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Fabrication of antibacterial silver nanoparticle-modified chitosan fibers using *Eucalyptus* extract as a reducing agent

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ABSTRACT: Green chemical method could be a promising route to achieve large scale synthesis of nanostructures for biomedical applications. Here, we describe a green chemical synthesis of silver nanoparticles (Ag NPs) on chitosan-based electrospun nanofibers using *Eucalyptus* leaf extract. A series of silver salt ($AgNO_3$) amounts were added to a certain composition of chitosan/polyethylene oxide aqueous acetic acid solution. The solutions were then electrospun to obtain nanofibrous mats and then, morphology and size of nanofibers were analyzed by scanning electron microscopy (SEM). Incubation of $AgNO_3$-containing mats into Eucalyptus leaf extract led to the formation of Ag NP clusters with average diameter of 91 ± 24 nm, depicted by SEM and transmission electron microscopy. Surface enhanced Raman spectroscopy also confirmed formation of Ag NPs on the nanofibers. The mats also showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* bacteria with bigger inhibition zone for extract-exposed mats against S. aureus. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42133.

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INTRODUCTION

Nobel metal nanoparticles have intensely been investigated due to their incomparable optical, electrical, mechanical, magnetic, and chemical properties.[1] Many different techniques such as salt reduction[2] and photochemical reduction in reverse micelle[3] have been used to prepare metallic nanoparticles. Recently, green synthesis has been used as a new strategy for production of nanoparticles. The green synthesis method provides researchers with nontoxic and safe chemicals from microorganisms and plant extracts and also, natural irradiation for nanoparticle production.[4] Different plant leaf extracts such as *Andrachnea chordifolia*[5] Alfalfa[6] *Aloevera*[7] *Geranium*[8] and *Achillea*[9] have been used for synthesis of metal nanoparticles.

Electrospinning as a favorable technique generates ultrafine fibers. This technique produces nonwoven fibrous mats through an external electrical field applied on a polymer solution or melt.[10] Many factors including solution and processing parameters in electrospinning affect the morphology and diameter of produced nanofibers.[11] Because of their enormous surface, nano-scale diameters, and functionalization ability, electrospun nanofibers have been applied in many fields such as biosensors,[12] tissue engineering,[13] membranes,[14] wound dressing, and enzyme carriers.[15]

Scientists have used different solid state synthesis methods to fabricate metal nanoparticles on electrospun nanofibers. In one study, silver nanoparticles on the Ag-NP/TCNQ (tetracyanoquinodimethane) composite nanofibers were produced using UV irradiation through an in situ reduction method.[16] Moreover, Li *et al.* applied photocatalytic approach to prepare gold nanoparticles on the electrospun nanofibers of titania.[17]

Recently, Gao *et al.* have reviewed antibacterial electrospun nanofibers and their composition with metal nanoparticles.[18] In one of the studies, electrospinning process has been used to fabricate chitosan/gelatin nanofibers containing silver nanoparticles.[19] In this method, chitosan plays as a reducing and stabilizing agent. In another research, An *et al.* successfully produced Ag/chitosan (CS)/polyethylene oxide (PEO) ultrafine electrospun fibers and their results demonstrated antibacterial activity against *Escherichia coli*.[20] They used $NaBH_4$ to reduce silver ions in the polymer solution. Also, Pant *et al.*[21] fabricated antibacterial electrospun naylon-6 nanofibers decorated with Ag NPs. In their one-step approach, the electrospinning solvents act as reducing agent to convert $AgNO_3$ into Ag NPs. In

Table I. Effect of Salt ($AgNO_3$) Concentration on the Size of Ag NPs Clusters

Exp. No	Type of mat	*Eucalyptus* Ext. in ethanol (% wt/v)	Salt amount (wt %)	Size of Ag NP clusters (nm)
A_1	CS/PEO	None	None	Inapplicable
A_2	CS/PEO/$AgNO_3$	None	5.3	Inapplicable
A_3	CS/PEO	0.1	None	Inapplicable
A_4	CS/PEO/$AgNO_3$	0.1	5.3	98 ± 15
A_5	CS/PEO/$AgNO_3$	0.1	10.6	270 ± 18
A_6	CS/PEO/$AgNO_3$	0.1	21.2	280 ± 16

another study, antibacterial Ag/polyacrylonitrile (Ag/PAN) nanofibers were prepared.[22] In this process, $AgNO_3$ precursor was reduced to metallic silver nanoparticles by atmospheric helium plasma treatment and then, electrospun into smooth nanofibers with embedded Ag nanoparticles.

This study followed a solid state production of Ag NPs on the surface of CS/PEO nanofibers via a green synthesis method. Chitosan is an antibacterial biopolymer and its bacteriostatic mechanisms have been investigated by researchers.[23] Silver nanoparticles also exhibit antibacterial effects.[24,25] In this work, aqueous acetic acid solution of chitosan (CS)/polyethylene oxide (PEO) having silver salt ($AgNO_3$) was electrospun and the obtained nanofibrous mats were then incubated by ethanolic leaf extract of *Eucalyptus* to form Ag NPs on the nanofibers. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and surface enhanced Raman spectroscopy (SERS) were used to analyze the obtained nanofibers and Ag nanoparticle-containing nanofibers.

EXPERIMENTAL

Materials

Low molecular weight chitosan (CS, degree of deacetylation 91.2%) was purchased from Easter Group (Dong Chen), China. Polyethylene oxide (PEO, MW: 600,000 g/mol) was obtained from Acros Organics, Belgium. The $AgNO_3$ and glacial acetic acid were supplied from Merck, Germany. All the materials were used without further purification. The *Eucalyptus camaldulensis* extract was provided by Herbarium section of College of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. *E. coli* (ATCC 29625) and *Staphylococcus aureus* (ATCC 25923) were purchased from Darvash, Iran. The electrospinning set up was supplied by Fanavaran Nano-Meghyas, Tehran, Iran.

Preparation of Electrospinning Solutions

Solutions of electrospinning were prepared according to our previous studies.[26,27] Briefly, CS and PEO solutions were prepared by dissolution of the polymers in the aqueous acetic acid (80%, v/v) to obtain a total polymer solution of 3.0% (w/v) and weight ratio of 80/20 for CS/PEO under overnight constant stirring at 50°C.

To obtain a mixture of CS/PEO/$AgNO_3$ for electrospinning, different volumes of a 0.10*M* $AgNO_3$ solution in 80% (v/v) aqueous acetic acid were added to 5.0 ml of the aforementioned 3.0% (w/v) CS/PEO solution under constant stirring for 15 min in 50°C. The proportions of salt related to the total solid (polymers and salt) in the solutions are given in Table I.

Electrospinning of Blended Solutions

Solutions of CS/PEO and CS/PEO/$AgNO_3$ were loaded into a standard 5 ml syringe that was connected to an 18 gauge needle with inner diameter of 0.8 mm. The electrospinning was performed by applying a high voltage of 15 kV between the needle and the rotating collector. The needle–collector distance and flow rate of the injection were set at 10 cm and 1.0 ml/h, respectively. All the spinning processes were performed at room temperature.

Reduction of Silver in Electrospun Mats

The prepared CS/PEO and CS/PEO/$AgNO_3$ nanofibrous mats were incubated into 20 ml of different concentrations of ethanolic *Eucalyptus* leaf extract solution for 24 h at room temperature as given in Table I. Then, electrospun mats were washed several times with 70% (v/v) ethanol and dried at room temperature for 2 h.

Characterization

Nanofibrous mats were sputter-coated with gold and examined by scanning electron microscope (FE-SEM, Hitachi S-4160) to investigate size and surface morphology of the nanofibers and Ag NPs. Micrographs were analyzed by the SemAfore software (version 5.2, JEOL) and average diameter of about 100 nanofibers was measured by Origin 6.0 Professional software. The average diameter of Ag clusters was also measured by the same method considering 100 clusters. Transmission electron micrographs of the nanofibers containing Ag NPs were acquired by a TEM instrument (Zeiss, Germany, model: em10c, applied voltage: 80 kV). To confirm the presence of the Ag NPs on the surface of the CS/PEO nanofibers, SERS was performed by a Raman instrument (BRUKER, Germany, Model: SENTERRA 2009, Laser wave number: 785 nm and Spectral Range: 200–3500 cm^{-1}).

Antibacterial Experiments

Antimicrobial effects of the electrospun fibers containing Ag NPs were investigated against the *E. coli* (gram negative) and *S. aureus* (gram positive) bacteria by the agar disk diffusion method.[28] The bacteria under experiment were uniformly swabbed in sterilized Luria–Bertani (LB) broth media across a culture plate then incubated overnight at 37°C under shaking. According to Table II, experiments of B_1–B_4 were done. Disks of samples of 15 mm in diameter were placed on the agar

Table II. Antimicrobial Activity of the Electrospun Nanofibrous Mats Against *E. coli* and *S. aureus* Bacteria

		Inhibition zone of a 15.0 cm disk for samples	
Exp. No	Sample type	*E. coli*	*S. aureus*
B_1	CS/PEO	Resistance	Resistance
B_2	CS/PEO/Ext.	Resistance	Resistance
B_3	CS/PEO/$AgNO_3$	15.4 mm	16.0mm
B_4	CS/PEO/$AgNO_3$/Ext.	18.2 mm	19.0 mm

medium. After 24 h, inhibition zones of the CS/PEO nanofibrous mat, as a control sample, also CS/PEO/$AgNO_3$ and *Eucalyptus* extract-treated CS/PEO/$AgNO_3$ mats were compared.

RESULTS AND DISCUSSION

Surface Morphology of Nanofibers and Formation of Ag Nanoparticles

Various parameters including solution concentration, viscosity, charge density, needle tip to collector distance, and applied voltage affect the morphology and diameter of electrospun nanofibers and are widely reviewed.[29,30] Through controlling these factors, bead-free and uniform ultrafine electrospun fibers would be obtained.

In this study, electrospun fibers of CS/PEO were prepared in the best conditions that we have performed in pre-examination tests and reported in our previous study.[26] Our results showed that CS/PEO non-woven fibers with a high weight ratio of chitosan to PEO could be achieved in 80% acetic acid without addition of any chemicals. These electrospun fibers were used as a platform to synthesize silver nanoparticles by a green process using *Eucalyptus* extract. This extract is recently used as a reducing agent to prepare cotton/silver composites by immersing cotton fibers in the solution of extract-reduced silver nanoparticles.[31] In this study, silver nanoparticles were deposited on the surface of the cotton fibers. However, in our study, CS/PEO solutions containing silver salt were electrospun and then, the obtained mats were treated with the *Eucalyptus* extract. Chitosan is previously reported for reducing silver salt to elemental silver.[19] It is proposed that when mixing $AgNO_3$ to chitosan, Ag^+ ions would bind to hydroxyl and glucosidic groups of chitosan through electrostatic interactions. Consequently, silver ions are reduced to elemental silver[32] and very small silver nanoparticles would be formed. In the second step, the plant extract could efficiently reduce silver salt inside the electrospun chitosan fibers and form silver nanoparticle clusters on the obtained fibers.

Morphology of CS-based fibers and formation of Ag nanoparticles on the surface of nanofibers after addition of $AgNO_3$ and reduction by *Eucalyptus* extract were investigated by scanning electron microscopy. Figure 1(a,b) depicts the SEM image and size distribution histogram of the CS/PEO nanofibers, obtained from Exp. A_1 (Table I). It could be found that the chitosan/PEO fibers have smooth, uniform, and bead-less morphology with size distribution ranging from 160 nm to 400 nm. Addition of $AgNO_3$ to chitosan/PEO solution leads to an increase in diameter of produced nanofibers. Figure 1(c) (Exp. A_2, Table I) shows SEM image of the CS/PEO/$AgNO_3$ fibers without

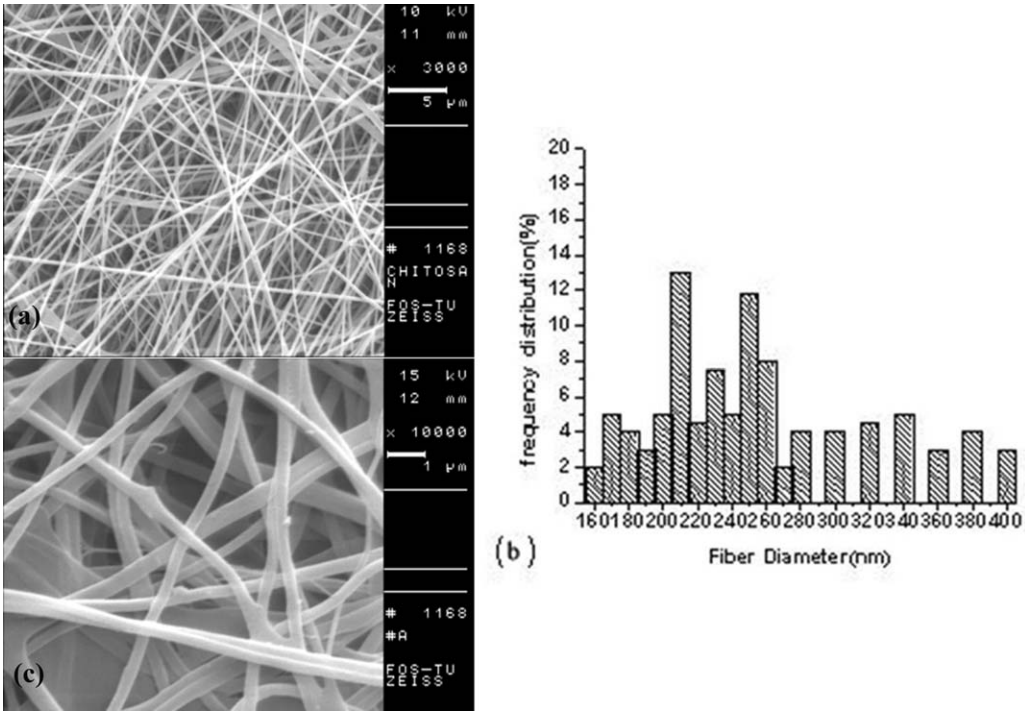

Figure 1. SEM image of the CS/PEO nanofibers from Exp. A_1 (a), its size distribution histogram of nanofibers (b), and SEM micrograph of the CS/PEO/$AgNO_3$ electrospun fibers of Exp. A_2 (c).

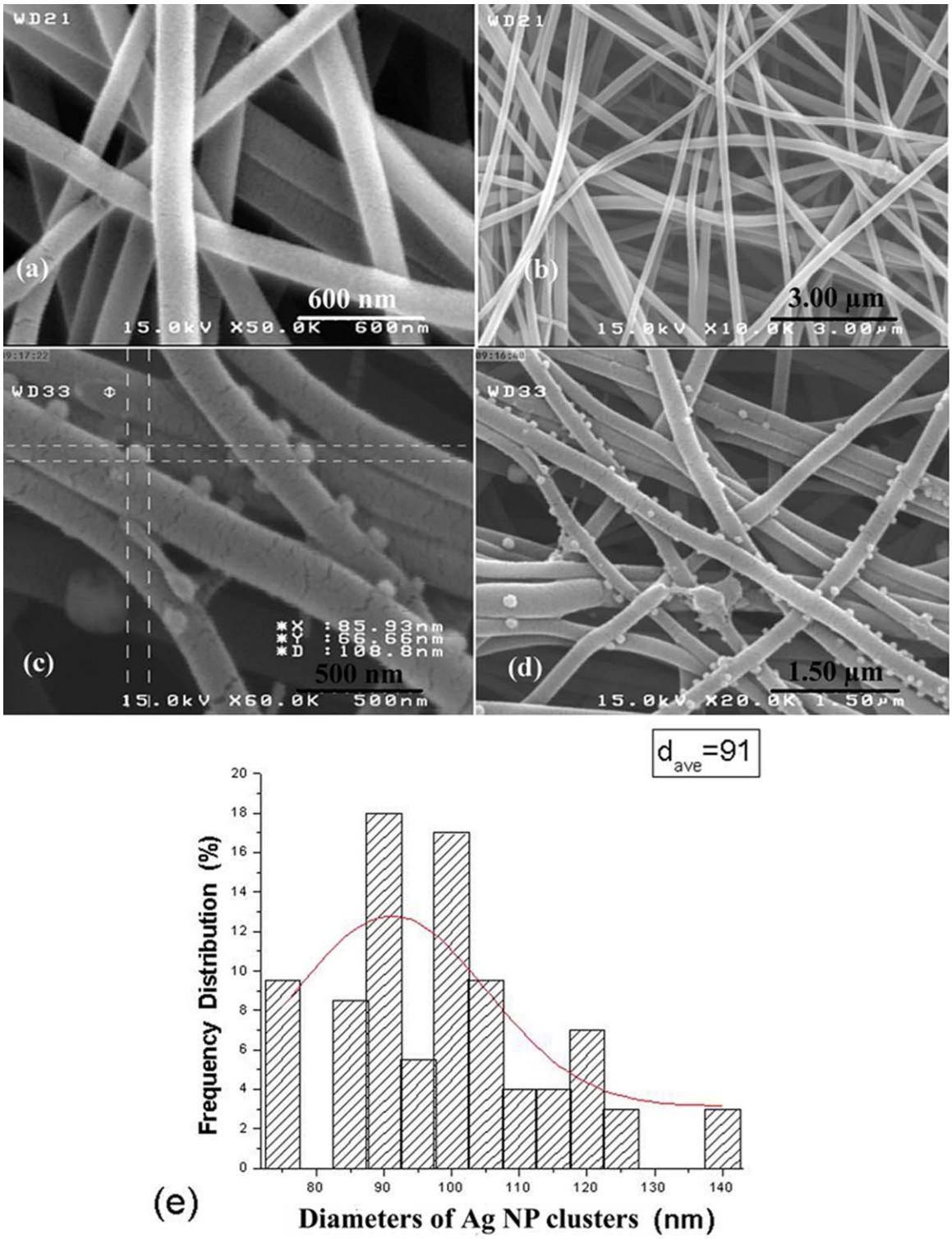

Figure 2. SEM micrographs of the CS/PEO nanofiber without $AgNO_3$ treated with *Eucalyptus* extract (Exp. A_3) in two different magnification (a, b), $AgNO_3$ embedded CS/PEO nanofiber treated with *Eucalyptus* extract (Exp. A_4) in two different magnification (c, d) and diameter distribution of Ag NP clusters of Exp. A_4 (e). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

subsequent treatment with the *Eucalyptus* extract. This image represents rather thicker electrospun fibers containing $AgNO_3$ (with average diameter of 305 ± 80), compared to corresponding solution lack of $AgNO_3$ [i.e., Figure 1(a), with average size of 254 ± 61]. As these figures show, there are no Ag clusters or beads on the produced nanofibers.

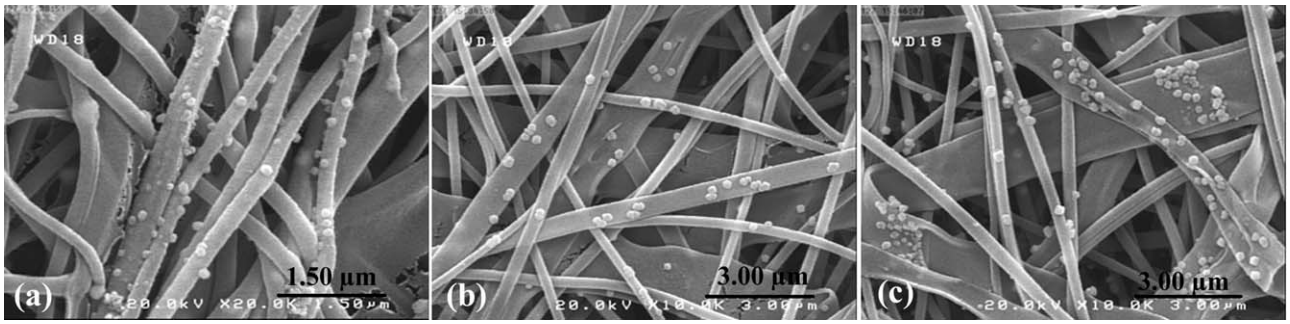

Figure 3. SEM micrographs of the CS/PEO/$AgNO_3$ nanofibrous mats with various salt concentrations, (a) 5.3 wt %, (b) 10.6 wt %, and (c) 21.2 wt % (Exp. A_4–A_6).

Figure 2 elucidates the SEM images of CS/PEO and CS/PEO/$AgNO_3$ nanofibers after incubation in *Eucalyptus* extract in Exp. A_3 and A_4 (Table I). Figure 2(a,b) are SEM images of CS/PEO nanofibers after performing Exp. A_3 and as they show, there are no Ag clusters due to the lack of $AgNO_3$ in the electrospinning solution. These extract-treated nanofibers have a smooth and defects-free morphology. Figure 2(c,d) depicts SEM micrographs of CS/PEO/$AgNO_3$ nanofibers after treatment with the extract (Exp. A_4). These figures demonstrate the formation of Ag NP clusters attached to the surface of the nanofibers. Comparatively, these results illustrate the reduction of silver salts by the extract and consequently, formation of Ag NPs. Size distribution histogram of the Ag NPs clusters formed on the surface of nanofibers is depicted in Figure 2(e). The clusters of Ag particles have an average diameter of 91 ± 24 nm in the range of 80–140 nm.

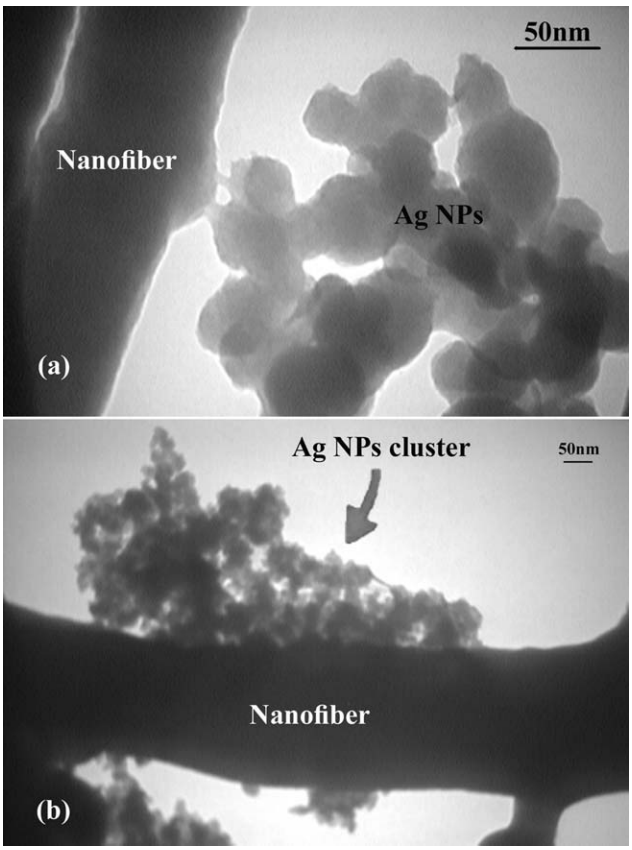

Figure 4. TEM images of the CS/PEO/$AgNO_3$ nanofibers incubated in *Eucalyptus* extract from Exp. A_4.

Investigation results of $AgNO_3$ concentration influence on size of obtained silver clusters, located on the electrospun nanofibrous mats, are shown in Table I. The results showed that increasing $AgNO_3$ concentration leads to an increased number and size of Ag NP clusters. The results are in the same line with other works. For example, Demir *et al.* have reported synthesis of palladium nanoparticles on electrospun copolymers of acrylonitrile and acrylic acid using hydrazine as reduction agent and obtained similar results.[33] SEM micrographs in Figure 3 depict the images of CS/PEO/$AgNO_3$ nanofibers in the conditions of different salt concentrations (i.e., Exp. A_4–A_6 from Table I). According to the SEM images in Figure 3, extract-treated CS/PEO/$AgNO_3$ fibers show a heterogeneous morphology, containing thin or thick ribbon-like fibers associated with Ag NP clusters on the surfaces, compared to the homogenous morphology of untreated CS/PEO/$AgNO_3$ electrospun fibers without any Ag NPs [i.e., Figure 1(c)]. It seems that some of the fibers are merged to each other after treating with the extract and formed a ribbon-like structure. These fibers show more accumulations of Ag NP clusters.

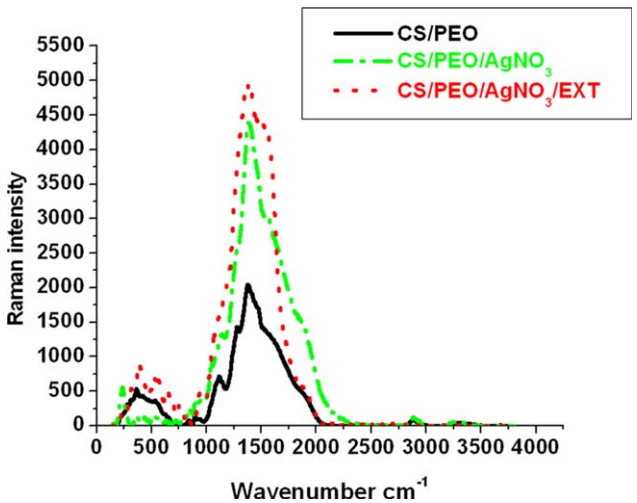

Figure 5. Raman spectra of CS/PEO (Exp. A_1), CS/PEO/$AgNO_3$ (Exp. A_2), and extract-incubated CS/PEO/$AgNO_3$ (Exp. A_4) nanofibrous mats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

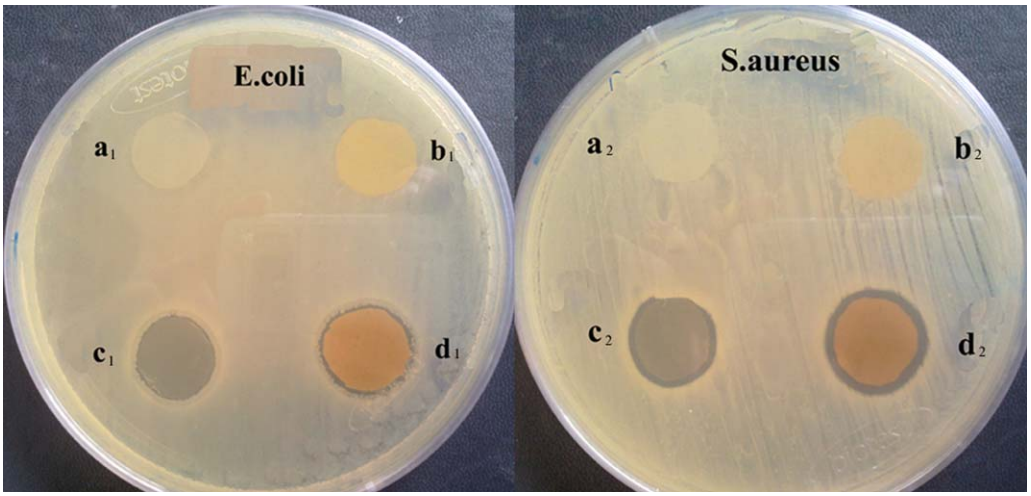

Figure 6. Inhibition zones of the disks against *E. coli* and *S. aureus*, according to the experiments in Table II: Pure CS/PEO nanofibrous mat (a_1 and a_2, Exp. B_1), pure CS/PEO mat treated with extract (b_1 and b_2, Exp. B_2), CS/PEO/$AgNO_3$ mat without extract incubation (c_1 and c_2, Exp. B_3), and CS/PEO/$AgNO_3$ with extract treatment (d_1 and d_2, Exp. B_4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Transmission Electron Microscopy

To investigate Ag NPs formation on and within the nanofibers as well as the NPs shape, the TEM image of CS/PEO/$AgNO_3$ after incubation in the extract was used. Figure 4 shows the TEM images of the CS/PEO/$AgNO_3$ electrospun fibers after incubated in *Eucalyptus* extract (Exp. A_4). From this figure, clustered silver nanoparticles were observed to be formed only on the surface of nanofibers and no particles were observed within the entire nanofiber matrix. These suggest that the reduction of $AgNO_3$ to Ag NPs by *Eucalyptus* extract only occurs on the surface of nanofibers. The images also depict that the Ag NPs have relatively spherical shapes and make clusters on the surface of nanofibers.

Raman Spectroscopy

SERS has been widely considered as one of the most important techniques for identifying interfacial properties and acquiring data from low concentration species.[34] Wang *et al.* also applied SERS signal for characterization of the Ag NPs that dispersed in polyacrylonitrile electrospun nanofibers.[35] We here applied SERS to demonstrate the formation of Ag NPs on the nanofibers. Figure 5 shows Raman spectra of the neat CS/PEO nanofiber mat (Exp. A_1) and also CS/PEO/$AgNO_3$ nanofibrous membranes with and without extract treatment (Exp. A_4 and A_2, respectively). The recorded Raman intensity peaks of the neat CS/PEO and CS/PEO/$AgNO_3$ mats are about 2000 and 4500, respectively, whereas that of extract incubated CS/PEO/$AgNO_3$ electrospun mat is about 5000. Since, surface-enhanced Raman scattering occurs at the metallic surfaces such as Ag and Au nanostructures and boosts the Raman scattering signal of surface-adsorbed molecules, therefore, the enhancement of the intensity peaks of the extract-exposed CS/PEO/$AgNO_3$ demonstrates formation of Ag NPs on the nanofibers.

Antibacterial Activities

Bactericidal effects of the CS/PEO/Ag NP mat were evaluated against two pathogenic bacteria including *E. coli* (gram negative) and *S. aureus* (gram positive) using agar disk diffusion method.[36] Figure 6 elucidates the inhibition zones of CS/PEO/$AgNO_3$ nanofibrous samples (with and without incubation in *Eucalyptus* extract) against *E. coli* and *S. aureus*. As the above-mentioned figure shows, although there are no significant inhibition zones in the case of pure CS/PEO and extract-exposed CS/PEO disks, the inhibition zones of CS/PEO/$AgNO_3$ nanofibrous samples, with or without *Eucalyptus* extract incubation, are measurable. Previous studies also show superior antibacterial activities in a combination of chitosan and Ag NPs.[20,32] In addition, the resultant inhibition zones of extract incubated CS/PEO/$AgNO_3$ disks are much bigger than that of without incubation. The details of the method are given in Table II. The technique proves formation of Ag NP clusters on the nanofibers using *Eucalyptus* extract as a green reduction agent and demonstrates that membranes containing Ag NPs have a higher antibacterial potential. Results also show that CS/PEO/$AgNO_3$ electrospun mats (with and without extract incubation) caused bigger inhibition zones against *S. aureus* compared to *E. coli*.

To follow the durability of antibacterial activity, CS/PEO and CS/PEO/$AgNO_3$ electrospun samples with and without

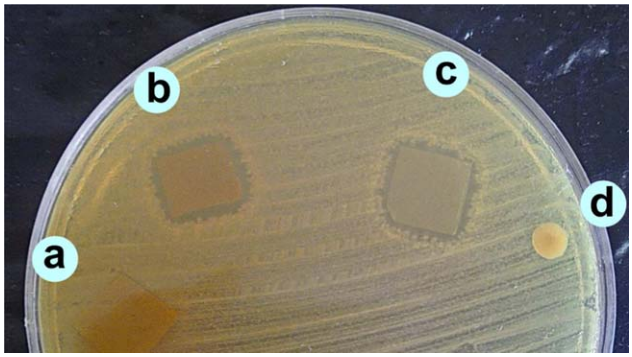

Figure 7. 20-Day following of bactericidal activity of: extract treated CS/PEO (a), extract treated CS/PEO/$AgNO_3$ (b), CS/PEO/$AgNO_3$ without extract treatment (c), and *E. coli* colony (d). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

treatment with Eucalyptus extract were investigated against *E. coli* for 20 days, as shown in Figure 7. This image clearly indicates that after 20 days, CS/PEO mat [Figure 7(a)] does not show antimicrobial activity while samples of CS/PEO/$AgNO_3$ with and without extract treatment [Figure 7(b,c)] represent strong bactericidal effects. Furthermore, extract-treated CS/PEO/$AgNO_3$ samples show slightly bigger inhibition zone than that of untreated ones.

As the prepared composites contain chitosan fibers and silver nanoparticles, two different mechanisms could be proposed for their bactericidal activity. Chitosan as a polycation is known to interact with negatively charged surfaces of the bacteria and lead to loss of membrane permeability, cell leakage, inhibition of DNA replication, and therefore, cell death.[38,39] Besides, fibrous structure of the composites provides an enormous surface area to interact with bacteria. Also, silver nanoparticles render even more powerful antibacterial activity to the obtained composites through binding to cell wall and disturbing its permeability and cell respiration.[20,37,39]

CONCLUSIONS

Antibacterial chitosan-based nanofibrous mats containing silver nanoparticles were prepared by combination of electrospinning and a green synthesis method using *Eucalyptus* leaf extract as reducing agent. Electron microscopies revealed that obtained fibrous mats have smooth and bead-less morphology with formation of Ag NPs clusters on the surface of fibers. Efficient reduction of silver ions and formation of Ag NPs on the electrospun mats were also confirmed by Raman spectroscopy. Bacteriostatic studies showed that CS/PEO/Ag NP nanofibrous mat has a stronger antibacterial activity on *E. coli* and *S. aureus* bacteria in comparison with the corresponding composites without the Ag NPs. Also, 20-day bactericidal following indicated that CS/PEO/$AgNO_3$ and CS/PEO/Ag NP mats preserve their activity against bacteria. The CS/PEO/Ag NP nanofibrous mat can be considered as an operational membrane in diverse biomedical applications such as tissue scaffolds, antibacterial wound dressing, and patches especial for diabetic bedsore and chronic infectious skin ulcers.

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