Preparation of Antimicrobial Poly(ε-caprolactone) Electrospun Nanofibers Containing Silver-Loaded Zirconium Phosphate Nanoparticles

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ABSTRACT: Antimicrobial nanofibers of poly(ε-caprolactone) (PCL) were prepared by electrospinning of a PCL solution with small amounts of silver-loaded zirconium phosphate nanoparticles (nanoAgZ) for potential use in wound dressing applications. The electrospun nanoAgZ-containing PCL nanofibers were characterized using field emission scanning electron microscopy, energy dispersive X-ray spectrum (EDX), X-ray diffraction analysis (XRD), antimicrobial tests, and biocompatibility tests. The SEM, EDX, and XRD investigations of the electrospun fibers confirmed that silver-containing nanoparticles were incorporated and well dispersed in smooth and beadless PCL nanofibers. The results of the antimicrobial tests showed that these fibers have maintained the strong killing abilities of Ag^+ existed in the nanoAgZ against the tested bacteria strains and discoloration has not been observed for the nanofibers. To test the biocompatibility of nanofibers as potential wound dressings, primary human dermal fibroblasts (HDFs) were cultured on the nanofibrous mats. The cultured cells were evaluated in terms of cell proliferation and morphology. The results indicated that the cells attached and proliferated as continuous layers on the nanoAgZ-containing nanofibers and maintained the healthy morphology of HDFs. The earlier results suggested that nanoAgZ-containing fibers may be expected to be a novel material for potential wound dressing applications because of the significant bacteriostatic activities and good biocompatibility. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 1208–1214, 2007

Key words: antimicrobial agent; electrospinning; composite; wound dressing

INTRODUCTION

Infections that develop in traumatic and surgical wounds remain a major problem and one of the key approaches for minimizing the possibilities of wound infections is the application of topical antimicrobial agents.[1,2] As we know, silver has long been recognized as a broad-spectrum and highly effective antimicrobial agent for treating wounds and burns.[3] Silver ion works by denaturating the proteins and nucleic acids of the bacteria by binding to their negatively charged components. Besides, silver acts in generating oxygen which in turn destroys the cell wall membranes of bacteria.[4] Interestingly, although silver is a kind of highly effective antimicrobial agent, it has been found that the use of silver-based dressings enhances epithelialization of wound in clean wounds of pigs, which indicates a beneficial effect of silver ions in wounds besides its antimicrobial activity.[5,6] Therefore, a considerable number of silver-based wound dressing products have been developed against the potentially wound infections.

Electrospinning technique which is able to produce continuous nonwoven nanofibers has been drawing great attention in recent years. When a strong electrostatic force is applied to the syringe containing a polymer solution, it will be ejected from the capillary and deposited as nonwoven nanofibers on a grounded collector. Electrospun nanofibers have the characteristics of huge surface area-to-volume ratio, high porosity, and fully interconnected pore network, which can mimic utmostly the nanosized features of natural extracellular matrix and make them viable options in the fields of wound dressings and tissue engineering scaffold materials.[7–9] Several studies have been reported about the wound dressings prepared by electrospinning, which proved that the electrospun nanofibers can meet the requirements such as higher gas permeation and protection of wounds.[10,11] Furthermore, the incorporation of functional nanoparticles into polymer nanofibers can also be achieved by blending electrospinning method conveniently. The resulting composite

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polymer nanofibers can exhibit the distinctive properties of the incorporated nanoparticles and it can even be expected that these electrospun blend nanofibers will exhibit stronger distinctive activities than conventional microfibers because of their high surface area-to-volume ratio.[12–14] Therefore, if silver ions can be incorporated into the nanofibers by blending electrospinning technique, these fibers can also be expected to show antimicrobial activities besides its unique characteristics of electrospun materials. A few silver-loaded electrospun materials have been reported in the literature.[15–18] However, among them, the silver-containing nanofibers were developed by electrospinning of polymer solution with silver nitrate followed by photoreduction. The resulted metallic silver is relatively inactive compared to the ionic silver and this method may cause the discoloration of nanofibers in appearance and the skin discoloration and irritation associated with the use of silver nitrate.[19,20]

Recently, some novel silver-containing compounds have been developed to have a steadily and prolonged release of silver ions and protect the host material from oxidation and discoloration such as Ag^+-loaded zirconium phosphate. It is prepared by ion-exchanging of silver ions into the three-dimensional skeletal structure of sodium zirconium phosphate (NZP).[21,22] The Ag^+-loaded zirconium phosphate was increasingly used as an additive in the manufacture of antimicrobial wound dressings, plastic containers and other plastic items, fibers, polymeric materials, and ceramics.[23,24] It can also be manufactured into nanoparticles, which can be blended with fiber forming polymers.

Poly(ε-caprolactone) (PCL) is a biodegradable aliphatic polyester, which has been regarded as nontoxic and tissue compatible and approved by FDA to produce a number of medical and drug delivery devices after extensive *in vitro* and *in vivo* tests. It has also been studied as a wound-management material and drug delivery system as far as the 1970s because of its excellent properties.[25–27]

To our knowledge, there has no report on the preparation of PCL nanofibers containing Ag^+-loaded zirconium phosphate nanoparticles (nanoAgZ). In this study, antimicrobial PCL nanofibers were prepared by incorporating nanoAgZ particles into PCL nanofibers by blending electrospinning technique. We expect to obtain a new kind of material that would combine the advantages of nanofibers and nanoAgZ together and have the potential use in wound healing applications.

EXPERIMENTAL

Materials

PCL pellets (M_n = 80,000, Sigma, USA) and nanoAgZ ($Ag_{0.16}Na_{0.84}Zr_2(PO4)_3$, silver 3.6%, average particle size = 63.73 nm, Conval, China) were purchased and used as received. NanoAgz (1%) was first dissolved in a mixture of 2, 2, 2-trifluoroethanol (Aldrich Chemicals) and 1% of Triton X-100 (Sigma, USA), and then treated ultrasonically for 1 h at room temperature. Therefore, PCL pellets were dissolved in the mixture with the concentration of 8% by vigorous stirring for 6 h. Here in this study, 1 wt % of nanoAgZ is the recommended and appropriate addition amount for polymer fibers for antimicrobial nonwoven fabric, disposable sanitary products and clothing according to the manuals of manufacturer.

Electrospinning system

A schematic diagram of the complete electrospinning apparatus is shown in Figure 1. It consisted of a syringe and stainless needle (ID = 0.84 mm), a grounded electrode, a copper plate covered by aluminum foil as a collector, and an adjustable high voltage supply (0–30 kV, BGG 40/2, Beijing BMEI, China). The prepared solution was filled into the syringe and the positive lead from the power supply was attached to the external surface of the metal syringe needle. When the applied electric charge overcame the force of surface tension and viscosity, the charged solutions in the syringe were ejected from the tip of the needle. The solvent evaporated during the time in which the polymer jet moved in the electric field and then nanofibers were formed and deposited on the grounded collector. In this study, the solutions were electrospun at 12 kV positive voltage, 10 cm working distance (the distance between the needle tip and the collector), and 3 mL/h solution flow rate controlled by a syringe pump. The thickness of the resulting fibrous mat was measured using a micrometer and it was about 80 μm. The

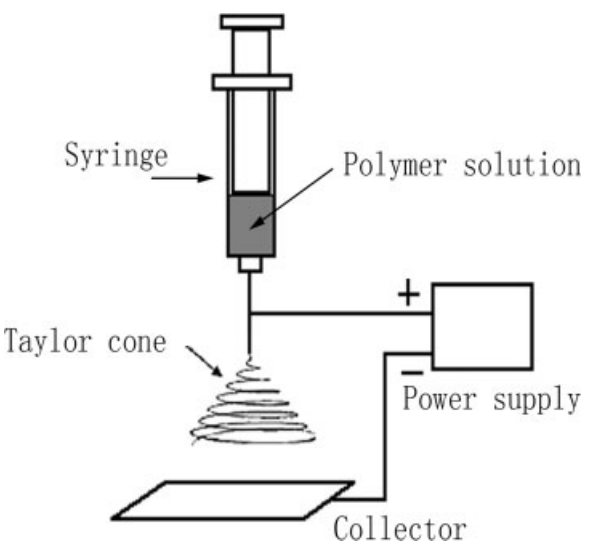

Figure 1 Schematic diagram of the electrospinning setup.

pristine PCL nanofibers and nanoAgZ-containing PCL nanofibers were electrospun at the same parameters. The fibrous mats were dried and kept in the desiccator.

Characterization of nanofibers

The morphology of the electrospun fibers was examined using a cold field emission scanning electron microscope (JSM- 6700F, JEOL, Japan) after gold coating. Energy dispersive X-ray spectrum (EDX) analysis was also conducted to investigate the existence and distribution of nanoAgZ particles in the nanofibers. The average diameters were determined by analyzing SEM images with ImageJ software (National Institute of Health, USA). X-ray diffraction (XRD) analyses of electrospun PCL, nanoAgZ-containing PCL, and original nanoAgZ powders were conducted by a X-ray diffractometer(D/max2400, Rigaku, Japan) using Cu Kα radiation at a scanning rate of 0.02°/s.

Antimicrobial assessment

The antimicrobial activity of the electrospun nanoAgZ-containing PCL nanofibers was tested against Gram-positive *Staphylococcus aureus* (ATCC 6538) and Gram-negative *Escherichia coli* (ATCC 25922) by the methods in the Chinese Industrial Standard.[28] Here, 0.2 mL solution of bacteria (5×10^5 CFU/mL) was added onto the samples and covered by a sterilized polyethylene (PE) film. The agar plates containing test samples were incubated at 37°C in a incubator for 24 h. After the incubation period the microorganisms are washed off the samples and 0.2 mL of the washing solution was added into the different dishes containing the nutrient agar. After 24-h of incubation under similar conditions, the active bacteria were counted. The reductions of bacteria were calculated according to the equation, $R(\%) = (B-C)/B \times 100$, where R is the antibacterial effect (%), B and C are the mean numbers of bacteria on the control samples and the electrospun samples (CFU/sample) respectively.

Cell culture

Primary human dermal fibroblasts (HDFs) were isolated and cultured by the sequential enzymatic treatment of prepuce tissues from the child foreskin circumcision. Briefly, the skin specimens were cut into small pieces and thoroughly cleaned. The epidermal layer and subdermal fat tissues were separated and removed. Then the specimens were minced and trypsinized. The fibroblasts were collected and grown in Dulbecco's modified Eagle medium (Sigma) containing 10% fetal bovine serum (Hyclone) in a humidified atmosphere of 95% air and 5% $C0_2$ at 37°C. Fibroblasts in passages 5–8 were used for the following experiments.

Cell proliferation assay

HDFs were seeded on the neat PCL and nanoAgZ-containing PCL nanofibers, which were deposited on the round glass cover slips in the 24-well tissue culture plate at a density of 25,000/cm^2. Cell proliferation on the electrospun nanofibers was studied after 1, 3, 5, and 7 days by MTT staining colorimetric assay.[29–32] Samples were sterilized by 75% ethanol and the kit (Sigma, USA) was used according to the manufacturer's directions. Before testing, old culture medium was pipetted from the 24-well tissue culture plate and 500 μL of fresh culture medium was added to every well. 200 μL of MTT solution (0.5 mg/mL) was then added to every well. After culturing for 4 h, the intense red colored formazan derivatives formed were dissolved in dimethyl sulphoxide (Xi'an Chemicals, China) and the absorbance was measured with a spectrophotometer (550 model, Bio-RAD Program, USA) at 570 nm. Samples with culture medium but without cells were set as the control to determine background absorbance to be subtracted.

Cell morphology

Cell morphology was also observed by SEM (JSM-6700F, JEOL, Japan). After 5 days of culture, a sample was rinsed twice with phosphate buffered saline and subsequently fixed in 3% glutaraldehyde for 2 h. After that, the samples was rinsed with distilled water and then dehydrated with graded concentration of ethanol, 50%, 75%, 95%, and 100% ethanol for 15 min, respectively. Finally, samples were dried naturally and coated with gold using sputter coating before the observation.

RESULTS AND DISCUSSION

Characterization of nanoAgZ-containing nanofibers

SEM photographs of neat PCL and nanoAgZ-containing PCL nanofibers were shown in Figure 2. From the images, it can be seen that both the nonwoven nanofibers have highly porous microstructure with interconnected pores and the fiber diameter has a wide distribution. In terms of neat PCL nanofibers, fibers were smooth and beadless. The fiber diameters ranged from 361 nm to 1.6 μm with average value of 649 nm. And for the PCL fibers blended with silver-loaded nanoparticles, granular particles can be observed on the surface of fibers and the diameters varied from 350 nm to 3.3 μm with average value of

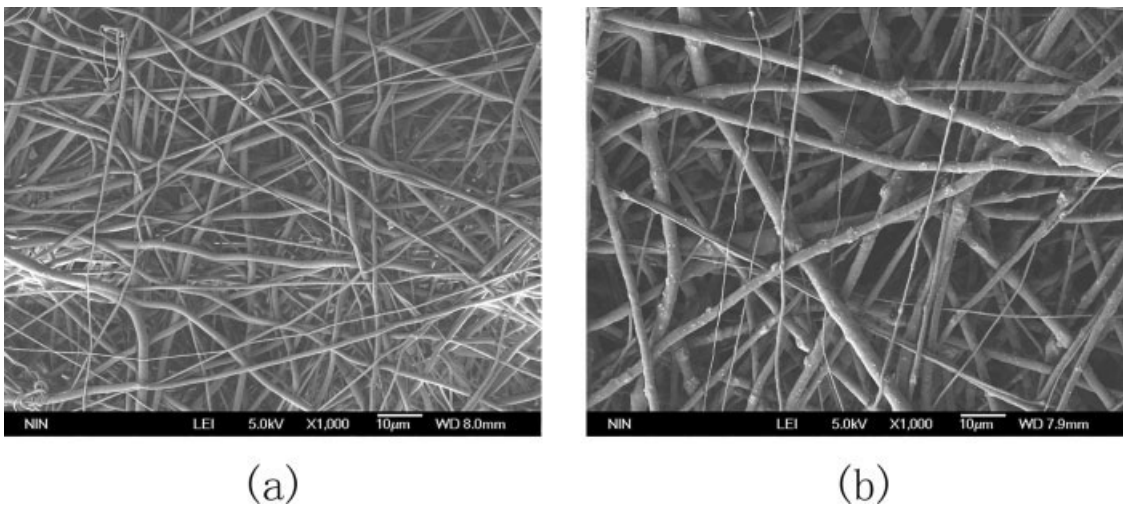

Figure 2 SEM photographs of neat PCL fibers (a) and nanoAgZ-containing composite PCL fibers (b).

909 nm. It seems that the addition of nanoparticles into the solution results in more granulated surfaces and larger distribution of fiber diameters. There were similar reports about it in the previous studies, which described that with the addition of particles, the fiber diameter distribution shifted toward larger values which may attribute to the increasing viscosity of solutions. And it is suggested that the addition of particles may increase viscosity of solution by the polymer absorption and bridging.[33–35] Besides, to confirm the existence of nanoparticles in the composite fibers. The EDX spectrum and the EDX mapping were conducted and shown in the Figure 3. The EDX spectrum of the exposed granules showed that the elements of silver, phosphorus, and zirconium were detected on the fibers which confirmed that nanoAgZ was added into the electrospun fibers. The EDX mapping of the element of silver proved the uniform dispersion of these silver-loaded particles. The earlier investigation of the electrospun fibers demonstrated that the nanoAgZ particles were incorporated into the as-spun composite fibers homogeneously.

The XRD pattern of nanoAgZ-containing PCL fibers, original nanoAgZ particles and the neat PCL fibers were shown in Figure 4. The strong and sharp crystalline peaks at $2\theta = 21.36^\circ$ and 23.68° correspond to the crystallographic planes of PCL crystal, respectively, as it is shown in Figure 4(a).[36,37] For the silver-containing fibers, the peaks of PCL shifted to lower which indicated that the crystallinity of the PCL nanofibers was slightly influenced by the presence of nanoparticles. And the other peaks at 19.28°, 20.1°, 23.24°, 28.02°, 30.94°, 33.38°, 34.74°, and 35.2° all corresponded to the NZP-type structure of nanoAgZ particles apart from the diffraction peaks of PCL [Fig. 4(b,c)].[38] These results also confirmed the nanoparticles existed in the composite material.

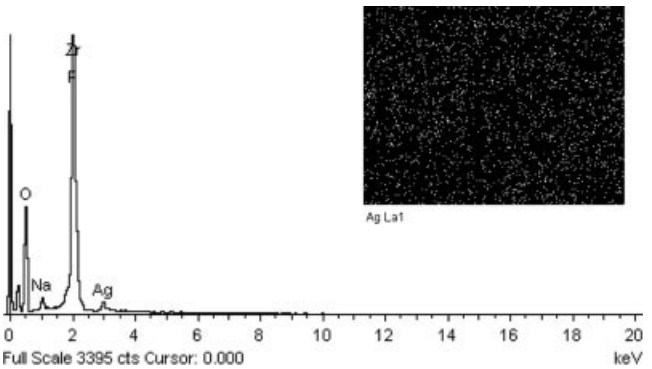

Figure 3 EDX spectrum and EDX mapping of nanoAgZ-containing composite PCL fibers.

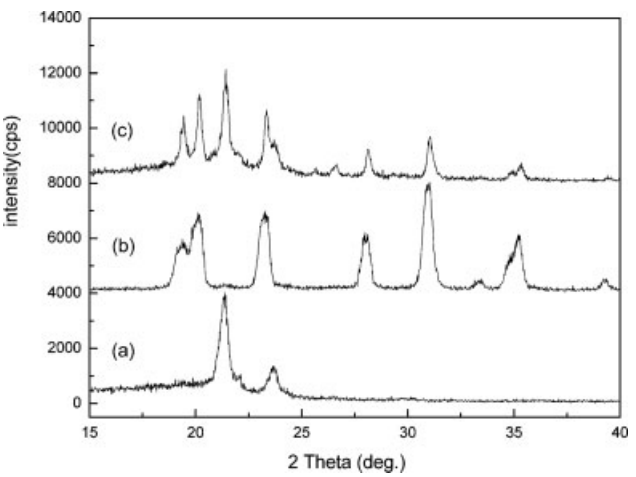

Figure 4 XRD patterns of neat PCL fibers (a), original nanoAgZ powders (b), and nanoAgZ-containing composite PCL fibers (c).

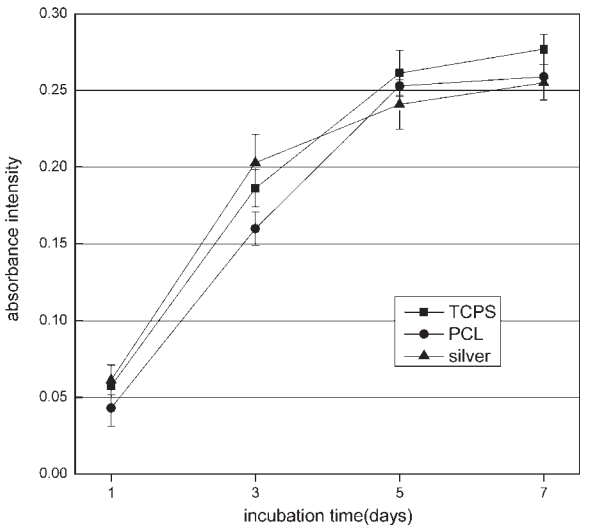

Figure 5 HDFs proliferation on TCPS, neat PCL nanofibers, and nanoAgZ-containing composite fibers.

Antimicrobial assessment

The antimicrobial activities of nanoAgZ-containing fibers were tested against *S. aureus* and *E. coli* according to the Chinese Industrial Standard mentioned earlier. The reductions of bacteria for as-spun silver-containing fibers were 99.27% and 98.44% for *S. aureus* and *E. coli*, respectively. The results showed that the nanoAgZ-containing fibers have maintained strong antibacterial activities against the test strains because of the Ag^{+} ions released from the carrier of highly crystalline zirconium phosphate. And it should be noted that the nanoAgZ-containing nanofibers can also protect the white physical appearances while providing a release of silver ions in the study. It is found that the added nanoAgZ had not caused discoloration on white PCL fibers even after 6 months.

In recent years, nanoAgZ has drawn the attention as the antimicrobial component of wound dressing products such as Avance (SSL International, UK). One of its

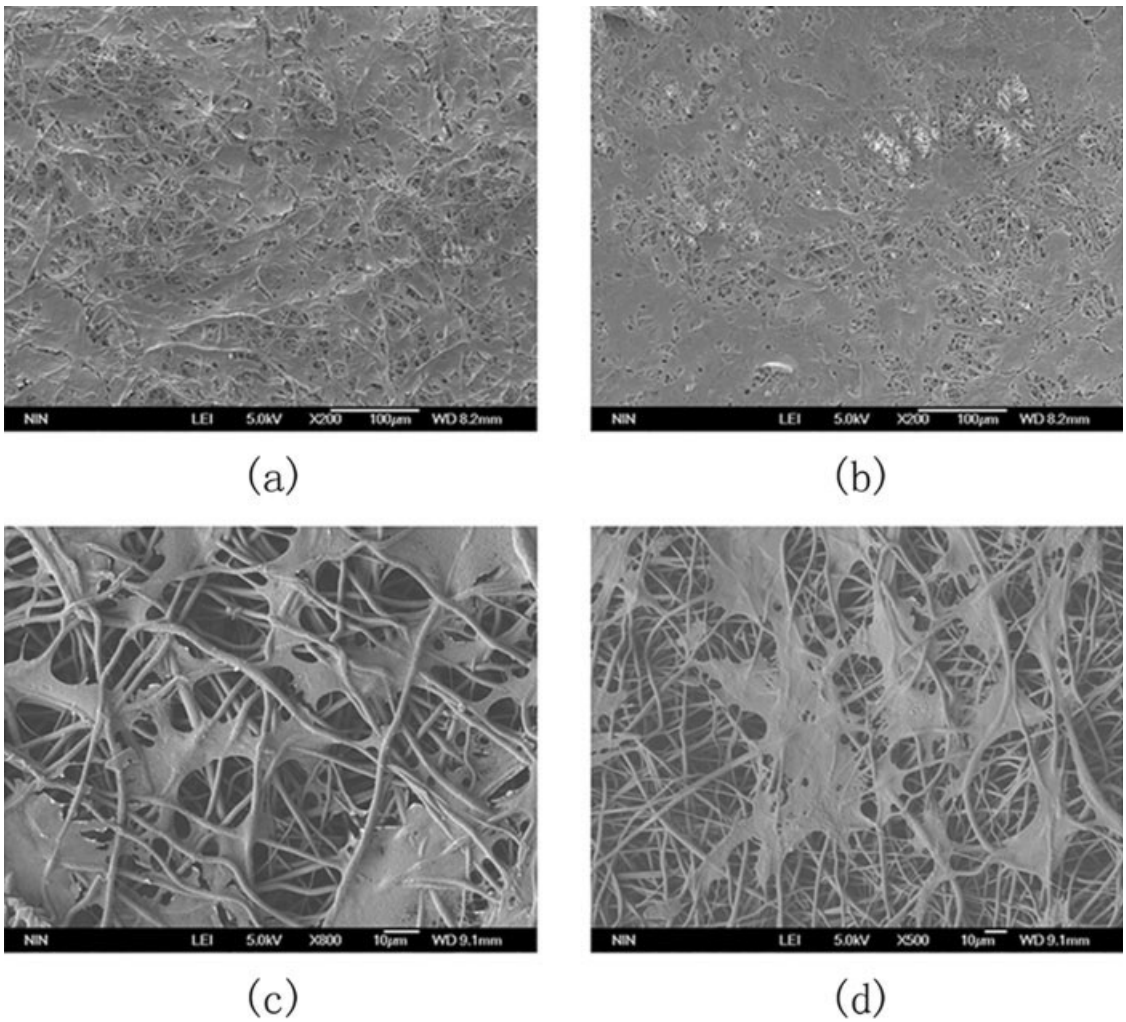

Figure 6 SEM photographs of HDFs on the neat electrospun PCL fibers (a and c) and the nanoAgZ-containing composite fibers (b and d).

main advantages of these dressings is that they provides a reservoir of silver ions within the dressing to release them more consistently, which overcomes the need for regular reapplication and become more acceptable to the patients. In addition, the nanoAgZ has color stability and heat resistance compared to the conventional silver-based antimicrobial agent. Therefore, it is chosen to be incorporated into the electrospun fibers. It was proved that the electrospun nanofibers have maintained the antimicrobial abilities of the silver ions of nanoAgZ and the white physical appearance.

Cell proliferation

Proliferation data of the dermal fibroblasts culturing in vitro were plotted in Figure 5. It showed that the absorbance intensity at 570 nm of silver-containing electrospun membrane was similar level to that of neat PCL nanofibers. It indicated that the introduction of nanoparticles did not influence the surface cellular biocompatibility of electrospun fibers and the addition of nanoAgZ has not been observed to have any cytotoxic effect on the cultured cells *in vitro*.

It is interesting that silver ions do not have similar cytotoxic effects also on eukaryotic cells. That may attribute to that much higher Ag^+ concentrations are required to achieve comparable toxic effects than for bacterial cells because eukaryotic cells are usually larger and show higher structural and functional redundancy than prokaryotic cells. This difference provides a ''therapeutic window'' in which bacterial cells are successfully attacked, whereas harmful effects on eukaryotic cells cannot be observed.[39] The results in the current study even showed that the proliferation of HDFs slightly increased on the silver-containing nanofibers after 3-days of culture. As we know, surface topography of material is one of the most important factors influencing the attachment and spreading of cells, and nanoparticles when coated or present have been shown to be effective in increasing the proliferation of cells.[40] In this study, the nanoparticles introduced into the fibers may improve the surface topography of material and cause the increase of the HDFs proliferation in the early stage.

Cell morphology

Figure 6 shows the morphology of cell attachment and growth on PCL neat nanofibers and PCL nanofibers with nanoAgZ particles after 5 days of *in vitro* culture till cell seeding. It can be seen that HDF adhered and spreaded similarly well on the surface of the nanoAgZ-containing fibers compared to the PCL nanofibers. The cells on both of the nanofibers were observed to proliferate and confluence to form continuous layer. From the SEM images with larger magnification, cells interacted and stretched very well with the surrounding silver-loading fibers. The cells have the spindle-like and flat shape and their filopodia also can be seen, which proved the healthy metabolizability and functionality of these cells. The result of cell behavior indicates that the HDFs can proliferate well on the silver-containing nanofibers.

CONCLUSIONS

PCL nanofibers containing Ag^+-loaded nanoparticles were prepared by electrospinning from the PCL solutions containing silver-zirconium phosphate nanoparticles. The nanoparticles were evenly distributed in the nanofibers, which were confirmed by the field emission scanning electron microscopy (FESEM), EDX, and XRD. The results of antibacterial tests showed that the fibers were bactericidal to the testing microorganisms because of the strong antibacterial ability of silver ions in the nanoAgZ and the nanoAgZ can maintain the physical appearance of nonwoven fabric. And from the results of MTT assay and SEM observations, the nanofibers also showed good cell attachment and proliferation manner in the absence of cytotoxicity. Thus, the nanoAgZ-containing PCL nanofibers prepared by electrospinning are believed to have great potential in the application of wound dressings, which combined with the advantages of biodegradable electrospun PCL nanofibers and strong silver ion antibacterial agents.

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