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Antibacterial Antiadhesion Membranes from Silver-Nanoparticle-Doped Electrospun Poly(L-Lactide) Nanofibers

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ABSTRACT: Electrospun fibrous membranes have been used frequently in biomedical applications, but their simultaneous use as antibacterial agents and in the prevention of cell adhesion on repaired tendons after injury has not been investigated. In this study, silver-nanoparticle (SN)-loaded poly(L-lactide) (PLLA) fibrous membranes were prepared by the electrospinning of SNs into PLLA fibers. Micrograph results showed that these membranes were composed of electrospun fibers and that the fibers were incorporated with SNs. From the results of X-ray diffraction and thermogravimetry, we concluded that the SNs were physically mixed into the fibers at the desired content. The mechanical properties were not significantly changed. The preliminary antibacterial effects on *Staphylococcus epidermidis* and *Staphylococcus aureus* and the synergistic antiproliferative effects of the SN-loaded PLLA fibrous membranes were observed. Taken together, these results demonstrate that SNs can be directly loaded onto a biodegradable PLLA fibrous membrane via electrospinning to achieve proper material properties with preliminary potential as antibacterial antiadhesion barriers for tendon injury. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 3459–3465, 2013

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INTRODUCTION

Adhesions between the tendon and the surrounding tissues are one of the common complications that occur after tendon injury.[1] Such peritendinous adhesions greatly restrict one's daily life. Nowadays, to prevent adhesion formation, physical barriers have been widely used.[2] However, these barriers usually focus on antiadhesion without preventing infection; this is a crucial etiology of adhesion formation to be taken into consideration. Furthermore, after the application of physical barriers to prevent adhesion, implants may increase the incidence of postoperative infection. However, the challenge to reduce peritendinous adhesion and, simultaneously, bacterial infection for surgeons has not been met.

Recently, electrospinning has aroused much interest as an attractive technique for producing polymer fibers with a large surface area-to-volume ratio, high porosity, and very small pore size.[3] According to these characteristics, electrospun fibrous membranes are widely used for tissue separation and drug delivery.[4] Furthermore, another advantage of these fibers as barriers in the prevention of peritendinous adhesion is the microporous structure, which allows the passage of nutrients from outside the tendon sheath to promote intrinsic healing.[5] However, these electrospun fibrous membranes can only reduce peritendinous adhesions and can leave the tendon sheath prone to infection.

Silver ions have frequently been used for their effective antibacterial abilities.[6] Nevertheless, the cytotoxicity of silver ions has been mentioned as a negative side effect.[7–9] However, such an antiproliferative activity of silver ions makes them suitable for antiadhesion treatments and also early prevention of infection if they were used after a proper manufactory process. Cao et al.[10] successfully introduced silver nanoparticles (SNs) onto the surface of sulfonated poly(ether sulfone) membranes with vitamin C as a reducing agent using the interaction between the sulfonated groups and silver ions. However, in their studies, the increased antibacterial ability was associated with a deterioration of the mechanical properties. The antibacterial membranes were

also fabricated by the adsorption of silver ions with subsequent hydrogen reduction to introduce SN onto their surfaces or into their fibers.[11,12] However, the antimicrobial agents on the surfaces of these fibers could fall off of the fiber surface after fiber degradation, and the fiber could eventually have no antibacterial activity after a certain period;[13] the fiber diameters are dramatically increased when $AgNO_3$ is added to incorporate SNs throughout the fibers during the electrospinning process.[14]

Nowadays, synthetic biodegradable poly(L-lactide) (PLLA) has drawn great attention because it can be used for important biomedical applications and has been approved by the U.S. Food and Drug Administration. SNs have been gradually recognized for their easy handling in implantable materials for antiinfection applications.[10] Therefore, in this study, they were directly incorporated into spin dopes and then electrospun into biodegradable PLLA fibers. We expected that the SNs could be incorporated into the PLLA fibrous membrane without significantly influencing the physical and chemical properties of the PLLA fibrous membrane. The initial *in vitro* effects of such membranes on the proliferation of fibroblasts and the spread of bacteria were assayed.

EXPERIMENTAL

Materials

PLLA (weight-average molecular weight = 50 kDa, weight-average molecular weight/number-average molecular weight = 1.6) was prepared by the bulk ring-opening polymerization of L-lactide with stannous chloride as an initiator (Jinan Daigang Co., Jinan, China). SNs (99.9% pure, 60–100 nm in size) were purchased from the Aladdin Regents Co. (Shanghai, China) and were used as an antibacterial agent in this study. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco (Grand Island, NY). All chemicals and solvents were analytical-reagent grade, were used without further purification, and were purchased from GuoYao Regents Co. (Shanghai, China) unless otherwise indicated.

Electrospinning of the SN-Loaded Fibrous Membranes

Dichloromethane (3.5 g) was used to dissolve 1.0 g of PLLA at a concentration of 25% w/w. Various amounts of SN (i.e., 0.04, 0.08, and 0.12 g) were completely dispersed in 1.5 g of *N,N*-dimethylformamide (DMF), respectively. Then, SN solution was then mixed with the PLLA solution and dispersed under continuous stirring. The SN/PLLA blend solutions with different weight ratios of SNs, 100 : 4, 100 : 8, and 100 : 12, were prepared for electrospinning and named PLLA–SN4, PLLA–SN8, and PLLA–SN12, respectively.

The electrospinning processes were performed as in a previous study.[15] Briefly, the electrospinning apparatus was set with a high-voltage statitron (Tianjing High Voltage Power Supply Co., Tianjing, China) with maximal voltage 30 kV. A precision pump (Zhejiang University Medical Instrument Co., Hangzhou, China) was used to charge the flow rate of the SN/PLLA blend solution to maintain a steady flow from the tip of the needle. PLLA was dissolved in dichloromethane to achieve a concentration of 25 wt %, and certain amounts of SNs (the final weight percentages of SNs were 4, 8, and 12%, respectively) were added to the solution. The solutions were drawn into 20-mL glass syringes fitted with needles 0.9 mm in diameter (feeding rate = 3.0 mL/h) to prepare the fibrous membranes at an applied voltage of 15 kV. The SN/PLLA electrospun fibrous membranes were collected on the surface of a piece of an aluminum sheet and used for further study after they were vacuum-dried at room temperature for 24 h.

Characterization of the SN-Loaded Fibrous Membranes

The morphology of the individual fibers was observed by scanning electron microscopy (SEM; FEI Quanta 200, Eindhoven The Netherlands). The fiber diameter of each specimen was measured according to more than five SEM images with 10,000× magnification. From each image, at least 20 respective fibers and 200 different segments were randomly chosen to determine the average fiber diameter with image-analytical software (Photoshop 8.0,San jose, United States).[16]

The mechanical properties of the obtained fiber were assessed by an all-purpose mechanical testing machine (Instron 5567, Norwood, MA) at a stretching speed of 0.5 mm/s (Sample number/group = 5). For the mechanical properties tests, all of the dry electrospun fibrous specimens were cut into small strips (70.0 $\times$7.0 $\times$ 0.6 mm^3). The stress–strain curves of the fibrous specimens were constructed from the load–deformation curves recorded. From the stress–strain curves, the Young's modulus, tensile strength, and elongation at break of the scaffolds could be determined.

The surface wettability of the individual specimens was detected by a water contact angle (WCA) method at room temperature (i.e., 25°C ± 1°C). The WCAs of different electrospun PLLA fibrous membranes with or without SNs were assessed by a Kruss GmbH DSA 100 Mk 2 goniometer (Hamburg, Germany) followed by the image processing of a sessile drop with DSA 1.8 software (Hamburg, Germany).

Each specimen was examined for the structure of SNs in the PLLA fibers by transmission electron microscopy (TEM; JEM-2100F, Joel, Japan).

To investigate the crystalline phase of the electrospun fibrous scaffolds, samples (20 $\times$ 20 mm^2) were analyzed with X-ray diffraction (XRD; Philips X'Pert PRO, Almelo, The Netherlands) over a 2θ range from 5 to 70° with a scanning speed of 0.35°/min (λ = 1.54060 Å).

Thermogravimetric analysis (TGA) measurements were performed with TGA equipment (Netzsch STA 449C, Bavaria, Germany). The samples were analyzed in perforated and covered aluminum pans under a nitrogen purge. Approximately 1 mg of sample was heated from 25 to 1000°C at a heating rate of 20°C/min.

In Vitro Cell Culture

C3H10T1/2 mouse fibroblasts were used to evaluate the antiproliferative effects of the PLLA electrospun fibrous membrane surfaces with or without SNs. The cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. After sterilization by immersion in 75% ethanol for 1.5 h, the

electrospun membranes in a 24-well plate were washed repeatedly with phosphate buffer saline to remove residual ethanol. A tissue culture plate was used as the control. Cells (2×10^5 cells/mL) were seeded into each well (100 μL/well). After 1 and 4 days of incubation, alarmar blue solution was added to each sample (25 μL/well) for an additional 4 h at 37°C. The absorbance at 570 nm was determined with a spectrophotometer (Synergy 2; BioTek, Winooski, VT).

Bacterial Inhibition Test

Staphylococcus epidermidis (ATCC12228) and *Staphylococcus aureus* (ATCC25923) were purchased in freeze-dried form from Chuangxiang Biotechnology (Shanghai, China). The stains were stored at −80°C in glycerol. The stains were cultured on trypticase soy agar (TSA; BD Biosciences, Franklin Lakes, NJ) medium at 37°C overnight, and a single stain was cultured at 37°C for 12 h in 10 mL of BBL trypticase soy broth (TSB).

The antimicrobial effects of the SN-loaded PLLA fibrous membrane were tested by the spread plate method.[17,18] After 12 h of culturing, each stain was adjusted to a concentration of 1 × 106 cfu/mL in TSB according to McFarland. Samples in a 24-well plate (Costar3548, Corelle, United States) were cultured in 1 mL of suspension at 37°C with agitation at 100 rpm. After 24 h of culturing, all of the samples were gently washed with phosphate buffer saline three times and replaced in 0.5 mL of 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 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 the control samples.

Statistical Analysis

The results are expressed as the mean plus or minus the standard deviation. The data were analyzed by a one-way analysis of variance test, where $p < 0.05$ is considered significant.

RESULTS

Morphology of the SN-Loaded Fibrous Membranes

The SEM and TEM micrographs of the fibers obtained are shown in Figures 1 and 2. The average diameters of the PLLA, PLLA–SN4, PLLA–SN8, and PLLA–SN12 fibers were 0.85 ± 0.21, 0.89 ± 0.38, 0.95 ± 0.42, and 1.06 ± 0.33 μm, respectively, and increased with increasing SN content. Furthermore, as shown in Figure 1, these fibers were round-shaped, bead-free, and randomly arrayed, and few SNs were observed on the different surfaces of the fibers in the PLLA/SN samples. The WCAs were 129.4 ± 5.3, 128.3 ± 3.7, 125.6 ± 4.5, and 123.2 ± 5.4° for fibrous membranes of PLLA and medicated PLLA–SN4, PLLA–SN8, and PLLA–SN12, respectively.

The TEM micrographs of the fibers obtained are shown in Figure 2. Because of the hydrophilicity of the SNs, the separated SNs were difficult to disperse uniformly on the electrospinning PLLA solution in dichloromethane and DMF. Aggregated SNs were observed on the TEM images (Figure 2) of the PLLA/SN.

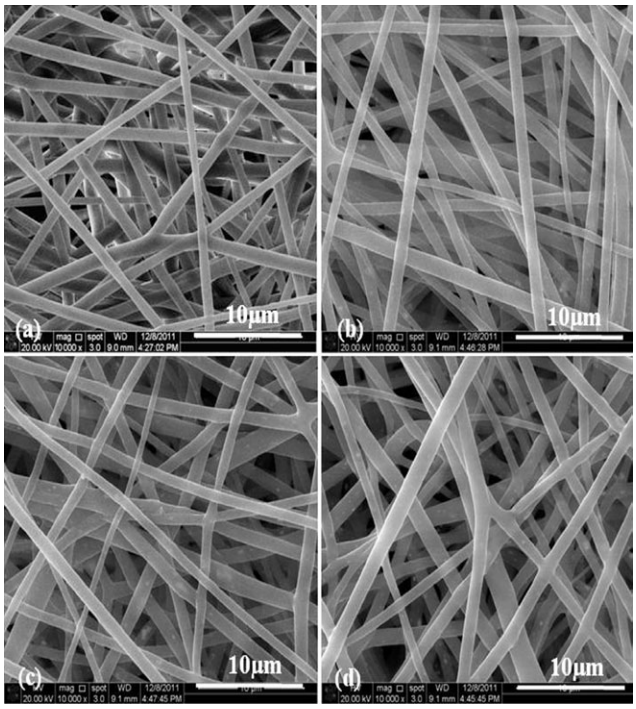

Figure 1. SEM images of the (a) electrospun PLLA, (b) PLLA–SN4, (c) PLLA–SN8, and (d) PLLA–SN12 fibers with 0, 4.0, 8.0, and 12.0% w/w, respectively, loaded SN.

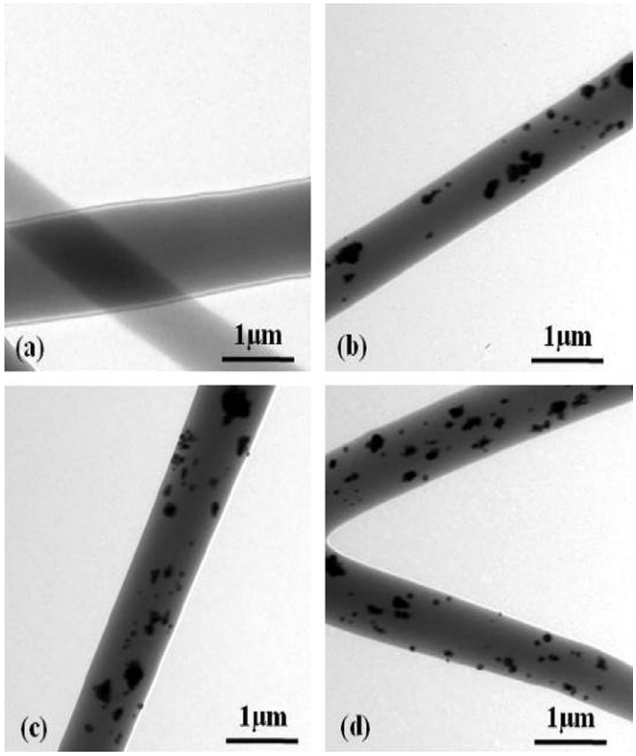

Figure 2. TEM images of the (a) electrospun PLLA, (b) PLLA–SN4, (c) PLLA–SN8, and (d) PLLA–SN12 fibers with 0, 4.0, 8.0, and 12.0% w/w, respectively, loaded SN.

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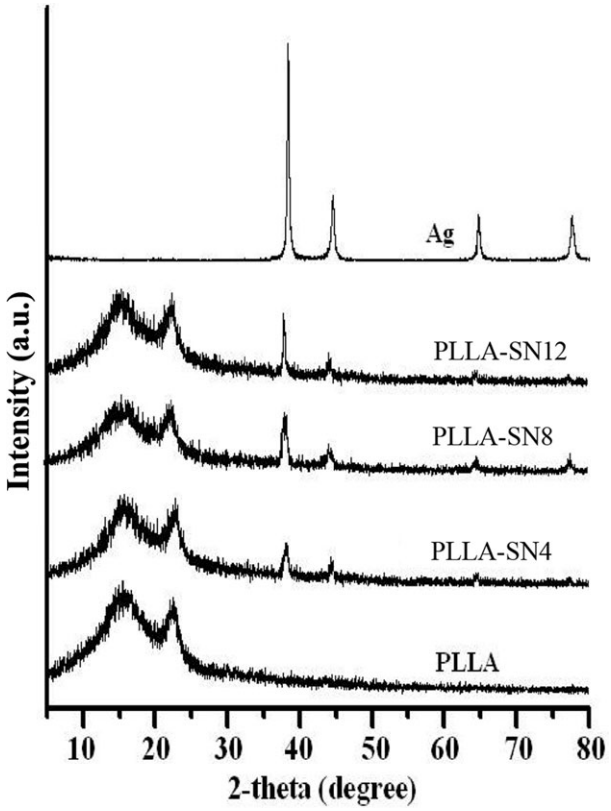

Figure 3. XRD patterns of the SN, PLLA fibers, and medicated PLLA–SN4, PLLA–SN8, and PLLA–SN12 fibers.

XRD and TGA of the SN-Loaded Fibrous Membranes

The XRD patterns of the SNs and PLLA, PLLA–SN4, PLLA–SN8, and PLLA–SN12 fibers are shown in Figure 3; these revealed that the SN-loaded fibrous membranes contained SNs. Furthermore, as shown in Figure 3, the intensities of the peaks of SN increased obviously from PLLA–SN4 to PLLA–SN12 with increasing SN content. To check the components of the SNs, TGA was conducted on the SN-loaded fibrous membranes. As shown in Figure 4, only SNs did not lose any weight with increasing temperature up to about 350°C. The residual weight percentages of PLLA–SN4, PLLA–SN8, and PLLA–SN12 was 4.1, 8.1, and 12.3%, respectively. The results were consistent with respect to the contents of the SN-loaded fibrous membranes.

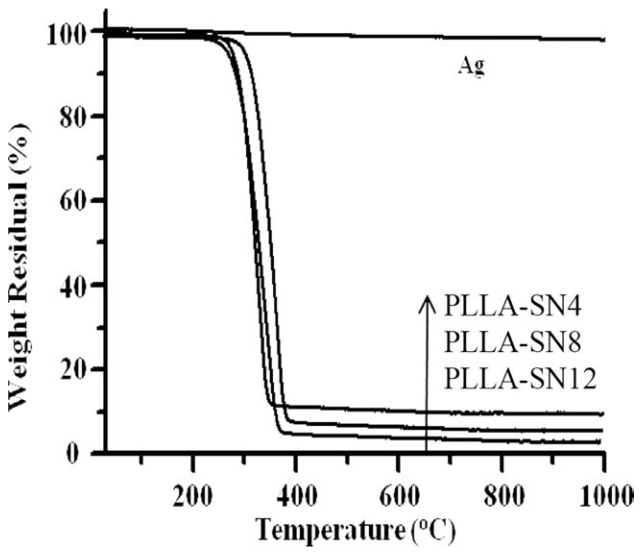

Figure 4. TGA curves of the Ag, PLLA–SN4, PLLA–SN8, and PLLA–SN12 fibrous membranes.

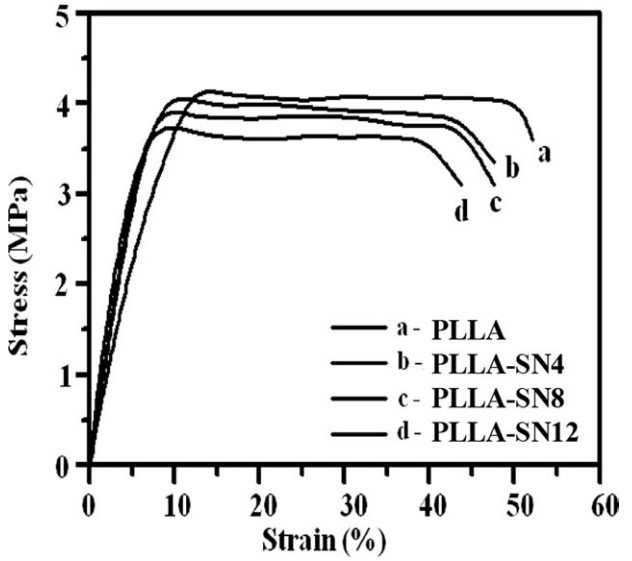

Figure 5. Stress/strain curves for the (a) electrospun PLLA, (b) PLLA–SN4, (c) PLLA–SN8, and (d) PLLA–SN12 fibers with 0, 4.0, 8.0, and 12.0% w/w, respectively, loaded SN.

Mechanical Properties of the SN-Loaded Fibrous Membranes

To clarify the effects of SNs on the mechanical properties of the PLLA electrospun fibrous membrane, strain–stress curves were plotted, and the stress–strain results are shown in Figure 5. The tensile strengths of the PLLA, PLLA–SN4, PLLA–SN8, and PLLA–SN12 fibrous membranes were 4.16 ± 0.33, 4.04 ± 0.27, 3.88 ± 0.29, and 3.77 ± 0.31 MPa, respectively, whereas the tensile moduli were 61.3 ± 5.6, 62.75 ± 5.5, 65.23 ± 4.98, and 68.87 ± 7.26 MPa, respectively. The statistical analysis indicated no significant difference between the PLLA and PLLA/SN fibrous membranes ($p > 0.05$). The mechanical analysis showed a higher modulus but a lower tensile strength and elongation at break for the SN-loaded PLLA fibrous membranes than for the PLLA fibrous membrane. These data also showed a decrease in the maximum mechanical tensile strength and maximum elongation with increasing SNs and increasing maximum modulus of elasticity with increasing SNs.

Cell Proliferation Assay

The antiproliferative effect of the PLLA fibrous membranes with or without SNs was compared after 1 and 4 days (Figure 6). We observed that cells grew on the surfaces of all of the specimens. However, the cells did not proliferated as well on the surfaces of SN-loaded PLLA fibrous membranes as on the surfaces of the PLLA fibrous membrane. Furthermore, the cell proliferation of the SN-loaded PLLA fibrous membranes decreased with increasing SN composition. By comparing the viability of cells on

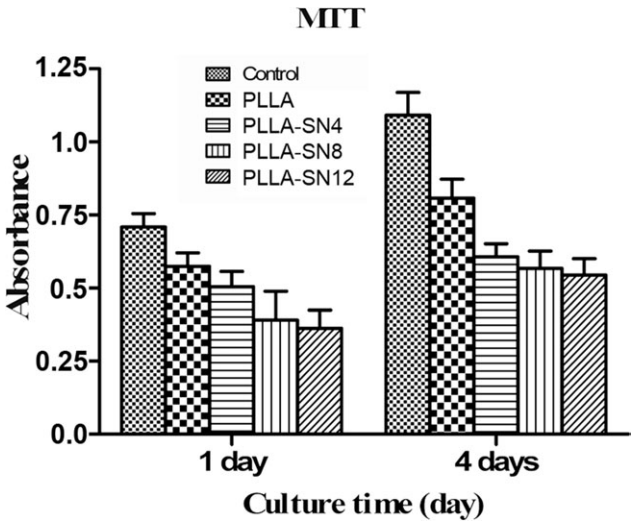

Figure 6. Cell attachment and proliferation on the specimens by alarmar blue assay for 1 and 4 days. The data are presented as the mean plus or minus standard deviation ($n = 5$, each group). MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

different surfaces after 1 and 4 days of culturing, we observed that the cell growth on the different surfaces after 4 days showed a similar trend with the cell adhesion after 1 day (Figure 6).

Antibacterial Test

As shown in Figure 7, the colony numbers of *S. epidermidis* and *S. aureus* for the SN-loaded PLLA fibrous membranes obviously decreased compared with those of the PLLA fibrous membrane. The number of viable bacteria was determined by the spread plate method and normalized to the count of the control sample. We observed that the SN-loaded PLLA fibrous membranes had fewer colony numbers of each tested stain ($p < 0.05$). No differences were observed in the suspension numbers between all three of the samples containing SNs ($p > 0.05$). No differences were also displayed among both stains ($p > 0.05$).

DISCUSSION

In this study, SNs were directly medicated into biodegradable PLLA fibers via electrospinning, and thus SNs were incorporated into PLLA fibrous membranes. Proper physical and chemical properties in the PLLA fibrous membrane were achieved

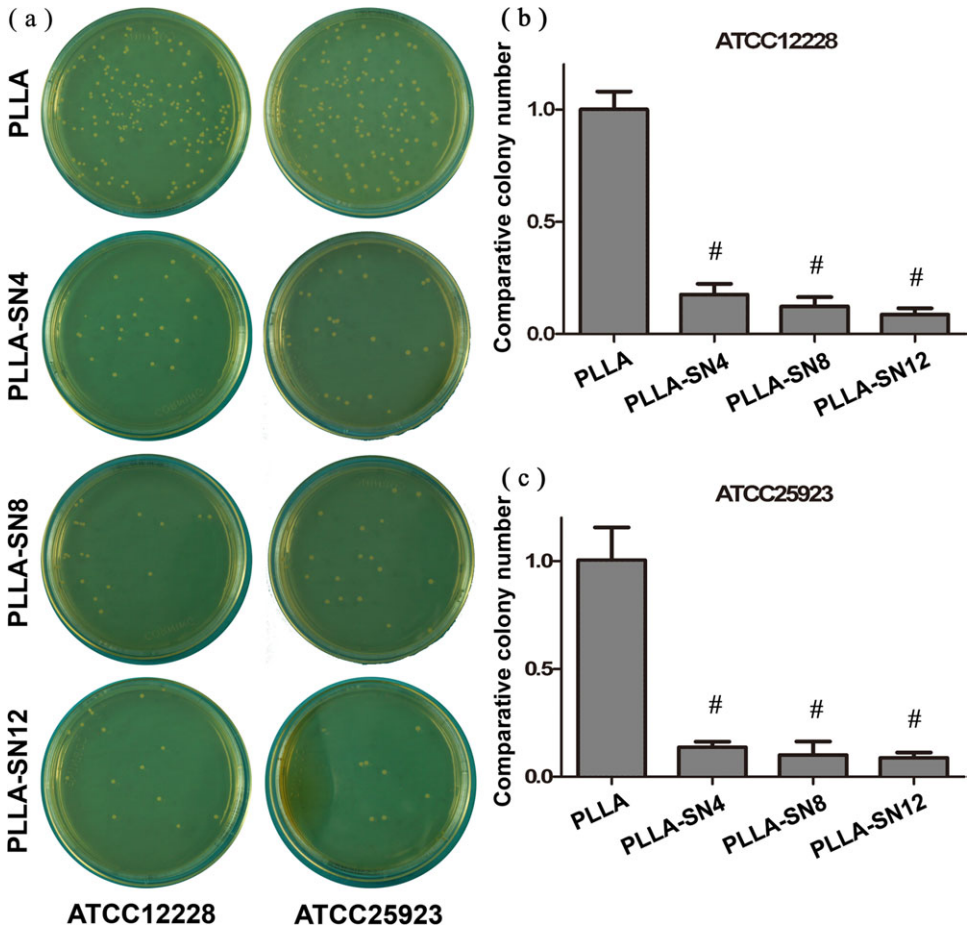

Figure 7. (a) Antimicrobial effects of the SN-containing PLLA fibrous membranes against *S. epidermidis* and *S. aureus*. (b,c) The number of viable bacteria on the surface of the samples after 12 h of culture were counted and normalized to the counts of the PLLA fibers. #, Significant difference compared with PLLA fibers for each stain ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and maintained after the incorporation of SNs. Furthermore, the SN-loaded PLLA fibrous membranes showed a combination of preliminary antibacterial effect on *S. epidermidis* and *S. aureus* and antiproliferation on fibroblasts.

Electrospun membranes are suitable for drug delivery because of their large surface area and controlled porous structure.[4,5] The porous structure of the electrospun membrane and the pores after diffusion out of drug molecules from the outer layer made it possible to release the drug further from the inner layer.[15] Zhang et al.[19] fabricated polyacrylonitrile fibrous membranes by electrospinning and then treated them in a hydroxylamine aqueous solution for the coordination of silver ions. Subsequently, the coordinated silver ions were converted into SNs. Thereafter, the SNs that were converted from the silver ions attached to the surfaces of the fibrous membranes, and thus only the fiber surfaces shown an admirable antimicrobial ability. In this study, from our TEM results, we observed that our preformulated SNs were successfully incorporated into the fibers by electrospinning. Furthermore, the incorporation of SNs into the polymeric matrices made the electrospun fibers wider, and thus the SNs were likewise a crucial factor influencing the diameter of the electrospun fibers. A possible reason for this may have been that the SNs were difficult to disperse uniformly in the electrospinning PLLA solution in the dichloromethane and DMF because of the phase separation of PLLA and SN.[12] This may also have been the reason for the changes in the mechanical properties, although the mechanical properties were not significantly changed. To assess the phases present, the mixture of fibers and SNs were subjected to XRD, and as a result, they did not have any chemical interaction. Therefore, the crystallinity was not affected. Therefore, the characteristics of the PLLA fibrous membranes as antiadhesion barriers were retained without significant changes after the incorporation of SNs by blending and electrospinning.

Ionic silver is widely used as an antimicrobial additive according to its physical stability, sustained activity, and intense antimicrobial properties, although it has possible toxicity to mammalian cells and tissues. The incorporation of silver into different materials results in broad-spectrum topical antimicrobial activity. Nowadays, there are different ways to introduce silver into coating materials, such as the sol–gel method, ion implantation, ion exchange, and sputtering; however, these leave the release not adequately accompanied by the overall process of degradation.[6,20–23] Specifically, SNs were loaded into materials by Yang et al.[11] and Wu et al.[24]. However, this was done through the adsorption of silver ions into the material first and then their reduction to SNs. The subsequent complex hydrogen reduction and therapy potential for applications in wound dressing and industry greatly limit their practical medical applications in reducing the incidence of postoperative infection with applied effects in antiadhesion formation. Li et al.[25] fabricated Ag/PLLA fibrous membranes having a weight ratio of SNs to PLLA at 5% w/w, but only *in vitro* antibacterial tests were performed with *Escherichia coli* and *S. aureus*. Antibacterial tests results, such as those on *S. epidermidis*, and the cytotoxicity of silver ions were negative effects mentioned in their study. In this study, SN-loaded fibers were successfully and easily fabricated by electrospinning from mixed aqueous solutions to prevent tendon adhesion and simultaneously infection. Furthermore, preliminary antiproliferation and antibacterial effects of the SN-loaded PLLA fibrous membrane were successfully detected.

During the electrospinning process, air entrapment between the fiber interfaces increased the surface hydrophobicity. As a result, the WCAs were 129.3 ± 3.3, 125.6 ± 4.5, and 121.8 ± 3.2° for fibrous membranes medicated with 4, 8, and 12% SNs, respectively. It is known that cells more readily adhere to and proliferate on moderately hydrophilic substrates than on hydrophobic or very hydrophilic substrates.[26] Therefore, cell growth was prevented by PLLA fibrous membranes in this study. From the better antiproliferation effect of the SN-loaded membranes compared to the PLLA fibrous membranes, we concluded that the silver ions seemed to play a key role in antiproliferation. Thereafter, we concluded that SN-loaded PLLA fibrous membranes may prevent cell proliferation through a synergistic effect of antiadhesion because of the hydrophobicity of electrospun membranes and the antiproliferation of SNs. Furthermore, the preliminary antibacterial effect of SN-loaded membranes was mainly due to the well-known therapy in which silver ions intercalate with double-stranded DNA molecules and thus prevent DNA polymerases from replicating; they also interact with thiol groups in proteins to induce the denaturation of bacterial proteins.[27]

CONCLUSIONS

In conclusion, SNs were directly medicated into biodegradable PLLA fibrous membranes via electrospinning without significant effects on the physical and chemical properties. The SN-loaded PLLA fibrous membranes not only prevented cell proliferation through a synergistic antiproliferative effect on the hydrophobicity of the electrospun structure and SNs but also simultaneously reduced infection because of the antibacterial effect of SNs. Thereafter, such SN-loaded PLLA fibrous membranes show preliminary potential as antibacterial antiadhesion barriers for tendon injury.

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