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Release modulation and cytotoxicity of hydroxycamptothecin-loaded electrospun fibers with 2-hydroxypropyl-β-cyclodextrin inoculations

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ABSTRACT

Hydroxycamptothecin (HCPT) is valid to various malignant tumors, but its insoluble and unstable lactone ring in physiological environment have restricted the clinic application. This work was aimed to formulate HCPT-loaded poly(DL-lactic acid)–poly(ethylene glycol) (PELA) fibrous mats through blend electrospinning with 2-hydroxypropyl-β-cyclodextrin (HPCD) to modulate the drug release and matrix degradation, and to enhance the structural integrity and cytotoxicity of the released HCPT. The entire drug fraction retained its active lactone form within electrospun fibers, and that was maintained over 85% during incubation for over 1 month. A biphasic release pattern was determined for HCPT-loaded electrospun fibers, which can be modulated by the addition of HPCD. HPCD served as solubilizer to maintain a large concentration gradient for HCPT between saturation and diffusion, and liberated HPCD created microstructure of ultrafine fibers, leading a faster release profile in the second phase. *In vitro* cytotoxicity test showed over 7 times higher inhibitory activity against cancer cells for HCPT-loaded electrospun fibers than free drug during 72 h incubation. Higher apoptosis rates and the arrest of the cell cycle during the S and G_2/M phases were detected through flow cytometry analysis. It indicated therapeutic potentials of HCPT-loaded electrospun fibers as implantable anti-cancer agents for local chemotherapy.

1. Introduction

Camptothecin (CPT) and derivates are known as topoisomerase-I inhibitors exhibiting high anti-tumor activity against a wide spectrum of human malignancies, such as lung, prostate, breast, colon, stomach, and ovarian carcinomas. However, the full therapeutic potential has not yet been achieved due to the insolubility in both water and physiologically acceptable organic solvents. Camptothecin was chemically modified, and 10-hydroxycamptothecin (HCPT) was indicated as one of the most effective analogues with higher activity and less toxicity than other CPT derivates (Thomas et al., 2004). Another challenge is the preservation of the lactone ring of CPT and its derivates, which is crucial for their anti-tumor activities. The lactone ring hydrolyzes under physiological condition and exists in a pH-dependent equilibrium with an open carboxylate form, which shows less anti-tumor activity and several unpredictable side effects (Gabr et al., 1997). In addition, this equilibration is also affected by the preferential binding of serum albumin to the carboxylate form, resulting in more rapid opening of the lactone ring under physiological environment (Opanasopit et al., 2005).

Many attempts have been made to increase the solubility of CPT and its derivates and stabilize the lactone ring, which involve chemical conjugate, and the formulations of liposome, micelle, hydrogel and nanoparticles (Lalloo et al., 2006; Zhang et al., 2007). The poor solubility of CPTs results in a limited loading effect and a sharp burst release in such drug delivery devices. Opanasopit et al. formulated CPT-loaded poly(ethylene glycol)-chitosan self-assembly micellar system, which showed an increase in the half-life of CPT from 94 min to 76.2 h. But low drug encapsulation efficiency and high initial burst release were detected. About 60% of CPT released from micelles in the first 20 h while 80% released after 4 d (Opanasopit et al., 2006). Moreover, all of these formulations were intravenously administered, which can be easily eliminated by the circulation of blood and highly enriched in reticuloendothelial system (RES) due to their size and the passive target effect. Only a fraction of the total dose reached the tumor site, and the remainder was distributed throughout healthy organs and tissues, leading to a variety of undesirable side effects ranging from neutropenia to cardiomyopathy (Crawford et al., 2004). Zhang et al. prepared HCPT-loaded nanoparticles showing higher anti-tumor efficiency. However, the HCPT-loaded nanoparticles displayed a

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significant distribution to RES organs, such as liver and spleen, whose concentrations were almost 100 times higher than those in tumor tissues (Zhang et al., 2007). Strategies have been applied to design multifunctional nanocarriers capable of prolonged residence in the blood, specific target recognition and intracellular penetration, however, systems like this still represent a challenge for cancer chemotherapy (Torchilin, 2006).

Currently, the most curative treatment option for solid tumor is surgical resection followed by adjuvant chemotherapy or radiation therapy to minimize the risk of recurrence. To expand the tumor exposure time and improve the outcome of chemotherapy, local regional delivery can be the optimal treatment of maximizing destruction to the tumor target by the enriched chemotherapeutic drugs, while limiting damage to the surrounding normal tissue by reducing the systemic dose (Pradilla et al., 2006). Electrospinning is a potential technique for polymeric biomaterials to formulate nano- or micro-scale fibers. Drug delivery with polymer nanofibers is based on the principle that the drug dissolution rate increases with increased surface area of both the drug and the corresponding carrier, and the drug release profile can be modulated by the fibrous morphology, porosity and composition. Electrospun nonwoven mats can be cut to almost any size and fabricated into other shapes using different target geometries for clinical applications (Verreck et al., 2003). Electrospun nanofibers with flexibility in choosing materials for drug delivery have great interests for releasing antibiotics, anti-cancer drugs, proteins and DNA (Kenawy et al., 2002). Xu et al. developed implantable carmustine-loaded polymeric fibers showed stronger anti-tumor activities over the whole experiment process, while that of pristine carmustine disappeared within 48 h (Xu et al., 2006). But the sustained release of carmustine was only achieved for 72 h, and the long-term delivery of antineoplastic drug may be essential in the development of the implantable device for cancer chemotherapy.

The extremely poor solubility and unstable lactone ring of HCPT under physiological conditions make it challenging to regulate of the release rate from polymer matrix to maintain suitable drug concentration within a therapeutic window for enough exposure time. As a promising implantable local delivery dosage, electrospun fibers have not been investigated to achieve sustained release and to enhance the structural integrity and anti-tumor activities of HCPT. In the current study, poly(ethylene glycol)–poly(DL-lactic acid) (PELA) copolymer was chosen to incorporate HCPT through blend electrospinning. 2-Hydroxypropyl-β-cyclodextrin (HPCD) was proposed to modulate the release profiles of HCPT by the addition of different amounts into electrospun fibers. The effects of HPCD addition were clarified on the structural integrity and release pattern of HCPT, and the matrix degradation behaviors of electrospun HCPT/PELA fibers. The cytotoxicity, apoptotic rate and cell cycle distribution were evaluated on human mammary gland cancer cells after treatment by the medicated fibers.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG, M_w = 6 kDa) was purchased from Sigma–Aldrich Inc. (St. Louis, MO). PELA containing 10% of PEG were prepared by bulk ring-opening polymerization using stannous chloride as initiator (Deng et al., 1990). The weight average molecular weight (M_w) of 46.4 kDa were determined by gel permeation chromatograph (GPC, Waters 2695 and 2414, Milford, MA) with a Styragel HT 4 column (7.8 mm × 300 mm) using polystyrene beads as standard. Tetrahydrofuran (THF, Fisher Scientific, Fair Lawn, NJ) was used as the mobile phase at a flow rate of 1.0 ml/min. HCPT was purchased from Sichuan Natural Product Co. (Chengdu, China) and stored at −20 °C. HPCD and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich Inc. (St. Louis, MO). Ultra-pure water from a Milli-Q biocel purification system (UPI-IV-20, Shanghai UP Scientific Instrument Co., Shanghai, China) was used. All other chemicals and solvents were of reagent grade or better, and purchased from Changzheng Regents Co. (Chengdu, China) unless otherwise indicated.

2.2. Preparation of HCPT-loaded electrospun fibers

HCPT and HPCD were dissolved in DMSO, while PELA was dissolved in dichloromethane at 20% (w/v) concentration. The blend solution of polymer and HCPT at a predetermined rate was transferred to a 2 ml syringe attached to a circular-shaped metal syringe needle as the nozzle. The electrospinning experimental was performed as described elsewhere (Cui et al., 2006). Briefly, the electrospinning apparatus was equipped with a high-voltage statitron (Tianjing High Voltage Power Supply Co., Tianjing, China) and the applied voltage was set as 19 kV. The flow rate was controlled by a microinject pump (Zhejiang University Medical Instrument Co., Hangzhou, China) to maintain a steady flow out of the capillary outlet. The distance between the needle tip and the receiving board was set as 20 cm. The collected fibrous mats were lyophilized overnight to remove residual solvents and stored at 4 °C, away from light, for further detection.

2.3. Characterization of HCPT-loaded electrospun fibers

The morphology of the electrospun fibers were observed by scanning electron microscope (SEM, FEI Quanta 200, The Netherlands) equipped with field-emission gun (20 kV) and Robinson detector after 2 min of gold coating to minimize charging effect. The fiber diameter was measured from SEM images as described previously (Cui et al., 2006). HCPT is highly fluorescent, and the drug inoculation and distribution in electrospun fibers were determined by fluorescence microscope (Leica DMR HCS, Germany) and laser confocal scanning microscope (LCSM, Leica TCS SP2, Germany). The fluorescence microscope was operated with a Cy2 filter with the excitation and emission wavelengths of 340/400 and 450/600 nm, respectively. For the LCSM analysis, the excitation and emission wavelengths were set as 418 and 515 nm, respectively. Blank fibers without HCPT loading were used as negative control, and all the parameters including the laser intensity and gain were adjusted until fluorescent signals could not be seen from the control sample. Then, without changing the settings, the same parameters were used to observe HCPT-loaded fibers.

The crystalline state of HCPT in electrospun fibers was analyzed by X-ray diffraction (XRD, Philips X'Pert PRO, The Netherland) over the 2θ range from 5° to 80° at a scanning rate of 5°/min, using Cu Kα radiation (λ = 1.54060 Å). HCPT-loaded PELA films were made for comparative purpose. Blend solutions of HCPT and PELA were cast onto glass Petri dishes, left at room temperature until the solvent was evaporated, and then dried under vacuum. The differential scanning calorimeter (DSC, Netzsch STA 449C, Bavaria, Germany) was used to determine the thermodynamic profiles of electrospun fibers and matrix polymers. The analysis was performed in perforated and covered aluminum pans under a nitrogen purge. Approximately 5 mg of PELA fibers and drug-loaded PELA fibers were heated from 25 to 150 °C with a heating rate of 5 °C/min.

The loading amount and encapsulation efficiency of HCPT were determined after extracting from electrospun fibers. Briefly, a known amount of fibers (ca. 2 mg) were dissolved in 1.0 ml chloroform and extracted three times with 20.0 ml pH 7.4 phosphate buffer saline (PBS). The HCPT content of the extracted solution was detected by fluorospectrophotometer (Hitachi F-7000, Japan) with the excitation wavelength of 380 nm and the emission wavelength

of 550 nm. The actual concentration was obtained using a standard curve from known concentrations of HCPT solutions in DMSO/PBS (pH 7.4). The extraction efficiency was calibrated by adding a certain amount of HCPT into polymer/chloroform solution along with the same concentration as above and extracted using the above-mentioned process. The loading amount of HCPT, determined in triplicate for at least five different batches and given as a percentage, indicated the amount (in milligrams) of HCPT encapsulated per 100 mg of electrospun fibers. The encapsulation efficiency indicated the percentage of HCPT encapsulated with respect to the total amount used for the fiber preparation.

2.4. Evaluation of the structural stability of HCPT

The structural stability of HCPT that remained in ultrafine fibers and in the release media was determined by reverse-phase high-performance liquid chromatograph (HPLC, Waters 2695, Milford, MA) (Li and Zhang, 1996). The mobile phase was 40/60 (v/v) of pure water and methanol (Fisher Scientific, Fair Lawn, NJ) at a flow rate of 1.0 ml/min, and HPLC was operated with a UV detector (Waters 2487, Milford, MA) set at 266 nm and a C18 column (4.6 mm × 150 mm, Waters, Milford, MA) at 40.0 °C. Briefly, electrospun fibers were removed from the release media, washed with pure water to eliminate the released drug and buffer salts, and lyophilized overnight. The drug-loaded fibers were dissolved in dichloromethane, extracted with the mobile phase, and the supernatant was analyzed by HPLC immediately. The retention time of the carboxylate form of HCPT was 1.7–2.0 min, while that of the lactone form was 3.0–3.5 min. The peak areas were measured, and the actual contents were calibrated using a standard curve from known concentrations of HCPT solutions. Stock solutions of HCPT in DMSO were diluted with buffer solutions of pH values of 3.0 and 9.0 to obtain HCPT of the lactone and carboxylate forms, respectively.

2.5. In vitro drug release and matrix degradation of HCPT-loaded electrospun fibers

The drug-loaded fibrous mat corresponding to 120.0 μg of HCPT was exactly weighted and immersed in 40.0 ml pH 7.4 PBS in order to obtain sink conditions. The suspension was kept in a thermostated shaking water bath (Taichang Medical Apparatus Co., Jiangsu, China) that was maintained at 37 °C and 120 cycles/min. At predetermined time intervals, 1.0 ml of the released solution was removed for analysis, and equal amount of fresh buffer solution was added back. The HCPT concentration in the release media was detected by fluorospectrophotometer as described above. To evaluate the effect of HCPT and HPCD incubations on the degradation profiles of electrospun fibers, a group of each fibrous mat was retrieved at predetermined time intervals, rinsed with pure water to remove residual buffer salts, and dried to constant weight in a vacuum desiccator. The morphology was observed with SEM as described previously. The mass loss was determined gravimetrically by comparing the dry weight remaining at a specific time with the initial weight. The molecular weight of recovered matrix polymer was determined by GPC as mentioned in Section 2.1.

2.6. In vitro cytotoxicity assay of HCPT-loaded electrospun fibers

The HCPT-loaded fibers and PELA fibers without drug loading were sterilized by ^{60}Co γ-ray at a dosage of 10 kGy. Human mammary gland cancer cells MCF-7 were used, and the cell viability was evaluated by Cell Counting Kit-8 reagent (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were cultured in RPMI 1640 (Gibco BRL, Rockville, MD) containing 10% fetal bovine serum (FBS, Gibco Invitrogen, Grand Island, NY), 100 U/ml pencillin (Sigma, St. Louis, MO) and 100 μg/ml streptomycin (Sigma, St. Louis, MO) at 37 °C in a humidified atmosphere of 5.0% CO_2. The cell density of 1×10^4 cells/ml was seeded in 48-well tissue culture plate (TCP, Costar, Corning, NY), and allowed 24 h to attach and grow in wells before drug treatment. HCPT stock solution was diluted in RPMI 1640, and drug-loaded electrospun fibers releasing equivalent amount of HCPT during 72 h (from *in vitro* release data) were applied. Cells proliferation without drug treatment was set as control. After incubation for 72 h, all the groups were washed by fresh media once, and filled with 300 μl of RPMI 1640 containing 30 μl CCK-8 in each well and incubated for 3 h according to the reagent instruction. An aliquot (150 μl) of incubated medium was pipetted into a 96-well TCP, and the absorbance at 450 nm was measured for each well by a microplate spectrophotometer (Elx-800, Bio-Tek Instrument Inc., Winooski, VT).

2.7. Flow cytometry analysis

MCF-7 cells were seeded in 6-well TCP with a cell density of 5×10^5 cells/well in 2 ml RPMI 1640 containing 10% FBS. The medium was removed after 24 h, followed by filling with RPMI 1640 containing HCPT or HCPT-loaded fibers. MCF-7 cells without any treatment were selected as negative control. The amount of free drug added was equivalent to HCPT released from electrospun fibers during 72 h. After 24 and 72 h of incubation, the adherent cells were washed twice in fresh cell medium, removed after trypsinization, and washed in ice-cold PBS. The cells were harvested by gentle centrifugation at 1500 × *g* for 3 min, fixed in ice-cold 70% ethanol, and deposited for 12 h at 4 °C to increase the penetrability of cell membrane. For cell cycle analysis, cells were harvested by centrifugation at 500 × *g* for 5 min and washed in PBS before being re-suspended in 0.5 ml of PBS with 50 μg/ml of propidium iodide (Sigma–Aldrich Inc., St. Louis, MO) and 100 mg/ml of RNase (Sigma–Aldrich Inc., St. Louis, MO). After incubation at 37 °C for 30 min, the cells were analyzed by FACScan flow cytometer (Epics Elite Esp, Beckman Coulter, CA).

2.8. Statistics analysis

Data are expressed as mean ± standard deviation (S.D.). The statistical significance of the data obtained was analyzed by the Student's *t*-test. Probability values of $p < 0.05$ were interpreted as denoting statistical significance.

3. Results and discussions

3.1. Characterization of HCPT-loaded electrospun fibers

Fig. 1 shows SEM morphologies of electrospun fibrous mats, which possessed the common feature of porous and bead-free randomly arrayed. The diameters were 780 ± 50 and 700 ± 20 nm for electrospun PELA fibers and HCPT-loaded PELA fibers, respectively. The inoculation of HCPT into electrospun fibers was analyzed by fluorescence microscope and LCSM. As shown in Fig. 1c, all the fibers emitted fluorescent light, suggesting the presence of HCPT in the electrospun fibers. Fig. 1d gives a representation of the same fiber with and without excitation superimposed on one another, showing that HCPT was well distributed in the electrospun fibers.

The crystalline state of incorporated drug in the electrospun fibers were examined by XRD, and Fig. 2A summarizes the results. Pure HCPT was crystalline with major peaks at $2\theta = 11.5^\circ$ and 13.5°, respectively (Fig. 2A, a1), while no HCPT peak was found in drug-loaded fibrous mats (Fig. 2A, a3 and a4). To inspect the effect of electrospinning process on the crystallization process, solvent casting of drug-loaded polymer films were prepared, and the main peaks of HCPT were detected (Fig. 2A, a2). During the electrospinning process, the large surface area associated with ultrafine fibers

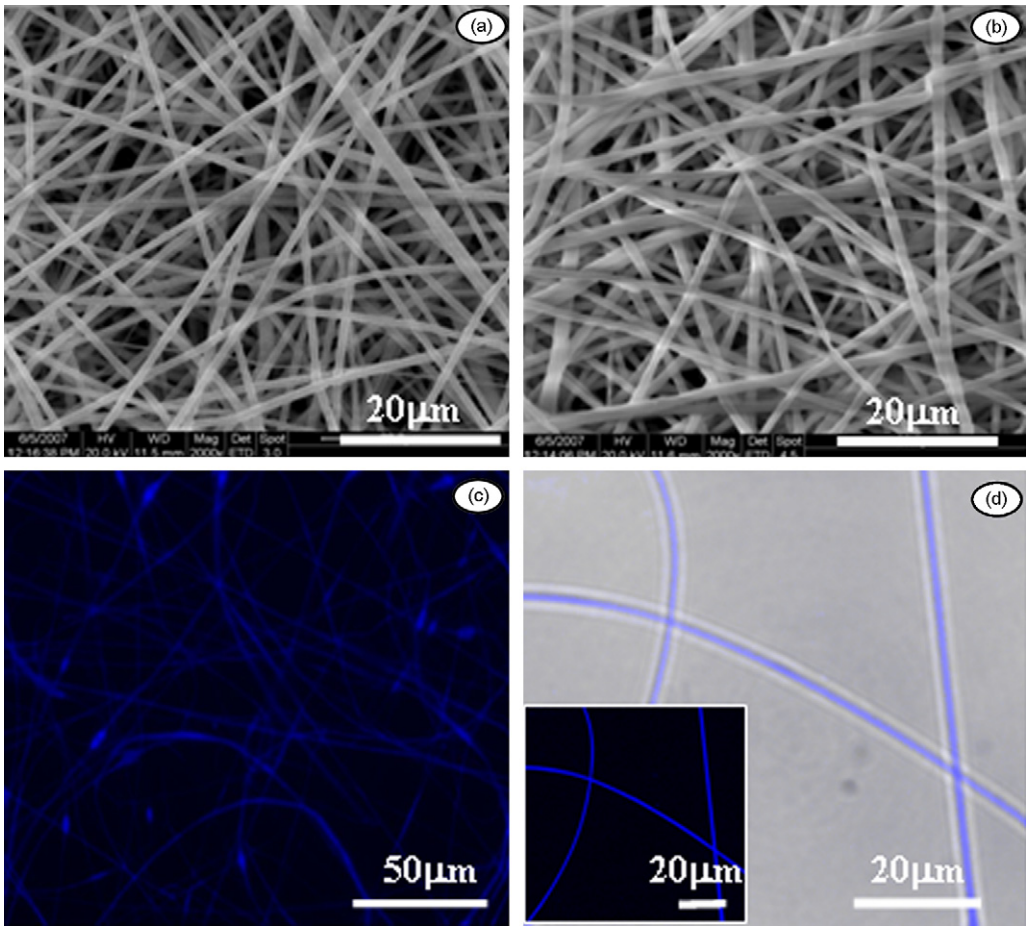

Fig. 1. SEM morphologies of electrospun PELA fibers (a) with and (b) without HCPT inoculation; (c) fluorescence microscope images of HCPT-loaded fibrous mats; (d) LSCM images of HCPT-loaded fibers with and without excitation superimposed on one another (the inset shows the dark field image).

allowed fast and efficient solvent evaporation, which gave incorporated drug limited time to recrystallize and favoring the formation of amorphous state.

Fig. 2B shows the DSC thermographs of polymer PELA, electrospun PELA fibers containing HCPT and/or HPCD. The glass transition temperatures (T_g) of PELA (Fig. 2B, b1) and electrospun PELA fibers (Fig. 2B, b2) were 52 and 42 °C, respectively. This was ascribed to the inner stress and the high degree of alignment and orientation of polymer chains caused by the electrospinning process. With the increase in temperature, the movement of the polymer chains driven by inner stress occurred at a lower T_g. The drug was usually thought as a plasticizer for the matrix polymer and formed a solid solution in the microspheres formulations, resulting in a lower T_g. However, as shown in Fig. 2B, the addition of HCPT and/or HPCD led to an increase in T_g of electrospun fibers. Blended fibrous mats of HPCD/PELA, HPCT/PELA and HPCD-HPCT/PELA showed T_gs of 46.0, 47.5 and 49.0 °C, respectively. The small molecules may act an irregular alignment on the molecular chains of the matrix polymer, which alleviated the elongation effect and inner stress during the electrospinning process.

Efficient encapsulation is one of the challenges in the preparation of HCPT-loaded micelles and microspheres. Attempts have been made to increase encapsulation efficiency through the design of matrix polymers and optimization of fabrication process (Opanasopit et al., 2006; Zhang et al., 2007). However, the HCPT loadings were determined to be close to the theoretic values for all the electrospun fibers. The loading amounts of 0.96 ± 0.14, 1.87 ± 0.17 and 4.61 ± 0.37% were detected for fibers with the initial addition of 1.0, 2.0 and 5.0%, respectively. The addition of HPCD showed little contribution on HCPT loading efficiency, which was no significant discrepancy from 90 to 96% with the increase in the HPCD concentrations up to 2.5%.

3.2. In vitro HCPT release profiles from electrospun fibers

For these extremely hydrophobic drugs, regulation of the release rate from polymer matrix is another challenge to maintain suitable drug concentration within a therapeutic window for enough exposure time. Strom et al. prepared CPT-loaded biodegradable polymer disc, and a large initial release occurred over the first 36–48 h followed by a steady but considerably slower rate of release over the next 25 d. Polymer discs with 20 and 50% of CPT loadings showed initial burst release of 5 and 22%, and additional release of 4 and 14% during the following 1 month, respectively (Storm et al., 2002). Attempts have been made to enhance the constant release of such hydrophobic antineoplastic agents through adjusting the matrix degradation and scaffold microstructure. Zeng et al. introduced the enzyme-catalysed hydrolysis, like proteinase K, and the acceleration of hydrophobic drug release was correlated to the polymer degradation rate (Zeng et al., 2005). Alternatively, Ong et al. demonstrated that the PLGA microporous foams fabricated by supercritical CO_2 gas foaming were able to provide sustained paclitaxel release with near zero-order kinetics and minimal initial burst over 1 month (Ong et al., 2009).

The current study focused on the release profiles of HCPT/PELA fibers with the addition of different amounts of HPCD. Fig. 3 shows the HCPT release profiles from blend electrospun fibers of HCPT loadings of 1.0, 2.0 and 5.0% and with HPCD inoculations up to 2.5%.

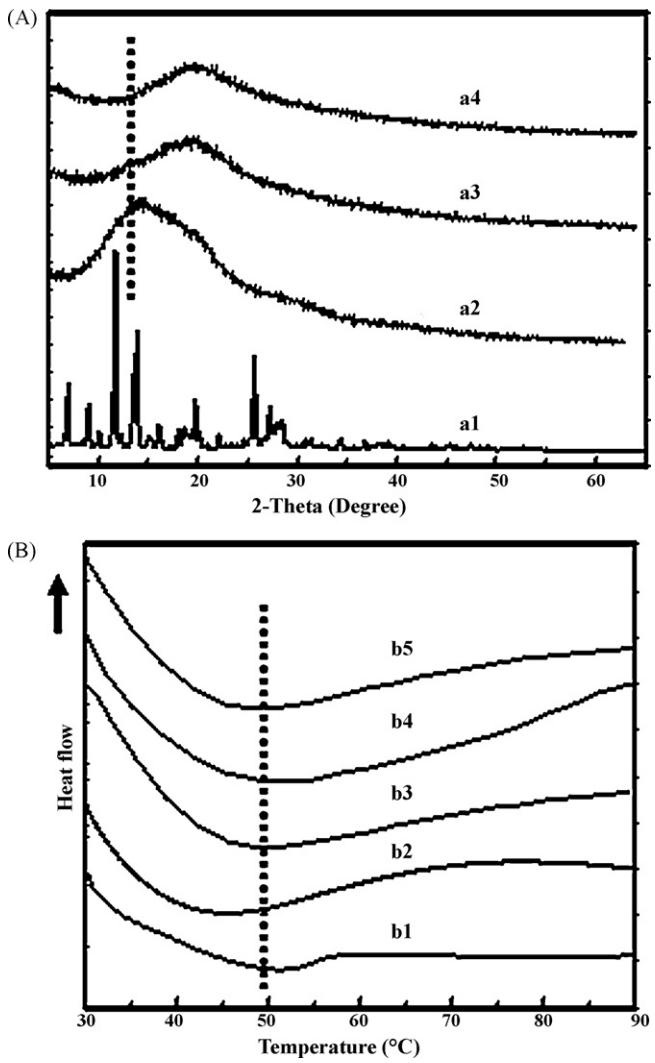

Fig. 2. (A) XRD patterns of free HCPT (a1), solvent-casting HCPT–HPCD/PELA films (a2), electrospun HCPT/PELA fibers (a3) and HCPT–HPCD/PELA fibers (a4); (B) DSC curves of PELA before electrospinning (b1), electrospun PELA fibers (b2), HPCD/PELA fibers (b3), HCPT/PELA fibers (b4) and HCPT–HPCD/PELA fibers (b5).

The release kinetics can be illustrated in two stages: an initial burst release followed by a constant slow release. As shown in Fig. 3a and b, there were around 15% of initial burst release for HCPT/PELA fibers without HPCD inoculation, and no more release was detected during the following 20 d incubation. Kunii et al. obtained similar results from the *in vitro* release of nanoparticles with 1.6% of CPT entrapment, showing an initial rapid release of approximately 20% within 1 h, and the remaining CPT was almost maintained in the nanoparticles over 24 h (Kunii et al., 2007). As shown in Fig. 3, significant higher amount of HCPT released out from HCPT/PELA fiber with higher HPCD inoculations. After 20 d incubation, 50.5 ± 3.8 and 58.2 ± 2.0% of HCPT released from the PELA fibers with 2.0% of the HCPT loading and HPCD loadings of 1.5 and 2.5%, respectively.

However, after the initial burst release of around 15%, a sustained release profile was observed for electrospun fibers with 5% of the HCPT loading and without HPCD inoculation, and over 95% of drug released during 20 d incubation (Fig. 3c). It was due to the micropores after the drug diffusion out of the electrospun fibers with higher HCPT loading, which made it possible that accelerated the further constant release from the inner part. However, a less significant release from polymer disc with 20% of the CPT loading was detected with the initial burst release of 5% followed by sustained release of additional 4% during 1 month (Storm et al., 2002). This indicated the advantages of electrospun fibrous mats over compressed disc to enhance the constant release of hydrophobic drugs due to the significantly higher surface area for the drug dissolution and carrier erosion.

The inner structure and the dispersion pattern of drug within the polymer matrix should be considered as critical factors controlling the release process. HCPT was highly hydrophobic, and HCPT/polymer solution would carry excess charges and move to the outer region of the electrospinning jet due to the charge repulsion. The initial burst release is due to the drug molecules enriched close to fiber surface during the electrospinning process or loosely associated with the fiber matrix. As shown in Fig. 3a and b, there is no significant difference in the amount of initial burst release for HCPT/PELA fibers with different HPCD inoculations ($p > 0.05$). HPCD was indicated to enhance the aqueous solubility and chemical stability of HCPT due to the formation of drug/cyclodextrin inclusion complexes (Saetern et al., 2004). It was suggested that the addition of HPCD should enhance the dissolution of hydrophobic HCPT located close to fiber surface and the desorption of HCPT loosely associated with the fiber matrix, leading a high initial burst release. However, the dispersion pattern of drug within the polymer matrix should be critical to modulate the initial burst release. During the electrospinning process, hydrophobic HCPT may move to the outer region of the electrospinning jet due to the charge repulsion. The addition of HPCD into the electrospinning solution provided a microenvironment into which HCPT may be included, and the movement of HCPT/HPCD complex in the electrospinning jet was considerably slower than HCPT/PELA polymer solution, thus inducing HCPT/HPCD complex embed within the fiber matrix (Kang et al., 2002). Therefore, the combined effects of HPCD on the dissolution enhancement and the preferential distribution of HCPT/HPCD complex within the fiber matrix resulted in no significant difference in the initial burst release for PELA fibers with 1.0 and 2.0% of HCPT loadings. But the effect of HPCD on the amount of HCPT embed inner the fiber matrix was limited with the increase in the HCPT loading, and the dissolution enhancement of HPCD led to higher initial burst release. As shown in Fig. 3c, there was 16.4 ± 2.8% of burst release from PELA fibers with 5.0% of the HCPT loading without HCPD inoculation. However, 26.7 ± 7.4 and 55.0 ± 4.8% of release

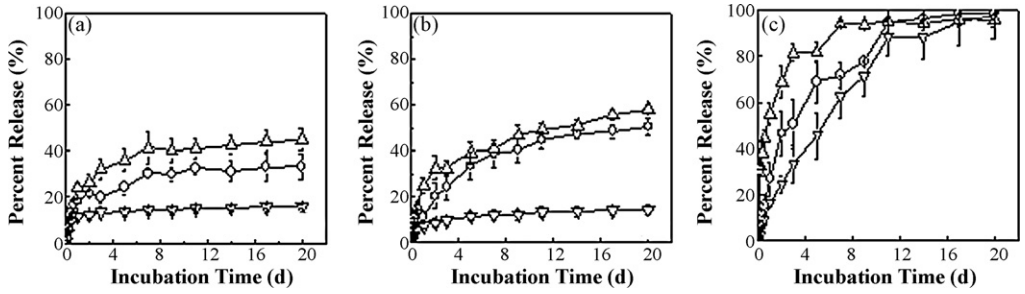

Fig. 3. *In vitro* drug release of electrospun fibers with HCPT loadings of 1% (a), 2% (b) and 5% (c) and containing 1.5% (○), 2.5% (△) of HPCD and no HPCD (▽).

were detected during the initial 24 h after inoculation of 1.5 and 2.5% of HPCD into the fibers, respectively.

For the delivery of antineoplastic drugs, a certain amount of initial burst is actually required to achieve enough initial dosage. Of course, for the cancer cells that survive the initial stage, sustained drug release is necessary. As shown in Fig. 3, significant differences were detected during the sustained release stage for HCPT/PELA fibers with the increase in HPCD inoculations ($p < 0.05$). HPCD, which is highly hydrophilic, can be easily diffused from fibers and liberated HPCD can be as effective solubilizing agent for HCPT to maintain a large concentration gradient for HCPT between the release media and the fiber matrix. Furthermore, the liberated HPCD can made lots of micropores and high surface area for diffusion throughout the ultrafine fibers allowed more HCPT to permeate from fiber matrix. Another possibility is that drug release would be the result of the combined diffusion of HCPT and HCPT/HPCD complex. The diffusivity of total drug release would be increased as the higher diffusion rates of HCPT/HPCD complex (Bibby et al., 2000). In conclusion, HPCD may serve as solubilizer to maintain a large concentration gradient for HCPT between saturation and diffusion, while the more HPCD liberated, the more porous have made and the microstructure of ultrafine fibers led a faster release profile in the second phase.

3.3. Structural stability of HCPT in electrospun fibers

The preservation of the lactone ring of CPTs is crucial for their anti-tumor activity. HCPT, like all CPTs, suffered rapid hydrolysis to its inactive carboxylate form with the half-life time ($t_{1/2}$) of 24 min under the physiological conditions. The structural stability of HCPT in the ultrafine fibers after electrospinning and incubation into the release medium was determined in the current study. As shown in Fig. 4a, the stability of HCPT was improved, and $t_{1/2}$ of around 40 min was obtained after the addition of different amounts of HPCD in the media. After electrospinning the entire drug fraction retained its active lactone form in ultrafine fibers, and the percentage of the lactone form was maintained above 85% after incubation for over 1 month (Fig. 4b). The surface of electrospun HCPT/PELA fibers was hydrophobic, and HCPT captured in the ultrafine fibers was prevented from exposing to the liquid environment. The active lactone ring was lipophilic, inclining to existence in hydrophobic polymer phase, and such insufficient water diffusion in electrospun fibers cannot dissolve HCPT that arrest kinetically lactone–carboxylate conversion by preventing the favorable reaction occurrence. In addition, it can be avoided for HCPT to attach human serum albumin in flowing blood by encapsulation into electrospun fibers. Moreover, there may present a low microenvironmental pH inside the ultrafine fibers with the polymer degradation, which can be a critical element for the shifting the lactone–carboxylate equilibrium. It suggested that electrospun fibers could effectively release the lactone form of HCPT, which was crucial to the anti-tumor activity.

3.4. In vitro degradation of HCPT-loaded electrospun fibrous mats

The degradation behaviors of HCPT–HPCD/PELA, HPCD/PELA and PELA fibers were determined in buffer solutions with regard to the morphological changes of the fibrous mats, the mass loss of the fiber matrix, and the molecular weight reduction of the matrix polymer. Fig. 5 shows SEM morphologies of HCPT–HPCD/PELA and HPCD/PELA fibers after incubation for 4 and 8 wk, and significant changes were observed compared to those shown in Fig. 1. The space between fibers shrunk for all samples, and curliness and congeries of fibers could be easily found. The electrospinning

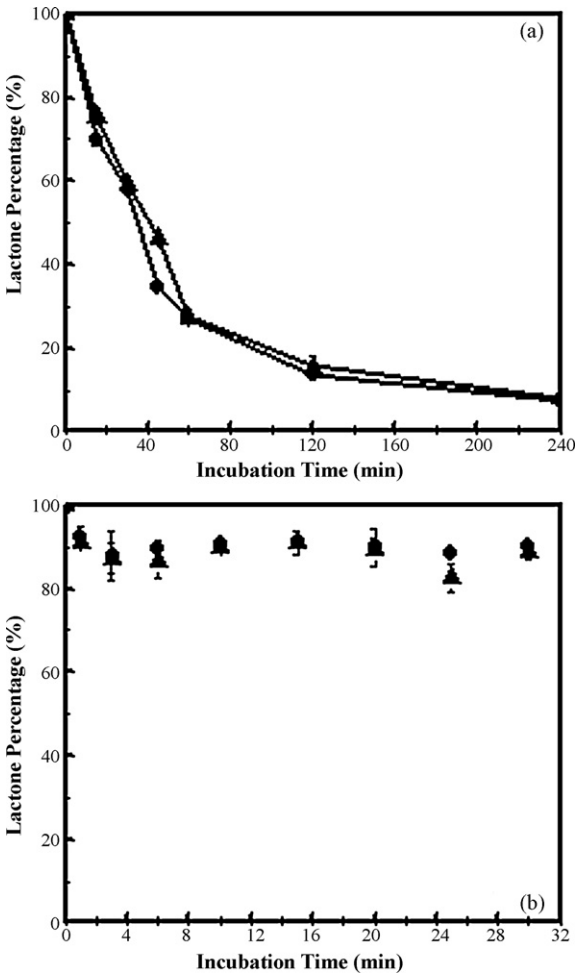

Fig. 4. The lactone percentage of (a) free HCPT after incubation at 37 °C in pH 7.4 PBS containing 1.5% (●) and 2.5% (▲) of HPCD and (b) HCPT extracted from electrospun fibers containing 1.5% (●) and 2.5% (▲) of HPCD after incubation in pH 7.4 PBS at 37 °C.

process caused the inner stress and the high degree of alignment and orientation of polymer chains, and the shrinkage was due to the chain relaxation of matrix polymers after incubated into the medium with elevated temperature. As shown in Fig. 5a and c, HPCD/PELA fibers were swollen and some vicinity regions adhered after immersed into the medium, and the average diameters of 2.43 and 3.84 μm were detected after incubation for 4 and 8 wk, respectively. The inoculation of HPCD improved the surface wettability, and the water diffusion into the fiber matrix was enhanced. HPCD/PELA mats changed from shrinking to puffing bigger than previously.

The mass loss and molecular weight reduction during the incubation of electrospun PELA fibers with inoculations of 1.0% of HCPT and 2.5% of HPCD are summarized in Fig. 6. There was no significant disparity of the degradation profiles among electrospun fibers containing different amount of HPCD (data no shown). There were 24.6 and 57.0% of mass loss and molecular weight reduction during 8 wk incubation of electrospun PELA fibers, respectively. But a slower degradation rate was detected for electrospun HPCD/PELA fibers, and there were 15.0 and 52.2% of mass loss and molecular weight reduction, respectively. The degradation of polylactide and its copolymers underwent different profiles depending on many more or less interdependent factors, such as the polymer components and the size of the device. For large size devices, the random hydrolytic cleavage of the main chain ester bonds was accelerated due to the autocatalytic action of the increas-

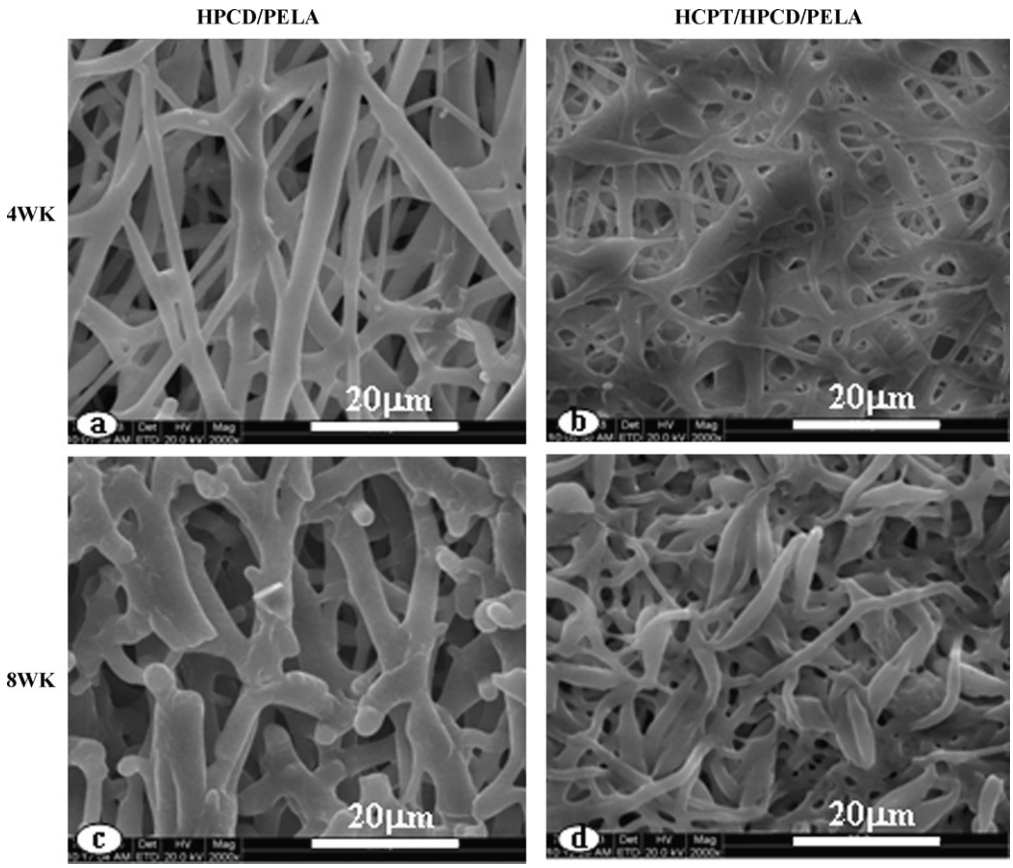

Fig. 5. SEM morphologies of electrospun HPCD/PELA fibers (a and c) and HCPT–HPCD/PELA fibers (b and d) after incubation in pH 7.4 PBS at 37 °C for 4 wk (a and b) and 8 wk (c and d).

ing number of carboxylic acid end groups during degradation and diffusion–dissolution–reaction phenomena involving the formed oligomers (Gfpferich, 1996). The autocatalysis that speeded up the degradation was not observed for tiny devices like thin films and particles smaller than a few micrometers (Dunne et al., 2000). As indicated in our previous study, the electrospinning process led to hydrophobic fibrous surface of electrospun PELA fibers with the water contacting angle of $114.0 \pm 1.3^{\circ}$, which was much higher than that of casting film of $70.1 \pm 2.3^{\circ}$ (Cui et al., 2008). The high hydrophobicity and water repellent properties of electrospun PELA fibers prevented the diffusion of oligomers with carboxylic acid end groups. Therefore, autocatalysis took place in electrospun PELA fiber even with diameters less than 1 μm. The addition of HPCD into the fiber matrix enhanced the water diffusion into and the oligomers diffusion out of the fiber matrix, which alleviated the autocatalysis effect and slowed down the degradation of electrospun HPCD/PELA fibers. As shown in Fig. 6, the entrapment of HCPT into the fibers matrix speeded up the degradation of the matrix polymer comparing to HPCD/PELA fibers, which may be attributed to the microstructure after drug release and the inner pH of fiber matrix after polymer erosion. More microporous frame existed after HCPT release from fibrous mats, and the high contact area of polymer matrix with the degradation medium caused faster mass loss for fibers than those without HCPT entrapped. Moreover, the entrapment of HCPT caused more hydrophobic fiber matrix, and the degradation end-products of oligomers and monomers may diluted slower by release medium than that in HPCD/PELA fiber, resulting in the autocatalytic decomposition of HCPT–HPCD/PELA fibers. On the other hand, the acidic microenvironment inside the electrospun fibers was beneficial to maintain the structural stability of the included HCPT (Fig. 4).

3.5. *In vitro cytotoxicity assay of HCPT-loaded electrospun fibers*

The cytotoxicity of PELA fibers of 1.0% of HCPT loading with different HPCD inoculations were tested on MCF-7 cells, and equivalent amount of HCPT was dosed corresponding to that released from drug-loaded fibers during 72 h. Free HCPT and electrospun HPCD/PELA fibers were chosen as controls. Fig. 7 summarizes the cell viability during 72 h incubation, and the half maximal inhibitory concentration (IC_{50}) was determined to show the effectiveness of HCPT-loaded fibers. As shown in Fig. 7a, IC_{50} of 1800 ng/ml was detected for free HCPT. The IC_{50} values of HCPT-loaded fibers without HPCD inoculation and with the HPCD addition of 1.5 and 2.5% were 116.5, 110.0 and 104.0 μg/ml, respectively. After correction, IC_{50} values of 148.7, 150.2 and 189.7 ng/ml of HCPT were achieved for above drug-loaded fibers, which indicated at least 7 times higher inhibitory activity against MCF-7 cells than free HCPT ($p < 0.05$).

Zhang et al. indicated free HCPT and HCPT-loaded polymeric nanoparticles at the equal concentration of 8 μg/ml showed the inhibition rate of 42.0 and 76.8% against human gastric cancer cells, respectively (Zhang et al., 2007). The higher anti-tumor activities for HCPT-loaded electrospun fibers may be attributed to the predominant protection of active lactone ring of HCPT during the electrospinning process and the sustained release of its active form from electrospun fibers. The *in vitro* release results of HCPT/PELA fibers indicated around 15% of burst release during the initial 24 h followed by no more release during 20 d incubation (Fig. 3a). The amount of HCPT dosed in the cell culture system was mainly due to the initial burst release for HCPT/PELA fibers. The retention of structural integrity was not achieved during the following 3 d incubation, and the suppression rate to MCF-7 cells of electrospun

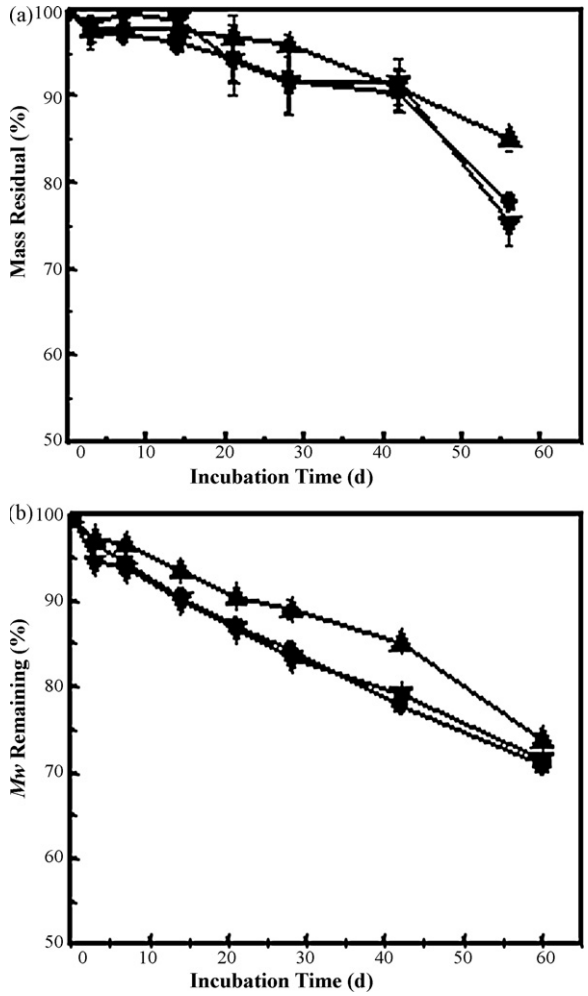

Fig. 6. The mass loss (a) and molecular weight reduction (b) of electrospun PELA fibers (▼), HPCD/PELA fibers containing 2.5% HPCD (▲) and HCPT–HPCD/PELA fibers containing 2.5% HPCD and 1.0% HPCT (●) after incubation in pH 7.4 PBS at 37 °C.

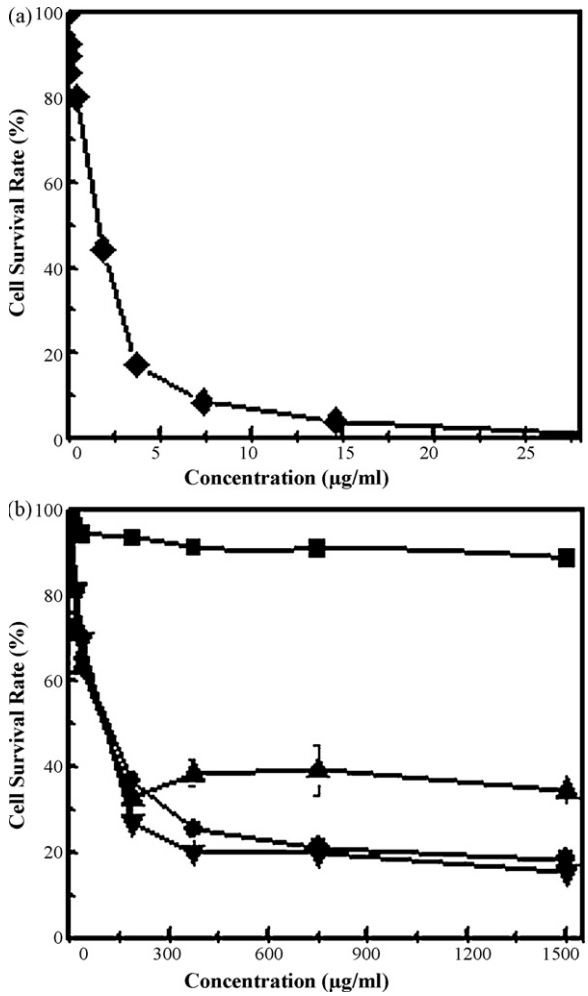

Fig. 7. *In vitro* cytotoxicity to MCF-7 cells after treated by (a) free HCPT, (b) HPCD/PELA fibers containing 2.5% HPCD (■), and HCPT–HPCD/PELA fibers with 1.0% of HCPT loading and containing 1.5% (●), 2.5% (▼) of HPCD and no HPCD (▲).

HCPT/PELA fibers without HPCD inoculation showed no significant increase at higher fiber concentrations (Fig. 7a). As shown in Fig. 7b, electrospun HPCD/PELA without drug entrapment was nontoxic at low concentration, but somewhat cytotoxic at high concentrations.

3.6. Flow cytometry analysis

Flow cytometry analysis of MCF-7 cells was performed to test the changes in the apoptosis rate and cell cycle distribution in response to the treatment of HCPT-loaded fibers mats for 72 h. The apoptosis rate after HCPT treatment is shown in Table 1. All the groups containing free HCPT or HCPT-loaded fibers exhibited significantly higher apoptotic rate compared to control. Apoptosis rates of HCPT-loaded fibers without HPCD inoculation and with HPCD addition of 1.5 and 2.5% were 2.3, 2.7 and 2.7%, respectively, at the first 24 h, but reached 19.2% for free HCPT group. That may be due to the low HCPT concentration diffused from fibers in the initial burst release stage. After incubation for 72 h, the apoptotic activity of HCPT–HPCD/PELA increased remarkably to over 90%, however, a slight increase to 35.5% was detected from the free HCPT group.

The cell cycle distributions after different treatment are summarized in Table 1, and Fig. 8 illustrates different phases of MCF-7 cells after treated with HCPT-loaded fibers with 2.5% of HPCD inoculation for 24 and 72 h. In the control group, the G_0/G_1, S, and G_2/M populations represented 53.5, 29.7, and 16.8% of cells after 72 h culture, respectively. The drug treatment caused the cycle gathered in the G_0/G_1 region in the first 24 h. With the sustained release of HCPT from fibers and the increase in the drug concen-

Table 1
The apoptotic rate and the cell cycle distribution for MCF-7 cells after treated by free HCPT and HCPT-loaded fibers with 1.0% of HCPT and different HPCD inoculations for 24 and 72 h.

Samples	24 h (%)				72 h (%)			
	Apoptosis	G_0/G_1	S	G_2/M	Apoptosis	G_0/G_1	S	G_2/M
Control	0.4	49.1	34.9	15.3	7.2	53.5	29.7	16.8
Free drug	19.2	75.6	12.9	12.9	35.5	33.5	30.7	35.8
Fibers without HPCD addition	2.3	51.4	48.6	8.3	92.7	41.5	33.5	25.0
Fibers with 1.5% of HPCD	2.7	53.5	42.2	4.6	96.0	43.9	27.4	28.7
Fibers with 2.5% of HPCD	2.7	56.1	35.1	8.8	97.4	44.9	20.7	34.4

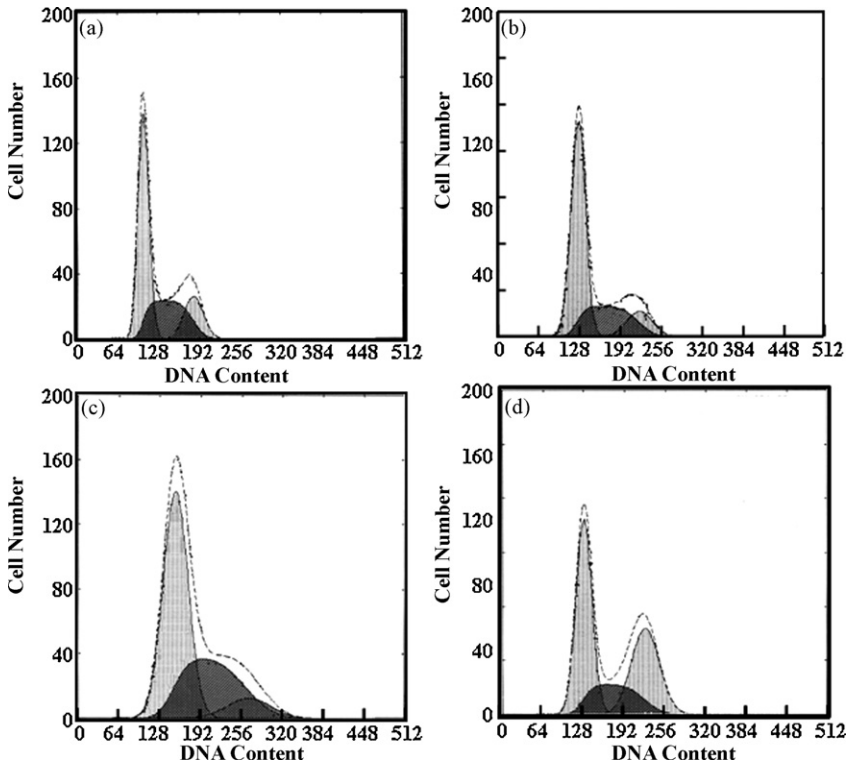

Fig. 8. Flow cytometry analysis of control cells (a), MCF-7 cells after treated by free HCPT for 24 h (b), HCPT–HPCD/PELA fibers containing 1.0% of HCPT and 2.5% of HPCD for 24 h (c) and 72 h (d).

tration, the accumulation of S phase and G_2/M delay can be found accompanied a decline of G_0/G_1 phase after 72 h incubation. The persistence of S and G_2/M phases can be contribute to the mechanism of HCPT binding to DNA-topoisomerase-I complexes, which stabilized single-strand DNA nicks, and facilitated double-strand breakage upon collision with the replication fork, thereby interfering with DNA synthesis and cell proliferation (Goldwasser et al., 1996). HCPT-loaded ultrafine fibers exhibiting higher apoptosis rates may attribute to sustainable release bioactive HCPT, which resulted in more cells' DNA fragmentation, the failure of DNA repair and subsequent arrest of the cell cycle during the S and G_2/M phases.

4. Conclusions

HCPT-loaded PELA fibrous mats were prepared by blend electrospinning. The active lactone form of HCPT was maintained after electrospinning process and incubation into the release media. The release profiles of HCPT from electrospun fibers and the matrix degradation behaviors can be modulated by the addition of different amounts of HPCD. Higher cytotoxicity and apoptosis rates and the arrest of the cell cycle during the S and G_2/M phases were detected for HCPT-loaded electrospun fibers, indicating therapeutic potentials as implantable anti-cancer agents for local adjuvant chemotherapy after surgical resection.

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