Three-layered electrospun PVA/PCL/PVA nanofibrous mats containing tetracycline hydrochloride and phenytoin sodium: A case study on sustained control release, antibacterial, and cell culture properties

Pouyan Askari, Payam Zahedi, Iraj Rezaeian
Department of Polymer, School of Chemical Engineering, College of Engineering, University of Tehran, Tehran, 11155-4563, Iran
Correspondence to: P. Zahedi (E-mail: phdzahedi@ut.ac.ir)

ABSTRACT: The main objective of this work was to prepare a tailor-made electrospun nanofibrous samples based on poly(e-caprolactone) (PCL) containing tetracycline hydrochloride (TC-HCl) as a middle layer and poly(vinyl alcohol) (PVA) including phenytoin sodium (PHT-Na) as lateral layers. The characterizations of the three-layered electrospun samples were carried out by using SEM, ATR-FTIR spectroscopy along with swelling/weight loss, UV–vis spectrophotometry as well as HPLC, antibacterial and MTT tests. The SEM micrograph images showed that the average diameter of PCL nanofibers was decreased from 243 ± 7 nm to 181 ± 5 nm by adding TC-HCl. The hydrolytic degradation of PVA nanofibers in the exposure of phosphate buffer solution (PBS) was confirmed by ATR-FTIR results in which a change at the intensity of the characteristic peak located at 3333 cm⁻¹ corresponding to hydroxyl groups (–OH) was observed. The UV–vis outcomes revealed a sustained control release of TC-HCl from the three-layered nanofibrous samples (PVA/PCL/PVA) with an amount of about 43% compared to the PCL nanofibers which had an ultimate release of the drug about 79%. Furthermore, the HPLC chromatograms showed the released PHT-Na from PVA nanofibers about 87%. Finally, the MTT assay along with the antibacterial evaluation exhibited that the surfaces of these electrospun three-layered nanofibrous samples have no cytotoxicity as well as the controlled release of TC-HCl from them enabled their prolonged use for preventing the bacterium growth such as \textit{S. aureus} during 24-h treatment time. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 2016, 133, 43309.

KEYWORDS: biocompatibility; biomedical applications; electrospinning; morphology

Received 1 October 2015; accepted 1 December 2015
DOI: 10.1002/app.43309

INTRODUCTION

Although biodegradable and biocompatible polymeric nanofibers produced by electrospinning as an efficient tool\textsuperscript{1–4} are widely used for biomedical applications such as bone tissue engineering\textsuperscript{5} and wound dressing materials,\textsuperscript{6,7} but the controlled release of drugs from them has remained the main problem so far. The electrospun polymeric nanofibers because of their unique properties such as high specific surface area and easy to produce have received a considerable attention as drug delivery systems (DDS) and a large number of reviews have been published on this subject.\textsuperscript{8–11}

Tetracycline hydrochloride (TC-HCl) as a well-known antibiotic is very suitable for the prevention of bacteria growth. In our previous works,\textsuperscript{12,13} the release rate profiles of TC-HCl loaded into electrospun nanofibers were investigated. In the first work,\textsuperscript{12} electrospun PCL nanofibers containing different concentrations of TC-HCl solution were prepared and then their antibacterial evaluations were assessed. The results showed that the samples containing 500 µg mL⁻¹ of the drug had a relatively rapid release rate with an initial burst release profile during 12 h and provided an effective inhibitory zone for \textit{Staphylococcus aureus} (\textit{S. aureus}) than that of \textit{Escherichia coli} (\textit{E. coli}) on the agar plates. In the second work,\textsuperscript{13} the role of egg albumin (EA) as a protein in the release behavior of TC-HCl from the PVA nanofibrous mats was studied. We concluded that not only EA enabled to sustain and control the release of TC-HCl from the fibers, but also the mechanical properties of the PVA nanofibers were increased in the presence of EA. Also, the brittleness behavior of EA loaded PVA nanofibers was more remarkable than those without EA. Recently, Karuppuswamy et al.\textsuperscript{14} investigated the controlled release of TC-HCl from electrospun PCL nanofibers. They showed that by using a mixture of chloroform and methanol (3:1) as the solvent system, the morphology of electrospun PCL nanofibrous samples led to a uniformity into the fibers structure. Also, their results showed that the burst release of the drug within the first hour was minimum and was followed by a sustained control release for a period of 8 days. Despite achieving a long-term release of TC-HCl from PCL nanofibers, a series of complementary tests for
the evaluation of cytotoxicity and antibacterial properties of the samples have not been carried out.

Phenytoin sodium (PHT-Na) is a potent anti-epileptic drug which was first noted in 1958 for healing periodontal wounds. Because of the remarkable characteristics of PHT-Na such as fibroblast proliferation, the production of extracellular matrix (ECM) and the activity of growth factor, it was used in this work. We have already studied the effect of this drug loaded into the electrospun PVA nanofibrous samples for investigating the proliferation and adhesion of the human umbilical cord matrix (hUCM) onto the surfaces of the samples.\(^6\) The observations resulted in hUCM cells were perfectly formed on the nanofibers containing PHT-Na and were spread with a suitable distribution after 6 days.

Limited research works have been published regarding controlled release of drugs by using multilayered electrospun polymeric nanofibers. For instance, Yoon and Kim\(^{15}\) have done a study of layered micro/nanofibrous mats containing PCL and poly(ethylene oxide) (PEO)/Rhodamine-B (RM), as an indicator for controlled release. They have prepared the samples by the use of electrospinning process in order to observe the release of RM from the layered mats. Their results showed that the release amount of RM from the three-layered samples (PCL/PEO-RM/PCL) was completely dominated by the thickness of outer PCL layers. In other words, thickness variation of outer layers of PCL nanofibers could control the drug release. In contrast to the preparation of multilayered electrospun polymeric nanofibers controlling the sustained release of the drug, a series of works have been carried out in the shape of single-layered nanofibers for the variety of drugs to control their release from these DDS along with the different methods such as altering the polymers, crosslinking, blending and so forth.\(^{16-18}\) The main advantage of the current work compared to the other efforts on the three-layered nanofibers is the application of two significant drugs to help the fibroblast cells growth and their proliferation with controlling the drugs release by fabricating three-layered nanofibrous mats.

The aim of this work, for the first time, was to prepare a three-layered electrospun nanofibrous samples with PCL containing TC-HCl and PVA containing PHT-Na and to compare their release behavior of TC-HCl loaded PCL nanofibers as the middle layer with the single-layered electrospun PCL nanofibers including this antibiotic. Moreover, the morphology, release kinetic, biodegradability, and antibacterial evaluation as well as fibroblast cells culture were carried out in order to investigate the potential of these samples as a wound dressing material in \textit{in vitro} applications.

**EXPERIMENTAL**

**Materials**

Poly(ε-caprolactone) (PCL) (a biodegradable polymer with the average molecular weight of 80 kDa) was purchased from Sigma–Aldrich, Pilsburgh, The Netherlands. Poly(vinyl alcohol) (PVA) (trade name GH-17R-GOHSENI, a white powder, average molecular weight of 98 kDa and about 88 mol % degree of hydrolysis, viscosity of the solution (4%) = 30 mPa s\(^{-1}\) and melting point 180°C) was provided from NIPPON GOHSEI, Japan. Tetracycline hydrochloride (TC-HCl) (an antibiotic, a yellowish powder with the purity of 99.99%) was supplied by Merck, Germany. Phenytoin sodium (PHT-Na) (white, fine powder, particle size max 180 μm, purity 99.99%) was obtained from Katwijk Chemie (The Netherlands). All the other chemicals were analytical reagent grades (Merck, Germany) and were used without further purification.

**Samples Preparation**

Weighed PVA powder was dissolved in distilled water at 80°C and the solution was stirred overnight at room temperature in order to provide PVA solution 8 (\%/v/v). Then, the weighed PHT-Na powder was added to the PVA solution and stirred for about 30 min for preparing the mixture with 1\% PHT-Na concentration. For the preparation of PCL solution with 9.5 (\%/v/v) concentration containing 500 μg mL\(^{-1}\) of TC-HCl, weighed PCL was completely dissolved in chloroform by stirring for 1 h. Then, TC-HCl-PCL powder was added to dimethylformamid (DMF) and stirred for about 30 min in order to reach the determined concentration. A mixture containing PCL 9.5 (\%/v/v) and TC-HCl 500 (μg mL\(^{-1}\)) was prepared by using (7/3 v/v) ratio of chloroform/DMF solvents.

For the preparation of the layered samples, an electrospinning device (model espinner NF/COEN/II) from Asian Nanostructure Technology, Iran, was used. The electrospinning conditions for laying each layer were as follows: PVA 8 (\%/v/v) nanofibers containing PHT-Na (1\%)—the solution flow rate = 0.8 mL h\(^{-1}\), applied voltage = 19.5 kV and the distance between the syringe needle tip and collector = 10 cm; PCL 9.5 (\%/v/v) nanofibers containing TC-HCl 500 (μg mL\(^{-1}\))—the solution flow rate = 0.45 mL h\(^{-1}\), applied voltage = 19.5 kV and the distance between the syringe needle tip and the collector = 14.5 cm. In the electrospinning process, the three-layered samples were designed as follows: first, the PVA nanofibrous mat containing 1\% PHT-Na was electrospun for 2 h and then a layer of PCL nanofiber containing 500 μg mL\(^{-1}\) of TC-HCl was electrospun upon the PVA layer for 2 h. At last, another PVA layer similar to the initial PVA layer covered the surface of PCL nanofibrous layer. The final layered sample was denoted as (PVA–PHT-Na/PCL–TC-HCl/PVA–PHT-Na).

For crosslinking the samples, they were exposed to saturated vapor of glutaraldehyde 25 (\%/v/v) in a vacuum chamber at 40°C for about 11 h in order to prevent them from dissolving in water or other aqueous solutions.

**Samples Characterization**

**Scanning Electron Microscopy (SEM) Studies.** The surfaces of nanofibrous samples on the aluminum foil were covered by a thin layer of gold via an auto sputter coater (model Emitech K450 X, United Kingdom). The morphology and mean diameter of the nanofibers containing the drugs individually and those as a layered structure were studied by using a scanning electron microscopy (SEM) (model AIS 2100, Seron Technology, Korea) with 10,000× magnification and ImageJ software, respectively. For reducing the statistical errors and calculating standard deviation (SD) for average diameter of the nanofibers, the diameters of five different sections for each sample were measured.
Degree of Swelling and Weight Loss. Degree of swelling and amount of weight loss of the prepared samples were calculated by using eqs. (1) and (2), respectively. Both experiments were done into phosphate buffer solution (PBS, pH = 7.2) as the drug release environment at 37°C for time periods of 1, 6, 24, and 48 h.

\[
\text{Degree of swelling} (\%) = \frac{M - M_0}{M_d} \times 100,
\]

(1)

\[
\text{Weight loss amount} (\%) = \frac{M_t - M_d}{M_t} \times 100,
\]

(2)

where “M” is the weight of the nanofibrous samples which were wiped-dried by using a filter, “M_d” is the weight of the nanofibrous samples which were completely dried in an oven at 50°C until a constant weight was attained. “M_t” is the initial weight of the nanofibrous samples before immersing into PBS.

Biodegradability Studies. The biodegradability of the layered nanofibrous samples (PVA/PCL/PVA) containing PHT-Na was utilized by an attenuated total reflectance Fourier transformed infrared (ATR-FTIR) (model EQUINOX 55, Bruker, United States). In this test, the samples were immersed in the PBS environment at temperature of 37°C for 24 h and then after they were dried the intensity and location of the characteristic peak of hydroxyl group (–OH) regarding the PVA hydrolytic degradation as well as altering the other major characteristic peaks of PCL were studied at wavenumbers ranging from 4000 to 400 cm⁻¹.

In Vitro Drugs Release Calculation

For the determination of TC-HCl release from the three-layered nanofibrous samples and also those samples with single layer, a UV–vis spectrophotometer (model mini-1240, Shimadzu, Japan) was used. First, the calibration curve for TC-HCl dissolved in DMF in different concentrations of 5, 25, 50, 100, 150 and 200 μg mL⁻¹ at maximum wavelength (λmax) of 254 nm was calculated based on Beer–Lambert law according to eq. (3):

\[
A = ε \times c \times l = 0.0359 \times εc,
\]

(3)

where “A” is the amount of absorbance, “ε” is the extinction coefficient of TC-HCl solution, “c” is the TC-HCl solution concentration, and “l” is the distance which the UV light travels through the drug solution (usually is equal to 1 cm). Afterward, for the determination of TC-HCl release from the single-layered PCL nanofibrous sample and three-layered PVA/PCL–TC-HCl/PVA nanofibrous sample, they were immersed into PBS environment at pH = 7.2 for a variety of times up to 48 h. Equation (4) shows the relationship between cumulative amounts of the drug release from the electrospun samples into PBS and release time.

\[
\text{Cumulative release percentage} = \sum_{i=0}^{t} \frac{M_t}{M_0} \times 100,
\]

(4)

where “M_t” and “M_0” are the cumulative amount of TC-HCl released at each sampling point and the initial weight of the drug loaded into the samples, respectively. The release kinetics of TC-HCl from the electrospun samples can be explained by using the well-known Korsmeyer–Peppas equation as follows:

\[
\frac{M_t}{M_\infty} = k t^n,
\]

(5)

where “M_t” is the cumulative amount of the drug released at time “t”, “M_\infty” is the initial drug loading, “K” is the constant characteristic of the polymer-drug system, and “n” is the diffusion exponent suggesting the nature of release mechanism. In addition, four more models were used to further analyze the profiles of the drug release including zero order, first order, and Higuchi (Table I).

To determine the PHT-Na release from the three-layered nanofibrous sample which was denoted by (PVA–PHT-Na/PCL/ PVA–PHT-Na), a high performance liquid chromatography (HPLC) (model SCL-10AVP, Shimadzu, Japan) supplemented with a Waters 486 UV detector (λ = 254 nm) (Waters Associates, Milford, MA) was used. The detector was coupled with a C18 analytical column (Bond clone 30 cm x 3.9 mm i.d., 5 μm, Phenomenex, Macclesfield Cheshire, United Kingdom) was utilized for separation. The buffer component of the derivatized solution was prepared with deionized water and the pH was adjusted to 7.2. The mobile phase consisted of MeOH/H2O/acetonicitrile/trimethylamine (1%)/CH3COOH (270:500:230:5:1) and was sonicated about 15 min. The mobile phase was delivered at the flow rate of 1 mL min⁻¹ and the eluent was evaluated at a wavelength of 254 nm. Equation (6) showed the calibration curve for PHT-Na dissolved in distilled water as follows:

\[
A = 4742.3 \times c + 9514,
\]

(6)

where “A” is the surface area under the HPLC chromatogram and “c” is the concentration of PHT-Na solution. Similar to calculating cumulative release of TC-HCl, the amount of PHT-Na was also estimated by eq. (4) in this study.

Antibacterial Evaluation. The antibacterial activity of the nanofibrous samples including PCL–TC-HCl, PVA/PCL–TC-HCl/ PVA, PVA/PCL/PVA, and the TC-HCl solution as a control for the S. aureus (Gram positive, ATCC 25023) as a usual bacterium for many biomedical applications such as wound healing was evaluated for 24 h. The assessments were conducted on the basis of the disc diffusion method of the US Clinical and Laboratory Standards Institute. This procedure was performed in a Luria-Bertani (LB) medium solid agar Petri-dish. The nanofibers were cut as circular discs with 2.5 cm in diameter and put on the agar medium consisted of S. aureus and the inhibitory zones, the place which is clear from the bacterium and there should have been no growth of microbes, around the disc specimens were photographed for report.

Cell Culture. In this work, in order to investigate the role of each drug used and also to observe the effect of the nanofibers’ morphology on proliferation of the fibroblast cells, two samples in terms of single-layered PCL nanofibers containing TC-HCl
and three-layered PVA/PCL/PVA nanofibers including both TC-HCl and PHT-Na were selected. After cutting the samples as square shapes with a surface area of 0.6 cm², they were sterilized under UV light for 3 h. Afterward, a solution with the determined volume containing the fibroblast cells with their maximum growth consisted of trypsin (0.5 g L⁻¹) and ethylene diamine tetra-acetic acid (EDTA) (0.2 g L⁻¹) was seeded on the surfaces of the samples. To observe the cell morphologies onto the surfaces of the nanofibrous samples, field emission scanning electron microscopy (FE-SEM) investigations at day-6 culture time were carried out. Briefly, the samples were fixed by using glutaraldehyde 2.5 (%v/v) for about 12 h at 4°C. After fixing, the samples were dehydrated with ethanol/distilled water mixture from 50 to 100% in steps of 10% at 10 min intervals. The resulting samples were observed by the use of a FE-SEM (model SU 8040, Hitachi, Japan) at 1000× and 3000× magnifications.

Cytotoxicity of the electrospun samples were evaluated according to ISO 10993-5 standard and viability of the cultured cells on the nanofibers was characterized after 6-day culture time were carried out. Briefly, the samples were fixed by using glutaraldehyde 2.5 (%v/v) for about 12 h at 4°C. After fixing, the samples were dehydrated with ethanol/distilled water mixture from 50 to 100% in steps of 10% at 10 min intervals. The resulting samples were observed by the use of a FE-SEM (model SU 8040, Hitachi, Japan) at 1000× and 3000× magnifications.

RESULTS AND DISCUSSION

The Morphology of the Single and Three-layered Electrospun Samples with and without the Drugs

Figure 1 (a–d) shows the SEM micrograph images of the neat electrospun PCL nanofibers [Figure 1(a)], the PCL nanofibers with TC-HCl [Figure 1(b)], the layered sample denoted by (PVA/PCL–TC-HCl/PVA) which was loaded with TC-HCl within the PCL nanofibrous mat as the middle layer [Figure 1(c)] and the layered sample denoted by (PVA–PHT-Na/PCL–TC-HCl/PVA–PHT-Na) consisted of two drugs [Figure 1(d)]. As it can be seen from Figure 1(a,b), by the addition of TC-HCl into the electrospun PCL nanofibrous sample, a reduction in the average nanofibers diameter from 243 ± 7 nm to 181 ± 8 nm was observed. On the other hand, the incorporation of TC-HCl into the PCL solution led to a uniformity in the nanofibers morphology along with the structures without beads. It can be related to the key role of the TC-HCl solution.

Figure 2. (a) The degree of swelling and (b) the weight loss percentage of the neat three-layered electrospun PVA/PCL/PVA nanofibers during 48 h into PBS.

Toxicity (%) = \( 1 - \frac{\text{mean OD of sample}}{\text{mean OD of control}} \) × 100, \( (7) \)

Viability (%) = 100 – Toxicity (%), \( (8) \)

Figure 1. The SEM micrograph images of the electrospun nanofibrous samples: (a) neat PCL, (b) PCL containing TC-HCl, (c) three-layered PVA/PCL–TC-HCl/PVA, and (d) three-layered PVA–PHT-Na/PCL–TC-HCl/PVA–PHT-Na (the scale bar is 5 μm).
as a plasticizer through the PCL molecular chains in which the viscosity of the PCL solution was decreased compared to the PCL solution without the drug before electrospinning. Despite the single layer electrospun PCL nanofibrous sample, the three-layered ones have nonuniformity in size distribution of the nanofibers diameter. Figure 1(c,d) depicts the morphologies of the nanofibers with the three-layered structures for PVA/PCL—TC-HCl/PVA and PVA—PHT-Na/PCL—TC-HCl/PVA—PHT-Na, respectively. As it is evident, by adding the PHT-Na into the PVA solution, the mean nanofibers diameter of electrospun PVA sample was decreased from 317 ± 8 nm to 258 ± 6 nm as far as a relative uniformity in the size distribution of two PVA and PCL nanofibrous samples with the drug was occurred [Figure 1(d)].

The Degree of Swelling and Weight Loss of the Electrospun Layered Nanofibrous Samples

For the investigation of the degree of swelling and weight loss of the neat layered electrospun samples, they were immersed into the PBS environment (pH = 7.2, at temperature of 37 °C). These properties were calculated based on eqs. (1) and (2) after 1, 6, and 24 h immersing times and the trends are illustrated in Figure 2(a,b). As it can be seen from Figure 2(a), initially after immersing the samples into PBS, the degree of swelling as a main criteria for the drug release mechanism was significant and the ultimate swelling percentage for the three-layered sample was 521% after 48 h. The variations in the weight loss of the samples with the conditions similar to those for degree of swelling were exhibited in Figure 2(b). In contrast to the swelling variations of the samples, their weight loss percentage has no significant changes during 48 h in the exposure to PBS. It can be concluded that although PVA owing to the hydrophilic property showed a high swelling percentage, its hydrolytic degradation in contact with water molecules as a function of weight loss alteration was quantitatively negligible.

Biodegradability of the Three-layered Electrospun Samples

Figure 3(a,b) shows the ATR-FTIR spectrum of the three-layered electrospun PVA/PCL/PVA nanofibrous sample containing PHT-Na before and after immersion in PBS (pH = 7.2 and temperature 37 °C).
Table II. The Regression Coefficient Results of Released TC-HCl and PHT-Na from Three-layered Electrospun PVA/PCL/PVA Nanofibrous Samples Calculated by Korsmeyer–Peppas (\(n = k^\prime t^n\), \(n = 0.5\))

<table>
<thead>
<tr>
<th>Sample</th>
<th>(R^2 \pm SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three-layered electrospun PVA/PCL/PVA nanofibrous sample containing TC-HCl</td>
<td>0.88 ± 0.04</td>
</tr>
<tr>
<td>Three-layered electrospun PVA/PCL/PVA nanofibrous sample containing PHT-Na</td>
<td>0.95 ± 0.04</td>
</tr>
</tbody>
</table>

of the drug for the single-layered electrospun PCL nanofibers was almost two folds compared to the sample with three-layered structure. The reason for this interesting behavior was referred to the presence of lateral PVA nanofibrous mats as a barrier to prevent the rapid diffusion of TC-HCl from PCL nanofibers. Subsequently, after 10 h the released TC-HCl was governed gradually with a gentle slope due to the PVA hydrolysis during 48 h. Overall, it can be concluded that the ultimate amount of TC-HCl release from single layer electrospun PCL nanofibers was about 79% whereas this value for three-layered electrospun PVA/PCL/PVA nanofibrous sample was only 43%. These observations confirmed that the layered nanofibrous samples were promising materials for biomedical applications especially wound dressings because they can reduce the bandage removal time onto the wound surface and hospitality costs.

Furthermore, the release of PHT-Na from the electrospun PVA nanofibrous sample was studied by HPLC. Figure 4(b) demonstrates the PHT-Na release profile along with its HPLC chromatogram as an example in order to show the drug molecules exited from the instrument column after 4.85 min via mobile phase. As it can be seen from Figure 4(b), the drug release from the sample diffused out with a burst initial release until 10 h and thereafter, the drug released with a gradual and slow trend. This can be due to the hydrophilic property of PVA in which the PVA nanofibers were rapidly swollen and degraded by water molecules. As a result, the ultimate PHT-Na release from the electrospun nanofibers was about 87%. It should be noted that besides the prolonged TC-HCl release, PHT-Na release behavior revealed that it can help the wound dressings to heal the wounds during 24 h post-treatment time.

Moreover, the four more release kinetic models in terms of Korsmeyer–Peppas, zero order, first order and Higuchi were investigated for both TC-HCl and PHT-Na release from PVA and PCL nanofibers. As it is shown in Table II, the regression coefficients regarding the released TC-HCl and PHT-Na from three-layered electrospun sample on the basis of Korsmeyer–Peppas were 0.88 and 0.95, respectively. Accordingly, the exponent index \(n\) after curve fitting was calculated to be 0.5 whereby it can be concluded that the main release mechanism for the drug-polymer systems was governed by Fickian diffusion.

Additionally, the regression coefficients of the released TC-HCl and PHT-Na from the samples for the other three release kinetic models were represented in Table III. Evidently, the Higuchi model has a more suitable correspondence to the experimental release data compared to the zero order and first order models which resulted in the governed mechanism was diffusion.

Table III. The Regression Coefficients of Different Mathematical Models Corresponding to the Experimental Release Data of TC-HCl and PHT-Na from the Three-layered Electrospun PVA/PCL/PVA Nanofibrous Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three-layered electrospun PVA/PCL/PVA nanofibrous sample containing TC-HCl</td>
<td>0.65 ± 0.02</td>
<td>0.67 ± 0.01</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Three-layered electrospun PVA/PCL/PVA nanofibrous sample containing PHT-Na</td>
<td>0.6 ± 0.02</td>
<td>0.62 ± 0.01</td>
<td>0.7 ± 0.03</td>
</tr>
</tbody>
</table>
The Antibacterial Evaluation of the Single and Three-layered Nanofibrous Samples Containing the Drugs

The inhibitory effect of TC-HCl released from the single-layered electrospun PCL and three-layered electrospun PVA/PCL/PVA nanofibrous samples and also their comparison with the neat layered nanofibers (negative control) as well as TC-HCl solution with 500 μg mL⁻¹ concentration (positive control) on a common bacterium so-called S. aureus was assessed. As it can be observed from Figure 5(a–d), the inhibitory zones sizes for the TC-HCl solution, the electrospun PCL nanofibers containing TC-HCl, the TC-HCl loaded into electrospun PVA/PCL/PVA nanofibers and the neat ones were 9, 6, 3, and zero in sizing inhibitory zones, respectively. The results obtained from the antibacterial evaluation had a direct relation with the TC-HCl released from the samples and confirmed the sustained control release of TC-HCl from the three-layered nanofibrous sample with respect to the single-layered electrospun PCL sample during 48-h treatment period.

The Cytotoxicity and Their Adhesion onto the Surfaces of the Single and Three-layered Nanofibrous Samples Containing PHT-Na and TC-HCl

The cell cytotoxicity of the surface of the nanofibers is the main parameter in order to use these materials for many biomedical applications such as wound dressings. The sustained control release of TC-HCl can dominantly affect the prevention of bacteria growth and anticipate helping the cell viability. Therefore, the fibroblast cells viability of the single-layered PCL nanofibers and also the three-layered electrospun PVA/PCL/PVA nanofibers

Figure 5. The inhibitory zones observations of S. aureus bacterium for (a) TC-HCl solution with 500 μg mL⁻¹ concentration as the positive control and the nanofibrous samples: (b) the single-layered PCL containing TC-HCl, (c) the three-layered PVA/PCL/PVA containing TC-HCl and (d) neat three-layered PVA/PCL/PVA as the negative control by using agar disc diffusion method after 48 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6. The viability of fibroblast cells onto the surfaces of the single-layered electrospun PCL nanofibers containing TC-HCl, the three-layered electrospun PVA/PCL/PVA nanofibers containing TC-HCl and PHT-Na and feeding environment of the cells as the control at days 1, 3, and 7 at 37 °C by using MTT test.

Figure 7. The cells adhesion and spreading onto the surfaces of the nanofibrous samples after 24-h culture time: (a) single-layered PCL containing TC-HCl and (b) the three-layered PVA/PCL/PVA containing TC-HCl and PHT-Na (the scale bars are 30 and 10 μm).
containing both PHT-Na and TC-HCl was carried out by MTT test and the results compared with growing the cells in the presence of their feed, DMEM-F12 (control) are shown in Figure 6. The MTT test was designed at three different days including days 1, 3, and 7. As it can be seen from Figure 6, the cells viability of the three-layered electrospun sample at day 3 was lower than that of the electrospun PCL nanofibers. Conversely, a different result was observed for these samples at day 7 as the cells viability regarding the layered electrospun sample was increased and the quantity of cells numbers was reached to the control sample. This can be due to the gradual release of TC-HCl from the middle layer PCL nanofibers to prevent any contaminations from the cells growth environment.

Figure 7(a,b) shows the skin fibroblast cells adhesion onto the surfaces of the electrospun PCL nanofibers containing TC-HCI and the three-layered electrospun PVA–PHT-Na/PCL–TC-HCl/PVA–PHT-Na nanofibers after 24-h culture time. As expected, the adhesion of the cells for the layered sample and their accumulation within the interconnected network of this sample [Figure 7(b)] was remarkably higher than that of the PCL nanofibers [Figure 7(a)]. The reason for this observation can be related to the presence of PHT-Na into the three-layered sample as a cell growth factor.

Furthermore, the cells dispersion which reveals the capability of the sample surface for viability and adhesion of the fibroblast cells was carried out by staining the cells with Hoechst color. Figure 8(a,b) illustrates the cells dispersion throughout the surface of the nanofibrous samples. It can be concluded that the three-layered electrospun sample had a more suitable environment in which the cells could uniformly be dispersed onto the polymer surface [Figure 8(a)] whereas the surface of the electrospun PCL nanofibers conducted the cells as aggregates without an appropriate dispersion [Figure 8(b)].

CONCLUSIONS

In this work, a three-layered nanofibrous sample (PVA/PCL/PVA) was prepared by electrospinning technique. The hydrophilic layer (PVA) containing PHT-Na and the hydrophobic layer (PCL) containing TC-HCl were produced. The TC-HCl release profile in the PBS medium showed that the drug release rate from a single-layered PCL nanofibrous sample was faster than the three-layered sample during 48-h period. Also, PHT-Na release rate from the three-layered sample was evaluated by HPLC. SEM micrograph images showed that the average diameter of nanofibers was reduced due to the drugs presence. ATR-FTIR spectrum showed that the presence of PCL layer in this sample reduced the degradation of PVA considerably. The swelling of this three-layered nanofibers during 48 h was about 500% while the weight loss was negligible. The release profiles of these two drugs corresponded to Korsmeyer–Peppas equation and the mechanism was Fickian diffusion. The antibacterial evaluation results also confirmed this drug release behavior. The bacterial inhibition zone of the single-layered nanofibers was nearly double the three-layered sample. The cell cytotoxicity studies showed that three-layered nanofibrous sample compared to single-layered had a better viability. Although, for the first and third day, the single-layered sample had relatively a better performance but gradually as culture time increased, the percentage of residual alive cells on the three-layered sample compared to the single-layered nanofibers and control sample was increased. The qualitative and quantitative adhesion values of fibroblast cells showed that the three-layered nanofibers were higher than single-layered PCL nanofibers. The FE-SEM images showed that the cultured cells in the three-layered nanofibrous sample developed as a function of time, but on the surface of PCL–TC-HCl nanofibers remained as spindle-like shape. The results of stained-cells with fluorescent material showed that the growth, proliferation, and dispersion of the cells on the surface of three-layered nanofibers (PVA/PCL/PVA) was better than single-layered PCL nanofibers.

REFERENCES

2. Ramakrishna, S.; Fujihara, K.; Teo, W.-E.; Lim, T.-C.; Ma, Z. An Introduction to Electrospinning and Nanofibers; World Scientific Publishing: Singapore, 2005.


