

# Co-spinning of Silver Nanoparticles with Nisin Increases the Antimicrobial Spectrum of PDLA: PEO Nanofibers

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**Abstract** Silver nanoparticles (AgNPs), synthesized using *N,N*-dimethylformamide (DMF), were electrospun with nisin in a 50:50 blend of 24 % (w/v) poly(D,L-lactide) (PDLA) and poly(ethylene oxide) (PEO). Addition of AgNPs decreased the average diameter of the nanofibers [silver nanofibers (SF)] from  $588 \pm 191$  to  $281 \pm 64$  nm, or to  $288 \pm 63$  nm when nisin was co-spun with AgNPs. Nanofibers containing AgNO<sub>3</sub> (SF) had a beads-on-string structure, whereas nanofibers with AgNPs and nisin [silver plus nisin nanofibers (SNF)], nanofibers with only nisin [nisin nanofibers (NF)], and nanofibers without AgNPs and nisin [control nanofibers] had a uniform structure. The irregular topography was confirmed by atomic force microscopy. No interactions occurred between silver, nisin, PDLA, and PEO, as confirmed with Fourier transform infrared spectroscopy. Most of the AgNPs ( $18 \pm 2.8$  ppm) and nisin ( $78.1 \pm 1.2$  µg/ml) were released within the first 2 h. SF and SNF inhibited the growth of gram-positive and gram-negative bacteria, whereas NF failed to inhibit gram-negative bacteria. A wound dressing with broad-spectrum antimicrobial activity may be developed by the incorporation of nanofibers containing a combination of AgNPs and nisin.

## Introduction

Inclusion of silver nanoparticles (AgNPs) in a polymer matrix and the controlled release of silver particles proved effective in the treatment of chronic topical infections [8, 21]. Silver binds to electron donor groups in molecules containing sulfur, oxygen, and nitrogen [6]. Randall et al. [16] reported cell membrane damage and growth inhibition of *Staphylococcus aureus* by silver particles. Little information is available on bacterial resistance to silver, possibly due to multiple target sites [7].

Nisin is a 34-amino acid lanthionine-containing polypeptide produced by certain strains of *Lactococcus lactis* [22]. The lantibiotic, classified as a class I bacteriocin, is active against a number of gram-positive bacteria such as *S. aureus*, *Streptococcus pneumoniae*, *Clostridium difficile*, *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium botulinum* [1, 5, 10]. Gram-negative bacteria are resistant to nisin, but some examples of growth inhibition were reported when cells were pre-treated with EDTA [17, 19]. EDTA chelates Mg<sup>2+</sup> and Ca<sup>2+</sup> in the outer membrane of gram-negative bacteria, resulting in destabilization of the phospholipid structure and migration of nisin to the peptidoglycan layer [12, 13].

Nisin alone and nisin incorporated with 2,3-dihydroxybenzoic acid in nanofiber dressings proved effective in the treatment of skin infections caused by *S. aureus* [10] and the prevention of biofilm formation by a methicillin-resistant strain of *S. aureus* [1]. The high surface-to-volume ratio of nanofibers renders them ideal as drug carriers [4, 20].

In this study, we report the antimicrobial potential of AgNPs, combined with nisin and incorporated in poly(D,L-lactide) (PDLA) and poly(ethylene oxide) (PEO) nanofibers, against *S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella*

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*pneumonia*, *Escherichia coli*, and *Salmonella typhimurium*.

## Materials and Methods

### Materials

Poly(D,L-lactide) (PDLLA, 75–120 kDa), poly(ethylene oxide) (PEO, 200 kDa), and *N,N*-Dimethylformamide (DMF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Silver nitrate was purchased from BDH laboratory (Poole, England). Nisin (Nisaplin<sup>®</sup>) was obtained from Danisco (Copenhagen, Denmark). All other reagents were of analytical grade. Bacterial strains were from Caliper Life Sciences (Hopkinton, MA, USA).

### Synthesis of Silver Nanoparticles

Silver nitrate particles (AgNPs) were synthesized in DMF, according to a modification of the method described by Pastoriza-Santos and Liz-Marzan [15]. Silver nitrate (AgNO<sub>3</sub>; 1 % w/v) was suspended in DMF containing 12 % (w/v) PDLLA and 12 % (w/v) PEO. The suspension was dissolved for 1 h at 25 °C, followed by heating at 40 °C for 30 min on a hot plate. Change in color was recorded by absorbance readings at 420 nm, using a SmartSpec<sup>™</sup> plus, UV–Vis spectrophotometer (BioRad, USA).

### Electrospinning of Nanofibers

Nisin was suspended in DMF (15 mg/ml), as described by Heunis et al. [10]. To this suspension, AgNO<sub>3</sub> (1 % w/v) was added and electrospun in a 50:50 blend of 24 % (w/v) PDLLA and PEO. Electrospinning was performed as described by Heunis et al. [10] and Ahire and Dicks [3]. Polymer solutions were loaded in glass tubes of 150 × 6 mm, tapered to a 1 mm internal diameter. A constant electric field of +10 kV was applied from a copper electrode, immersed into the polymer solution, to a –5 kV collector. The nanofibers collected at the anode were dried for 8 h in a laminar flow at 25 °C. Nanofibers containing AgNO<sub>3</sub> and nisin were labeled silver plus nisin nanofibers (SNF), nanofibers containing only nisin were labeled nisin nanofibers (NF), and those with only AgNO<sub>3</sub> were labeled silver nanofibers (SF). Nanofibers without nisin or AgNO<sub>3</sub> were labeled control nanofibers (CF).

### Characterization of Nanofibers

The structure of nanofibers was studied using a Leo 1430VP (Zeiss) scanning electron microscope (SEM),

according to the method described by Ahire and Dicks [3]. The diameter of the nanofibers was determined using ImageJ Software (version 1.46; Scion Corporation). AgNPs in the nanofibers were visualised using a JEOL 1200-EX II (JEOL USA, Inc., MA) transmission electron microscope (TEM), set at an acceleration voltage of 120 kV. The concentration of AgNPs in nanofibers was determined by energy-dispersive X-ray (EDX) analysis of SEM (EVO<sup>®</sup> MA15VP, Zeiss), using an Oxford Instruments<sup>®</sup> detector and Oxford INCA software. Surface topology was studied using an Easyscan 2 atomic force microscope [atomic force microscopy (AFM); Nanosurf Inc., CA, USA]. X-ray diffraction patterns were recorded with a Bruker AXS D8 advance X-ray diffractometer (Bruker AXS, Frankfurt, Germany), operated in locked coupled mode and equipped with a Vantec-1 position-sensitive detector optimized for Cu-K $\alpha$  radiation ( $\lambda = 1.5406 \text{ \AA}$ ). The X-ray tube was operated at 40 mA and 40 kV, and measurements were recorded at a scanning rate of 1 s/step, with a step size of 0.0275° in a  $2\theta$  range that extended from 4° to 69.99°. All the silver peaks were compared with the standard powder diffraction card of Joint Committee on Powder Diffraction Standards (JCPDS), silver file No. 04-0783. Interaction of silver with nisin, PDLLA, and PEO was determined using a Thermo Nicolet Avatar 330 (Thermo Scientific, Waltham, MA, USA) Fourier transform infrared (FT-IR) spectroscope with a smart performer Zn/Se ATR accessory.

### Release of Nisin and AgNPs from Nanofibers

The release of nisin and AgNPs from SNF, NF, and SF was determined by suspending 1 mg of the nanofibers in 1 ml sterile phosphate buffer saline (PBS, pH 7.3) and placing the suspension at 37 °C. The level of AgNPs released from the nanofibers was determined directly after immersion, and 2, 4, 6, and 24 h later, using an atomic absorption spectrophotometer (SpectrAA-250 Plus, Varian), equipped with an automatic dual beam. The release of nisin was estimated from protein concentrations recorded using the Micro Bicinchoninic Acid (BCA) kit (Pierce, Rockford, IL).

### Antimicrobial Activity of Nanofibers

The antimicrobial activity of the electrospun nanofibers was determined as described by Ahire et al. [2]. In brief, *P. aeruginosa* strains PA01 and Xen 5, *K. pneumoniae* Xen 39, *E. coli* Xen 14, and *S. typhimurium* Xen 26 were grown overnight at 37 °C in Luria Broth (LB). *S. aureus* Xen 29, Xen 30, Xen 31, and Xen 36 were cultured in brain heart infusion (BHI). The Xen strains were obtained from Bio-ware<sup>™</sup> Microorganisms (Caliper Life Sciences, USA). Sections (0.5 cm<sup>2</sup>) of SNF, SF, NF, and CF were placed on

the surface of soft agar (1 % w/v) plates (LB or BHI, depending on the strain), each seeded with  $10^5$  CFU/ml of a specific strain. Zones of growth inhibition (diameter in mm) were recorded after 24 h of incubation at 37 °C.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism [version 6.03 (Trial) for Windows, GraphPad Software Inc, USA]. A *P* value <0.05 was considered statistically significant.

### Results and Discussion

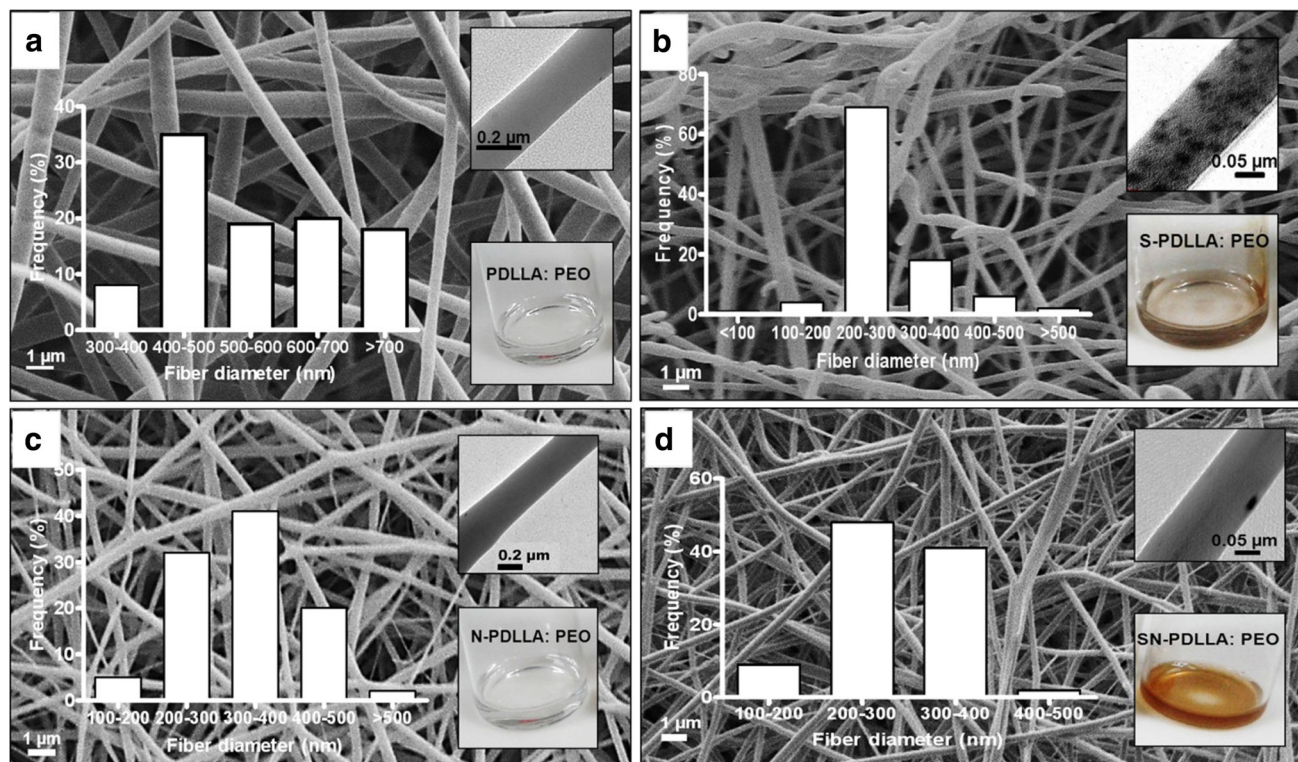
A change from white to yellow after heating of  $\text{AgNO}_3$  in the presence of PDLLA, PEO, and DMF (shown in the image inserted in Fig. 1) indicated that the silver ions ( $\text{Ag}^+$ ) were reduced to metal ( $\text{Ag}^0$ ) [14]. A twofold increase in absorption readings was recorded for SNF and SF, confirming the reduction of  $\text{Ag}^+$  (not shown).

The average diameter of nanofibers electrospun in the absence of silver and nisin (CF) was  $588 \pm 191$  nm (Fig. 1a). Thinner nanofibers were produced with the incorporation of  $\text{AgNO}_3$  (SF =  $281 \pm 64$  nm, Fig. 1b), nisin

(NF =  $337 \pm 80$  nm, Fig. 1c), and  $\text{AgNO}_3$  plus nisin (SNF =  $288 \pm 63$  nm, Fig. 1d). Nisin and  $\text{AgNO}_3$  increased the conductivity of the PDLLA: PEO suspension, which resulted in the formation of elongated fibers [18]. Similar results were reported when 2,3-dihydroxybenzoic acid (DHBA) was electrospun into PDLLA: PEO [1].

Nanofibers containing  $\text{AgNO}_3$  (SF) had a beads-on-string structure, whereas SNF, NF, and CF had a uniform structure. The irregular shape is ascribed to the higher conductivity and stronger elongation forces in the presence of  $\text{Ag}^+$  [18]. The presence of AgNPs in SF and SNF was clearly visible in images recorded with TEM (insert image in Fig. 1b, d). The average size of the AgNPs in SF was  $21.81 \pm 5.5$  nm, similar to that observed in SNF ( $24.15 \pm 5.7$  nm). According to EDX analysis, the concentration of AgNPs in SF was  $1.26 \pm 0.09$  %, but slightly higher in SNF ( $1.54 \pm 0.09$  %, S1 a, b). Similar levels of silver were embedded in carboxymethyl chitosan/polyethylene oxide nanocomposites [9].

The root mean square roughness (Rq) recorded for SF, SNF, NF, and CF was  $191 \pm 0.06$ ,  $0.246 \pm 0.08$ ,  $0.262 \pm 0.07$ , and  $0.294 \pm 0.11$  nm, respectively, as recorded with AFM (S2). Corresponding values for roughness average (Ra) were recorded ( $164 \pm 0.05$  nm for SF,  $0.218 \pm 0.07$  nm for SNF,  $0.234 \pm 0.06$  nm for NF,



**Fig. 1** Scanning electron microscopy (SEM) images and size distribution of **a** control nanofibers (CF), **b** silver nanofibers (SF), **c** nisin nanofibers (NF), and **d** silver plus nisin nanofibers (SNF). Inserted

images of nanofibers were taken with a transmission electron microscope (TEM). The color of the PDLLA: PEO suspension is shown

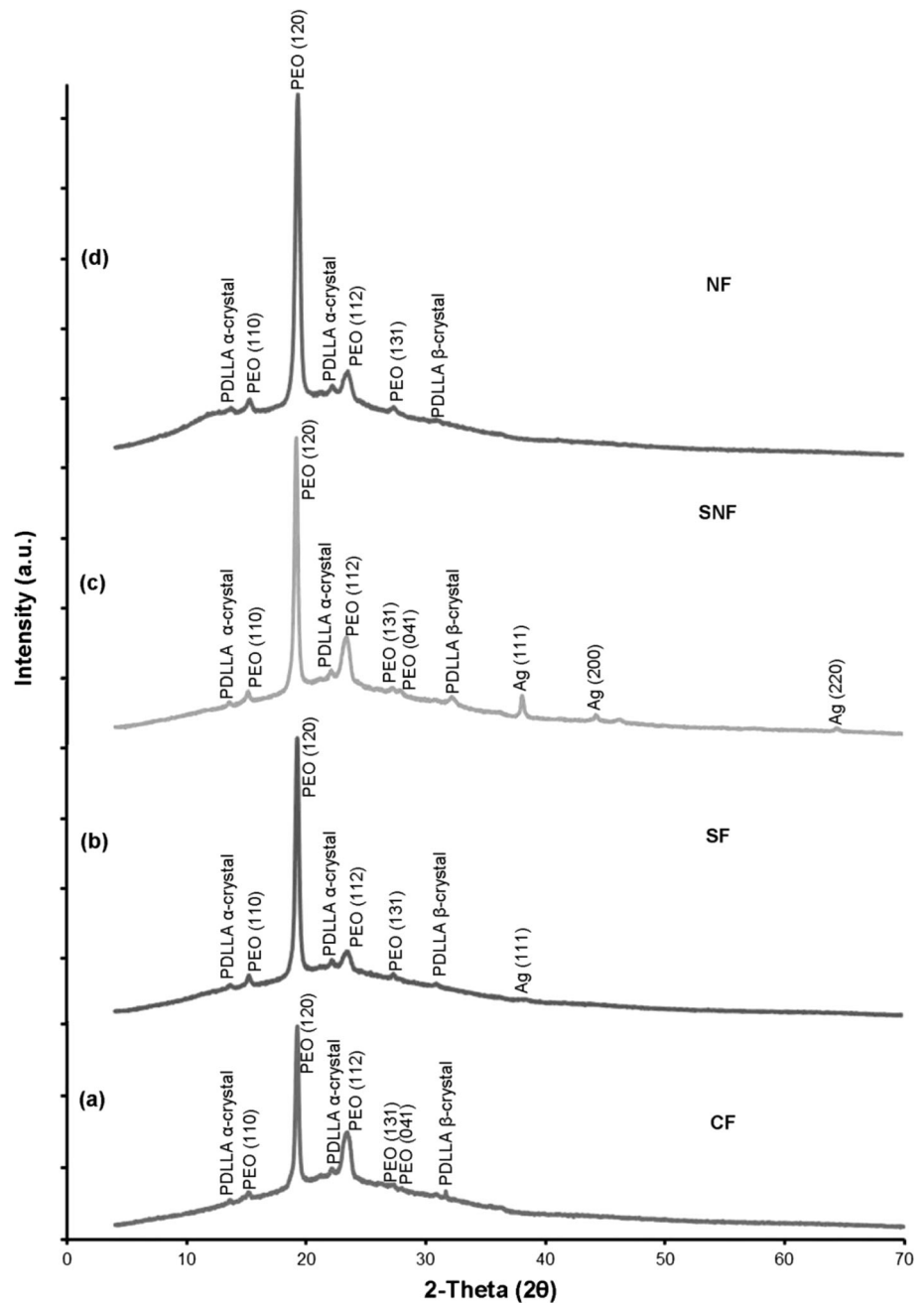
and  $0.258 \pm 0.10$  nm for CF; S2). This indicated that the addition of nisin, silver, or a combination thereof, produced nanofibers with an irregular topography.

The XRD analysis of SF, SNF, NF, and CF showed characteristics  $\alpha$  and  $\beta$  crystal peaks of PDLLA at  $2\theta$  values of  $12^\circ$ ,  $21^\circ$ , and  $31^\circ$  (Fig. 2). Peaks appeared at  $2\theta$  values of  $14^\circ$ ,  $19^\circ$ ,  $23^\circ$ ,  $26^\circ$ , and  $27^\circ$ , corresponding to crystal planes (110), (120), (112), and (131) of PEO. Nanofibers with AgNPs showed characteristic peaks at  $37^\circ$ ,  $43^\circ$ , and  $63^\circ$  for (111), (200), and (220) crystal planes,

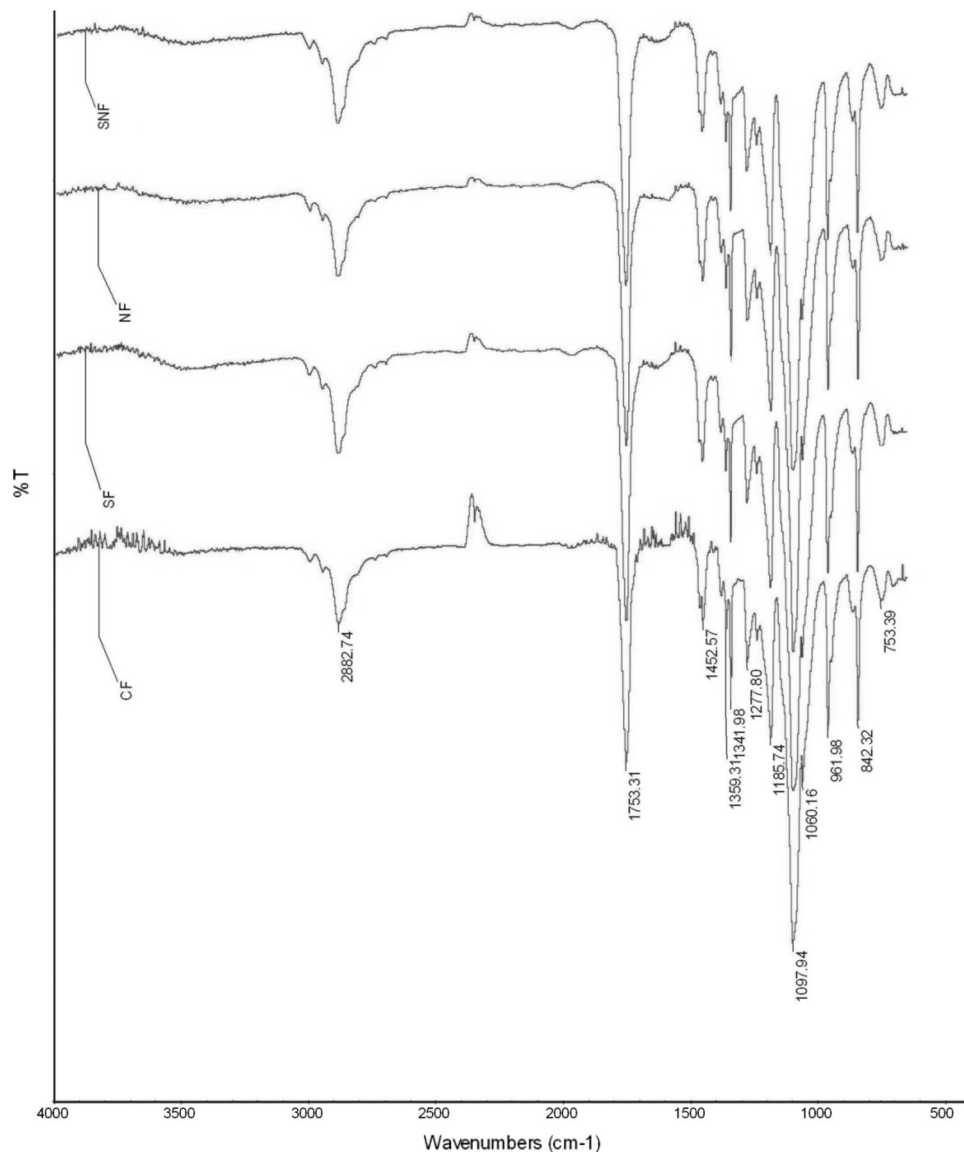
respectively, for the face-centered cubic structure of silver (Fig. 2).

Identical stretching vibration patterns of PDLLA: PEO were observed with CF, SF, NF, and NSF (Fig. 3), suggesting that no interactions occurred between silver, nisin, PDLLA, and PEO. Peaks at  $\nu$  positions  $2883$ ,  $1751$ ,  $1453$ , and  $751$   $\text{cm}^{-1}$  (Fig. 3) are characteristic of  $\text{CH}_3$ ,  $\text{C}=\text{O}_{\text{ester}}$ ,  $\text{CH}_2$  scissoring mode, and  $\text{CH}_2$  rocking mode, respectively. Positions  $\nu$   $1241$  and  $1185$   $\text{cm}^{-1}$  (Fig. 3) are characteristic of C–O bonds in PDLLA: PEO.

**Fig. 2** XRD patterns of control nanofibers (CF), silver nanofibers (SF), nisin nanofibers (NF), and silver plus nisin nanofibers (SNF)



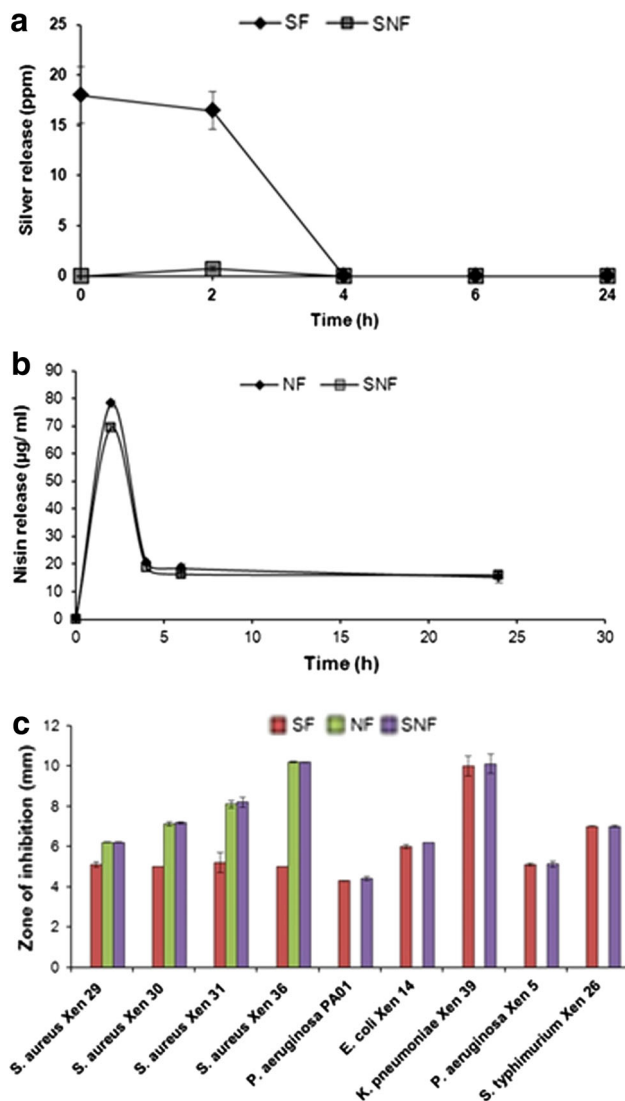
**Fig. 3** FT-IR spectra of control nanofibers (CF), silver nanofibers (SF), nisin nanofibers (NF), and silver plus nisin nanofibers (SNF)



Controlled release of entrapped or encapsulated molecules is an important property of nanofiber wound dressings [11]. An initial burst release of AgNPs ( $18 \pm 2.8$  ppm) was recorded immediately after the immersion of SF in PBS (Fig. 4a). Levels decreased to  $16.5 \pm 1.9$  ppm after 2 h of immersion in PBS (Fig. 4a). No detectable levels of AgNPs were recorded during the remaining 22 h (Fig. 4a). AgNPs were completely released from SNF ( $0.71 \pm 0.2$  ppm) after 2 h in PBS (Fig. 4a). Silver concentrations as low as 0.0001 ppm inhibit the growth of most bacteria [18]. Maximum release of nisin ( $78.1 \pm 1.2$  and  $69.4 \pm 1.0$   $\mu\text{g/ml}$ ) was recorded from NF and SNF, respectively, after 2 h of immersion in PBS (Fig. 4b). The

release of nisin declined to 16  $\mu\text{g/ml}$  over the following 22 h (Fig. 4b). Similar results were recorded in a previous study [1].

Nanofibers containing AgNPs (SF and SNF) inhibited the growth of all microorganisms included in this study, whilst NF failed to inhibit gram-negative bacteria. The largest zone of growth inhibition (10 mm in diameter) was recorded with SF and SNF against *K. pneumonia* (Fig. 4c). Nisin diffused from NF inhibited the growth of *S. aureus* Xen 36, as recorded with a 10-mm-diameter inhibition zone (Fig. 4c). The antimicrobial activity observed with NF against *S. aureus* correlates with that reported in a previous study [1].



**Fig. 4** **a** Release of silver from silver nanofibers (SF) and silver plus nisin nanofibers (SNF). **b** Release of nisin from nisin nanofibers (NF) and SNF. **c** Antimicrobial activity of SF, NF, and SNF. No antimicrobial activity was recorded with control nanofibers (CF)

## Conclusion

AgNPs, prepared using DMF and co-electrospun with nisin into PDLA: PEO (SNF), were smaller in diameter compared to NF, thereby increasing the surface-to-volume ratio. By incorporating AgNPs with nisin, gram-negative bacteria were also inhibited. A wound dressing with broad-spectrum antimicrobial activity may be developed by the incorporation of SNF.

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