International Journal of Pharmaceutics 427 (2012) 379–384

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Lysozyme-loaded, electrospun chitosan-based nanofiber mats for wound healing

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ARTICLE INFO

Article history:
Received 21 November 2011
Received in revised form 4 January 2012
Accepted 7 February 2012
Available online 14 February 2012

Keywords:
Electrospinning
Lysozyme
Chitosan
Ethylenediaminetetraacetic acid

ABSTRACT

In this study, a blend mixture of chitosan–ethylenediaminetetraacetic acid (CS 2 wt%–EDTA) at a weight ratio of 30/70 and polyvinyl alcohol (PVA) solution (10 wt%) was electrospun to produce fibrous mats with lysozyme (10, 20 and 30 wt%) used for wound healing. The morphology and diameter of the electrospun fiber mats with and without lysozyme were analyzed by scanning electron microscopy (SEM). The amount of lysozyme loaded in the nanofiber mats was measured by HPLC. The cell lysis activity of the lysozyme was investigated with *Micrococcus lysodeikticus* cells as a substrate. The wound healing activity was performed in vivo using male Wistar rats. The SEM images of all lysozyme-loaded fibers show a smooth fiber without beads with an average diameter of 143–209 nm. The amount of lysozyme loaded in the nanofiber mats was slightly decreased when the initial concentration of lysozyme was increased. The rapid lysozyme release from the nanofiber mats was obtained and is dependent on the lysozyme-loading amount. In animal wound healing, lysozyme loaded CS–EDTA nanofiber mats accelerated the rate of wound healing when compared to the controls (gauze). In conclusion, our experiments demonstrated that biomaterials composed of lysozyme loaded CS–EDTA nanofibers have a potential for wound healing.

1. Introduction

Electrospinning is a versatile technique for creating nanofibers from many materials. The fibers produced by this method have shown unique characteristics, such as a very large surface to volume ratio and a high porosity with a small pore size (Bhardwaj and Kundu, 2010; Huang et al., 2003). In the electrospinning process, a high voltage is applied to a capillary containing a polymer solution. A droplet of the polymer solution drive fluid forms at the tip of the capillary, and as electrostatic forces overcome the surface tension of the polymer solution, the solution is ejected from the tip. The charged jets of polymer solution move toward a collector, the solvent rapidly evaporates and a non-woven fiber mat is collected on the collector (Reneker and Yarin, 2008). The morphology of the electrospun nanofibers can be easily controlled by adjusting the electrospinning parameter (Baji et al., 2010; Deitzel et al., 2001). Because of this characteristic, electrospun nanofibers have been applied in many fields, including biomedical sciences, filtration, and optical sensor fields. In biomedical applications, electrospun nanofibers can be used as a tissue engineering scaffold and are also valuable in drug delivery and wound dressing (Agarwal et al., 2008; Sill and Recum, 2008).

Materials for wound dressing made from the electrospinning product have been increasingly investigated. The electrospun nanofiber is appropriate for use as a wound dressing material due to its useful properties, including oxygen-permeable high porosity, variable pore-size distribution, high surface to volume ratio, and most importantly, morphological similarity to the natural extracellular matrix (ECM) in the skin, all of which promote cell adhesion, migration and proliferation (Jayakumar et al., 2011). For wound dressing applications, the electrospun nanofiber can be used with or without agents that promote wound healing and the polymer must be biocompatible, biodegradation and low toxicity. Only few polymers were used to prepare electrospun nanofiber for wound healing such as polyvinyl alcohol (PVA), poly(L-lactic acid) (PLA), polycaprolactone (PCL), gelatin and chitosan (CS) (Venugopal and Ramakrishna, 2005; Zahedi et al., 2010).

CS is produced by the alkaline deacetylation of chitin that is a copolymer of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN). CS is soluble in acidic media and insoluble in neutral and alkaline media. Because it is biodegradable, biocompatible, and non-toxic, CS has been proposed as a safer material for use in biomedical applications (Rinaudo, 2006). CS has been widely investigated as a wound dressing material and is available in proliferation, antibacterial and activates macrophages. Moreover, chitosan will gradually depolymerize into *N*-acetyl-D-glucosamine, which initiates fibroblast proliferation, assists in ordered collagen deposition and stimulates increased levels of natural hyaluronic acid synthesis at the wound site (Jayakumar et al., 2011; Paul

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doi:10.1016/j.ijpharm.2012.02.010

and Sharma, 2004). Recently, CS nanofibers have been successfully generated from the electrospinning of pure CS, CS derivatives and CS blends with other polymers. However, some organic solvents or toxic acids, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Min et al., 2004), trifluoroacetic acid (TFA) (Sangsanoh and Supaphol, 2006) and acetic acid (Geng et al., 2005), may form residues in the chitosan nanofibers. To reduce the toxicity of solvents, water soluble CS were used to prepare nanofibers such as carboxyethyl chitosan/PVA (Zhou et al., 2008) and quaternary chitosan (Ignatova et al., 2007) for wound dressing applications. Moreover, in our research, CS was also prepared in aqueous salt to form nanofibers without the use of organic solvents or toxic acids such as CS–hydroxybenzotriazole (HOBt)/PVA (Charernsriwilaiwat et al., 2010) and CS–ethylenediaminetetraacetic acid (EDTA)/PVA (Charernsriwilaiwat et al., 2011).

In this study, we used electrospinning to fabricate nanofiber mats from a CS–EDTA blend with PVA solution. In a previous study, the best weight ratio of CS–EDTA and PVA was 30/70 CS–EDTA/PVA, which produced a smooth fiber without beads in the structure. To enhance the wound healing effect, lysozyme (LZ) was chosen to be loaded into these CS–EDTA/PVA nanofibers due to its antibacterial properties (Hughey et al., 1989; Mecitoflu et al., 2006), solubility in water and synergistic antibacterial effects with EDTA (Branen and Davidson, 2004). Moreover, the enzymes that are effective in promoting the healing process (muramidase or *N*-acetylmuramide glycanhydrolase) are those that break down the bacterial cell walls and depolymerize CS to release *N*-acetyl-D-glucosamine (Reshetov et al., 2004). Therefore, the aim of this study was to prepare CS–EDTA/PVA nanofiber mats loaded with LZ for wound dressing applications. The morphology and structure of the nanofiber mats were characterized by scanning electron microscopy (SEM). The composites of the LZ loaded 30/70 CS–EDTA/PVA nanofiber mats were characterized by differential scanning calorimetry (DSC). The cytotoxicity tests for the CS–EDTA/PVA nanofiber mats were performed with MTT assays using human fibroblast cells. Finally, in vivo wound healing activities were evaluated using an animal model.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation 0.85, MW 110 kDa), ethylenediaminetetraacetic acid and lysozyme from chicken egg whites were purchased from Sigma–Aldrich Chemical Company, USA. Polyvinyl alcohol (PVA) (degree of polymerization ≈ 1600, degree of hydrolysis ≈ 97.5–99.5 mol%) was purchased from Fluka, Switzerland. Normal human foreskin fibroblast (NHF) cells were obtained from the American Type Culture Collection (ATCC) in Rockville, MD, USA. Dimethyl sulfoxide (DMSO) was obtained from BDH Laboratories, UK. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Trypsin–EDTA, and penicillin–streptomycin were purchased from Gibco BRL Rockville, MD, USA. All other reagents and solvents were commercially available and were of analytical grade.

2.2. Electrospinning

The 2% (w/v) CS solution was prepared by dissolving CS and EDTA in distilled water at a weight ratio of 2:1. In the case of CS–acetate, a 2% (w/v) CS solution was prepared by dissolving CS and a 2% (v/v) acetic acid solution. The 10% (w/v) PVA solution was prepared by dissolving PVA in distilled water at 80 °C and then allowing the solution to stir for 4 h. The CS–EDTA solution was mixed with a PVA solution at a weight ratio of 30/70. LZ (10, 20 and 30 wt% to polymer) was added into the 30/70 CS–EDTA/PVA solution. The viscosity, conductivity and surface tension of the solutions were measured. The electrospinning solution was contained in a 5-mL glass syringe connected with a 20-gauge stainless steel needle (diameter = 0.9 mm) at the nozzle. The needle was connected to the emitting electrode of positive polarity of a Gamma High Voltage Research device. The electric potential was fixed at 15 kV. The nanofibers were collected as-spun on an aluminum sheet that was wrapped on a rotating collector. The solution was electrospun at room temperature, and the collection distance was fixed at approximately 20 cm. The solution feed was driven by a syringe pump, with the rate fixed at 0.25 mL/h during spinning.

2.3. Characterization of LZ loaded CS-based nanofibers

The morphology and diameter of the nanofiber mats were determined using scanning electron microscopy (SEM; Camscan Mx2000, England). For this process, a small section of the electrospun fiber mats was sputtered with a thin layer of gold prior to SEM observation. The average diameter of the nanofiber mats were analyzed by randomly measuring the diameters of the nanofibers at 100 different points from the SEM images.

The thermal behavior of the nanofiber mats was evaluated by differential scanning calorimetry (DSC, Pyris Sapphire DSC, PerkinElmer instrument, USA) in an atmosphere of nitrogen. DSC traces were recorded from 25 to 250 °C at a heating rate of 5 °C/min.

2.4. Determination of lysozyme content

The content of LZ in the 30/70 CS–EDTA/PVA nanofiber mats was analyzed by HPLC (Agilent Technology, USA). A VertiSep® AQS C18 column (250 mm × 4.6 mm, 5 μm particle size) with a C18 guard column was used. The elution was carried out with gradient solvent systems consisting of 1% acetonitrile, 0.2% trifluoroacetic acid and 98.8% water (mobile A) and 70% acetonitrile, 0.2% trifluoroacetic acid and 29.8% water (mobile B) with a flow rate of 1 mL/min at ambient temperature. The gradient was programmed as follows: 100% A for 0–3 min, constant at 45% A for 9 min, 45% A to 20% A in 3 min, and 20% A to 100% A in 5 min. The wavelengths of the spectrofluorimetric detector were set at *Ex* = 276 nm and *Em* = 345 nm (Riponi et al., 2007). The nanofiber mats (5 mg) were dissolved in 5 mL of distilled water at room temperature and continuously stirred for 24 h. Then, the amount of LZ in the nanofiber mats was analyzed. The LZ content was calculated using Eq. (1).

$$\text{LZ content}\,(\%) = \left(\frac{L_a}{L_t}\right) \times 100, \tag{1}$$

where L_a is the amount of LZ embedded in the nanofibers, and L_t is the theoretical amount of LZ (obtained from feeding condition) incorporated into the nanofibers.

2.5. Lysozyme activity assay

The biological activities of the LZ loaded CS–EDTA/PVA nanofiber mats were measured with an EnzChek lysozyme assay kit (E-22013). *Micrococcus lysodeikticus* cells were used as a substrate. The samples were prepared by dissolving the nanofiber mats in pH 7.4 phosphate buffer (50 μL) at room temperature and continuously stirred for 24 h and mixed with 50 μg/mL (50 μL) of *M. lysodeikticus*, labeled with fluorescein at each LZ concentration in a 96-well plate. The mixtures were incubated at 37 °C for 30 min, and the fluorescence intensity of each sample was measured in a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). The fluorescence absorption maxima and emission maxima of the digested

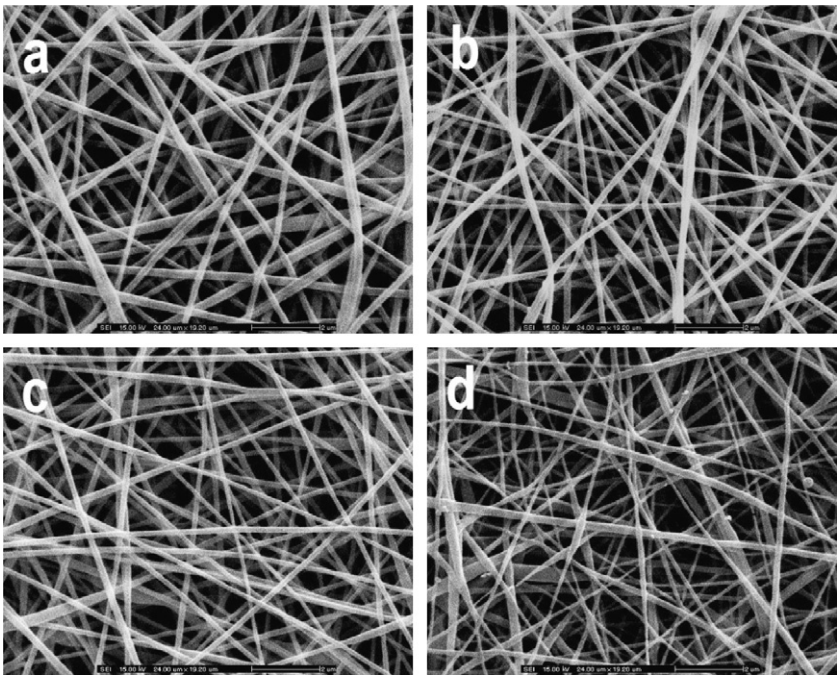

Fig. 1. The SEM image of the LZ loaded CS–EDTA/PVA nanofiber mats with different concentrations of LZ: (a) 0%, (b) 10%, (c) 20% and (d) 30%.

products from the substrate were at 494 nm and 518 nm, respectively. Specific activity was defined in terms of units of activity per milligram of protein (U/g). All samples were assayed in triplicate.

2.6. In vitro release of lysozyme

The release characteristics of LZ from the LZ loaded CS–EDTA/PVA nanofiber mats were investigated using Franz's diffusion cells with a water jacket connected to a water bath at 37 °C, each cell having a 6.5 mL volume and 2.43 cm^2 effective diffusion area. The receiver compartments were filled with acetate buffer (pH 5.5) and stirred with a Teflon magnetic stirrer at 600 rpm. The nanofiber mats were cut with an equal diameter effective diffusion area and were mounted between two half cells of the diffusion cell. At a given time interval, an aliquot (1.0 mL) of the receiver solution was withdrawn and replaced with the same volume of fresh medium to keep the volume constant. The concentration of LZ in the samples was assayed by HPLC (Agilent Technology, USA) using the same conditions. The concentration of LZ and their cumulative amounts were plotted against time. The experiments were carried out in triplicate.

2.7. Indirect cytotoxicity evaluation

The cytotoxicity of the nanofiber mats was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact) (Chen et al., 2008). The LZ-loaded nanofiber mats were sterilized by UV radiation for 1 h. The mats were then extracted in a serum-free medium (SFM; containing DMEM, 1% (v/v) L-glutamine, 1% (v/v) lactalbumin, 1% (w/v) antibiotic and antimycotic formulation) in an incubator for 24 h to produce extraction media of varying concentrations (10, 7.5, 5, 2.5 and 1 mg/mL). Normal human foreskin fibroblast (NHF) cells were plated in 90 μL of DMEM, supplemented with 10% FBS, at a density of 8000 cells/well in 96-well plates. When the cultures reached confluency (typically 48 h after plating), the tested extraction media with varying concentrations were replaced, and the cells were re-incubated for 24 h. After treatment, the tested extraction solutions were removed. Finally, the cells were incubated with 100 μL of a MTT-containing medium (1 mg/mL) for 4 h. The medium was removed, the cells were rinsed with PBS (pH 7.4), and the formazan crystals that had formed in the living cells were dissolved in 100 μL DMSO per well. Cell viability (%) was calculated based on the absorbance at 550 nm using a microplate reader. The viability of the non-treated control cells was arbitrarily defined as 100%.

2.8. Wound healing test

Male Wistar rats (240–280 g) were used in this study, and the study was approved by an Investigational Review Board (Animal Studies Ethics Committee, Faculty of Pharmacy, Silpakorn University, Approval No. 2-2553). After anesthetization, two full-thickness rectangular wounds with a surface area of 0.8 cm^2 were cut from the back of each of rat's neck. The wound was covered with a nanofiber mat equal size to its size, gauze and commercial antibacterial gauze dressing (Sofra-tulle®, Sanofi Aventis, UK) ($n = 6$). The area of the wound was measured every day using the planimetry method until the wound completely healed. The percentage of wound healing is defined by Eq. (2).

$$\text{Wound area}\,(\%) = \left(\frac{A}{A_i}\right) \times 100, \tag{2}$$

where A_i is the initial wound area, and A is the wound area after a fixed time interval.

2.9. Statistical analysis

Data were collected from triplicate samples and were depicted as the mean ± standard deviation (S.D.). Statistically significant differences were examined using Student's t-test. The significance level was set at $p < 0.05$.

Table 1
Solution parameters of LZ loaded 30/70 CS–EDTA/PVA solution and average diameter at each concentrations.

LZ concentration (%)	Viscosity (mPa s)	Conductivity ($\mu S\ cm^{-1}$)	Surface tension ($mN\ m^{-1}$)	Fiber diameter (nm)
0	254.74 ± 0.76	1016.3 ± 0.66	49.46 ± 1.43	209.91 ± 35.68
10	221.27 ± 0.76	1366.0 ± 0.66	49.01 ± 0.44	168.72 ± 24.16
20	204.27 ± 1.36	1689.7 ± 1.36	49.31 ± 0.48	160.50 ± 26.84
30	198.82 ± 0.66	2013.0 ± 0.76	49.60 ± 0.21	143.65 ± 50.41

3. Results and discussion

3.1. Electrospinning

LZ was added to the CS–EDTA/PVA (weight ratio of 30/70) electrospinning solution. The maximum concentration of LZ for the preparation of electrospun nanofibers was 30 wt%. When the LZ concentration was greater than 30 wt%, nanofibers could not be obtained via electrospinning. Fig. 1 shows the morphology of 0–30 wt% LZ loaded CS–EDTA/PVA fiber mats. The SEM images of all concentrations of LZ show a smooth fiber without beads. When the concentrations of LZ were increased, the diameter of the nanofibers decreased from 209.91 ± 35.68 to 143.65 ± 50.41 nm. The decrease in diameter may be due to the change in the solution parameter (Table 1). The conductivity of the electrospun solution increased when the concentration of LZ increased, whereas the viscosity and surface tension of the solution were slightly decreased and did not change at each concentration. Therefore, the decrease in the diameter of the nanofibers is mainly due to the conductivity of the solution. When the conductivity of a solution increases, the diameter of the nanofiber decreases. LZ is a protein that can provide charge when dissolved in water. Because of the amine groups in its structure, an increase in the conductivity of the solution occurs when the concentration of LZ increases. This result corresponds to our previous study, which showed that the diameter of the CS–HOBt/PVA nanofiber mats decreased when the conductivity of the aqueous solution increased (Charernsriwilaiwat et al., 2010).

3.2. Characterizations

Fig. 2 shows the DSC thermograms of the CS–EDTA/PVA nanofiber mats with and without LZ and pure LZ powder. The endothermic curves of the bare-nanofiber mats (0% LZ) showed that the melting point slightly decreased from approximately 218.5 °C to 217.3, 216.7 and 216.1 °C when the concentration of LZ increased to 10, 20 and 30%, respectively. For the pure LZ powder, the melting point was observed at approximately 200 °C, which is lower than that of the CS–EDTA/PVA nanofiber mats. This indicates that the LZ content in the nanofiber mats does not affect the thermal behavior of the mats.

Table 2
% LZ content and activity of LZ loaded 30/70 CS–EDTA/PVA nanofiber mats at each concentrations.

LZ concentration (%)	% LZ content	LZ activity (U/g)
10	95.44 ± 2.54	4.40 ± 1.23
20	93.86 ± 1.91	8.89 ± 1.56
30	92.39 ± 1.76	16.30 ± 2.39

3.3. LZ content and activity

The LZ content and activity in the CS–EDTA/PVA nanofiber mats are shown in Table 2. The content of LZ in the nanofiber mats was 92.39–95.44%, which shows the excellent incorporation of LZ in the nanofiber mats. When the concentration of LZ increased, the % content slightly decreased. This might be the capacity of nanofiber mats was maximum at 10% LZ. The activity of LZ in the CS–EDTA/PVA nanofiber mats was between 4.4 and 16.3 U/g. LZ is a water-soluble enzyme that homogenously mixes with the hydrophilic polymer. Therefore, it can be readily dissolved in CS–EDTA/PVA solution and provides high content and activity. This result shows that LZ is not eliminated during the electrospinning process under high electric voltage conditions (Kim et al., 2007).

3.4. In vitro release study

Fig. 3 shows the LZ release profiles from CS–EDTA/PVA nanofiber mats with different concentrations. It can be observed that the lysozyme is rapidly released. The percentage of cumulative LZ release reaches approximately 80% within 30 min. This result reveals that the LZ was burst released from the nanofiber mats. The maximum total amount of LZ release from the nanofiber mats within 4 h was approximately 90%. This result was similar to the

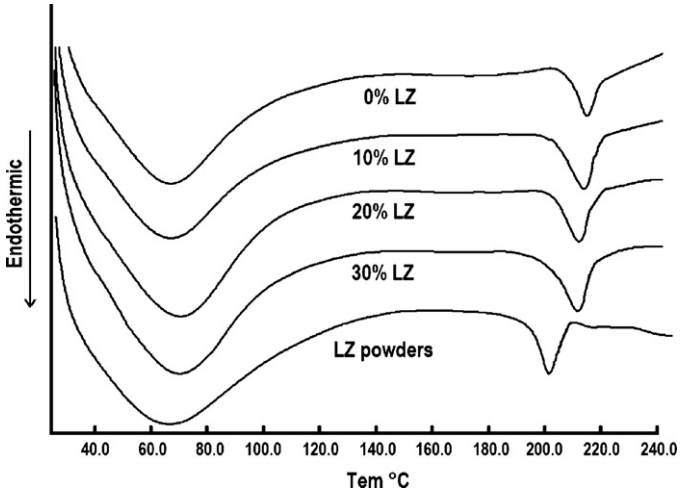

Fig. 2. DSC thermogram of the LZ loaded CS–EDTA/PVA nanofiber mat with different concentrations of LZ and pure LZ powders.

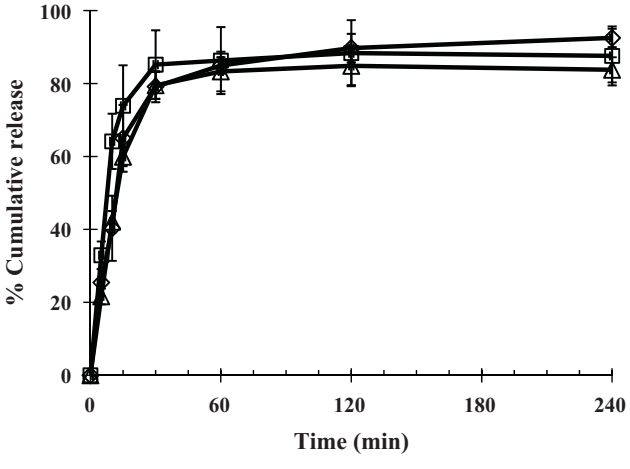

Fig. 3. Lysozyme release profiles from CS–EDTA/PVA nanofiber mats with various initial lysozyme concentrations: (◊) 10%, (□) 20% and (△) 30%.

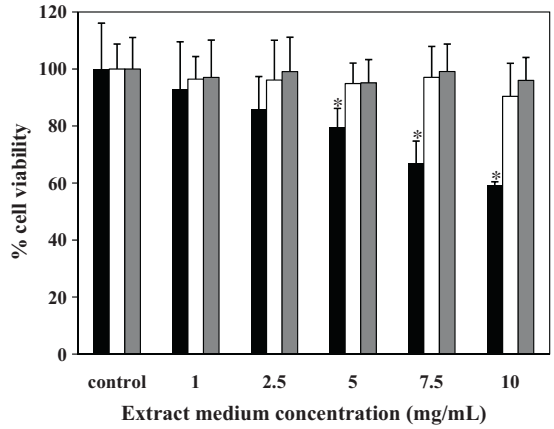

Fig. 4. Cell viability of the extract of nanofiber mats (■) bare-CS–acetate/PVA (□) bare-CS–EDTA/PVA and (■) 30% LZ loaded CS–EDTA/PVA nanofiber mats at various concentrations in NHF cells. Difference values * were statistically significant ($p < 0.05$) compared with control. The data are presented as mean ± S.D. ($n = 5$).

release of LZ from the electrospun hydrophilic PVA/gelatin scaffold (Yang et al., 2008). This result indicates that LZ could release well from the CS–EDTA/PVA nanofiber mats, and the LZ release patterns may be explained by both the mechanism of polymer erosion and lysozyme diffusion (Kim et al., 2007).

3.5. Indirect cytotoxicity

The cytotoxicity of various concentrations of the extract medium from 30% LZ loaded CS–EDTA/PVA, bare-CS–EDTA/PVA and bare-CS–acetate/PVA nanofiber mats were shown in Fig. 4. There was a significant decrease in cell viability when the NHF cells were incubated with higher concentrations (5–10 mg/mL) of the extraction media of the nanofiber mats of bare- CS–acetate/PVA nanofiber mats when compared with the control ($p < 0.05$). The average cell viability was decreased when the weight of the nanofiber mats increased. However, cell viability remained similar to that of the control non-transfected cells in all concentrations of the CS–EDTA/PVA extract medium and 30% LZ loaded CS–EDTA/PVA nanofiber mats. This may be because the acetic acid that was used as a solvent remained in the nanofiber mats. Our results indicate that the CS–EDTA/PVA nanofiber mats with and without LZ is safe at the concentrations used (1–10 mg/mL).

3.6. Wound healing test

In the wound healing test, two full-thickness round wounds with surface areas of 0.8 cm^2 were created on the back of each rat. Fig. 5 shows the representative images of the wound healings at 1, 4, 7 and 10 days after treatment with 30% LZ loaded CS–EDTA/PVA nanofiber mats, gauze (negative control) and commercial antibacterial gauze dressing (positive control). Wound closure in all treatments was recovered within 10 days. The healing treatment with 30% LZ loaded CS–EDTA/PVA nanofiber mats was faster than that of the gauze and commercial antibacterial gauze dressing treatments at 4 and 7 days after operation. Fig. 6 shows the changes in wound areas at different healing times. The wound areas decreased gradually and achieved approximately 2% after 10 days when treated with three different wound dressings. In the first 1–5 days, the healing effect of 30% LZ loaded CS–EDTA/PVA nanofiber mats was better than that of the gauze ($p < 0.05$) and was similar to that of the commercial antibacterial gauze dressing. This may be because the LZ was rapidly released from the nanofiber mats, which assists in the acceleration of the healing process by deactivating bacterial and depolymerizing the CS (Mecitoflu et al., 2006). Byproducts from CS depolymerization such as EDTA and *N*-acetyl-D-glucosamine can also enhance the healing rate because of their antibacterial properties and aid in fibroblast proliferation (Reshetov et al., 2004). After that, the % wound area of all treatments between day 5 and recovery was similar. This revealed that wound healing was processed by a mechanism of the body, independent from the effects of the treatment. Chen et al. (2008) also reported similar results for electrospun collagen/chitosan nanofibrous membranes, which proved to be better than gauze and commercial collagen sponge for wound healing.

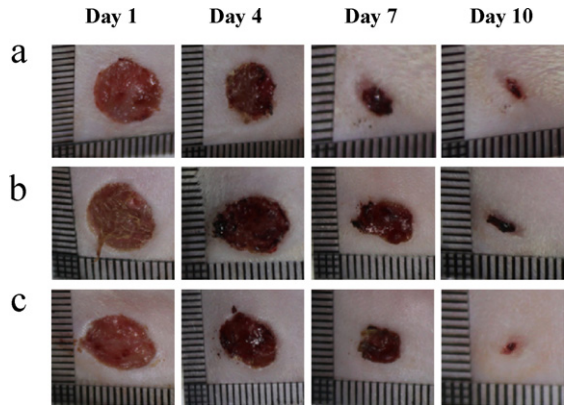

Fig. 5. Appearance of would healings at 1, 4, 7 and 10 day after treat with (a) 30% LZ loaded CS–EDTA/PVA nanofiber mats, (b) gauze (negative control) and (c) commercial antibacterial gauze dressing (Sofra-tulle®) (positive control).

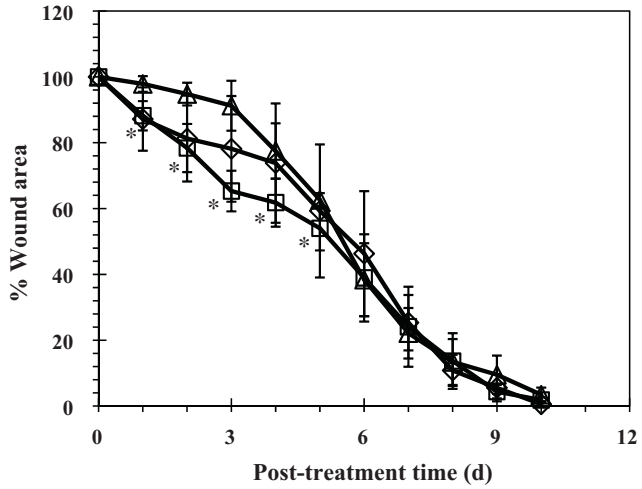

Fig. 6. Wound healing tests of (□) 30% LZ loaded CS–EDTA/PVA nanofiber mats, (△) gauze (negative control) and (◊) commercial antibacterial gauze dressing (Sofra-tulle®) (positive control). Difference values * were statistically significant ($p < 0.05$) compared with gauze. The data are presented as mean ± S.D. ($n = 6$).

4. Conclusion

An ideal wound dressing should be biocompatible, biodegradable and able to enhance the healing process. LZ was successfully loaded into CS–EDTA/PVA nanofiber mats using the electrospinning method. The 30% LZ loaded CS–EDTA/PVA nanofiber mats are in the nanometer range, are non-toxic and rapidly released, retains the LZ activity, and enhance the healing process. These biodegradable, biocompatible, electrospun chitosan-based nanofiber mats have great potential for use as wound dressings.

Acknowledgements

The authors wish to thank the Commission of Higher Education (Thailand), the Thailand Research Funds through the Golden Jubilee Ph.D. Program (Grant No. PHD/0183/2550) and Project No. DBG5480004 for financial support.

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