IJAMBR 5 (2017) 40-51 ISSN 2053-1818

International Journal of Applied Microbiology and Biotechnology Research

www.bluepenjournals.org/ijambr

Effects of gentamicin-loaded PCL nanofibers on growth of Gram positive and Gram negative bacteria

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Article History
Received 23 February, 2017
Received in revised form 20 April, 2017
Accepted 04 May, 2017

Keywords:
Gentamicin, PCL, Electrospinning, Nanofibers, Antibacterial.

Article Type:
Full Length Research Article

ABSTRACT

Poly-ε-caprolactones (PCLs) incorporated with gentamicin of different concentrations (0, 2.5, 5 and 10 wt.%) were electrospun under various conditions, and the resultant nanofibers of different thicknesses (1, 2 and 4 layers) were used against the growth of Gram-negative and Gram-positive bacteria, such as *Escherichia coli*, *Salmonella* sp. and *Staphylococcus epidermidis*. PCL polymer was selected mainly because of its biodegradable aliphatic polyester characteristics and also, it plays a critical role in tissue engineering and pharmaceuticals. Scanning electron microscopy (SEM) images showed that the resultant fibers were in the range of 50 to 200 nm with an average diameter of 100 nm. Bacterial test results revealed that the gentamicin molecules in the nanofibers were gradually released from the PCL nanofibers during the *in vitro* tests and prevented bacterial growth at different inhibition zones and kinetics. Overall, this work provides a detailed explanation of how to improve the antibacterial properties of new drug delivery systems for many biomedical fields, such as scaffolding; drug, DNA, and protein delivery; and wound healing.

INTRODUCTION

Electrospinning has been gaining much attention worldwide because of the size, shape, flexibility, integrity, and cost of the resultant products in various scientific and technological applications, compared to other techniques and their products. Electrospinning allows scientists to produce fibers in the range of micro to nano size and in the forms of beaded and non-beaded texture. Using different type of polymers helps researchers/scientists to fabricate hydrophobic or hydrophilic fibers of various kinds, which may be useful for many industrial applications (Asmatulu et al., 2011; Nuraje et al., 2011, 2013).

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Essentially, nanofibers are fabricated by an electrostatically driven jet of a polymeric solution. The polymer jet undergoes bending instability due to the interaction of charged particles with the electrostatic field before it is collected on a grounded collector, placed some distance from the capillary tube. The solvent is evaporated during this process. Depending on the types of collectors, either solidified fiber in an alignment or non-woven fiber forms are obtained from this process (Huang et al., 2003; Sill and von Recum, 2008). Electrospinning technology opens up unique opportunities for the generation of fiber-based biocide materials, including antimicrobial biopolymers. Electrospun nanofibers have been used for many biomedical applications such as tissue engineering, wound dressing, drug delivery (Sill and von Recum, 2008), sensors (Dersch et al., 2005), and filters (Gopal et al., 2007, 2006).

Polycaprolactone (PCL) is a Food and Drug Administration (FDA)-approved synthetic polymer as a suture material in clinical practice (Yu et al., 2008). Many investigations also confirmed its benign biocompatibility for use in tissue engineering and drug-delivery devices (Yu et al., 2008). It is commonly used for medical purposes because PCL has both biocompatibility and slow biodegradability, which can promote the bone growth (Agarwal et al., 2008). By combining PCL's fundamental hydrophobic property with the great characteristics of a nanofiber structure, a promising material can be obtained for biomedical use (Duan et al., 2007). Nanofiber structures show a nanoscale diameter, high surface-to-volume ratio, small pore size, high porosity, and unique physical properties, which make them appropriate for an extensive range of medical applications (Kang et al., 2012; Xie et al., 2008). Numerous studies have been done on the electrospinning of PCL. However, it is difficult to generate bead-free fibers via electrospinning (Lowery et al., 2010). PCL has a hydrophobic property, which slows down drug release rates. At the same time, beaded formation helps to slow down the drug delivery process. Generally, hydrophobic materials usually release drugs over a longer period of time compared to the hydrophilic materials (Joosten et al., 2004; Ruckh et al., 2012; Takechi et al., 1998; Yohe et al., 2012).

Recent developments

A study by Liu et al. (2010) involved PCL nanofiber yarns containing ampicillin sodium salt. The author noticed that ampicillin sodium salt affects more *S. aureus* than *Klebsiella pneumoniae.* Moreover, Francis et al. (2011) prepared poly(3-hydroxybutyrate) gentamicin-encapsulated microspheres to investigate the release of gentamicin and found that it was bimodal - an initial burst release followed by a diffusion-mediated sustained release. Sirc et al. (2012) fabricated polyvinyl alcohol and polyurethane nanofibers with the addition of gentamicin by using needleless technology. They observed the gentamicin release and reported the zone of inhibition area on the *S. aureus* and *Pseudomonas aeruginosa* cultures. Shawki et al. (2012) produced dextran nanofibers with the addition of moxifloxacin by using electrospinning. Using *E. coli* and *S. aureus*, they observed a zone of inhibition for both cases. Sezer et al. (2012) prepared PCL as the main matrix material, and gentamicin-loaded microspheres composed of β-tricalcium phosphate (β-TCP) and gelatin. Their aim was to use this material as a support for bone tissue. The author also examined the material with *E. coli* and *S. aureus*. Their microsphere material exhibited a zone of inhibition for both bacteria. The results in this study showed that the PCL nanofibers without antibiotic did not show any antibacterial activity. As a result, any antibacterial activity of PCL nanofibers could only be applicable to the gentamicin incorporated fibers. These results confirmed that PCL-gentamicin nanofibers were potential candidates for biomedical application. These findings further encourage future work in both classical and nontraditional electrospinning fibers, as well as their morphology, mechanical properties, and porosity.

This study investigated PCL-based electrospun fibers and developed antibiotic-loaded nanofiber structures displaying modified release characteristics in functional dressings for wound-healing applications. To design the PCL-based nanofiber system, gentamicin was chosen as the incorporated drug because of its well-known antibiotic function of inhibiting or killing bacteria that are common in most typical post-surgical infections. Gentamicin has been used to treat osteomyelitis caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Joosten et al., 2004; Takechi et al., 1998). Antibacterial effectiveness against *E. coli*, *Salmonella*, and *S. epidermidis* have also been tested to establish the appropriateness of these applied electrospun fibers for decreasing frequency and severity of post-surgery infections.

MATERIALS AND METHODS

Materials

PCL pellets with a molecular weight of 70,000 were purchased from Scientific Polymer Products, Inc. (Ontario, NY USA), while acetonitrile was purchased from Thermo Fisher Scientific (Waltham, MA USA). Gentamicin sulfate powder was provided by National Fish Pharmaceuticals (Vero Beach, FL USA). These materials were used in the present study without any further purifications or modifications. Three different bacteria (*E. coli*, *Salmonella* and *S. epidermidis*) were reproduced in the Department of Biological Sciences at Wichita State University and used in the present study.

Fabrication of electrospun nanofibers

Electrospinning was used to fabricate PCL nanofibers (Asmatulu et al., 2011; Nuraje et al., 2013). First, PCL (15 wt. %) was dissolved in acetonitrile and then different concentrations of gentamicin (0, 2.5, 5 and 10 wt. %) were added to the PCL solution. The solutions were mechanically stirred at 700 rpm and 55°C for 24 h. Each prepared polymeric solution was transferred to a 10 ml plastic syringe and electrospun at various conditions to produce nanofibers. Figure 1 shows the schematics of an electrospinning process. SEM (ZEISS SIGMA VP) was used to characterize the morphology of the PCL electrospun fibers. Additionally, Fourier transform infrared

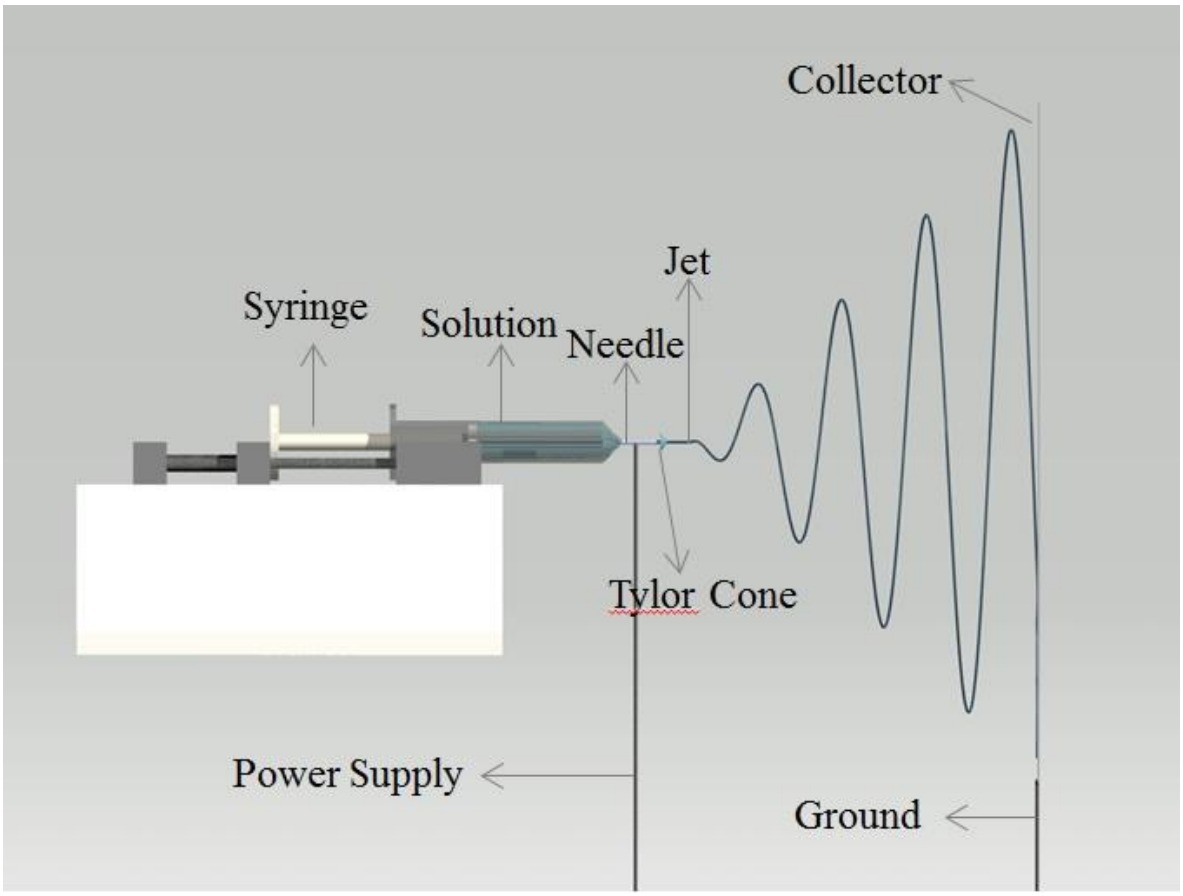

Figure 1. Schematic view of a conventional electrospinning process.

(FTIR) spectroscopy, thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) tests were conducted on the samples to characterize their physical and chemical properties.

Antibacterial tests

The bacteria inhibition assay was conducted based on evaluating clear zones of inhibition of *E. coli*, *Salmonella,* and *S. epidermidis* growth around PCL nanofibers loaded with gentamicin. These tests were adapted from the Kirby-Bauer disk-diffusion method. Agar and lysogeny broth (LB) were mixed with distilled water in an Erlenmeyer flask and autoclaved. After autoclaving, the solution was placed into Petri dishes, 8.5 cm in diameter, to allow the agar to harden. *E. coli, Salmonella*, and *S. epidermidis* were cultured, and 200 µL of the bacteria was diluted with 4000 µL of the LB solution. An amount of 200 µL of the diluted bacterial solution was spread evenly in each prepared Petri dish. The prepared PCL nanofibers were cut to approximately 0.8 cm in diameter and placed in each Petri dish forming a three-by-three plypattern of fibers. The first row had one layer of nanofibers, whereas the second row had two layers of nanofibers. Finally, the third row had four layers of nanofibers. Petri dishes were incubated for 37°C to encourage bacteria growth. Digital photographs were taken for up to seven days to monitor the bacterial growth. The antibacterial activity of the gentamicin-loaded PCL nanofibers was assessed by measuring the mean diameter of the zone of inhibition to the nearest millimeter. All tests were repeated three times and the results were averaged.

Statistical analysis

Statistical analysis among groups was performed by SPSS (version 16, Chicago, IL USA). One-way ANOVA test with the Schafer formula for post hoc multiple comparisons was used to compare the means among groups. A p-value of less than 0.05 was considered as significant difference. Data are expressed as Mean ±

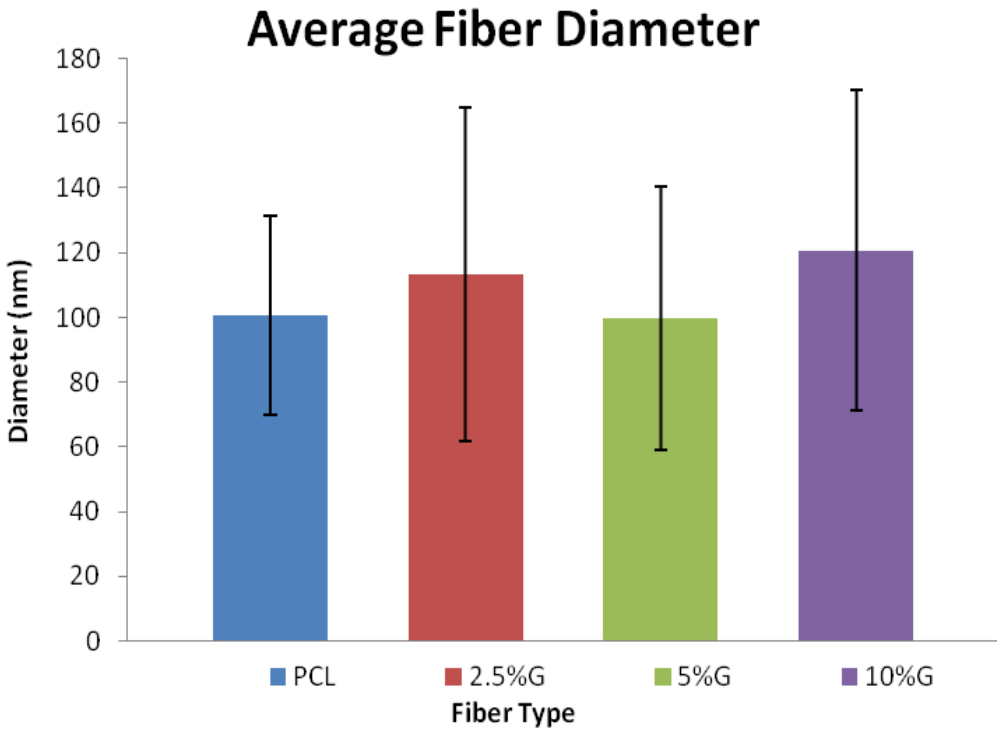

Figure 2. Average fiber diameter of PCL fibers with different concentrations of gentamicin.

Standard Deviation.

RESULTS AND DISCUSSION

Characterization of PCL nanofibers

Gentamicin-loaded PCL nanofibers were successfully fabricated and tested. Figure 2 summarized the estimated pore sizes of the PCL-gentamicin nanofibers based on the SEM images. There was no significant changes on the diameters of the nanofibers with various concentration of gentamicin loaded (2.5%, 5% and 10%), although the 5wt. % gentamicin-loaded PCL fibers exhibited the narrowest distribution and smallest mean fiber diameters.

The test results showed that *Salmonella* was more susceptible to gentamicin than *E. coli* and *S. epidermidis*. This finding is comparable to that demonstrated by Ruckh et al. (2012), although they prepared encapsulated rifampicin-PCL nanofiber scaffolds using the electrospinning method. The author observed that the release of rifampicin from the PCL nanofiber scaffold towards to *P. aeruginosa* and *S. epidermidis.*

Figure 3 shows the SEM images of PCL nanofibers with different wt. % of gentamicin. As can be seen, the morphology of fibers consists of pores and beaded structures. These PCL nanofibers were investigated with FTIR spectroscopy. Figures 4 and 5 show the spectra of gentamicin, PCL, and PCL nanofibers with the addition of 0, 2.5, 5 and 10 wt. % gentamicin. The PCL and 2.5, 5, and 10wt. % gentamicin additions indicate a strong sharp peak around 1717 cm^{-1}, which is due to C=O vibrations (Chakrapani et al., 2012). C-H peaks also are present around 2,942 cm^{-1} (Bassi et al., 2011). Gentamicin is absorbed in the vicinity of 1,616 cm^{-1} wavelength. The different additions of gentamicin to PCL nanofibers show similar peaks in the FTIR spectroscopy.

Figure 6 illustrates the results of TGA data of the electrospun PCL nanofibers with the inclusion of gentamicin. These results demonstrated a similar behavior, even though different weight percentages of gentamicin were added into the PCL nanofibers. Determination of the weight losses around 380°C will be conducted in the future studies (Chakrapani et al., 2012). In addition to the TGA tests, a number of DSC tests were also conducted on the same samples. Figure 7 reveals the DSC results of PCL electrospun nanofibers with different percentages of gentamicin. As can be seen, the peaks around 60°C correspond to the melting point of PCL.

Antibacterial properties of PCL nanofibers

Antibacterial properties were evaluated by taking pictures

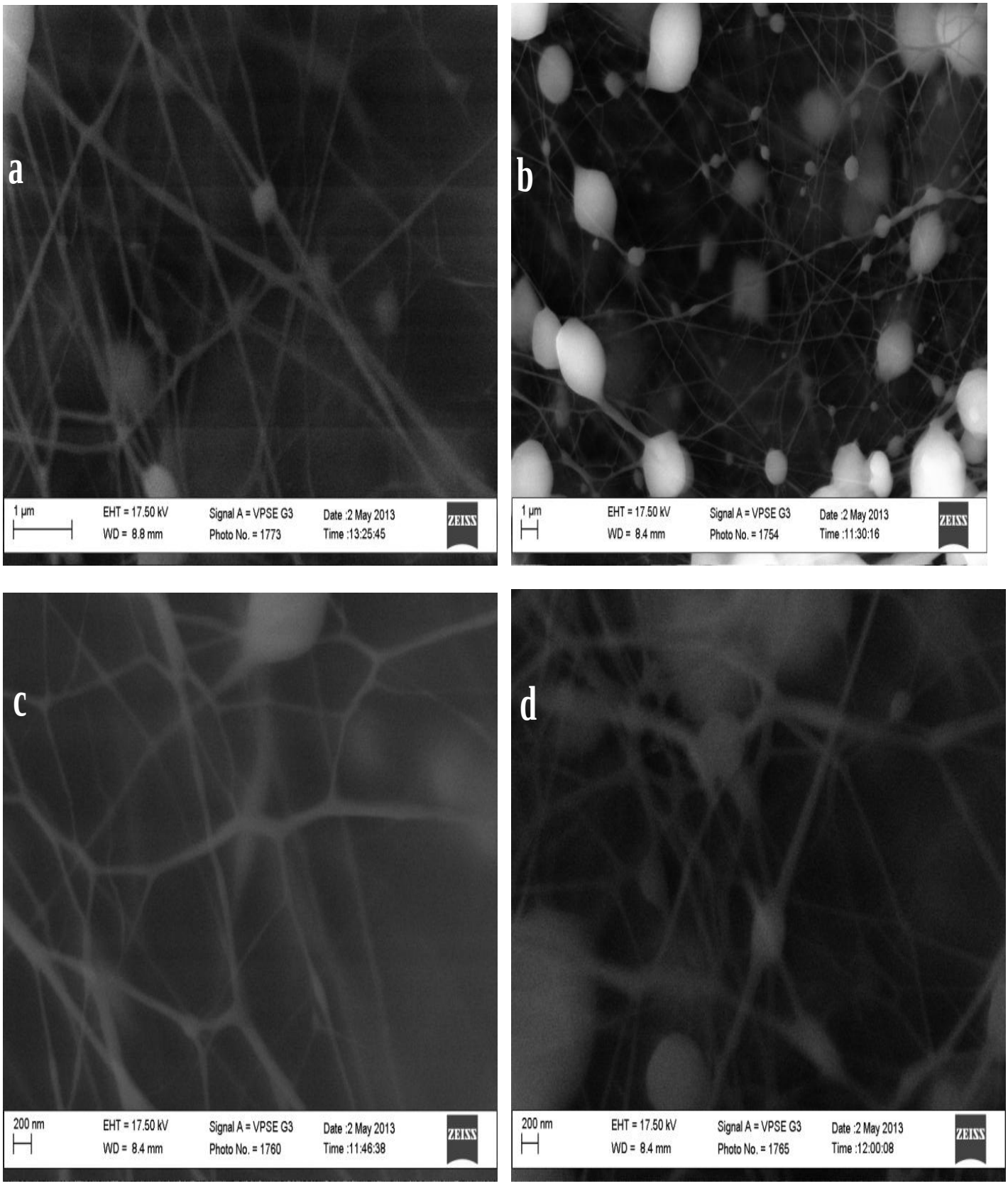

Figure 3. SEM images of PCL nanofibers with different concentrations of gentamicin: **a,** 0 wt. %; **b,** 2.5 wt. %; **c,** 5 wt. %; **d,** 10 wt. %.

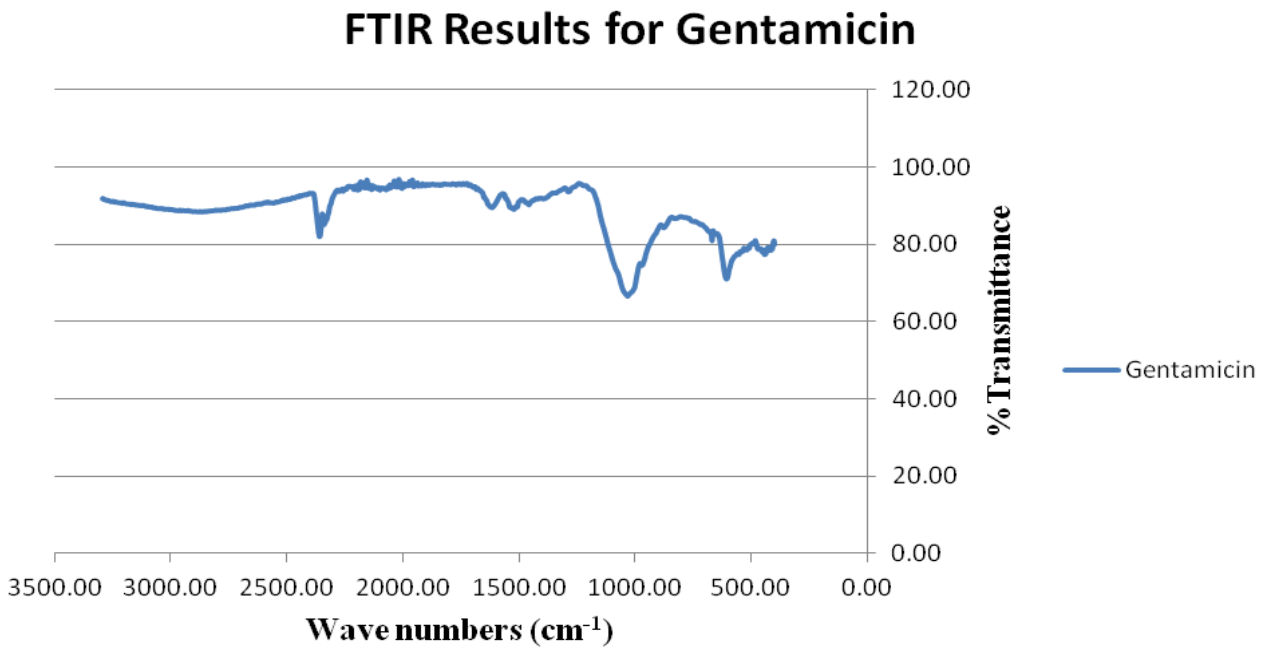

Figure 4. FTIR spectroscopy graph for gentamicin only sample.

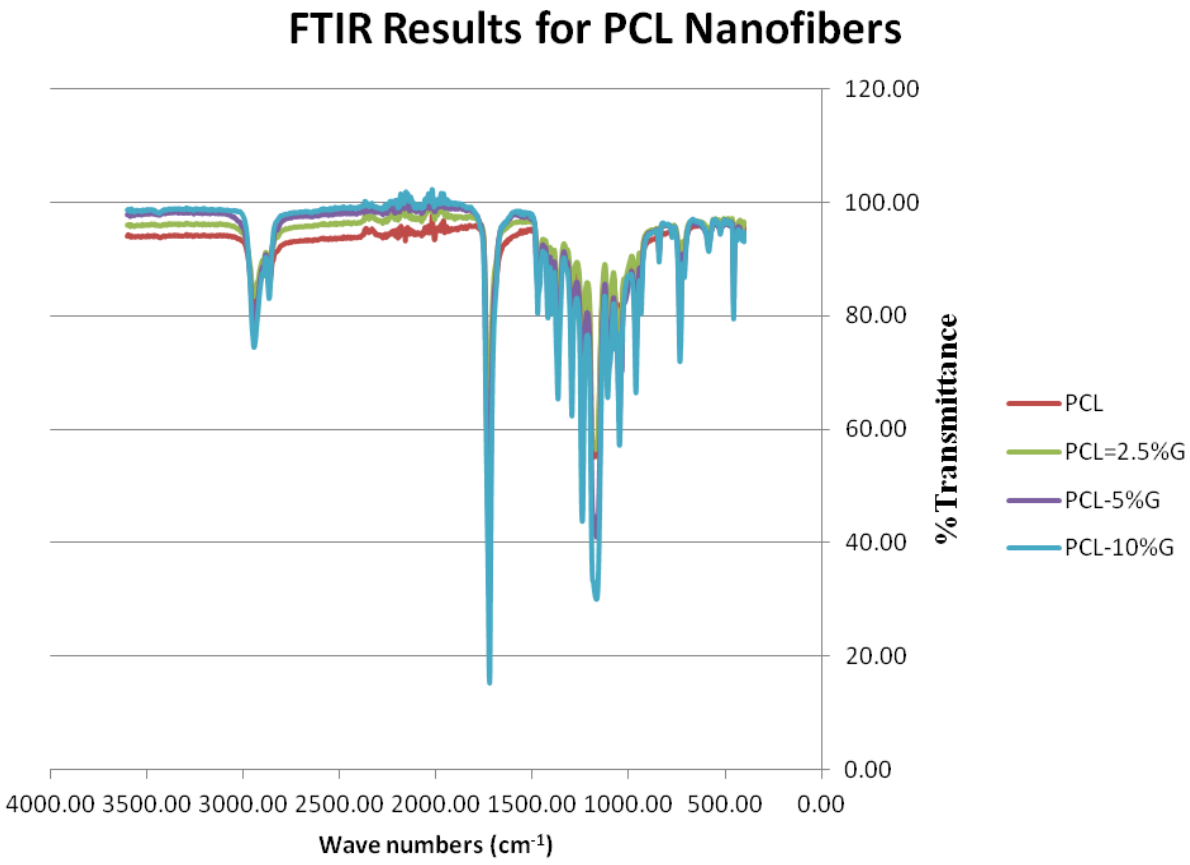

Figure 5. FTIR spectroscopy graph for PCL nanofibers with different wt. % concentrations of gentamicin.

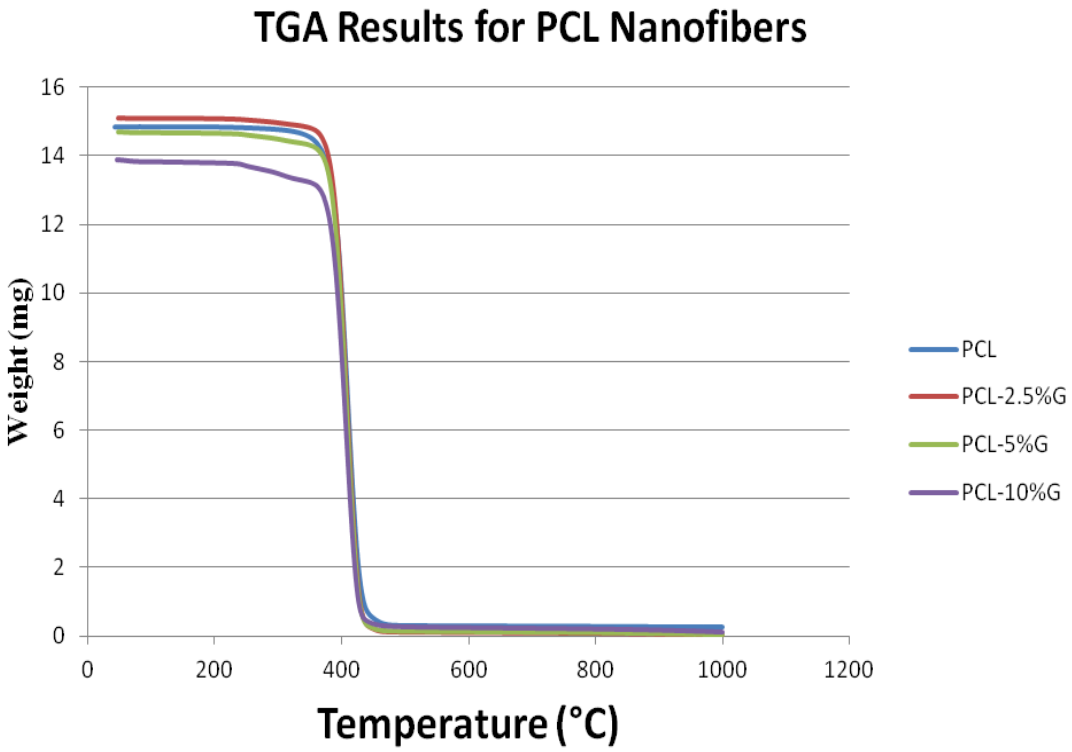

Figure 6. TGA graph for PCL nanofibers with different wt. % concentrations of gentamicin.

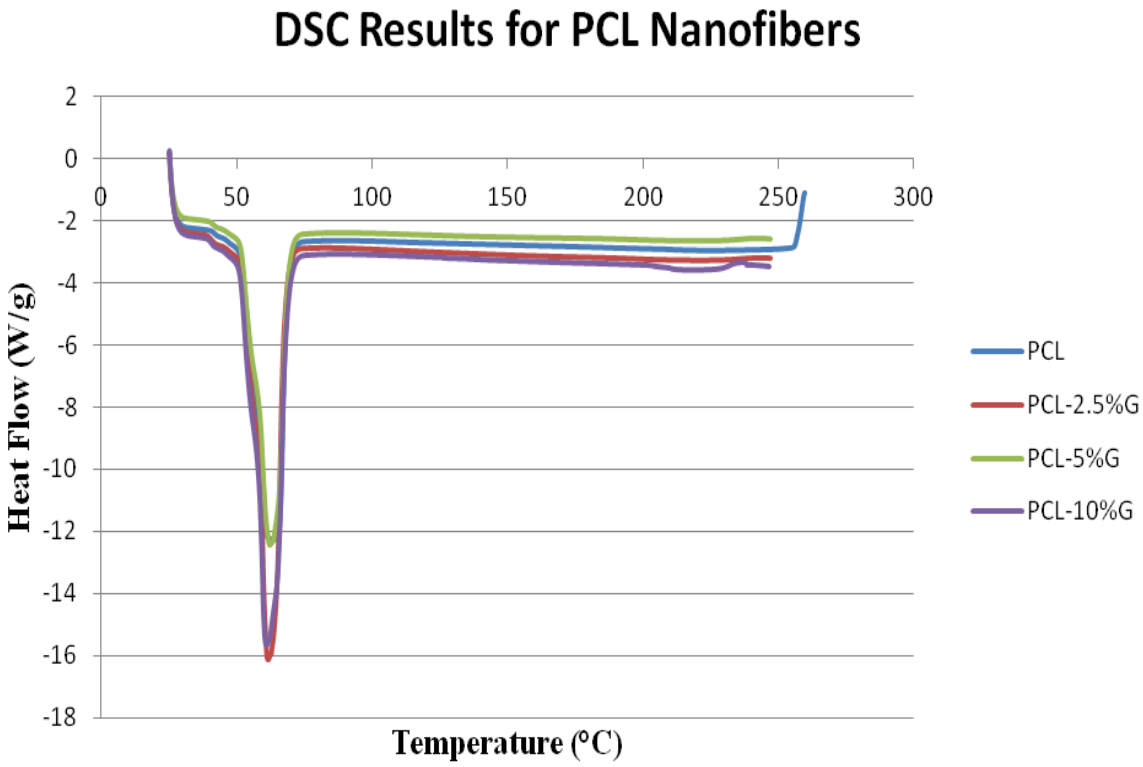

Figure 7. DSC graph for PCL nanofibers with different wt. % concentrations of gentamicin.

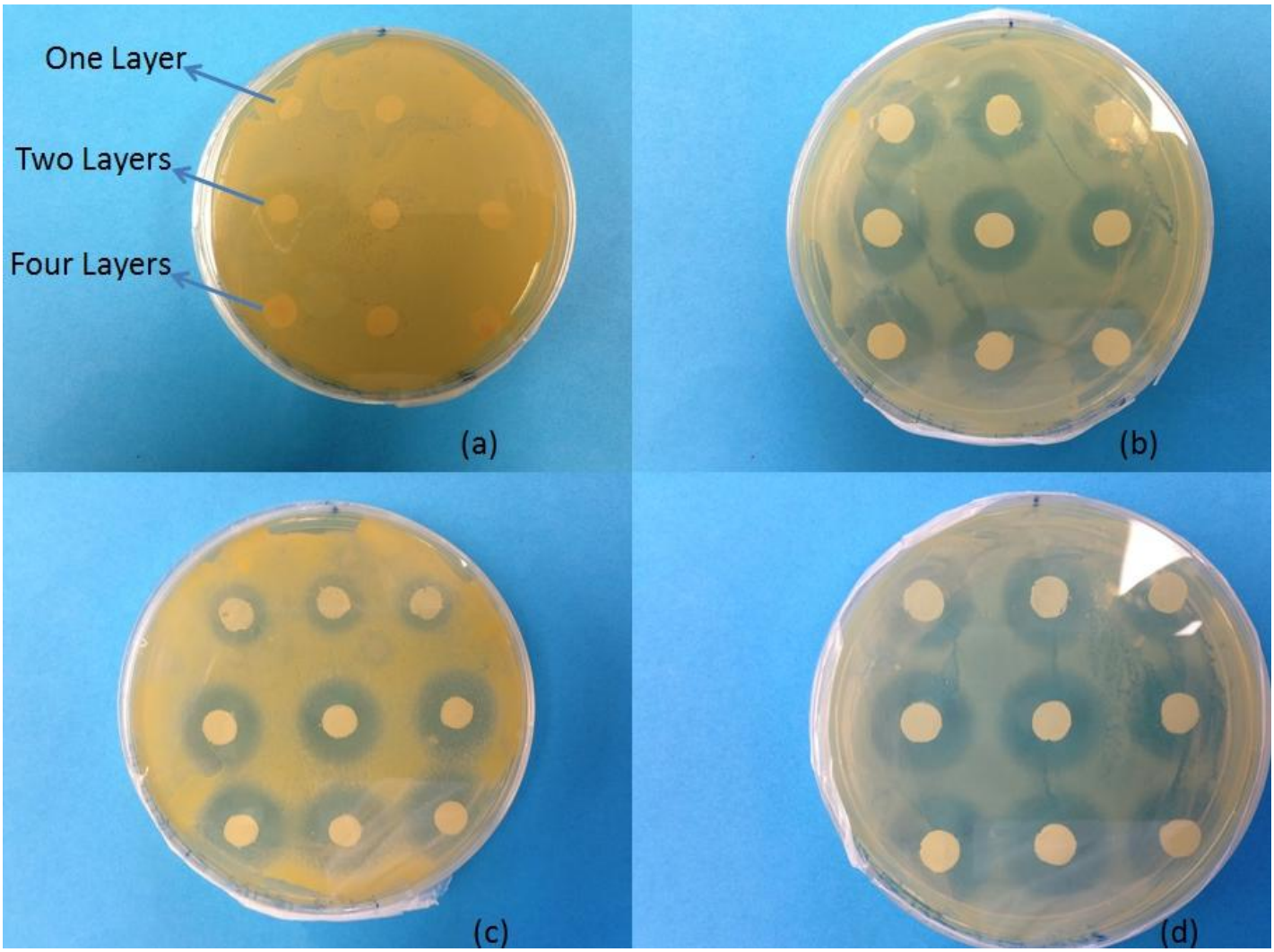

Figure 8. Antibacterial test results of PCL fibers with different concentrations of gentamicin: **a,** 0 wt. %; **b,** 2.5 wt. %; **c,** 5 wt. %; **d,** 10 wt. %; after the seven days of in vitro tests. Top row samples have one layer, middle row samples have two layers, and bottom row samples have four layers of PCL nanofibers.

of electrospun-nanofibers at 1, 3, 5 and 7 days after the initial bacterial culture of *E. coli*, *Salmonella*, and *S. epidermidis*. The electrospun PCL nanofibers did not show any significant antibacterial function. These sets of the PCL nanofibers did not contain any gentamicin, so bacteria grew all around the petri dish. Since PCL-only nanofibers did not show any zone of inhibition, it can be stated that gentamicin addition forms a zone of inhibition.

With the increase of gentamicin concentrations and increasing number of layers of fibers, it was found that antibacterial properties of the nanofibers were significantly improved. Figure 8 show the PCL fibers with 0%, 2.5 wt. %, 5 wt. % and 10 wt. % gentamicin after seven days. The optical images were taken after the bacterial study started at 3 h, 6 h, 12 h, day 1, day 2, day 3, day 4, day 5, day 6 and day 7. As can be seen, 0% gentamicin does not show a zone of bacterial inhibition–bacteria can be seen all over the petri dish. However, 2.5, 5 and 10 wt. % gentamicin clearly show the zones of inhibition, and by increasing the number of layers of fibers, the inhibition zone was drastically higher. When the gentamicin concentration was increased to 10 wt. %, as shown in Figure 8d, the zone of inhibition was increased.

Figure 9 displays the *E. coli* bacterial inhibition area of nanofibers containing 2.5, 5 and 10 wt. % gentamicin. Zones of inhibitions can be seen due to the fact that all samples contain gentamicin. The 10 wt. % gentamicin sample shows a larger inhibition zone compared to the 2.5 wt. % and 5 wt. % gentamicin samples. The 2.5 wt. % sample shows a larger inhibition zone when compared to

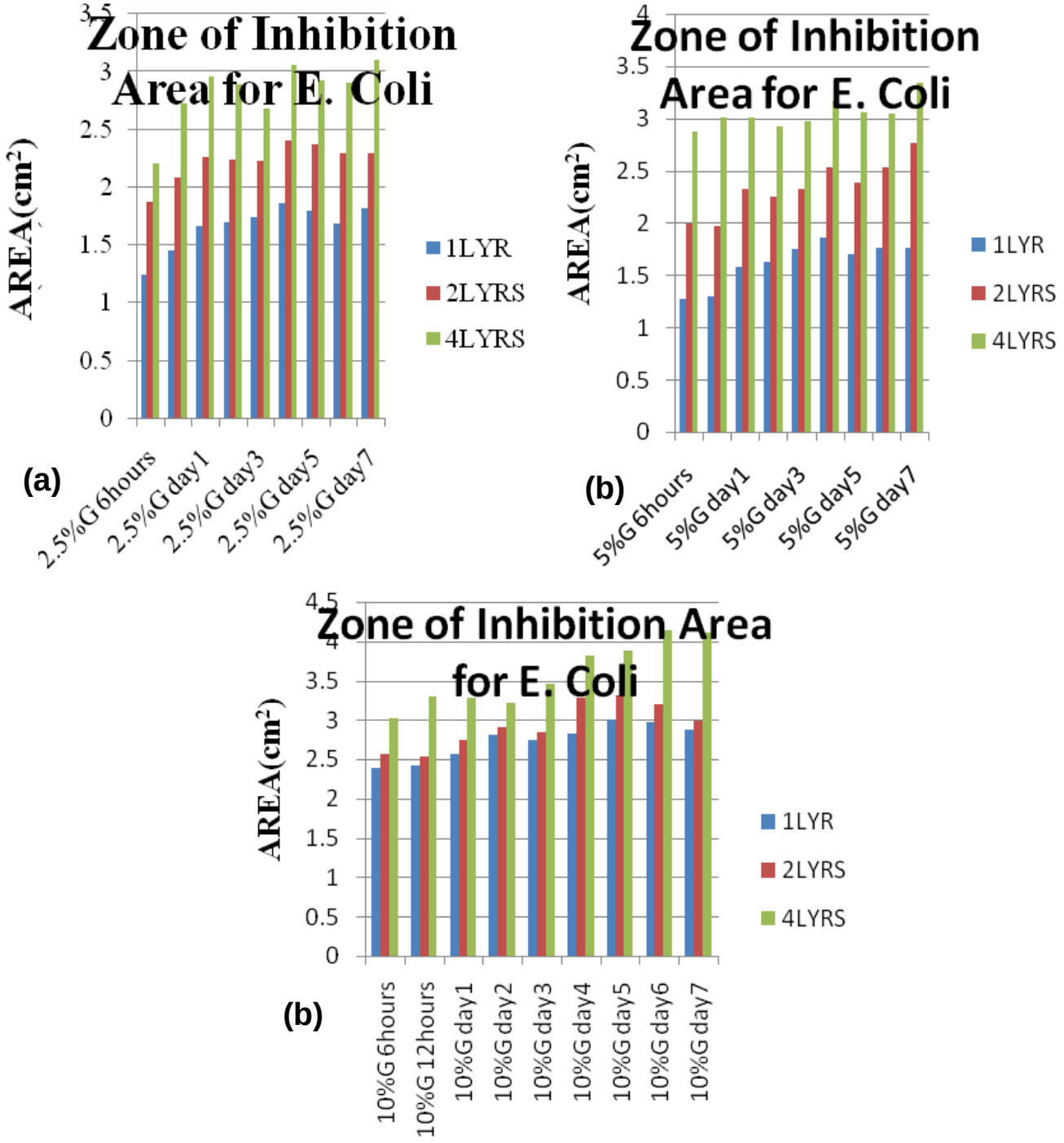

Figure 9. *E. coli* bacterial inhibition zones/areas of PCL nanofibers containing different concentrations of gentamicin: **a,** 2.5 wt. %; **b,** 5 wt. %; **c,** 10 wt. %.

the 5 wt. % sample, which could be attributed to non-uniformity in thickness of the fiber layer.

Figure 10 shows the *Salmonella* bacterial inhibition areas of nanofibers containing 2.5, 5 and 10 wt. % gentamicin contents. Zones of inhibitions against the *Salmonella* illustrate the antibacterial behaviour of the nanofibers containing gentamicin. The area with 10 wt. % gentamicin shows a larger inhibition zone compared to the areas with 2.5 wt. % and 5 wt. % gentamicin.

Figure 11 exibites the *S. epidermidis* bacterial inhibition

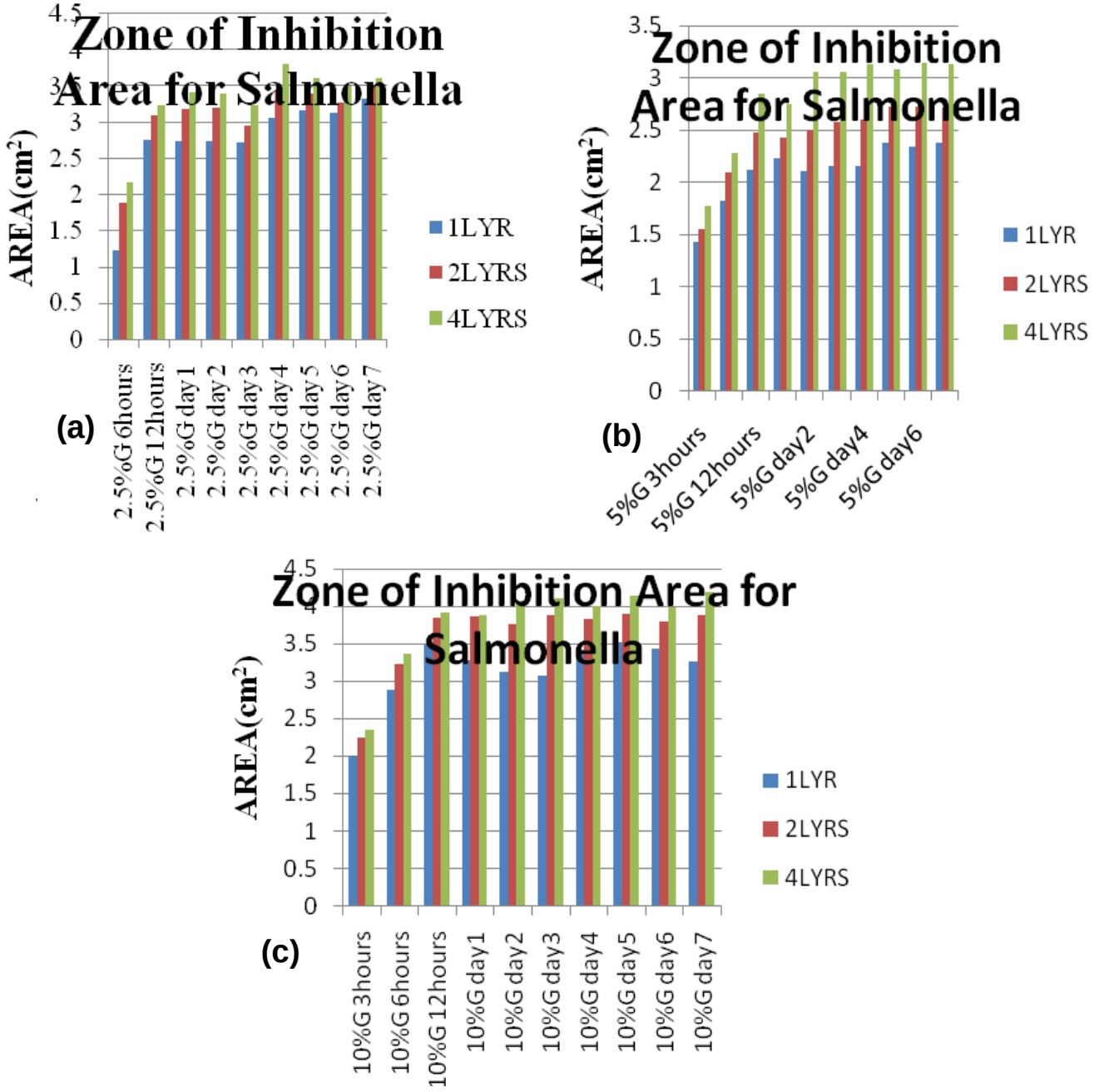

Figure 110. *Salmonella* bacterial inhibition zones/areas of PCL nanofibers containing different concentrations of gentamicin: **a,** 2.5 wt. %; **b,** 5 wt. %; **c,** 10 wt. %.

area of nanofibers containing 2.5, 5 and 10 wt. % gentamicin contents. This set of experiments also provided clear zones of inhibitions due to the gentamicin contents in the fibers. As expected, the 10 wt. % gentamicin sample gave the highest inhibition zones compared to the other samples. These tests indicated that gentamicin loaded PCL fibers are effective ways of protecting nanofibers against *E. coli*, *Salmonella* and *S. epidermidis* for the future drug delivery applications.

Conclusion

Gentamicin-loaded PCL nanofibers were successfully fabricated via the electrospinning process and characterized using SEM, FTIR, TGA, DSC and optical

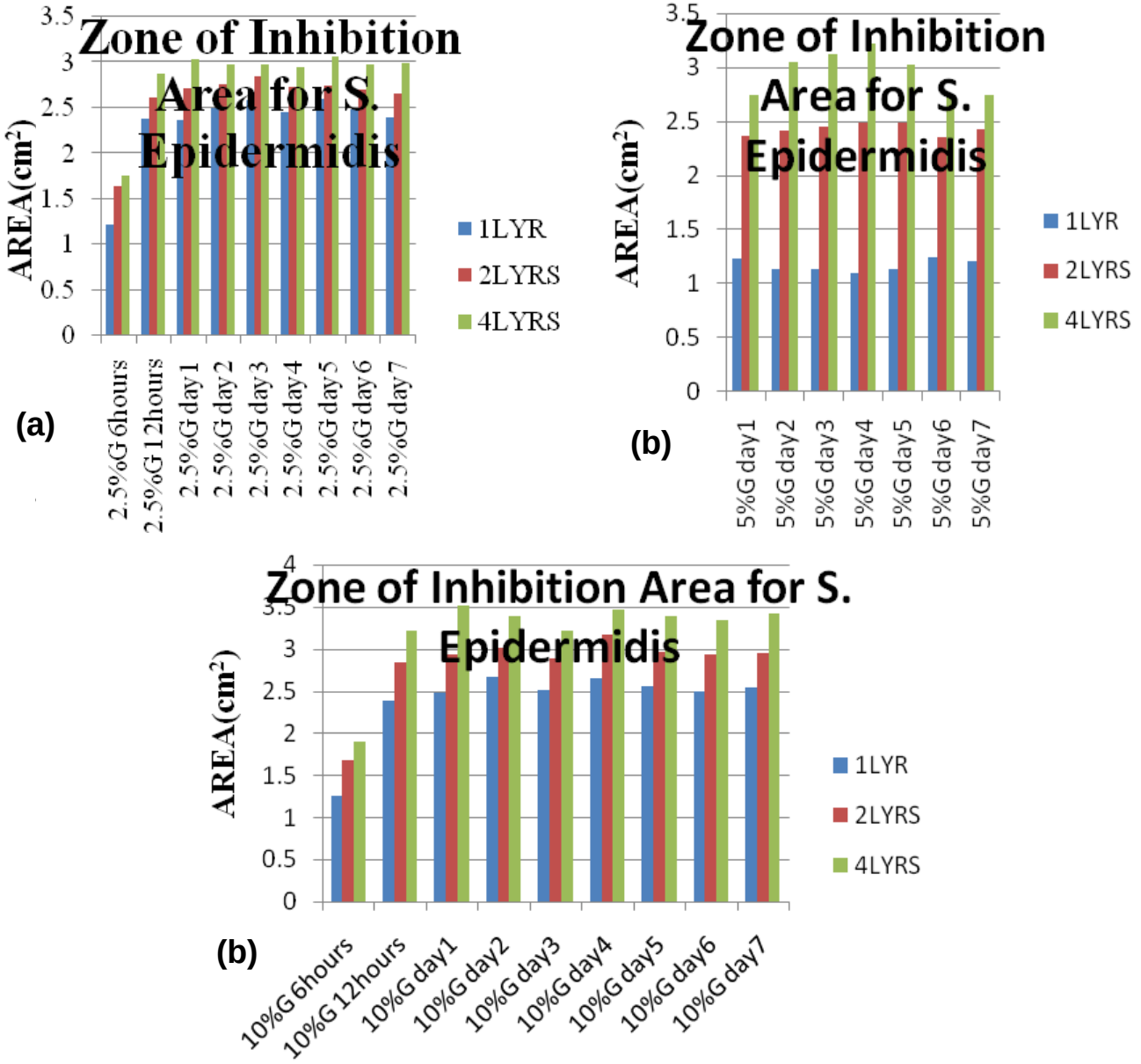

Figure 11. *S. epidermidis* bacterial inhibition zones/areas of PCL nanofibers containing different concentrations of gentamicin: **a,** 2.5 wt. %; **b,** 5 wt. %; **c,** 10 wt. %.

goniometer. The PCL nanofibers (~100 nm) at different thicknesses (1, 2, and 4 layers) were used against the *E. coli*, *Salmonella* and *S. epidermidis* bacteria. These bacterial studies indicated that the gentamicin molecules in the nanofibers were gradually released from the PCL nanofibers during the *in vitro* tests and prevented the bacterial growth for more than seven days. The increase in gentamicin concentration and layers of fibers showed better antibacterial properties on the PCL nanofibers. As a result, this study confirmed that antibacterial agent concentrations, thicknesses of nanofiber layers, and time were important factors, which may be useful for several biomedical applications of the present PCL nanofibers, such as scaffolding; drug, DNA, and protein delivery; wound healing; and other tissue engineering.

ACKNOWLEDGEMENTS

The authors greatly acknowledge the Flossie E. West Foundation and Wichita State University for the financial support of this work. The authors also wish to thank Ms.

Zheng Song, Jimmy Nguyen, Dr. Jianhao Jiang, Velda B. Soydaş, and Jacqueline Weber for their excellent technical assistance and advice.

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