, compared to the control cells cultured in extract free medium.

2.6. Bacterial inhibition test

2.6.1. Bacteria culture

Staphylococcus epidermidis (ATCC12228), *Staphylococcus aureus* (ATCC25923) and *Pseudomonas aeruginosa* (ATCC27853) were purchased in freeze-dried form from Chuangxiang Biotechnology (Shanghai,

China). The stains were stored at −80 °C in glycerol. For testing, the stains were cultured on Trypticase soy agar (TSA; BD Biosciences, Franklin Lakes, NJ) medium at 37 °C overnight and the single stain was cultured in 10 ml BBL Trypticase soy broth (TSB) at 37 °C for 12 h. After 12 h of culture, each stain was adjusted to a concentration of 1×10^6 CFUs/ml in TSB according to McFarland. Samples in a 24-well plate (Costar3548, USA) were cultured in 1 ml of suspension at 37 °C with agitation at 100 rpm.

2.6.2. Confocal laser scanning microscope

A LIVE/DEAD Baclight bacteria viability kit (Invitrogen; Eugene, OR) was used to analyze the adherence of bacteria on the samples. After 24 h of culture, samples were gently washed by PBS three times and immersed in 300 μl stain solution for 10 min. The viable and nonviable bacteria were assessed by using confocal laser scanning microscope (CLSM, Leica TCS SP2; Leica Microsystems, Heidelberg, Germany) since the viable bacteria with intact cell membrane stain fluorescent green and nonviable bacteria with damaged membranes stain fluorescent red. Quantitative analysis of each image was performed by determination of mean fluorescence intensity using Image-Pro Plus. Results were gained based on three CLSM images and normalized to the mean fluorescence intensity of control samples.

2.6.3. SEM

SEM was also used to observe the attachment of bacteria. After 24 h of culture, samples were gently washed three times with PBS to remove no adherent bacteria and fixed in 2.5% glutaraldehyde for 2 h at 4 °C. After dehydration with increasing concentrations of ethanol, samples were subsequently freeze-dried, sputter coated with gold, and observed using a scanning electron microscope (SEM, FEI Quanta 200, Netherlands). After 24 h of culture, duplicate samples were used for quantitative analysis by spread plate method [22,23]. All samples were gently washed with PBS three times and replaced in 0.5 ml TSB followed by ultrasonication in a 150 W ultrasonic bath (B3500S-MT, Branson ultrasonics Co., Shanghai China) at a frequency of 50 Hz for 5 min. The suspension was serially diluted by 10-fold, plated in triplicate on TSA and then cultured at 37 °C for 24 h. The numbers of surviving colonies on TSA were counted and bacteria in the biofilm were calculated and normalized to the counts of control samples.

2.7. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical software SPSS 10.0 (Chicago, IL) was used to analyze the data by one-way analysis of variance; $P<0.05$ considered significant.

3. Results

3.1. Characterization of the Ag-loaded fibrous membranes

The SEM and TEM micrographs of the fibers obtained are shown in Fig. 1. These fibers possessed the common feature of being round-shaped, bead-free, randomly arrayed and very porous, and few AgNPs was shown on the surface of fibers in the PLLA/Ag samples. The average diameters of PLLA fibers and medicated PLLA/Ag4%, PLLA/Ag8%, PLLA/Ag12% fibers are 0.85 ± 0.21, 0.89 ± 0.38, 0.95 ± 0.42 and 1.08 ± 0.32 μm, respectively, increasing as the increase of AgNPs. Few aggregated AgNPs were shown on the TEM images.

3.2. Ag release study

The Ag ion release profiles of the PLLA electrospun fibrous membranes containing 4%, 8% and 12% AgNPs were shown in Fig. 2. During the initial 2 days, burst release of Ag ions from the medicated PLLA/

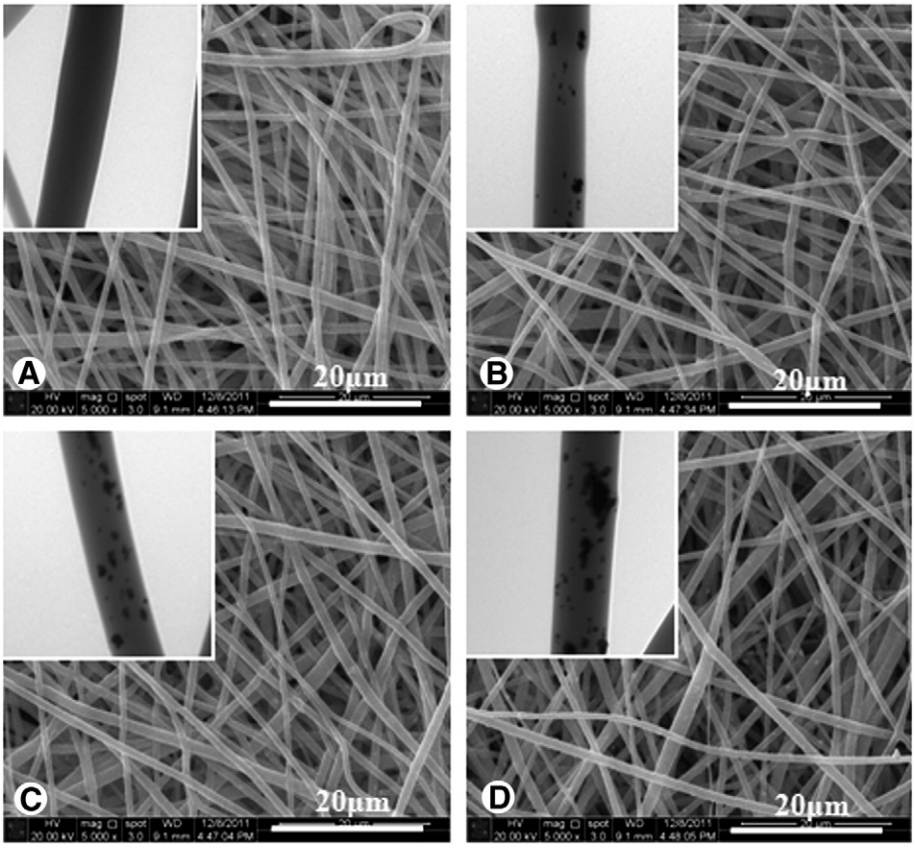


Fig. 1. SEM and TEM (inset picture) images of electrospun PLLA (A), PLLA/Ag4% (B), PLLA/Ag8% (C) and PLLA/Ag12% (D) fibers with 0%, 4.0%, 8.0% and 12.0% AgNPs (w/w), respectively.

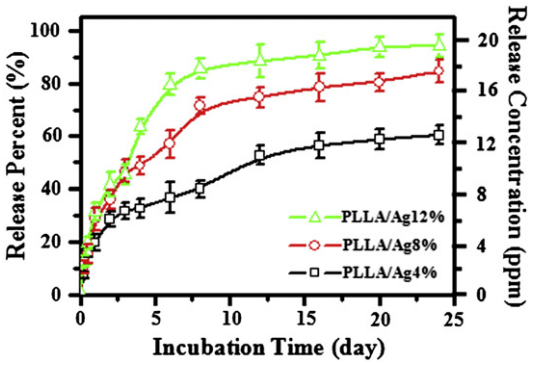
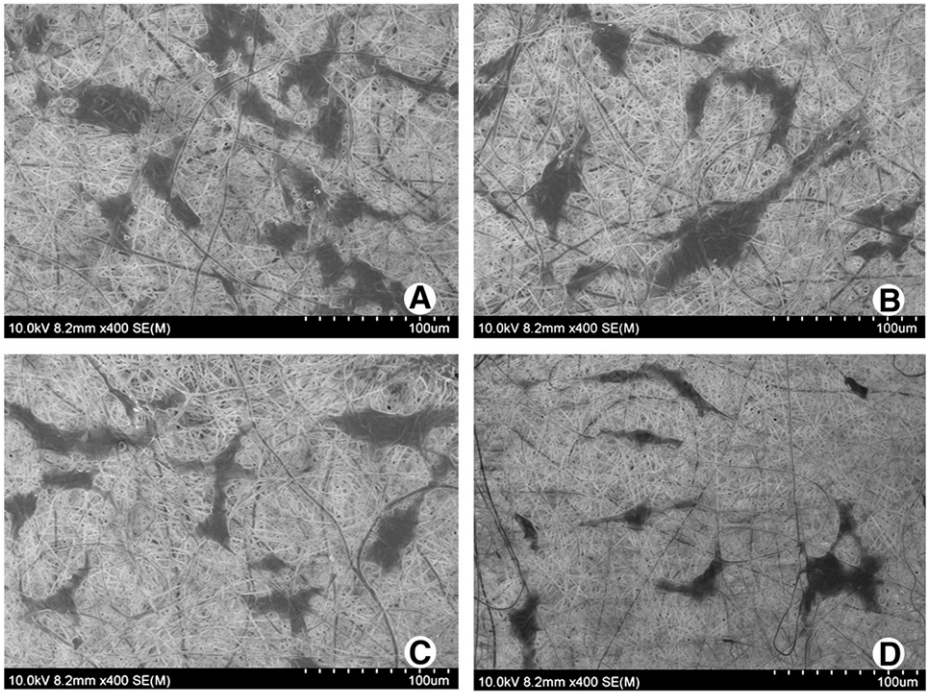


Fig. 2. In vitro Ag ion release cumulative percentage and release concentration from electrospun fibers with 4.0%, 8.0% and 12.0% AgNPs after incubating in PBS at 37 °C.

Ag4%, PLLA/Ag8% and PLLA/Ag12% electrospun fibrous membranes are 28%, 35% and 41%, respectively. During the following 10 days, the samples of PLLA/Ag4%, PLLA/Ag8% and PLLA/Ag12% took on a sustained release phase and, especially, Ag ions were completely released from PLLA/Ag12% at the last time point. In vitro Ag release cumulative concentration (ppm) from PLLA/Ag electrospun fibrous fibers was also summarized in Fig. 2. The concentration of Ag ions released during the initial 24 days was about 13.4, 17.6 and 19.5 ppm in the fibrous membranes with 4.0%, 8.0% and 12.0% AgNP entrapment, respectively.

3.3. Cell proliferation assay

The proliferation of C3H10T1/2 cells on the surface of PLLA fibrous membrane with or without AgNPs was compared after 1 and 4 days (Fig. 3A–D). It was observed that cells grew on the surfaces of all



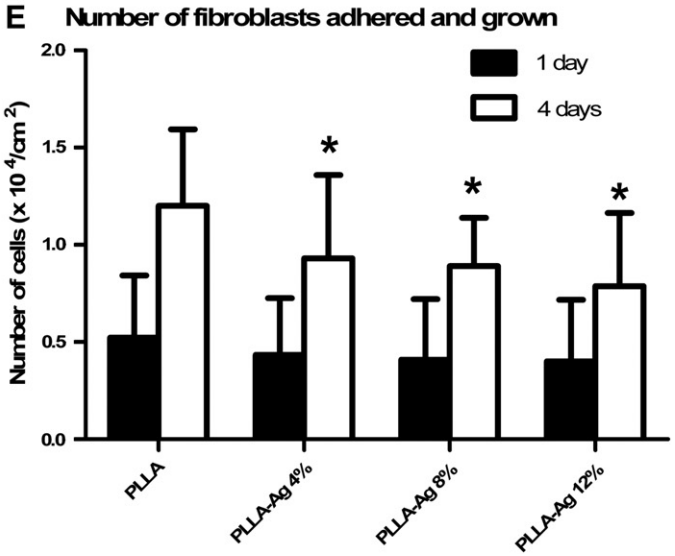


Fig. 3. SEM observation of cells growing on the surface of PLLA (A), PLLA/Ag4% (B), PLLA/Ag8% (C), PLLA/Ag12% (D) on day 4. Cell counts (E) on PLLA, PLLA/Ag 4%, PLLA/Ag 8% and PLLA/Ag 12% at day 1 and day 4. *$P<0.05$ compared with cells grown on the PLLA fibrous membrane. Data are expressed as mean ± SD (each group, n = 3).

specimens. However, fewer cells were detected on the surfaces of AgNP-loaded PLLA fibrous membranes compared with the surface of PLLA fibrous membrane. Furthermore, the cell proliferation on the surfaces of AgNP-loaded PLLA fibrous membranes decreased with increasing AgNPs composition. By comparing the number of cells on different surfaces after 1- and 4-day culture, the cell growth on the different surfaces after 4 days showed a similar trend with those after 1 day; the cells were proliferated better on the surfaces of PLLA fibrous membranes after 4 days (Fig. 3E).

3.4. Cytotoxicity

The cytotoxicity of PLLA fibrous membrane with or without AgNPs was evaluated by the MTT assay. Based on the reduction of MTT absorption at 490 nm compared to the DMEM medium control, the relative enzymatic activity of PLLA fibers and PLLA/Ag 4%, PLLA/Ag 8%, PLLA/Ag 12% fibers are 99.31 + 3.22%, 97.2 ± 6.31%,96.25 ± 8.29% and 93.12 ± 9.35%, respectively. Extracts of PLLA fibrous membranes with or without AgNPs were not cytotoxic.

3.5. Anti-bacterial test

3.5.1. CLSM observation

The adherent of bacteria for 24 h was observed by CLSM after staining. The image revealed a clearly difference among the fluorescence of bacteria on the surfaces of each sample (Fig. 4). The bacteria on the surface of PLLA fibrous membrane showed the most intense fluorescence of bacterial adherent ($P<0.05$) (Fig. 4A–C). CLSM imaging also showed almost exclusively the green viable bacteria on the surface of PLLA fibrous membrane, while the bacteria decreased in an obvious fashion on the surfaces of AgNP-loaded PLLA fibrous membranes. Consequently, the materials with AgNPs had a better antibacterial ability against these three stains. However, no differences of mean intense fluorescence were observed between all three samples containing AgNPs ($P>0.05$) and between the three bacterial strains ($P>0.05$).

3.5.2. SEM observation

SEM was also used to further examine the attachment of three bacterial strains on different surfaces after 24 h of culture. The images

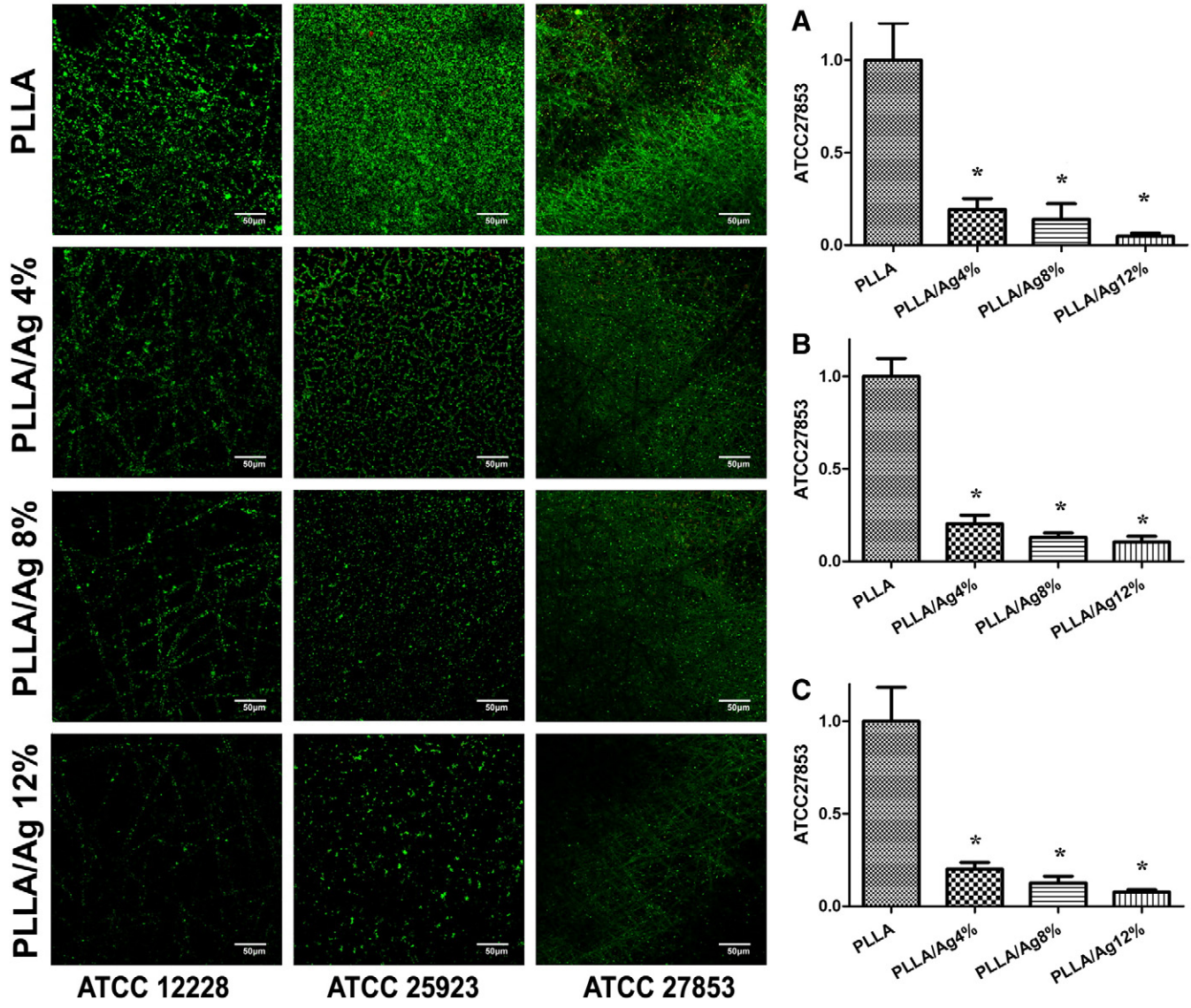


Fig. 4. Confocal laser scanning microscopy image of bacteria adhered to the samples after 24 h of culture with a Baclight dead/live stain. The statistic results of *S. epidermidis* (A), *S. aureus* (B) and *P. aeruginosa* (C). *$P<0.05$ compared with bacteria adhered to the PLLA fibrous membrane. Data are expressed as mean ± SD (each group, n = 3).

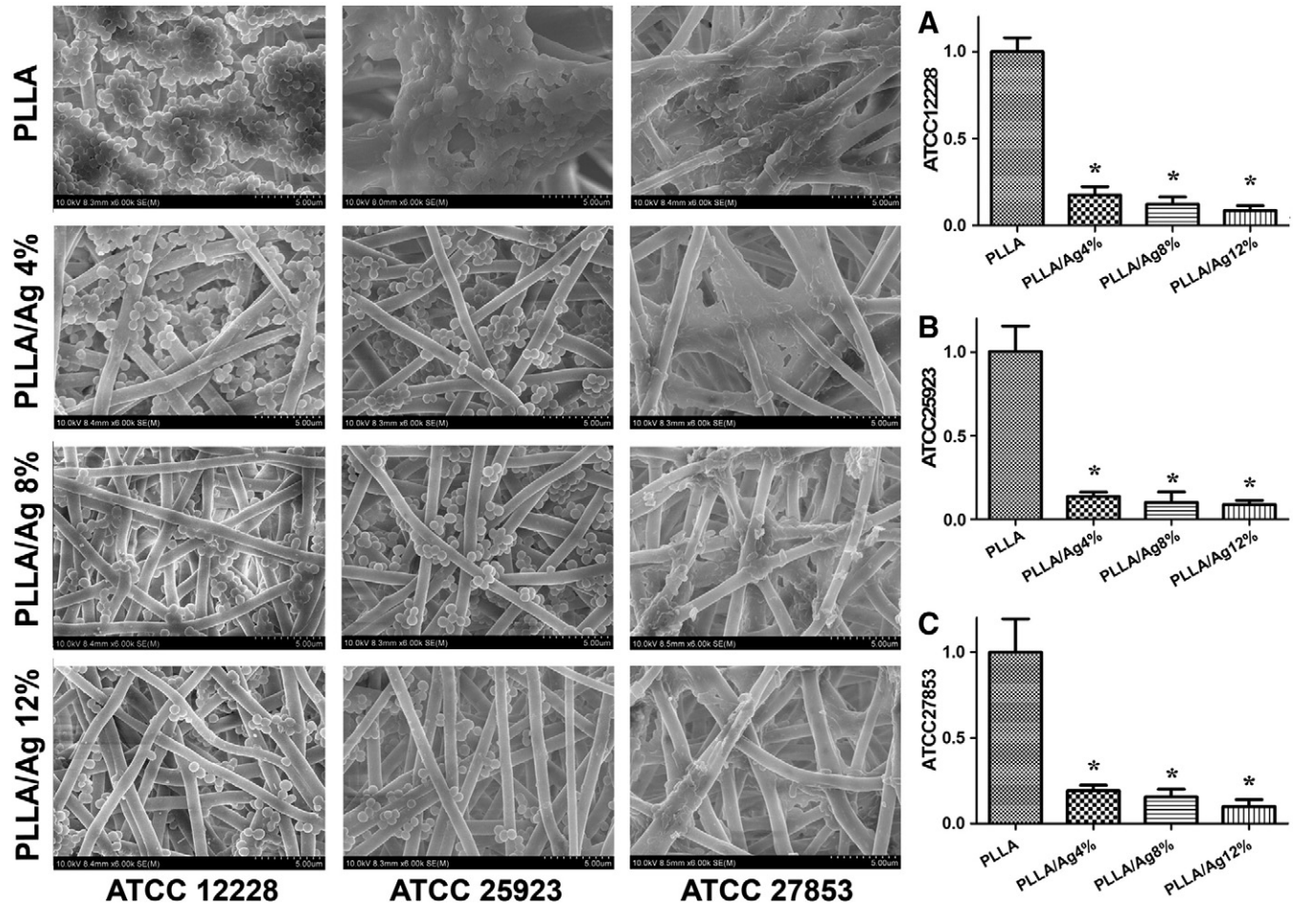


Fig. 5. The SEM images of bacteria on the surface of different nanofibers after 1 day of culture. The statistic results of *S. epidermidis* (A), *S. aureus* (B) and *P. aeruginosa* (C). *$P<0.05$ compared with bacteria adhered to the PLLA fibrous membrane. Data are expressed as mean ± SD (each group, $n=3$).

revealed a clearly difference among the number of all bacteria on the surfaces of each sample. SEM images showed that the bacteria on pure PLLA fibers were dense, while those on PLLA/Ag fibers were sparse. There were fewer bacteria on AgNP-loaded PLLA fibrous membrane surface suggested the material with AgNPs had a better antibacterial ability against these three stains (Fig. 5). The number of surviving colonies of AgNP-loaded PLLA fibrous membranes is less than that of PLLA fibrous membrane ($P<0.05$). No differences were observed in number between all three samples containing AgNPs ($P>0.05$).

4. Discussion

In this study, biodegradable AgNP-loaded PLLA fibrous membranes were fabricated via electrospinning. The TEM micrographs of the fibers showed that the AgNPs were successfully electrospun into the PLLA fibers in different contents with ability of Ag ion release. The effect of anti-proliferation on fibroblasts of the AgNP-loaded PLLA fibrous membranes was observed. Furthermore, no cytotoxicity was detected. The broad-spectrum topical antimicrobial activity of AgNP-loaded PLLA fibrous membranes on *S. epidermidis* (ATCC12228), *S. aureus* (ATCC25923) and *P. aeruginosa* (ATCC27853) was certificated.

Electrospun fibrous membranes can only prevent peritendinous adhesions leaving tendon sheath infection not adequately figured out. Silver ions have long been used due to the powerful antibacterial activity [7]. Nevertheless, an anti-proliferative activity is frequently and negatively mentioned as the cytotoxicity of silver ions [8–10]. However, such properties make silver ions just suitable for prompting anti-adhesion treatment and simultaneously early prevention of infection. Zhang et al. [24] fabricated polyacrylonitrile (PAN) nanofibrous membranes by electrospinning and then treat them in hydroxylamine aqueous solution for coordination of Ag ions. Subsequently, the coordinated Ag ions were converted into AgNPs. However, the AgNPs that converted from the Ag ions attached to the surfaces of the nano-fibrous membranes and thus only the admirable antimicrobial functionality on the nanofiber surfaces may be acquired. From our TEM results, we can learn that antimicrobial agents (AgNPs) are distributed throughout the nanofibers. The porous structure of the electrospun AgNP-loaded PLLA fibrous membranes and the pores after diffusion out of drug molecules from the surface layer made it possible to further release the Ag ions from the inner layer in this study [25]. Furthermore, the release behavior mainly depended on not only polymer matrix degradation but also drug diffusion of the samples. Therefore, we can see that the drug release rate increases with silver content increase in PLLA electrospun fibers. However, as the amount of AgNP entrapment increases, the drug molecules may aggregate more greatly in the fibers, which would lead to an even larger initial burst of Ag ions.

As a physiologically important part of formation of adhesion, extrinsic healing occurs through the chemotaxis of the specialized fibroblasts into the defect from the ends of the tendon sheath [18]. Thereafter, recent study focused on how to effectively resist the fibroblasts adhesion and proliferation on the anti-adhesion membranes. The anti-adhesion effect of hydrophobic PLLA chains of PLLA fibrous membrane has been investigated [19–21]. From the results we can learn that the cell proliferation on the surfaces of AgNP-loaded PLLA fibrous membranes was worse than on the surface of PLLA fibrous membrane. Furthermore, it decreased with increasing AgNP composition. Thereafter, it is the

anti-proliferation effect of silver ions that reduced the cell viability and proliferative activity. However, the traditional negative effect that prevents cell proliferation was now treated as the positive effect that inhibits adhesion formation. To specify the bio-safety of the AgNP-loaded PLLA fibrous membranes, cytotoxicity test was performed and the results were considered as no cytotoxicity according to the ISO standards (1992). However, the in vivo biosafety and the effect on tendon healing should be further studied.

The prevention of bacterial adhesion should assist in reduction of device associated infection. One goal of this work was to evaluate the antibacterial effect of AgNP-loaded PLLA fibrous membranes. The test was conducted with Gram-positive *S. aureus* and *S. epidermidis* and Gram-negative *P. aeruginosa*, because these bacteria are frequently responsible for tendon and tendon sheath infections in hand surgery [26,27]. The surfaces of AgNP-loaded PLLA fibrous membranes were observed to significantly reduce the bacterial adhesion, confirming the antibacterial activity of them regardless of cell wall composition of the examined bacterial strains. It is because of the mechanism of Ag as antimicrobial active agent. Firstly, Ag ions avidly bind to sulfhydryl groups in the cell wall, thereby causing dysfunction of these components in various enzymes that involved in transmembrane energy generation and electrolyte transport. Thereafter, adenosine triphosphate synthesis was interrupted [28,29]. Next, Ag ions block the respiratory chain of bacteria such as thiol groups in the cytochrome oxidase and NADH-succinate-dehydrogenase region and thus bacterial respiration and adenosine triphosphate synthesis were interrupted [30,31]. Finally, Ag ions can bind to nucleotide bases and thus intercalate with double stranded DNA molecules preventing DNA polymerases to replicate them by displacing hydrogen bonds between adjacent purines and pyrimidines [32]. Thereafter, these suggested mechanisms are the reasons of broad spectrum of anti-microbial activities of silver ions. However, the anti-bacterial effect in an in vivo study should be performed before clinic use.

5. Conclusion

The AgNPs can be directly electrospun into biodegradable PLLA fibrous membrane for Ag ion release. In vitro anti-adhesion study showed that the AgNP-loaded PLLA fibrous membranes have a significant effect of preventing cell adhesion and proliferation without significant cytotoxicity. Moreover, bacterial adhesion inhibition study indicated a significantly inhibited effect of *S. aureus*, *S. epidermidis* and *P. aeruginosa* strains on AgNP-loaded PLLA fibers compared with PLLA fibers alone. Thus, the AgNP-loaded PLLA fibrous membranes can find convenient practical medical potential in reducing the incidence of infection and adhesion formation after tendon injury.

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