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Research paper

Ultrafine PEG–PLA fibers loaded with both paclitaxel and doxorubicin hydrochloride and their in vitro cytotoxicity

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ABSTRACT

By means of "emulsion-electrospinning", both hydrophobic and hydrophilic drugs, paclitaxel (PTX) and doxorubicin hydrochloride (DOX), were successfully loaded into PEG–PLA nanofiber mats to realize multi-drug delivery. The release behaviors of both the drugs from the same fiber mats were ascribed to their solubility properties and distribution status in the fibers. Due to its high hydrophilicity, DOX was easy to diffuse out from the fibers, and its release rate was always faster than that of hydrophobic PTX. Moreover, the release rate of PTX was accelerated by DOX's release from the same drug-loaded fibers. In vitro cytotoxicity against rat Glioma C6 cells indicated that the dual drug combination showed a higher inhibition and apoptosis against C6 cells than a single drug-loaded system, which suggests the promise for multi-drug delivery on combination therapy.

1. Introduction

In clinical treatment, patients suffering from cancer take antitumor drugs orally or by systematic injection. Although drugs can be delivered to the site of cancer, the amount of delivered drug decreases against initial drug dose because drugs also spread to the healthy site. Therefore, patients need to take excessive amount of drugs, which may induce undesirable severe side effects. It is desirable that minimized amount of drug is locally delivered to the disease site and effectively absorbed at the disease site. Locally controlled release systems have numerous advantages compared to conventional dosage forms, such as improved therapeutic effect, reduced toxicity, reduced frequency of drug administrations, and convenience [1–4].

Generally, the rate of drug uptake by human body rises with the decrease in the size of drug and its carrier, because of the increasing surface area of the drug or carrier [4]. Hence, drug delivery systems have been developed using polymeric materials in the form of nano or microparticles, hydrogels and micelles [4–7]. Recently, drug-loaded electrospun fibers with higher drug encapsulation efficiency and better stability than other drug formulations have attracted a great deal of attention, because they have a very strong drug efficacy due to their high surface area-to-volume ratio, and the composite electrospun nanofiber webs afforded the prospect of preparing useful polymer systems for controlled release of drugs with bioactivity [7–10].

Paclitaxel (PTX) and doxorubicin hydrochloride (DOX) are among the most common anticancer drugs in clinical use nowadays, due to their significant anticancer activity against various solid tumors [11,12]. PTX is highly hydrophobic and it can interact with tubulin dimers in the G_2 phase of cell mitotic cycle to promote microtubule polymerization resulting in the formation of highly stable microtubules, and thus preventing cell division [13–15]. DOX is water soluble and it can interfere with the growth of cancer cells by intercalating into the DNA strands, inhibiting further DNA and RNA biosynthesis, and eventually causing cell death [16–18]. In principle, cytotoxicity can be maximized if the two anticancer drugs with distinct characteristics and action mechanisms can be delivered simultaneously to the same cell [11]. Recent clinical studies have shown that the drug formulations which combine DOX with PTX increase tumor regression rate relative to the individual drugs [11,19–22]. Therefore, here we load water soluble DOX and hydrophobic PTX into biodegradable electrospun fibers to realize multi-drug delivery for combination therapy.

It is generally accepted that a single carrier for both the drugs should better ensure that both the drugs target the same cell and maximize cytotoxicity while minimizing the chances of cell resistance to any one of them [11]. However, successful incorporation and sustained release of the drugs having distinct properties such as solubility generally need multiple carriers or solvents, limiting the likelihood of simultaneous delivery [11]. "Emulsion-electrospinning" is a novel process to incorporate a hydrophilic drug into hydrophobic polymer fibers [23–25]. Here, we suppose that if a

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hydrophobic drug is also dissolved into the oily phase to prepare a water-in-oil (W/O) emulsion, in which the aqueous phase contains a hydrophilic drug, thus an emulsion containing both drugs with different solubility will be obtained. After the composite W/O emulsion is electrospun, both the drugs can be incorporated into the same hydrophobic polymer fibers.

Therefore, in this study, amphiphilic poly(ethylene glycol)–poly(L-lactic acid) (PEG–PLA) diblock copolymer and "emulsion-electrospinning" method were chosen to realize perfect incorporation and controlled release of PTX and DOX. Ultrafine PEG–PLA fibers containing both the drugs were successfully electrospun from W/O emulsions, in which the aqueous phase contained DOX, and the oily phase was a chloroform solution of PEG–PLA and PTX. In vitro release profiles and cytotoxicity of the medicated PEG–PLA fibers were investigated in detail.

2. Experimental section

2.1. Materials

PTX of 99% purity was purchased from Bio-Sep Bio-technique Co. Ltd., Xi'an Jiaotong University. DOX was a gift from Zhejiang Hisun Pharmaceutical Co. Ltd. Triethyl benzyl ammonium chloride (TEBAC), propidium iodide (PI), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were obtained from Sigma and were used without further purification. Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY). Tris–hydroxymethylaminomethane (Tris–base) was supplied by Merck, and was used without further purification to prepare Tris–HCl buffer solution of pH = 8.6. Proteinase K and sodium azide were purchased from Sigma.

Diblock copolymer PEG–PLA (prepared from PEG750 and L-lactide) was synthesized in our laboratory. In a typical preparation process, L-lactide was polymerized at 125 °C with 0.025 wt% $Sn(Oct)_2$ as a catalyst and in the presence of PEG as a macromolecular initiator under argon atmosphere and anhydrous condition for 50 h. Then the polymerization product was dissolved in chloroform, precipitated into anhydrous alcohol, and finally dried in vacuum at 60 °C for 48 h to get purified PEG–PLA diblock copolymer. Its molecular weight and polydispersity (Mn/PD) determined by GPC were 84,800 and 1.36, respectively.

2.2. Preparation of W/O emulsions containing PTX and DOX

PEG–PLA was dissolved in chloroform to prepare a 6 wt% solution. Five weight percentage of TEBAC with respect to the polymer used was added into the polymer solution as an emulsifier. Then 0.5 and 1.0 wt% of PTX with respect to PEG–PLA were dissolved into the organic solution, respectively, forming homogeneous and transparent PTX/PEG–PLA/chloroform solutions.

Aqueous solutions (2 ml) of 0.5 and 1.0 wt% DOX with respect to PEG–PLA solid used were slowly dropped into the above-mentioned solutions (25 ml), correspondingly, and were emulsified at a rotating rate of around 6500 r/min for about 15–20 min. Ultimately, stable and homogenous W/O emulsions with PTX and DOX in their oily and aqueous phases, respectively, were obtained. The total drug contents in the emulsions were 1.0 wt% (0.5 wt% DOX + 0.5 wt% PTX) and 2.0 wt% (1.0 wt% DOX + 1.0 wt% PTX), respectively.

Two control samples were prepared as follows: (1) Under the same operation conditions, 2 ml aqueous solutions of 0.5, 1.0 and 2.0 wt% of DOX with respect to the polymer carrier were emulsified into 25 ml of 6 wt% chloroform solution of PEG–PLA, respectively, forming the W/O emulsions only containing DOX. (2) 0.5, 1.0 and 2.0 wt% of PTX with respect to the polymer used were added into 7.5 wt% chloroform solution of PEG–PLA. 5 wt% of TEBAC (with respect to PEG–PLA) was added into the PTX/PEG–PLA/$CHCl_3$ solutions as a surfactant to improve the electrospinning process.

All the resultant emulsions were exposed to ultrasonic treatment for several minutes before electrospinning in order to (a) remove the air bubbles which possibly formed during emulsification process and (b) make the emulsions more stable.

2.3. Electrospinning

The W/O emulsions containing DOX or DOX/PTX and the PTX/PEG–PLA/chloroform control solutions were electrospun using conventional electrospinning setup depicted in reference [26]. In this study, typical electrospinning parameters were as follows: electric field strength: 2.53.0 kV/cm; air gap distance: 20 cm; inner diameter of spinneret: 0.4 mm; and flow rate of solution: 26 ml/h. The electrospinning parameters were all kept constant, and all the experiments were conducted at room temperature in air. In order to remove the residual chloroform and water, the fiber mats collected were vacuum-dried at room temperature for about 48 h.

2.4. Morphology analysis

The morphology of the medicated electrospun fibers was observed with an environmental scanning electron microscope (ESEM, Model XL 30 ESEM FEG from Micro-FEI Philips), and its accelerating voltage was 20 kV. Samples were mounted on metal stubs using a double-sided adhesive tape and vacuum-coated with a platinum layer prior to examination.

2.5. In vitro drug release

A piece of drug-containing fiber mat (20–30 mg) was placed in a vial filled with 20 ml of 0.05 mol/L Tris–HCl buffer solution (pH = 8.6) containing 4 μg/ml of proteinase K. In the case of control test, no proteinase K was added. The vial was incubated at 37 °C in a thermostated shaker. At appropriate intervals, the mat was transferred to another 20 ml of fresh buffer solution for further release test, and the released DOX in the original buffer solution was monitored by a UV–visible spectrophotometer at the wavelength of 483.5 nm. The UV absorbance of DOX detected was converted to its concentration according to the calibration curve of DOX in the same buffer. Then, the accumulative weight and relative percentage of the released DOX were calculated as a function of incubation time.

To determine the released amount of PTX, the release solution in the sample cell for DOX detection was added back into the -original release solution, and HPLC (high pressure liquid chromatography) was performed as follows. Two milliliters of dichloromethane was added to the release solution to extract PTX. The extraction was repeated three times, thus 6 ml extracted solution was obtained. Then, 2 ml acetonitrile–water (50:50, v/v) mixture was added to the 6 ml of extractant solution. A nitrogen stream was introduced to evaporate the dichloromethane until a clear solution was obtained. Finally, the resultant solution was analyzed by HPLC. The accumulative percentage of the released PTX was calculated as a function of incubation time.

The HPLC was conducted by using a Waters HPLC system equipped with a column (Symmetry Shield™ RP18, $\varphi 3.9 \times 150$ mm, Waters), a UV-2487 UV-detector (Waters) and an LC-10AT pump (Shimadzu). The mobile phase was a mixture of acetonitrile and water (50:50, v/v), the flow rate was 1.0 ml/min, and the detection wavelength was 227 nm.

Correction of the extraction efficiency was needed for calculating the cumulative released percentage of PTX. The recovery efficiency of the extraction procedure was determined as follows.

Known mass of PTX-loaded fiber mat was fully degraded by proteinase K in 20 ml of the buffer solution, and then was extracted three times by 2 ml dichloromethane. Afterwards, 2 ml of acetonitrile–water (50:50, v/v) was added. The same procedure as described above was carried out. The determined extraction efficiency was 80.0%, which means that 80.0% of the original amount of PTX could be detected. The released amount of PTX was corrected accordingly.

Drug loading efficiencies of both the drugs were calculated using the formula:

$$\text{Drug loading efficiency} = (A - B)/A \times 100\%$$

where A stands for the drug contents in the electrospinning emulsion with respect to PEG–PLA and B for the drug content in the electrospun fibers. B was determined in the same detection procedure as mentioned above, after three original known masses of drug-loaded fiber mats were degraded thoroughly in the Tris–HCl buffer solution in the presence of proteinase K. Drug loading efficiency of each drug was given as the average of three fiber mats.

2.6. In vitro cytotoxicity of drug-loaded fiber mats

2.6.1. MTT assay

The PEG–PLA fiber mats and drug-loaded PEG–PLA fiber mats were disinfected by ^{60}Co γ-ray at a dosage of 15 kGy.

Rat Glioma C6 cells were grown in DMEM containing 7 g/L $NaHCO_3$, 100 U/ml penicillin, and 10% calf serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, dissociated with 0.25% trypsin in PBS (pH 7.4) and centrifuged at 1000 r/min for 5 min at room temperature. The cells were collected and dispersed in 20 ml of PBS. Two hundred microliters of the dispersion was used for cell counting in a hemacytometer.

The experimental cells were inoculated to each well of five 96-well plates with a cell density of 2×10^4 cells/well in 200 μl DMEM, and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 24 h to allow the cells to adhere to the bottom of the well. Then the DMEM was renewed, and the fiber samples were added into the wells of each plate in three groups. The five plates were used for five different incubation periods of time.

The first group was control group, and consisted of two subgroups (6 wells for each): test cells sub-group (nothing was added except test cells) and blank fiber sub-group (only PEG–PLA fiber mat was added). The second group was "1.0% group" and it consisted of four sub-groups (a) to (d) (6 wells for each). Following fiber samples were added, respectively: (a) 1.0 wt% PTX/PEG–PLA fiber mat (abbreviated as "1.0% PTX" hereafter), (b) a mixture of 0.5 wt% DOX/PEG–PLA fiber mat ("0.5% DOX") and 0.5 wt% PTX/PEG–PLA fiber mat ("0.5% PTX"), (c) (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat ("composite of 0.5% DOX + 0.5% PTX"), and (d) 1.0 wt% DOX/PEG–PLA fiber mat ("1.0% DOX"). The total drug contents were all 1.0 wt% with respect to the fiber mass, and were all 80 μg/ml with respect to the culture medium. The third group was 2.0% group, consisting of following four subgroups (6 wells for each): (a) 2.0 wt% PTX/PEG–PLA fiber mat, (b) a mixture of 1.0 wt% DOX/PEG–PLA fiber mat and 1.0 wt% PTX/PEG–PLA fiber mat, (c) (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fiber mat, and (d) 2.0 wt% DOX/PEG–PLA fiber mat. The total drug contents were all 2.0 wt% with respect to the fiber mass, and were all 80 μg/ml with respect to the culture medium.

After incubation for a predetermined time, a plate was withdrawn for morphology observation by a microscope (Eclipse TE-2000-U, Nikon) with an attached camera (digital camera DXM1200F, Nikon), and for MTT assay.

The MTT assay was as follows: 20 μl of MTT solution (5 mg/ml) in PBS (pH 7.4) was added to each well. The incubation was continued for another 4 h, and then the solution was aspirated cautiously from each well. Thereafter, the MTT derivative was dissolved with 100 μl DMSO completely, and the optical density (OD) of the solution was measured with a microplate reader (Multiskan MK3, Thermo Electron Corporation, USA) at 492 nm. The OD values in one sub-group were averaged. The relative cell viability was calculated by dividing the OD value of the test sub-group by that of the "test cells sub-group" in the same plate. All the experiments were performed in triplicate.

2.6.2. Flow cytometry analysis

The samples were divided into the following five groups (6 wells for each): (a) control group: only test cells were added, (b) 1.0 wt% PTX/PEG–PLA fiber mat, (c) a mixture of 0.5 wt% DOX/PEG–PLA fiber mat and 0.5 wt% PTX/PEG–PLA fiber mat, (d) 1.0 wt% DOX/PEG–PLA fiber mat, and (e) (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat. For each drug-loaded group, the total drug loading was 1.0 wt% with respect to the fiber mass, and was 40 μg/ml with respect to the culture medium.

C6 cells were seeded (4×10^5 cells/well) in 6-well plates and cultured in DMEM plus 20% FBS for 24 h, followed by a 24 h cultivation in the culture media. The cells were then treated with the above-mentioned samples for 72 h, respectively. After being dissociated with 0.25% trypsin and washed twice by PBS, the cells were harvested, fixed with ice-cold 70% ethanol and deposited at −20 °C overnight to increase the penetrability of cell membrane. Then the cells were harvested by centrifugation from ice-cold 70% ethanol/cell suspension, re-suspended with 100 μl RNase (0.1 mg/ml) at 37 °C for 30 min and 0.2 ml PI (0.05 mg/ml, containing 0.03% Triton X-100) and incubated at 4 °C in the dark for 30 min, followed by flow cytometric analysis at 492 nm.

3. Results and discussion

3.1. Electrospun PEG–PLA fiber mats containing both PTX and DOX

Fig. 1 shows the ESEM images of (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fibers and (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers, respectively. They looked uniform. Their sur-

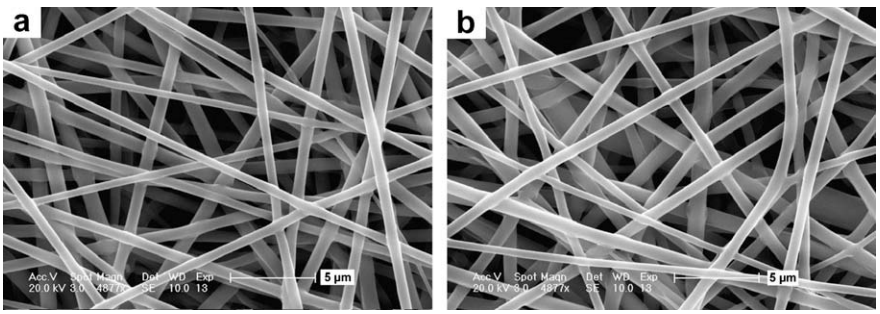

Fig. 1. ESEM photographs of (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fibers (a) and (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers (b).

faces were smooth, and no drug crystals were detected. All these indicated that both the drugs were finely incorporated into the electrospun fibers. Moreover, it seemed that the medicated fibers of different drug contents did not show appreciable difference in morphology and average diameter. Their average diameters were all about 730 nm. Drug loading efficiency was found to be 90.6% for DOX and 97.0% for PTX in both drug-loaded fibers. The residual solvent entrapped in the fiber mats was removed completely by vacuum-drying, and its absence was demonstrated by TGA measurement up to 120 °C (data not shown).

3.2. In vitro drug release

Fig. 2 gives the drug release profiles of (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mats (curves a and d) and (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers (curves b and c) in Tris–HCl buffer solution without proteinase K, respectively. It is seen that DOX (curves a and b) was released much faster than PTX (curves c and d). For example, in the first 11 h, about 75 wt% and 58 wt% of DOX were released from the "0.5% + 0.5%" and "1.0% + 1.0%" fibers, respectively, while only 9 and 12 wt% of PTX were released. This is because the release occurs at the interface of the fiber and the buffer solution, and there are three pairs of interactions among the drug, the fiber matrix and the buffer solution. All of them may affect the diffusion rate of the drug, and thus affect the drug release. Firstly, polymer PEG–PLA has some extent of water affinity because of the PEG block, water molecules may diffuse into the interior of the fibers, resulting in more opportunities for the drug molecules to contact with the water medium. Compared to paclitaxel, DOX is much more hydrophilic and is easier to diffuse into water, leading to a faster release. Interaction between the matrix polymer and the drugs may influence the diffusion and the release of the drugs. For example, wholly speaking, the matrix polymer is hydrophobic and has more affinity with paclitaxel than with DOX. This may be another reason for DOX to release faster than paclitaxel.

Curves a, b, c, and d were re-plotted against square root of time in order to (1) further assess the release mechanisms of DOX and PTX from dual drug-loaded fibers and to (2) compare the release behaviors of both drugs. As shown in curves e and f, the release profiles of DOX from both the fiber mats consisted of diffusion-controlled three sequential stages [27]. In the first 1 h, that is the first stage, DOX diffused out from the fibers in a relative slow rate. Then DOX was released out at a relative faster rate than that in the first stage with an approximately linear relationship between M_t/M_∞ and $t^{1/2}$. After 14 h (the third stage), the release curves of DOX deviated from the linearity, and the rates became slower and slower. This release kinetic was in agreement with that of the reservoir-type drug release system. Moreover, when comparing curve a with curve b, it was found that the release rate of DOX decreased with increasing DOX content in the fibers. Therefore, it is believed that DOX was molecularly distributed at the central region of the fibers during "emulsion-electrospinning", forming a core-sheath structure [27,28]. In the first stage, DOX diffused firstly into the pure PEG–PLA sheath surrounding the DOX-containing core, and then it diffused out through the sheath layer with more and more DOX molecules entering this layer. In the second stage, DOX molecules diffused out in such a way that a molecule behind always followed the pass-way of the one in front. So the release rate of DOX became faster than that in the first stage. The interpretations for DOX's release behavior in the third stage could include (1) the decrease of the total content of DOX in the fibers with release time, (2) the longer distance for the drug molecules located in the center of fibers to diffuse through, and (3) the increase of the crystallinity of PEG–PLA with release time [29].

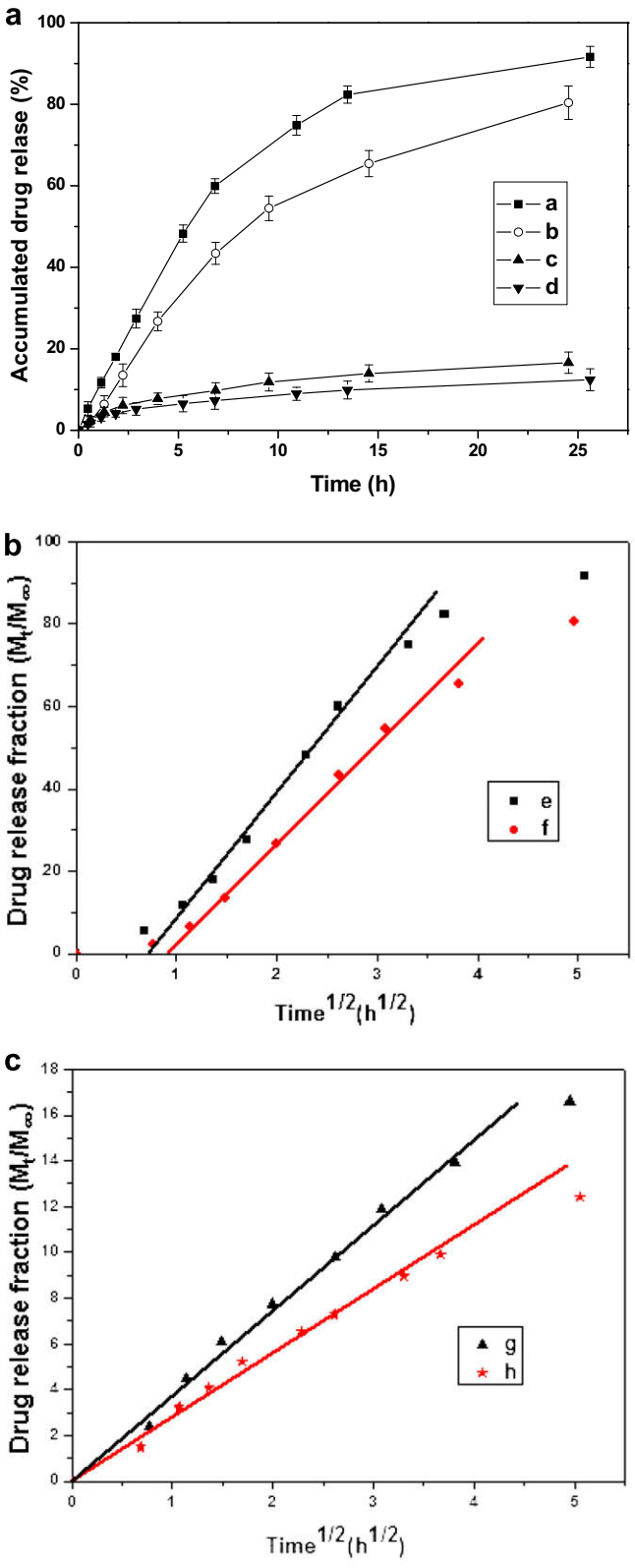

Fig. 2. Release profiles of DOX (curves a and b) and PTX (curves c and d) from (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fibers (curves a and d) and (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers (curves b and c) in 0.05 mol/L Tris–HCl buffer solutions without proteinase K at 37 °C. Curves a, b, c and d were re-plotted against square root of time to get curves e, f, g and h, respectively.

However, comparing curves g and h with curves e and f, it could be seen that the plot of released fraction of PTX vs. $t^{1/2}$ fitted a lin-

ear relationship from the very beginning until 16 h, and in the whole drug release period, the release rate of PTX increased with increasing PTX content in the fibers. These results suggested that PTX was dispersed throughout the whole PEG–PLA fiber, confirming a matrix-type formulation.

Figs. 3(a) and (b) displayed the drug release profiles of (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers in Tris–HCl buffer solution in the presence of proteinase K. The release rate of DOX (curve a) is still faster than that of PTX (curve b). In the first 15 h, about 84 wt% of DOX and 38 wt% of PTX were released, respectively. Comparing Fig. 3(a) with Fig. 2(b), it could be seen that in the presence of proteinase K, the release rate of DOX was accelerated. For example, at 15 h, about 84% and 66% of DOX released from the fibers in the presence and in the absence of proteinase K, respectively. This is because the release profile of DOX was determined by the combination of diffusion and enzymatic degradation in the presence of proteinase K [30].

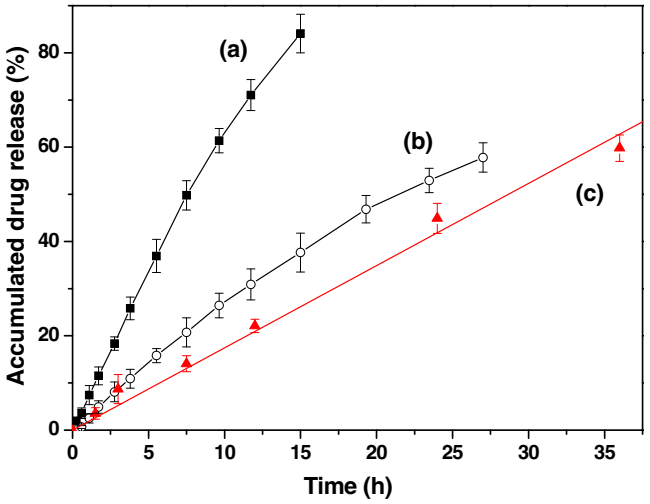

Fig. 3. Release profiles of DOX (a) and PTX (b) from (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fibers, and release profile of PTX from 1.0 wt% PTX/PEG–PLA fibers (c) in 0.05 mol/L Tris–HCl buffer solutions in the present of proteinase K (4 μg/ml) at 37 °C.

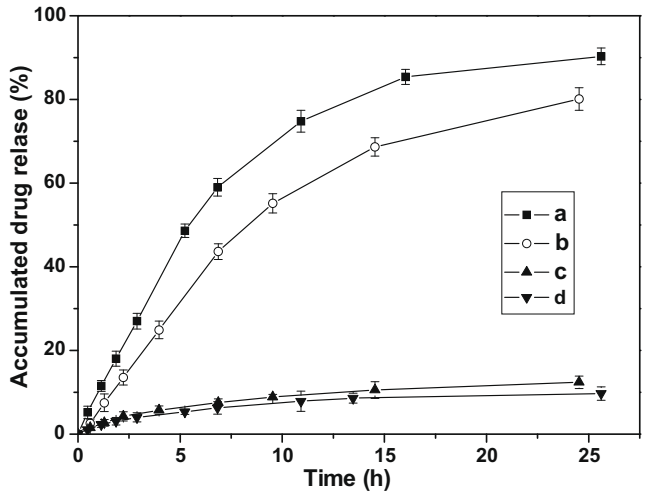

Fig. 4. Release profiles of DOX (curves a and b) and PTX (curves c and d) from 0.5 wt% DOX/PEG–PLA fibers (a), 1.0 wt% DOX/PEG–PLA fibers (b), 1.0 wt% PTX/PEG–PLA fibers (c), and 0.5 wt% PTX/PEG–PLA fibers (d) in 0.05 mol/L Tris–HCl buffer solutions without proteinase K at 37 °C.

Fig. 3(c) shows the release profile of paclitaxel from 1.0 wt% PTX/PEG–PLA fibers in the presence of proteinase K. It is almost a straight line, similar to that in reference [30,31] for paclitaxel/PLLA fibers, implying a polymer-degradation controlled release of paclitaxel. The release rate in curve 3(b) is faster than that in curve 3(c). For example, at 15 h, accumulative release ratio of paclitaxel was 38% in curve 3(b) and 26% in curve 3(c). The sole difference between these two samples was whether DOX was present in the fibers. Therefore, it could be concluded that the release of paclitaxel was accelerated by the release of DOX. Actually, similar results were obtained in the absence of proteinase K by comparing Figs. 4(c) and (d) with Figs. 3(c) and (d). This is explained as follows: many pass-ways and nano-pores were created in the fibers as a result of DOX diffusion and release through them [7], thus the buffer

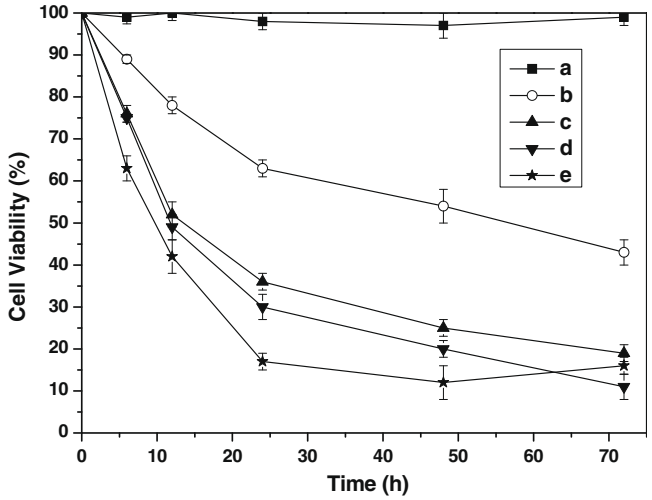

Fig. 5. Cytotoxicity of various drug-loaded fiber mats to the rat Glioma C6 cells vs. time: (a) PEG–PLA blank fiber mat, (b) 1.0 wt% PTX/PEG–PLA fiber mat, (c) mixture of 0.5 wt% DOX/PEG–PLA fiber mat and 0.5 wt% PTX/PEG–PLA fiber mat, (d) (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat, and (e) 1.0 wt% DOX/PEG–PLA fiber mat. The total drug contents in the culture media were kept at 80 μg/ml for all tests.

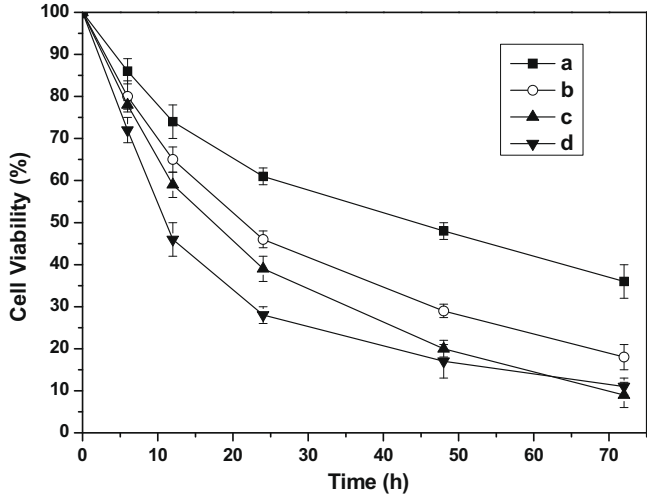

Fig. 6. Cytotoxicity of various drug-loaded fiber mats with 2.0 wt% total drug loadings to the rat Glioma C6 cells vs. time: (a) 2.0 wt% PTX/PEG–PLA fiber mat, (b) mixture of 1.0 wt% DOX/PEG–PLA fiber mat and 1.0 wt% PTX/PEG–PLA fiber mat, (c) (1.0 wt% DOX + 1.0 wt% PTX)/PEG–PLA composite fiber mat, and (d) 2.0 wt% DOX/PEG–PLA fiber mat. The total drug contents in the culture media were kept at 80 μg/ml for all tests.

solution was easier to penetrate into the fibers, accelerating the degradation rate of the fibers and even exposing more paclitaxel molecules to the buffer solution. Therefore, the release rate of PTX increased. In contract, the release of DOX was not affected by the co-existence of paclitaxel, as seen by comparing Figs. 4(a) and (b) with Figs. 2(a) and (b).

The drug release profiles of (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fibers in the same medium were similar to those shown in Fig. 3(a) and (b) (data not shown).

In conclusion, the release behaviors of both drugs were steady and sustained, and DOX's release rate was always higher than PTX no matter in the absence or in the presence of proteinase K. The faster release rate of DOX was due to its better hydrophilicity. The concentration dependence of DOX release and its release behavior provided evidence for its reservoir-type distribution in the fibers. The presence of DOX accelerated the release of PTX, while the presence of PTX did not show appreciable effect on DOX's release.

3.3. Cytotoxicity tests

3.3.1. MTT assay

In this study, the cytotoxicities to rat Glioma C6 cells of various fiber mats were examined. For the sake of comparison, the total

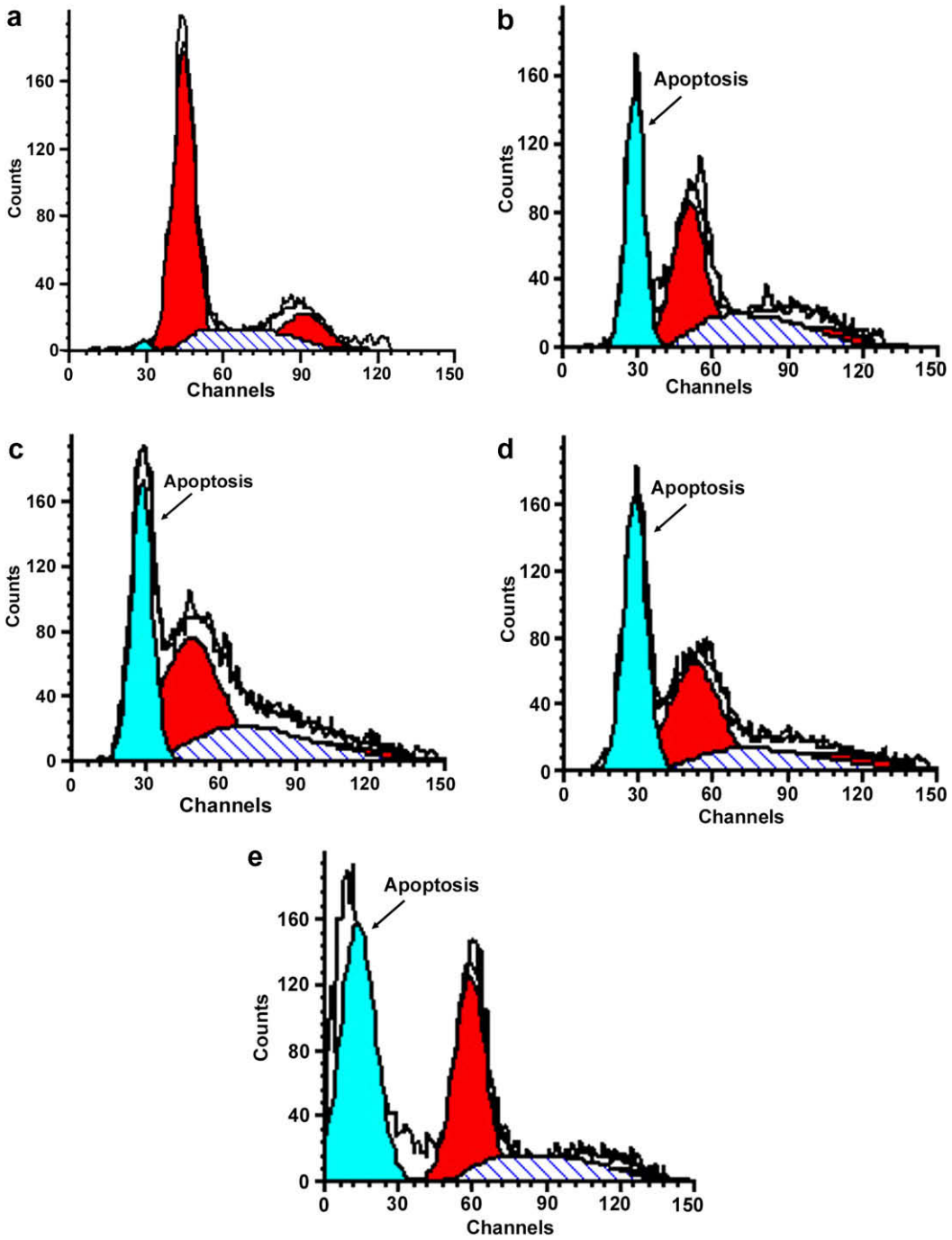

Fig. 7. Flow cytometric analysis of C6 cells treated by various fiber mats with different drug loading amounts for 3 days: (a) control cells, (b) 1.0 wt% PTX/PEG–PLA fiber mat, (c) the mixture of 0.5 wt% DOX/PEG–PLA fiber mat and 0.5 wt% PTX/PEG–PLA fiber mat, (d) 1.0 wt% DOX/PEG–PLA fiber mat, and (e) (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat.

drug contents in the culture media were kept at 80 μg/ml for all tests.

The cell growth inhibition ratios of various fiber mats with 1.0 wt% total drug loadings against C6 cells are shown in Fig. 5. As can be seen, the blank fiber mats without drugs did not display any cytotoxicity to C6 cells (curve a). All medicated fibers showed decreasing cell viability with culture time except 1.0 wt% DOX/PEG–PLA fiber mat after 48 h. The cell viability order was "1.0% PTX" (b) > mixture of "0.5% PTX" and "0.5% DOX" (c) > "composite of 0.5% PTX + 0.5% DOX" (d) > "1.0% DOX" (within 48 h) (e). Obviously, it was in agreement with their release rates discussed in the previous section and in Fig. 2. Among them, "1.0% DOX" exhibited much higher inhibition ratio than "1.0% PTX", because of the much faster release of DOX from the fibers. The composite fibers showed higher inhibition ratio than the fiber mixture because the presence of DOX accelerated the release of PTX. It is interesting to notice that the cell viability for "1.0% DOX" fibers went up after 48 h of incubation. This is because the DOX was almost exhausted within 48 h, and thus the cell growth could not be inhibited any longer. Comparatively, other samples displayed longer cytotoxicity due to more sustained release of PTX.

The cytotoxicities of various fiber mats with total drug loading of 2.0 wt% (Fig. 6) were similar to the case of total drug loading of 1.0 wt%, that is, cell viability decreased with incubation time, inhibition ratios remained of the same order and the "2.0% DOX" fibers lost their activity after 48 h. Because the release rate of DOX decreased with increasing DOX content in the fibers, the cells treated with the mixture of "1.0% PTX" and "1.0% DOX" (b), "composite of 1.0% PTX + 1.0% DOX" (c) and "2.0% DOX" (within 48 h) (d) fibers displayed higher viabilities than the corresponding "1.0%" cases.

From these results, we can conclude that the cytotoxicities of medicated fiber mats were closely related to the release behaviors of drugs. The fiber mats loaded with DOX and PTX showed the strongest cell growth inhibition after 72 h. Because of the relatively faster release of DOX and the acceleration of PTX release by DOX, the co-electrospun-medicated fibers display both faster drug release at the early stage and sustained drug release at the later stage, and thus they display high enough inhibition ratio at the early stage and the cytotoxicity lasts for a long time. This release and activity profile is suitable for postoperative therapy of tumors.

3.3.2. Flow cytometry analysis

Flow cytometry analysis of C6 cells was performed to test the possible changes in the cell cycle distribution in response to the treatment of various drug-loaded fiber mats for 3 days. As shown in Fig. 7, various medicated fibers could induce C6 cell apoptosis. Compared with untreated case (Fig. 7a), significant apoptotic peaks were observed before G_1 phase for the cells treated with medicated fibers (Fig. 7b–e). Among them, the apoptotic peak of the cells treated with the co-electrospun fibers (composite of "0.5% PTX + 0.5% DOX") was the strongest. Because the area of apoptotic peak can directly reflect the number of apoptotic cells, the co-electrospun fibers showed the most effective antitumor activity.

The analytical results are listed in Table 1. The cell cycle distribution of control C6 cells was 54.7 ± 1.9%, 30.4 ± 2.0%, and 14.9 ± 3.8% in the G_0/G_1, S, and G_2/M phases, respectively, after 3 days of incubation. Treatment of the cells with medicated fibers induced a prominent change in cell cycle distribution. The percentage of the cells in G_0/G_1 phase increased, and the percentages of the cells in S and G_2/M phases decreased. Most C6 cells accumulated in G_0/G_1 phase would inhibit their progression through the cell cycle, i.e., cell apoptosis was induced. (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat showed strongest anticancer activity, after 3 days treatment, the cell apoptosis rate reached 46.0 ± 2.5% and the percentages of the cells in G_0/G_1, S, and G_2/M phases were 79.7 ± 1.8%, 13.4 ± 2.2%, and 7.0 ± 1.4%, respectively.

Table 1
The cell cycle distribution and the percentage of apoptotic cells for C6 cells treated by various fiber mats[A] for 3 days (mean ± (standard deviation)).

Group[A]	Drug content in the media (μg/ml)	Percentage of apoptotic cells (%)	Cell cycle distribution (%)		
			G_0/G_1	S	G_2/M
a	0	1.62 ± 0.06	54.7 ± 1.9	30.4 ± 2.0	14.9 ± 3.8
b	40	31.2 ± 3.0*	67.0 ± 1.2*	21.8 ± 1.9*	11.2 ± 4.6*
c	40	40.0 ± 3.0*	69.0 ± 1.7*	23.8 ± 1.7*	7.3 ± 4.3*
d	40	41.4 ± 2.7*	74.6 ± 2.6*	18.0 ± 2.1*	7.4 ± 2.1*
e	40	46.0 ± 2.5*	79.7 ± 1.8*	13.4 ± 2.2*	7.0 ± 1.4*

[A] (a) control cells, (b) 1.0 wt% PTX/PEG–PLA fiber mat, (c) mixture of 0.5 wt% DOX/PEG–PLA fiber mat and 0.5 wt% PTX/PEG–PLA fiber mat, (d) 1.0 wt% DOX/PEG–PLA fiber mat, and (e) (0.5 wt% DOX + 0.5 wt% PTX)/PEG–PLA composite fiber mat.
* $P < 0.05$ vs. control.

The results showed a marked increase in cell growth inhibition and apoptosis against C6 cells when the cells were treated with the co-electrospun fibers.

4. Conclusions

Both DOX and PTX were successfully co-electrospun into PEG–PLA nanofibers by "emulsion-electrospinning". They were distributed in the fibers in a reservoir-type and in a matrix-type, respectively. The solubility properties and distribution status of both drugs were responsible for their release behaviors. The release rate of DOX was always faster than that of paclitaxel. Although the release behavior of DOX was not obviously affected by PTX, the release rate of PTX was always accelerated by DOX. In vitro cytotoxicity assay showed that the co-electrospun medicated fiber mats showed the strongest cytotoxicity against rat Glioma C6 cells. The cell growth inhibition rate, apoptosis rate, and the percentage of the cells in G_0/G_1 phase were higher than a single drug-loaded system. Here, the promising anticancer outcomes suggested that a single carrier for multi-drug delivery may be useful in the development of new implantable polymeric device for combination therapy. Studies on anticancer activity in vivo have been performed and will be reported further.

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References

[1] R. Langer, Drug delivery and targeting, Nature 392 (1998) 5–10.
[2] J.R.B.J. Brouwers, Advanced and controlled drug delivery systems in clinical disease management, Pharm. World Sci. 18 (1996) 153–162.
[3] K.E. Uhrich, S.M. Cannizzaro, R.S. Langer, K.M. Shakesheff, Polymeric systems for controlled drug release, Chem. Rev. 99 (1999) 3181–3198.
[4] S. Ramakrishna, K. Fujihara, W. Teo, T. Lim, Z. Ma, An Introduction to Electrospinning and Nanofibers, World Scientific Publishing Co. Pvt. Ltd., Berlin, 2005. pp. 285–290.
[5] K. Kim, Y.K. Luuc, C. Chang, D. Fang, B.S. Hsiao, B. Chua, M. Hadjiargyrou, Incorporation and controlled release of a hydrophilic antibiotic using poly(lactide-co-glycolide)-based electrospun nanofibrous scaffolds, J. Control. Release 98 (2004) 47–56.
[6] L. Brannon-Peppas, Polymers in controlled drug delivery, Med. Plast. Biomater. 4 (1997) 34–44.
[7] W. Cui, X. Li, X. Zhu, G. Yu, S. Zhou, J. Weng, Investigation of drug release and marix degradation of electrospun poly(DL-lactide) fibers with paracetanol inoculation, Biomacromolecules 7 (2006) 1623–1629.
[8] E.R. Kenawy, G.L. Bowlin, G.E. Wnek, Release of tetracycline hydrochloride from electrospun poly (ethylene-co-vinylacetate) poly (lactic acid) and a blend, J. Control. Release 81 (2002) 57–64.
[9] J. Zeng, X.Y. Xu, X.S. Chen, Q.Z. Liang, X.C. Bian, L.X. Yang, X.B. Jing, Biodegradable electrospun fibers for drug delivery, J. Control. Release 92 (3) (2003) 227–231.

[10] H. Jiang, Y. Hu, Y. Li, P. Zhao, K. Zhu, W. Chen, A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents, J. Control. Release 108 (2005) 237–243.
[11] F. Ahmed, R.I. Pakunlu, A. Brannan, F. Bates, T. Minko, D.E. Discher, Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors inducing apoptosis in proportion to accumulated drug, J. Control. Release 116 (2006) 150–158.
[12] E. Briasoulis, G. Pentheroudakis, V. Karavasilis, E. Tzamakou, D. Rammou, N. Pavlidis, Weekly paclitaxel combined with pegylated liposomal doxorubicin (Caelyx(TM)) given every 4 weeks: dose-finding and pharmacokinetic study in patients with advanced solid tumors, Ann. Oncol. 15 (2004) 1566–1573.
[13] E. Harper, W. Dang, R.G. Lapidus, R.I. Garver, Enhanced efficacy of a novel controlled release paclitaxel formulation for local-regional therapy of lung cancer tumor nodules in mice, Clin. Cancer Res. 5 (1999) 4242–4248.
[14] E.R. Gariepy, M. Shive, A. Bichara, M. Berrada, D.L. Garrec, A. Chenite, J.C. Leroux, A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel, Eur. J. Pharm. Biopharm. 57 (2004) 53–63.
[15] K. Obara, M. Ishihara, Y. Ozeki, T. Ishizuka, T. Hayashi, S. Nakamura, Y. Saito, H. Yura, T. Matsui, H. Hattori, B. Takase, M. Ishihara, M. Kikuchi, T. Maehara, Controlled release of paclitaxel from photocrosslinked chitosan hydrogels and its subsequent effect on subcutaneous tumor growth in mice, J. Control. Release 110 (2005) 79–89.
[16] A. Di Marco, Adriamycin (NSC-123127): mode and mechanism of action, Cancer Chemother. Rep. 6 (1975) 91–106.
[17] H.S. Schwartz, DNA breaks in P-288 tumor cells in mice after treatment with daunorubicin and adriamycin, Res. Commun. Chem. Pathol. Pharmacol. 10 (1975) 51–64.
[18] M.F. Goodman, G.M. Lee, N.R. Bachur, Adriamycin interactions with T4 DNA polymerase: two modes of template-mediated inhibition, J. Biol. Chem. 252 (1977) 2670–2674.
[19] D.L. Gustafson, A.L. Merz, M.E. Long, Pharmacokinetics of combined doxorubicin and paclitaxel in mice, Cancer Lett. 220 (2005) 161–169.
[20] E. Briasoulis, V. Karavasilis, E. Tzamakou, D. Rammou, K. Soulti, C. Piperidou, N. Pavlidis, Interaction pharmacokinetics of pegylated liposomal doxorubicin (Caelyx) on coadministration with paclitaxel or docetaxel, Cancer Chemother. Pharmacol. 53 (2004) 452–457.
[21] D. Mavroudis, C. Kouroussis, S. Kakolyris, S. Agelaki, K. Kalbakis, N. Androulakis, J. Souglakos, G. Samonis, V. Georgoulias, Phase I study of paclitaxel (taxol) and pegylated liposomal doxorubicin (caelyx) administered every 2 weeks in patients with advanced solid tumors, Oncology 62 (2002) 216–222.
[22] J. Gehl, M. Boesgaard, T. Paaske, B.V. Jensen, P. Dombernowsky, Combined doxorubicin and paclitaxel in advanced breast cancer: effective and cardiotoxic, Ann. Oncol. 7 (1996) 687–693.
[23] E.H. Sanders, R. Kloefkorn, G.L. Bowlin, D.G. Simpson, Gary E. Wnek, Two-phase electrospinning from a single electrified jet: microencapsulation of aqueous reservoirs in poly(ethylene-co-vinyl acetate) fibers, Macromolecules 36 (2003) 3803–3805.
[24] X.L. Xu, L.X. Yang, X.Y. Xu, X. Wang, X.S. Chen, Q.Z. Liang, X.B. Jing, J. Zeng, Ultrafine medicated fibers electrospun from W/O emulsions, J. Control. Release 108 (2005) 33–42.
[25] H. Qi, Ping Hu, Jun Xu, Aijun Wang, Encapsulation of drug reservoirs in fibers by emulsion electrospinning: morphology characterization and preliminary release assessment, Biomacromolecules 7 (2006) 2327–2330.
[26] J. Zeng, X.S. Chen, X.B. Jing, Ultrafine fibers electrospun from biodegradable polymers, J. Appl. Polym. Sci. 89 (4) (2003) 1085–1092.
[27] X.L. Xu, X.S. Chen, P. Ma, X.R. Wang, X.B. Jing, The release behavior of doxorubicin hydrochloride (Dox) from medicated fibers prepared by emulsion-electrospinning, Eur. J. Pharm. Biopharm. 70 (1) (2008) 165–170.
[28] X.L. Xu, X.L. Zhuang, X.S. Chen, X.R. Wang, L.X. Yang, X.B. Jing, Preparation of core-sheath composite nanofibers by emulsion electrospinning, Macromol. Rapid Commun. 27 (2006) 1637–1642.
[29] X.L. Xu, X.S. Chen, X.Y. Xu, T.C. Lu, X. Wang, L.X. Yang, X.B. Jing, BCNU-loaded PEG-PLLA ultrafine fibers and their in vitro antitumor activity against Glioma C6 cells, J. Control. Release 114 (2006) 307–316.
[30] J. Zeng, L.X. Yang, Q.Z. Liang, X.F. Zhang, H.L. Guan, X.L. Xu, X.S. Chen, X.B. Jing, Influence of the drug compatibility with polymer solution on the release kinetics of electrospun fiber formulation, J. Control. Release 105 (2005) 43–51.
[31] J. Zeng, X.S. Chen, Q.Z. Liang, X.L. Xu, X.B. Jing, Enzymatic degradation of poly(ι-lactide) and poly(ε(-caprolactone) electrospun fibers, Macromol. Biosci. 4 (2004) 1118–1125.