International Journal of Pharmaceutics 382 (2009) 215–221

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Electrospun shikonin-loaded PCL/PTMC composite fiber mats with potential biomedical applications

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ARTICLE INFO

Article history:
Received 20 May 2009
Accepted 27 July 2009
Available online 4 August 2009

Keywords:
Electrospinning
PCL/PTMC composite fibers
Drug release
Shikonin

ABSTRACT

Novel electrospun poly(ε-caprolactone) (PCL)/poly(trimethylene carbonate) (PTMC) ultrafine composite fiber mats were prepared and used as drug-carrying materials to encapsulate the herbal medicine shikonin isolated from the plant *Lithospermum erythrorhizon* Sieb. et Zucc. The PCL/PTMC blended solutions in various ratios (9:1, 7:3, and 5:5, w/w) containing 1 and 5 wt.% shikonin were studied for electrospinning into nanoscale fiber mats. With good drug stability and high drug-loading efficacy, incorporation of shikonin in the polymer media did not appear to influence the morphology of the resulting fibers, as both the drug-free and the shikonin-loaded composite fibers remained unaltered, microscopically. The average diameter of the composite fibers decreased, and the morphology of the fibers became finer with the increasing content of PTMC. *In vitro* drug release studies demonstrated an initial rapid release of shikonin followed by a plateau after 11 h. It was found that the release behavior could be tailored by the PCL/PTMC blend ratio and drug-loading content. Moreover, the free radical scavenging activity and the antibacterial effects of the shikonin-loaded fiber mats indicated that it could act not only as a drug delivery system but also in the treatment of wound healing or dermal bacterial infections.

1. Introduction

Electrospinning is a convenient and versatile technique for generating consistent ultrafine fibers from polymer solutions or melts and has received increasing interest in recent time (Dalton et al., 2007; Sill and von Recum, 2008). It provides a promising way to fabricate infinite continuous fibers with diameter ranging from nanometers to microns. This property, with large surface area-to-volume ratio and small pore size, made the electrospun fibers optimal candidates for many important applications such as separation filters (Aussawasathien et al., 2008), carbonaceous materials (Ji and Zhang, 2009; Zhou et al., 2009), biosensors (Patel et al., 2006) and biomedical devices including tissue engineering scaffolds (Mo et al., 2004), wound dressing materials (Powell et al., 2008), and drug delivery platforms for delivering various bioactive agents (Luu et al., 2003; Maretschek et al., 2008; Zeng et al., 2005a; Kim et al., 2007; Suwantong et al., 2007; Xu et al., 2009).

Polymeric drug delivery systems are able to improve therapeutic efficacy, reduce toxicity, and enhance compliance of the patients by delivering drugs at a controlled rate over a period of time to the site of action (Kenawy et al., 2002; Nie et al., 2009). Numerous biodegradable and/or biocompatible polymeric materials have been electrospun into nanoscale fibers and demonstrated their potential as effective carriers for drug delivery. Xie and Wang (2006) developed PLGA-based electrospun micro- and nanofibers as implants for the sustained delivery of paclitaxel to treat C6 glioma *in vitro*. The release profiles suggest that paclitaxel sustained release could achieve for more than 60 days. Xu et al. (2008) utilized PEG-PLA diblock copolymer electrospun nanofibers to encapsulate doxorubicin hydrochloride in a core–sheath structure. The drug release behavior showed a diffusion-controlled mechanism and obeyed Fick's second law. Recently, many studies have focused on encapsulating drugs in blended polymer-based electrospun fibers, as the property of the fiberous material and drug release behavior could be tailored by adjusting the blend ratio of polymers (Kim et al., 2007; Yang et al., 2007; Kenawy et al., 2009).

Poly(ε-caprolactone) (PCL) and poly(trimethylene carbonate (PTMC) are both biodegradable and biocompatible polymers but with different biodegradation rates and different biomedical applications. The synthetic copolymer, P(εCL-TMC), has been investigated as a biomaterial for use in surgery and neuronal repairs. This is in part due to its high biocompatibility and the advantages of controllable mechanical property and degradation rate (Fabre et al., 2001; Jia et al., 2005). Thus PCL and PTMC polymers possess considerable potential for many biomedical applications. However to date, nanofibers have not been electrospun with the blend of PCL

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doi:10.1016/j.ijpharm.2009.07.027

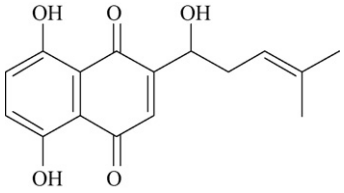

Fig. 1. Chemical structure of shikonin.

and PTMC, and there are no reports that address the application of their blended fibers as biomaterials.

Shikonin (Fig. 1) is a naturally occurring component found abundant in the root of the species of boraginaceae family, including *Lithospermum erythrorhizon* Sieb. Et Zucc., *Alkanna tinctoria*, *Arnebia euchroma* (Royle) Johnst, and *Arnebia guttata* Bunge. It is well known for its dyeing property as well as a wide spectrum of bioactivities, such as anti-tumor (Singh et al., 2003a,b), antioxidant (Han et al., 2008), antibacterial (Shen et al., 2002) and anti-inflammatory (Singh et al., 2003a,b) properties. Moreover, it has been shown that shikonin could enhance the proliferation of granulation tissue induced by cotton pellet in rats (Ozaki et al., 1998). Another study demonstrated that the incorporation of shikonin into microcapsules enhanced its hydrophilicity and stability (Assimopoulou and Papageorgiou, 2004).

In this investigation, the blend of PCL and PTMC was used to fabricate electrospun nanofibers and employed as a drug-carrier to encapsulate shikonin for the first time. The release behaviors of the drug from the fibers were examined using various formulation parameters such as polymer blend ratio and drug-loading content. The antioxidant and antibacterial activities of the resulting fiber mats were also studied for their biomedical applications.

2. Materials and methods

2.1. Materials

PCL (Mw ~ 100,000) and PTMC (Mw ~ 100,000) was provided by Minghe Functional Polymer Co., Ltd. (Qingdao, China). Shikonin was isolated and purified using the procedures previously reported (Han et al., 2008). The chemical structure of shikonin was confirmed by thin-layer chromatography (TLC), ^{1}H NMR, ^{13}C NMR and mass spectrometric techniques. The purity of the compound was greater than 98% based on the percentage of total peak area by HPLC analysis. Dichloromethane (DCM) and N,N-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical quality and used without further purification.

2.2. Nanofiber fabrication

The co-dissolving method was used to formulate the spinning dope. Different blend ratios of PCL and PTMC (9:1, 7:3 and 5:5, w/w) were dissolved in a mixture of DCM/DMF (3:1, v/v) to prepare the blank electrospinning solutions at a concentration of 8 wt.%. Shikonin-loaded PCL/PTMC solutions were obtained by dissolving shikonin in the amount of 1 and 5 wt.% based on the weight of PCL/PTMC powders in the solutions. Prior to electrospinning, the solutions were stirred for 2 h and then degassed with a ultrasonator (59 Hz, 350 W, Shanghai Jinghong Instrument Co., Ltd., Shanghai, China) for 30 min to obtain the homogeneous co-dissolved spinning dopes. The drug-free and shikonin-loaded PCL/PTMC solutions were carefully placed into a 5 mL syringe which included a metallic needle with an internal diameter of 0.5 mm. Electrospinning was carried out under a fixed electric field of 14 kV/18 cm. The solution feeding rate was controlled at 0.8 mL/h by means of a single syringe pump (Cole-Parmer®, USA). All experiments were carried out at room temperature and relative humidity of 40%. The resultant fibers were further dried for 24 h at ambient temperature in a vacuum drying oven (320 Pa, Shanghai Laboratory Instrument Work Co. Ltd., Shanghai, China) to remove the residual organic solvent and moisture.

2.3. Nanofiber characterization

Morphology of the nanofiber mats was observed using scanning electron microscope (SEM; JSM-5600LV, JEOL) at an accelerated voltage of 10 kV. A ^{1}H NMR spectrometer (Bruker DRX 400 MHz NMR spectrometer, Germany) was used to investigate the chemical stability of the loaded drug, using $CDCl_3$ as solvent.

2.4. In vitro drug release

2.4.1. Drug-loading determination

The actual content of shikonin in the fibers was quantified by dissolving each sample in a mixture of DCM/DMF (3:1, v/v) and measuring them in a UV–vis spectrophotometer (UV-2102PC Unico, Shanghai, China) at 516 nm. The amount of shikonin in the fibers was back-calculated from the obtained data against a predetermined calibration curve for the drug. Drug-loading content (DLC) was defined as follows:

$$\text{DLC}\ (\%) = \frac{\text{actual content of shikonin (mg)}}{\text{fiber sample weight (mg)}} \times 100 \quad (1)$$

The theoretical drug-loading content (TDLC) was calculated:

$$\text{TDLC}\ (\%) \frac{\text{added amount of shikonin (mg)}}{\text{added amount of shikonin and polymer (mg)}} \times 100 \quad (2)$$

The drug-loading efficacy (DLE) could be determined:

$$\text{DLE}\ (\%) = \frac{\text{DLC}}{\text{TDLC}} \times 100 \quad (3)$$

2.4.2. Shikonin release assay

A piece of drug-containing fiber mat (20–30 mg) was first placed in a vial filled with 20 mL of release medium solution; shikonin release studies were carried out at 37 °C and 100 rotation/min (rpm) in a thermostatical shaking incubator (85-2, Shanghai Minhang Hongpu Instrument Co., Ltd., Shanghai, China). Due to the solubility limitation of shikonin in aqueous solutions, the releasing medium was prepared by adding 1 vol.% of Tween 80 and 20 vol.% of methanol in the PBS solution as previously described (Taepaiboon et al., 2007) with minor modifications. In this case, 1.5 mL of samples was taken from the medium after 0, 0.5, 1, 3, 5, 8, 11, 24 and 48 h, and then the same volume of fresh release medium solution was added as replacement. The content of shikonin in the medium was determined by a UV–vis spectrophotometer at the wavelength of 516 nm. The results were presented in term of cumulative release as a function of release time:

$$\text{Cumulative amount of release}\ (\%) = \frac{M_t}{M_\infty} \times 100 \quad (4)$$

where M_t was the amount of shikonin released at time t, the actual amount of shikonin found in electrospun fibers was regarded as M_∞ in this paper. Three samples were tested for each electrospun fiber mats and the results were reported as average values.

2.5. Antioxidant activity

The antioxidant activity of shikonin before and after electrospinning was measured with 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radicals using the method previously reported (Han et al., 2008).

Briefly, 10 mg of 5 wt.% shikonin-loaded fibers were first dissolved in 5 mL of DCM/DMF (3:1, v/v) and 0.2 mL was added to 2.8 mL of 1×10^{-4} M methanol solution of DPPH•. The absorbance at 517 nm was measured after solution had been allowed to stand in dark for 60 min. The corresponding amount of shikonin in as-loaded PCL/PTMC solution (i.e., before electrospinning) was also assayed for comparison purpose. Lower absorbance of the reaction mixture indicates higher DPPH scavenging activity. DPPH• scavenging activity was calculated using the formula:

$$\text{DPPH}^{\bullet}\ \text{scavenging activity}\ (\%) = \left[\frac{1-(S-SB)}{C-CB}\right] \times 100 \qquad (5)$$

where *S*, *SB*, *C* and *CB* were the absorbance of sample, blank sample, control and blank control, respectively.

2.6. Antibacterial activity

The antibacterial activity of the electrospun fiber mats against two typical bacteria commonly found on burn wounds: *Escherichia coli* (*E. coli*, Gram-negative) and *Staphylococcus aureus* (*S. aureus*, Gram-positive) were investigated. The assessment was conducted based on the disc agar diffusion method (Rujitanaroj et al., 2008). A 100 μL aliquot of bacteria reconstituted in nutrient broth and previously subcultured was spread onto an agar plate. Both the drug-free and shikonin-containing PCL/PTMC fiber mats were cut into circular discs (15 mm in diameter) and placed on the top of the agar plate. The plates were inverted and incubated at 37 °C for 24 h. If inhibitory concentrations were reached, there would be no growth of the microbes, which could be seen as a clear zone around the disc specimens. The zone was then recorded as an indication of inhibition against the microbial species. Duplicate experiments were conducted and the results were reported as average values.

3. Results and discussion

3.1. Morphology of drug-free and shikonin-loaded PCL/PTMC fiber mats

Fig. 2 shows the SEM morphologies of electrospun fiberous mats. They possess the common features of being round-shaped, randomly arrayed and highly porous. PCL and PTMC were well blended in the fiber. Both the drug-free and the shikonin-loaded PCL/PTMC fibers appeared smooth and there no drug crystals were detected on the polymer surface. This suggested that shikonin was dispersed homogeneously in the electrospun fibers. Furthermore it seemed that incorporation of the drug in the PCL/PTMC solutions did not affect the morphology of the resulting fibers. However, it should be noted that the fiber morphology was significantly affected by the PCL/PTMC blend ratios. Average diameter of 302 ± 109, 266 ± 101, and 203 ± 77 nm were obtained for drug-free and shikonin-loaded fibers with PCL/PTMC mass ratio of 9:1, 7:3 and 5:5, respectively. It was found that increasing the amount of PTMC greatly decreased the fiber diameter and the diameter distribution. In the PCL/PTMC 9:1 fibers (Fig. 2a and d), some stump-like structures were observed in the white circles, reflecting the fragility of the polymer fibers at this blending ratio. When the content of PTMC was raised to 30% (PCL/PTMC 7:3), fewer stump-like structures were noted and the fibers appeared more continuous and smoother (Fig. 2b and e). The most consistent fiber morphology was obtained when PCL and PTMC were equally blended (PCL/PTMC 5:5) (Fig. 2c and f). This could be related to the overall property of the polymer solution. That included viscosity, surface tension and conductivity. Increasing the content of PTMC could assist in achieving thinner and more uniform medicated fibers in this study, probably due to the increased suitability of the polymer solution used for electrospinning.

3.2. Chemical stability of shikonin

The stability of shikonin during processing is crucial for its effectiveness as a pharmaceutical agent. It has been reported that shikonin is relatively stable against heat and light at acidic pH (Cho et al., 1999). However the stability of shikonin under a high electrical potential as used here in electrospinning is yet unknown. To verify this, shikonin-loaded PCL/PTMC electrospun fibers were dissolved in $CDCl_3$ and the resulting solutions were investigated by ^{1}H NMR. Solutions of both the drug-free PCL/PTMC electrospun fibers and shikonin in $CDCl_3$ were used as references. Fig. 3 shows ^{1}H NMR spectrum of the drug, drug-free PCL/PTMC fibers and the drug-loaded fibers that were obtained from a solution containing 5 wt.% of shikonin. Evidently, the chemical integrity of the as-loaded-shikonin was sustained after the electrospinning process, as the peaks corresponding to both PCL/PTMC and shikonin

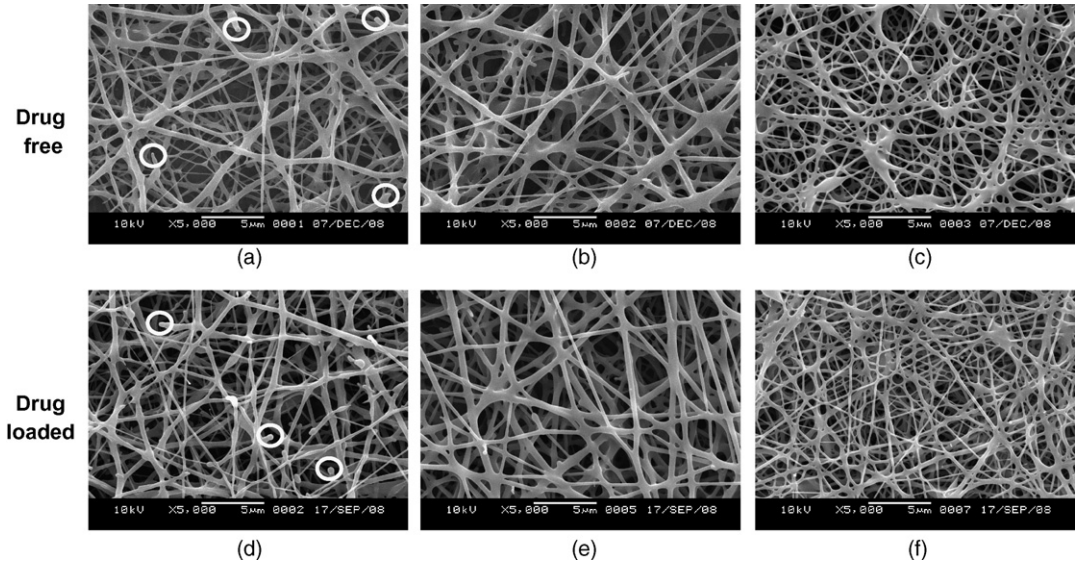

Fig. 2. SEM morphologies of drug-free and 5 wt.% shikonin-loaded electrospun PCL/PTMC fibers with various PCL/PTMC blend ratios: (a and d) PCL/PTMC 9:1; (b and e) PCL/PTMC 7:3; (c and f) PCL/PTMC 5:5.

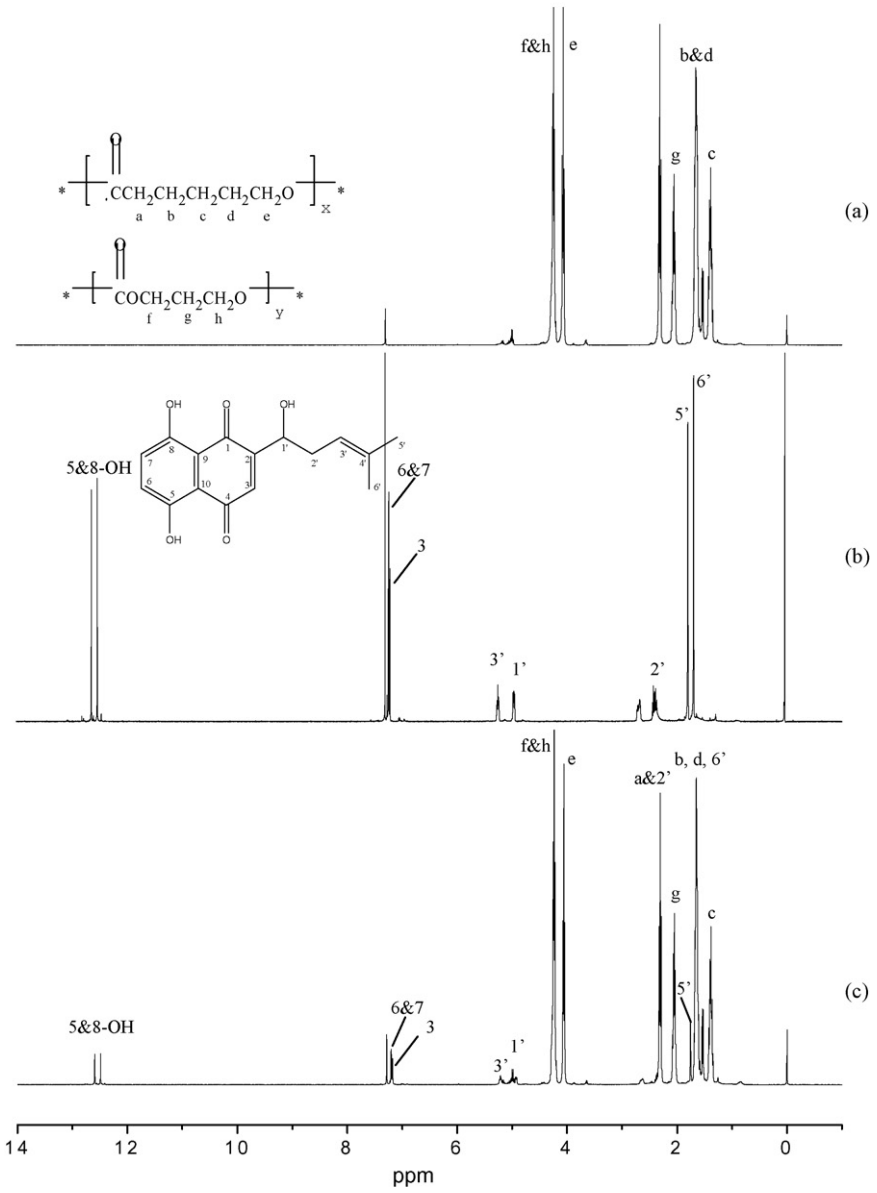

Fig. 3. ^{1}H NMR spectra of: (a) drug-free electrospun PCL/PTMC fibers; (b) shikonin; (c) shikonin-loaded electrospun PCL/PTMC fibers.

could be observed in the ^{1}H NMR spectrum of the drug-loaded fiber mats.

3.3. Drug-loading determination

In order to control the amount of drug-loading within the fibers and determine the drug release characteristic from the fiber matrix, it appears necessary to investigate the actual drug content in the resulted fibers. It can be observed from Table 1 that high drug-loading efficiency was successfully achieved. The actual amount of shikonin in the drug-loaded fiber mats was determined to be more than 93% and this increased with the increasing of initial shikonin added. Apart from the even surface of the shikonin-loaded fibers (Fig. 2c–f), this result further proved that shikonin was well encapsulated within the fibers. This could be due to the high solubility of shikonin in the polymer (PCL/PTMC)/solvent (DCM/DMF) solution. Therefore shikonin appeared to have good compatibility with the matrix/solvent system. When the solution jet was rapidly elongated and the solvent evaporated quickly, phase-separation was difficult to achieve, as the drug tended to remain inside the fiber where sufficient solvent remained (Zeng et al., 2005b). Thus, when the fiber became dry, shikonin could be totally encapsulated.

Table 1
Actual amount of shikonin in the electrospun PCL/PTMC fiber mats.

Initial amount of shikonin in PCL/PTMC fibers (wt.%)	Actual amount of shikonin based on the initial amount of shikonin-loaded	
	DLC (%)	DLE (%)
1.056	0.984 ± 0.032	93.2 ± 3.0
5.024	4.858 ± 0.171	96.7 ± 3.4

3.4. In vitro drug release studies

Shikonin release profiles from electropun fiber mats with different PCL/PTMC blend ratios and drug-loading contents were

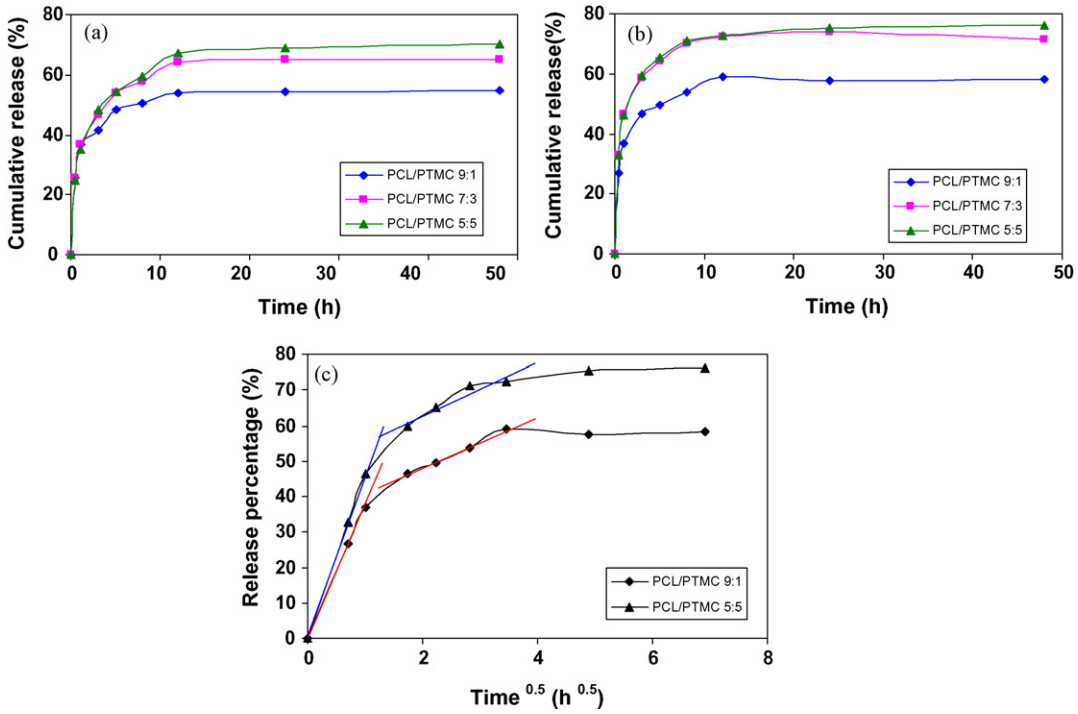

Fig. 4. The shikonin release profiles from the electrospun PCL/PTMC fibers with various PCL/PTMC blend ratios: (a) 1 wt.% shikonin-loaded; (b) 5 wt.% shikonin-loaded; (c) the corresponding release curves in (b) re-plotted against square root of time.

demonstrated in Fig. 4a and b. Generally the drug release curves were similar in that: an initial fast release was shown followed by a gradual release during the rest time. It was found that shikonin was released more rapidly when the amount of PTMC increased in the fibers. For the 9/1 blend fiber mats, about 55% and 58% were released from the 1 and 5 wt.% shikonin-loaded fibers after 48 h release, respectively. While for the 5/5 blend fiber mats, the release of shikonin could reach to 70% and 76% during the same period. These results could be correlated to the average diameter of the electrospun fiber mats. It has been mentioned above that the average fiber diameter was 302, 266 and 203 nm for 9:1, 7:3 and 5:5 PCL/PTMC blend fiber, respectively. Since the area-to-volume ratio of fiber is dependent on the fiber diameter, the narrower the fiber diameter, the larger the total surface area will be for the fiber mats with the same mass. Thus, more shikonin would be able to diffuse from the fiber matrix with small diameter (PCL/PTMC 5:5) in the same release time and this also resulted in a fast release rate. On the other hand, Fig. 5a and b demonstrated that after 48 h release, no significant change occurred and fiberous structure were still remained for 9:1 and 7:3 PCL/PTMC blends. This probably due to a higher PCL percent prevented the blend fibers from swelling to some extent. Whereas for the 5:5 PCL/PTMC blend (Fig. 5c), it was apparent that fibers were swollen and dissolved, and thus enable the sample to be in a porous-film state. This could be due to that amorphous PTMC is more hydrophilic than the semi-crystallized PCL. Thus higher the PTMC content in the fibers results in increasing water-absorption and so the fibers easily dissolved in the release medium. This phenomenon would also account for 5/5 blend fiber releasing more shikonin than the other two polymer materials in this study.

To further assess the mechanism of drug release, the release fraction of shikonin (M_t/M_∞) was re-plotted against the square root of time based on the *Higuchi* model (Wang et al., 2008). According to this model, if a drug release is controlled by a diffusion mechanism, the plot should show a linear arrangement (Suwantong et al., 2007; Xu et al., 2008). As demonstrated in Fig. 4c, the drug release profiles consisted of three sequential stages. Linear relationships ($R^2 > 0.928$) between M_t/M_∞ and $t^{0.5}$ were obtained for both the first and the second stages. The slope of the straight lines for the fist stage was steeper than that for the second stage. This is probably because drug molecules dispersing close to the surface of polymer fibers and adsorbing or loosely binding near the surface, would diffuse out quickly in initial release time. The slower release for the second phase may be attributed to the drug being encapsulated in the inner core of the fiber matrix, which would definitely need a long distance to diffuse through and so take longer time to be released. Further, due to the decline of the diffusion driving force induced by the reduction of the drug molecules inside the inner

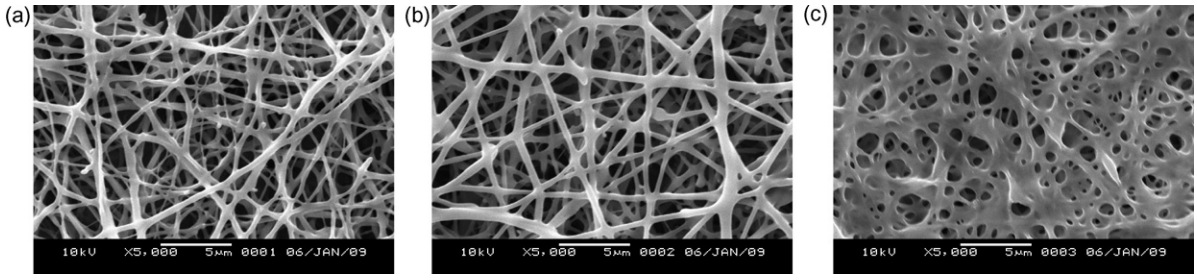

Fig. 5. SEM morphologies of shikonin-loaded electrospun PCL/PTMC fibers after drug release of 48 h. (a) PCL/PTMC 9:1; (b) PCL/PTMC 7:3; (c) PCL/PTMC 5:5.

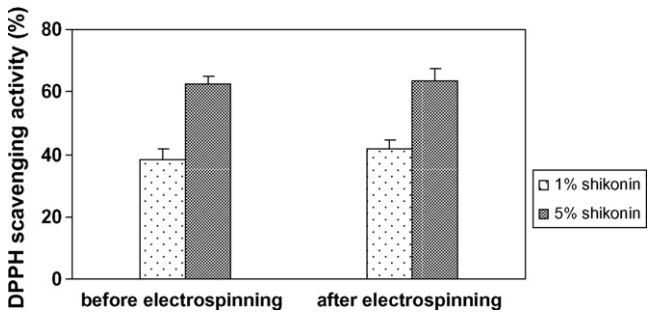

Fig. 6. Antioxidant activity of loaded-shikonin before and after electrospinning. Vertical bars represent the standard deviation for each dada point ($n = 2$).

space, the release rate became slower and slower as showed in the third phase. Moreover, it also could be found from Fig. 4a and b that the release rate of shikonin increased with the increasing drug content in the fibers. According to previous reports (Wang et al., 2008; Xu et al., 2009), the obtained results suggested that shikonin was distributed throughout the whole PCL/PTMC fibers and formed a matrices-type drug release system.

3.5. Antioxidant and antibacterial activity

The antioxidant activity of the loaded-shikonin in the electrospun fibers before and after electrospinning was investigated using DPPH• assay. DPPH• is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant (Lu and Yeap Foo, 2001). As indicated in Fig. 6, the antioxidant activity of shikonin before and after electrospinning was 38%, 62% and 42%, 64% for 1 and 5 wt.% shikonin-loaded, respectively. The result showed that there was no significant difference for DPPH• scavenging activity of shikonin after the electrospinning treatment and confirmed that the drug material could still retain antioxidant activity, even after it had been subjected to a high electrical potential during the fiber producing process.

The antibacterial activity of shikonin-loaded PCL/PTMC fiber mats were assessed against two typical pathogenic bacteria *E. coli* and *S. aureus* to evaluate their biomedical application potentials. The activity of drug-free PCL/PTMC fiber mats against these bacteria was used as control. As reported in Table 2, after 24 h of contact intervals, the 5 wt.% shikonin-loaded specimen gave a 21.3-mm diameter zone for *S. aureus* and a 16.9-mm for *E. coli* (the diameter of specimen is 15 mm), respectively. Moreover there was a complete lack of growth beneath the test fiber mats. Previous studies have indicated that shikonin was an effective antibacterial agent (Tabata et al., 1982; Shen et al., 2002). Sekine et al. (1998) reported that the antimicrobial activities of shikonin were related to the formation of semiquinone radicals and that these radicals may exhibit the antimicrobial activities *via* the generation of endogenous superoxide anion radical ($O_2^{\bullet -}$) (Kalyanaraman et al., 1980). In addition, it should be noted that the *S. aureus* was more sensitive to shikonin than *E. coli* in our experiment. This is consistent with some reports (Brigham et al., 1999; Shen et al., 2002; Dhandapani and Sarkar, 2007) and could be explained as: (i) the selective inhibition of shikonin to some bacterial strains (Brigham et al., 1999); and/or (ii) the different cell wall structures of the bacteria. A major structural difference between Gram-negative bacteria and Gram-positive bacteria is that the cell wall of the former is overlaid with an outer membrane that consists of lipopolysaccharide, and this region offers a supplementary barrier that inhibits the penetration of antimicrobial agents (Tan and Obendorf, 2007).

Table 2
Antibacterial activity of shikonin-loaded electrospun PCL/PTMC fiber mats.

Shikonin loading content	Mean zone of inhibition (mm)/sample diameter (mm)	
	Staphylococcus aureus	*Escherichia coli*
Control	15/15	15/15
1 wt.%	19.6/15	16.6/15
5 wt.%	21.3/15	16.9/15

4. Conclusions

In this present study, a herbal medicine shikonin regarded as an effective compound associated with healing of wounds, scar, and burns, was successfully encapsulated in PCL/PTMC nanofibers using electrospinning for the first time. Shikonin was homogenous distributed throughout the fibers forming a matrices-type drug loaded system. The fiber diameter was found to be dependant on the content of PTMC, with increasing PTMC content leading to a decrease in fiber diameter. A sustained release of shikonin could be achieved over 48 h with the release diffusionally controlled. In the whole release period, shikonin release rates increased with the increasing PTMC concentration and drug-loading content in the fibers. Furthermore, the loaded-shikonin retained its biological functionality even after it had been subjected to a high electrical voltage, indicating that the medicated fibers developed by our system would have the great potential in drug delivery, wound healing as well as promising materials for treating surfaces that contain pathogenic microorganisms.

Acknowledgments

This work was financially supported by UK-CHINA Joint Laboratory for Therapeutic Textiles based in Donghua University, in part by Biomedical Textile Materials “111 Project” from Ministry of Education of China (No. B07024).

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