Doxorubicin-Loaded Ultrafine PEG-PLA Fiber Mats Against Hepatocarcinoma

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ABSTRACT: The purpose of this study was to evaluate both cytotoxicity *in vitro* and *in vivo* anticancer activities of implantable doxorubicin hydrocloride (Dox)-loaded diblock copolymer poly(ethylene glycol)-*b*-poly(L-lactic acid) (PEG-PLA) fiber mats (hereafter medicated mats). For *in vitro* evaluation, SMMC7721 cells were directly exposed to the medicated fiber mats, followed with MTT assay. For *in vivo* evaluation, the medicated mats were locally implanted into H22 tumor-bearing mice, followed with detection of Fas protein and flow cytometry analysis. The results showed that *in vitro* cytotoxicity of medicated fibers were sustained throughout the whole experiment process with the degradation of fiber matrix, while that of pure Dox was reduced in six days; medicated fibers had superior *in vivo* anticancer activities to pure Dox ($P < 0.01$). In conclusion, Doxorubicin-loaded PEG-PLA electrospun fiber mats had the properties of loading doxorubicin hydrochloride (Dox) and sustained release of Dox from medicated fiber matrix and apparently retained both *in vitro* cytotoxicity and *in vivo* anticancer activities in a long term. Medicated fiber mats are therefore suitable for a local therapy device. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 209–217, 2012

Key words: doxorubicin; PEG-PLA fibers; electrospinning; drug delivery; anticancer

INTRODUCTION

Doxorubicin, an anthracycline antibiotic, has been widely used to treat many solid tumors in clinical practice.[1–3] 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.[4–6] However, like many other drugs used to treat cancers, doxorubicin hydrochloride (Dox) by systematic administration has short circular retention time to reduce bioavailability and causes acute toxicity (e.g., hairloss, nausea, and vomiting) as well as irreversible cardiac toxicity, which restricts the repeated clinic administration.

Block copolymer poly (ethylene glycol)-b-poly (L-lactic acid) (PEG-PLA), characterized by good biocompatibility, biodegradability, amphipathic property, appropriate mechanical strength, ease of processing, high drug loading, and potential to sustained drug release, has been involved in lots of pro-drugs synthesized for the compound of Dox-PEG-PLA as novel drug delivery systems (in the formulation of nanoparticles, micelles, and fiber mats) to improve the drug efficacy, reduce toxic side effects and achieve stable storage, selective targeting, prolonged blood circulation time, and low interactions with reticular endothelial system (RES).[7–14]

Recently, our group has developed a technique "W/O emulsion electrospinning" to successfully encapsulate hydrophilic Dox into electrospun PEG-PLA fibers.[9,15] Hydrophilic Dox is hard to dissolve in the solvent for PEG-PLA, thus difficult to coelectrospin into PEG-PLA fibers. The technique "W/O emulsion electrospinning" helps to prepare ultrafine fiber mats to load hydrophilic Dox.[9,11,15-17] This form of drugs has numerous advantages than other Dox formulations: (1) convenient, soft, available for local implantation; (2) an strong drug efficacy due to their high surface area-to-volume ratio and localized drug release, i.e., not only a high local drug concentration by using a small amount of drug, but also minimization of severe side effects; (3) the local sustained Dox release potentially prolongs the period of tumor's exposure to the drug; (4) higher drug encapsulation efficiency; (5) better stability; (6) degrade to be physically adsorbed without residuals. These Dox-loaded fiber mats are therefore prospective in biomedical applications, especially in postoperative

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local chemotherapy, namely implanting the Dox-loaded fiber mats into tumor-removing foci to kill residual tumor by released drug.

At present, these medicated fiber mats exhibited satisfactory *in vitro* cytotoxicity against mouse-derived glioma cells (C6 cell lines) in a short term. However, there were two issues: (1) the display of superior *in vitro* cytotoxicity resulted from sustained release of Dox from medicated fiber mats in a long term; (2) the anticancer activity *in vivo*.

In this study, the time for *in vitro* evaluation was lengthened; these Dox-load fiber mats were locally implanted into the site of tumors in mice, and the *in vitro* cytotoxicity and *in vivo* anticancer activity of Dox-loaded fiber mats was evaluated.

EXPERIMENTAL

Materials

Doxorubicin hydrochloride (Dox, pure degree 98%) was a gift from Zhejiang Hisun Pharmaceutical Co, Ltd., China; Sodium dodecyl sulfate (SDS) was obtained from Sigma, and used without further purification; diblock copolymer PEG-PLA (prepared from mPEG of 750 Da and L-lactide) were polymerized using 0.025 wt.% $Sn(Oct)_2$ catalyst in our lab,[9] its molecular weight and polydispersity (Mn/PD) determined by GPC were 84,800/1.36. Trihydroxymethyl aminomethane (Tris-base, 99.5 wt.% purity) was supplied by Roche (Shanghai, China), and was used without further purification to prepare Tris-HCl buffer solution of pH = 8.6; RPMI1640 medium and calf bovine serum were purchased from Gibco (Grand Island, NY); dimethyl sulfoxide (DMSO, analytic purity) was supplied by Shenyang 5th Reagent Factory (Shenyang, China); MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide) and propidium iodide (PI) were purchased from Sigma; Fas Detection Kit was supplied by Wuhan Boster (Wuhan, China); proteinase K and sodium azide were purchased from Merck (Darmstadt, Germany).

Preparation and morphology of Dox-loaded fiber mats

The concentration of the aqueous drug solution was about 40 mg mL^{-1}. PEG-PLA was dissolved in chloroform to prepare a 5.5 wt % solution. Totally, 5 wt. % of SDS with respect to the polymer used was added into the polymer solution as emulsifier. First, the SDS/polymer solution was stirred with a high-shear-mixing homogenizer at a speed of 6,500 rpm for 5 min at room temperature. Then the aqueous drug solution was slowly dropped into the polymer solution (the volume ratio of polymer solution to drug solution was 24 : 1), and emulsified for about 20 min. In the whole process, the emulsion container was kept in an ice/water bath to avoid the evaporation of chloroform.

Then 1.7 wt. % of Dox-loaded PEG-PLA fiber mats (thereafter called medicated fibers) were prepared as references.[9,15–17] Briefly, the stable W/O emulsion was transferred to a 30-mL syringe with a right angle-shaped needle of 0.4 mm in inner diameter attached to it. A pressure was applied to the emulsion in syringe to maintain a steady flow of the emulsion from the needle outlet in the range of 50–70 Al min^{-1}. The electric field strength was 2.5–2.8 kV cm^{-1}. The distance between the needle tip and the grounded target was 18 cm.

The surface morphology and size distribution of electrospun fibers were investigated by Environmental Scanning Electron Microscope (Model XL 30 ESEM FEG from Micro FEI Philips), and its accelerating voltage was 25 kV. The samples were mounted on metal stubs using a double-sided adhesive tape and vacuum-coated with a platinum layer prior to examination. The medicated fiber mats were disinfected for 8 h by 15 kGy ^{60}Co γ- ray prior to biological use.

Accumulated drug release *in vitro*

A piece of medicated fiber mats (70 mg, 3 cm [length] 3 cm [width] · 0.25 mm [thickness]) was placed in a jar filled with 200 mL of 0.05*M* Tris-HCl buffer solution (pH = 8.6) containing 1 μg mL^{-1} of proteinase K and 0.01 wt % sodium azide. Another piece was placed in Tris-HCl buffer solution without proteinase K. Samples were shake at 90 rpm, 37°C in a thermostated shaker. On a predetermined time point, 2 mL of release solution was taken out, followed with measurements on an UV-vis spectrophotometer (UV-2401PC, Shimadzu) 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 each time. Then the accumulative weight and relative percentage of the released Dox were calculated.

Cells and animals

SMMC cells (human hepatocarcinoma SMMC-7721 cell lines) were supplied by Shanghai Institute of Life Science, Chinese Academy of Science. Cells were grown in RPMI 1640 media (containing 10% calf serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2, dissociated with 0.25 wt.% trypsin in 0.2*M* PBS (pH 7.4), and centrifuged at 1,000 rpm for 5 min at room temperature. The cells were collected and suspended in 20 mL of serum-free media. 20 μL of the suspension

was used for cell number counting by trypan blue staining in a hemacytometer.

C-57BL mice and H22 (mouse hepatocarcinoma) cells were kept by School of Public Health, Jilin University, China. The animals were acclimatized at a temperature of 25 ± 2°C and a relative humidity of 70 ± 5% under natural light/dark conditions.

Cytotoxicity

SMMC cells were inoculated to each well of eight 96-well plates at a cellular density of 5×10^3 cells in 200 μL of complete RPMI 1640 media, and incubated for 24 h to allow the cells to attach to the bottom of wells. Then the media was refreshed with 100 μL of complete RPMI 1640 media. Meanwhile, wells in each plate were divided into groups, five replicates for each group: (1) control group: only test cells were added; (2) pure Dox group: Dox-containing culture media of calculated concentration was added into each well, the final concentration was 0.82 μg ml^{-1} (IC50 dose, see surpoting information for details); (3) medicated fiber mats group: Dox-loaded PEG-PLA fiber mats were immersed into culture media. The Dox content in fiber mats was 1.7 wt. % with respect to the polymer weight used. Actually, Dox concentration was 0.82 μg mL^{-1} too; (4) blank fiber group: only 0.01 mg of PEG-PLA fiber mats were immersed in media.

At a predetermined time, one plate was withdrawn for morphology observation using an optical microscope (Eclipse TE-2000-U, Nikon, equipped with an attached camera, digital camera SXM1200F, Nikon), followed with MTT assay. It was here clarified that in four days additional 100 μL of complete RPMI 1640 media were supplemented to the left three plates.

MTT assay was performed as follows. The culture media was discarded, 200 μL of MTT solution (0.5 mg mL^{-1} in PBS pH 7.4) was then added to each well. Cells were incubated for another 4 h, and then the solution was cautiously aspirated from each well. Thereafter the MTT derivatives (blue formazan crystals) were completely dissolved with 150 μL of DMSO by swirling for 10 min. Then the optical density (OD) was measured with a microplate reader (Multiskan MK3, Thermo Electron, USA) at 540 nm. Cell viability was calculated by dividing the mean OD value of the test group by that of the control group, and cell inhibition equaled to a hundred percent minus cell viability.

Anticancer activity *in vivo*

A preliminary test shows SMMC cells can not survive to form solid tumor in C-57BL mice body, therefore the H22 cells were adopted to continue anticancer experiment *in vivo*. Anticancer activity *in vivo* was evaluated against H22-bearing C-57BL mice as follows. An experienced experimentalist performed all animal experimental procedures. All animal protocols were approved by the Animal Experimental Center in Bethune Medical School of Jilin University Institutional Animal Care and Use Committee.

0.1 mL of the diluted ascites containing 2×10^6 H22 cells were subcutaneously injected to each of 40 C-57BL mice (male and female evenly, six-week-old, body weight of about 20 g) in the thigh muscle of left hind limb.[18] The tumor sizes (major axis and minor axis) were measured using a micrometer caliper on the predetermined time, and the tumor volume was calculated to an approximate formula: 0.5· (major axis) · (minor axis).[2] As the solid tumor grew to 44–49 mm^3 (mean 46 ± 2 mm^3, Table I), 40 tumor-bearing C-57BL mice were randomly divided into four groups, 10 mice for each group, male and female evenly: (1) control group: 0.1 mL of physiological saline was injected by vena tail; (2) injection i.v. group: a single injection dose of Dox in physiological saline was 0.1 mL of 1 mg mL^{-1} per mouse, which was administered by vena tail; (3) injection *in situ* group: equivalent dose of Dox as injection i.v. group was administered by local injection into tumor, 2–3 mm in depth; (4) medicated fiber mats group: 6 mg of Dox-loaded PEG-PLA fiber mats were implanted locally at site of tumor. The final dose of Dox was 0.1 mg per mouse, 0.33 mg·$(kg\ weight)^{-1}$. Briefly, anesthetized mice were fixed in lateral position and routinely disinfected. After tumors were exposed by dissection, tumor mass was crossing incised along major axis and minor axis. The medicated fiber mats were prepared into a ball of 2–3 mm in diameter, and were implanted into mass center (2–3 mm in depth). Tumor incisions were left free, and then the skin was sutured. During the experiment, animals were allowed to eat and drink freely.

On predetermined time, all mice were sacrificed, followed with tumor excision. A portion of excised tumors were treated with paraffin imbedding. The paraffin-embedded specimens were sectioned into continuous slices of 2 μm thick. Conventional immunohistochemical staining was performed using Fas antibodies, horse radish peroxidase (HRP)-labeled secondary antibodies and chromophore diaminobenzidine (DAB) in accordance with Fas Expression Detection Kit's instruction. Microscopic observation with 200X magnifications was performed. The percentage of Fas-positive cells in one random microscopic field was calculated.

Another portion of excised tumors were digested 20 min by 0.5 wt.% trypsin in 0.2*M* PBS (pH 7.4) to prepare single cells, and washed two times by PBS.

TABLE I
Tumor Volume Mean at Each Time Points in Each Group (Mean ± SD, $n = 8$)

	Tumor volume (mm^3, $n = 8$)				
	Time (day)				
Group	1	3	6	9	13
Medicated fiber mats	47 ± 26	190 ± 82	272 ± 108	215** ± 60	398* ± 182
Injection *in situ*	45 ± 19	209 ± 54	304 ± 90	420 ± 127	779 ± 463
Injection i.v.	44 ± 17	179 ± 73	351 ± 143	474 ± 235	871 ± 396
Control	49 ± 16	299 ± 82	376 ± 90	674 ± 283	1020 ± 480

** $P < 0.01$, * $P < 0.05$ compared with injection *in situ* group.

10^6 cells were precipitated for 5 min by centrifugation at 1,000 rpm. The precipitated cells were fixed with ice-cold 70% ethanol solution and deposited in 4°C overnight. Centrifugation was performed at 1,000 rpm, and then fixative solution was discarded. Cells were re-suspended with 200 μL RNase (0.1 mg mL^{-1}) in 37°C for 30 min. After cells were washed two times by PBS, staining was performed with 1 mL of 0.05 mg mL^{-1} PI (containing 0.03% TritonX-100) in darkness at 4°C for 30 min. Stained cells were detected by flow cytometry (FCM; excitation wavelength: 488 nm, emission wavelength: 630 nm) according to the instruction of BD Carlibur Instrument, USA. The apoptosis% = hypodiploid/total diploid ×100%.

All data were given as mean ± SD and statistically analyzed by Spss12.0.

RESULTS AND DISCUSSIONS

Preparation and morphology of Dox-loaded fiber mats

SDS was added into the polymer solution prior to emulsification as a surfactant to lower down the surface tension of the oily phase. The aqueous solution was added into the oily phase drop-by-drop to ensure the atomization of the aqueous phase. The size of medicated fibers was well-distributed about 750 nm in average diameter (Fig. 1), and the faces were smooth. Dox had been successfully encapsulated in fibers (see Refs.[9,16] to acquire more characterization of these medicated fiber mats).

Accumulated drug release *in vitro*

Proteinase K tends to degrade PLA fast, and thus was used in this study to examine the enzyme-promoted Dox release from fiber matrix. Figure 2 showed the release profiles of Dox from 1.7 wt. % medicated fibers. The medicated fiber mats in solution containing proteinase K released 100% Dox within 12 h. This Dox release profile was a combination of diffusion at the first stage and subsequent enzyme-promoted degradation of fiber matrix. The initial burst release in the first three hours (more than 50% accumulated Dox) was mainly due to the diffusion of Dox near fiber surfaces. A certain amount of initial burst is actually required, for the delivery of antineoplastic drugs to achieve enough initial dosage to kill tumors. Thereafter the enzyme-promoted degradation of medicated fiber matrix predominated Dox release. The sustained drug release is necessary for killing cancer cells that survived the initial stage.

The medicated fiber mats in nonproteinase K buffer released about 40% Dox in the first 3 h. It was the initial burst release, mainly due to the diffusion of Dox near the fiber surfaces. Four hours later and even longer when the degradation of fiber matrix slowed down by the lack of proteinase K to promote degradation of fiber matrix, the accumulated release kept stable in about 50%. These results were consistent with the ones reported in references[9,17] (see references[17] to acquire more information about the release behavior of Dox from medicated fibers).

Cytotoxicity

IC50 was 0.82 ± 0.03 μg mL^{-1} (See supporting information for details) and used for both medicated fiber mats and pure Dox for cytotoxicity experiments. Usually, 10% of IC50 value was the concentration for experiment *in vitro*. However, in this study, medicated fiber mats only loaded 1.7% Dox, the cytotoxicity experiment need a trace of Dox, and it was hard to operate medicated fiber mats with 10% of IC50 value. IC50 value was therefore used in consideration of convenience. In addition, IC50 value can present better cytotoxicity than 10% of IC50, which can apparently demonstrated the advantages of medicated fibers.

Usually, 5×10^4 cells and 200 μL of complete culture media were added to each well of culture plates. However, in this study, low initial cell density (5×10^3 cells in each well of eight plates) was

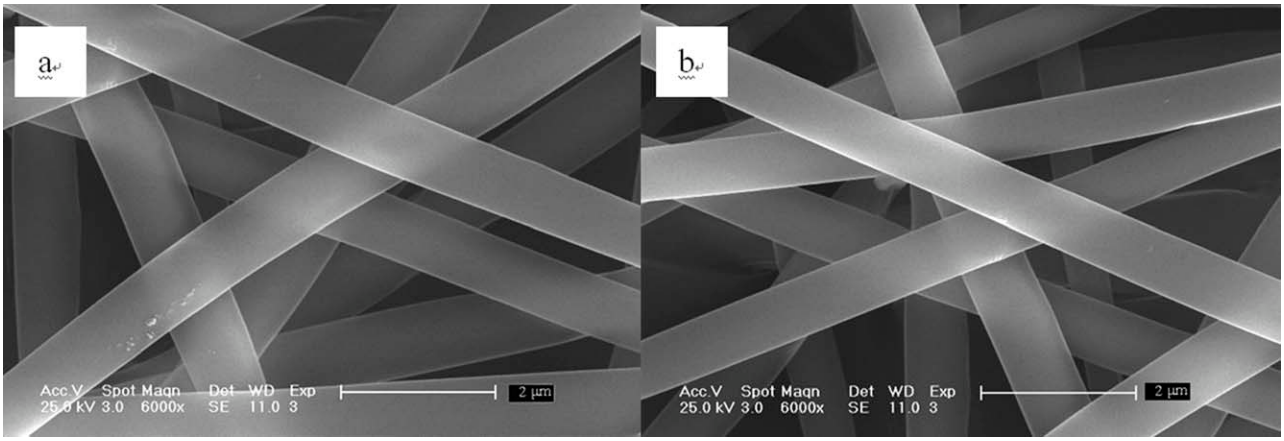

Figure 1 ESEM morphology of electrospun fibers of 750 nm in diameter presented smooth surface. (a) Medicated fibers; (b) PEG-PLA fiber blank.

used, meanwhile, 100 μL of complete culture media were added to supply cells with nutrients. In four days, when five culture plates (time points of 0, 1, 2, 3, and 4 day) had been taken out for detection, cells in the left three plates, especially cells in control group required fresh nutrients, and then additional 100 μL of complete culture media were supplemented to each well of left three plates. Thus Dox concentration at time points of 5, 6, and 7 day was half of the initial Dox concentration. At that time, adding culture media containing fresh Dox was inappropriate because Dox with incubation would decrease in toxicity and adding fresh Dox would artificially heighten the cytotoxicity of pure Dox. In this study, cells in all groups in the left three plates were additionally treated with the same procedures, which could minimize the impacts of additional treatments on the experimental effects.

In Figure 3, cell inhibition was plotted to incubation time for medicated fiber mats, pure Dox and blank PEG-PLA fiber mats. The blank fiber mats had no anticancer effect because their cell inhibition was around 0%. The cell inhibition for both medicated fiber mats and pure Dox appeared in two days, and increased with incubation time in five days. In six days, cell inhibition of pure Dox began decreasing, and that of medicated fiber mats kept increasing (reached 96.8 ± 2.2% in seven days). It indicates that the medicated fiber mats exhibited better and more sustained cytotoxicity than pure Dox *in vitro*.

It was interesting to notice that cell inhibition of medicated fiber mats was always higher than that of pure Dox through the experimental time, except at the time point of 0.25 day ($P > 0.05$). It was easy to explain why cell inhibition of medicated fiber

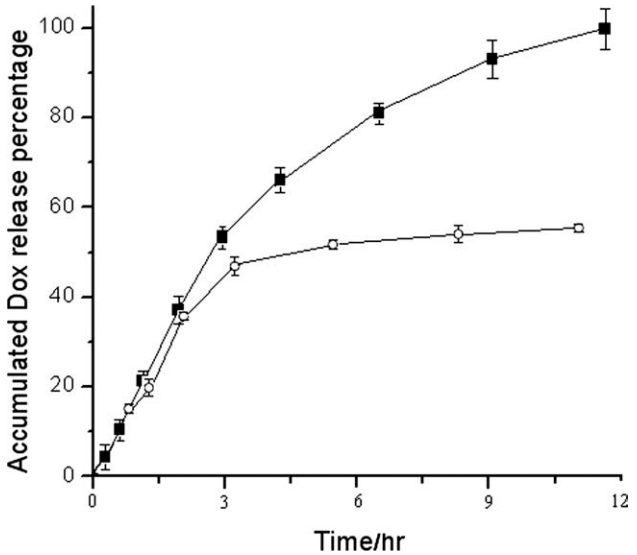

Figure 2 Accumulated release of Dox from Dox-loaded fibers in 0.05*M* Tris-HCl buffer containing 1 μg mL^{-1} of proteinase K (■) and without proteinase K (○). Dox content loaded on fibers was 1.7 wt. %.

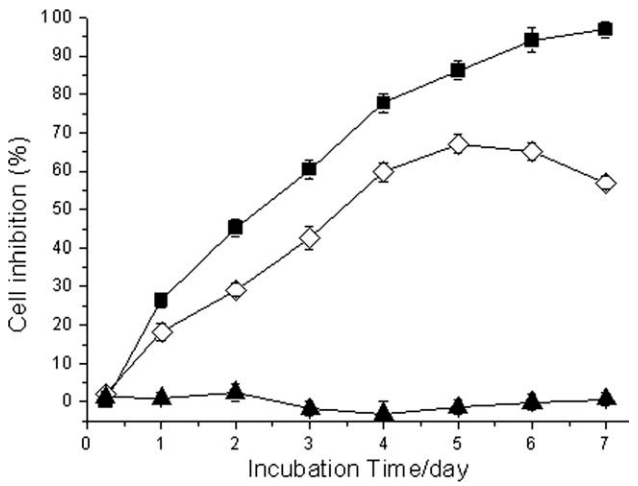

Figure 3 *In vitro* anticancer activity of midecated fiber mats killing SMMC cells. Test materials: medicated fiber mats (■), free Dox (◇), and PEG-PLA fiber mats blank (▲).

mats was a little lower ($P > 0.05$) than that of pure Dox at the earlier time point: Dox release from the medicated fiber mats was delayed while pure Dox worked once they were added. At other time points, it was inferred that the experiment was a static cell culture process and the medicated fiber mats lay on the well bottom where cancer cells adhered and proliferated. Once Dox released from the medicated fiber mats, they could reach cancer cells easily. Therefore, there might be a high static local drug concentration in the neighborhood of cancer cells to cause higher cytotoxicity. Pure Dox was exhausted in six days, and cell inhibition was reduced. In comparison, medicated fiber mats had an increase in cell inhibition because of the sustained release of Dox from the medicated fiber matrix.

Optical microscopic observation confirmed above results. The numbers and the growth status of cancer cells in medicated fiber mats-containing wells were always worse than the case of pure Dox (see supporting information for details).

In addition, as shown in Figure 2, little further release of Dox occurred after 10 h in Tris-HCl buffer solution. However, *in vitro* cancer cell inhibition results indicated that Dox level could be maintained for many days. The appropriate interpretation to this phenomenon may be that the culture media greatly helps *in vitro* release of Dox from the medicated fiber mats.

In this study, the drug loading of 1.7 wt. % was enough to kill the tumor cells. Actually, higher or lower drug loading can also kill the tumor cells efficiently. For instance, in 2005, Xu *et al.*[9] coelectrospun the PEG-PLA medicated fiber mats containing 3.18 wt. % of Dox, and the resultant cytotoxicity to rat glioma C6 cells in 48 h by exposure to 0.32 μg mL^{-1} of Dox totally in each well was 80%. In 2009, Xu *et al.*[11] coelectrospun ultrafine PEG-PLA medicated fiber mats loaded with 1 wt. % or 2 wt. % of Dox, and the resultant cytotoxicity to rat glioma C6 cells in 48 h by exposure to 0.08 μg mL^{-1} of Dox totally in each well were 79% and 87%, respectively. And in this study, the cytotoxicity to SMMC cells in 48 h by exposure to 0.82 μg mL^{-1} of Dox totally in each well were 45%. However, these data seems incommensurable, because the medicated fiber mats load different dose of Dox, and the experiments used both different type of cell lines and different final concentration of Dox in each well. More researches for the accuracy therefore may be needed.

Tumor volume measurement

Animal tests were performed to examine the differences in tumor volume, apoptosis and cell cycle between various treatments of tumor-bearing mice models.

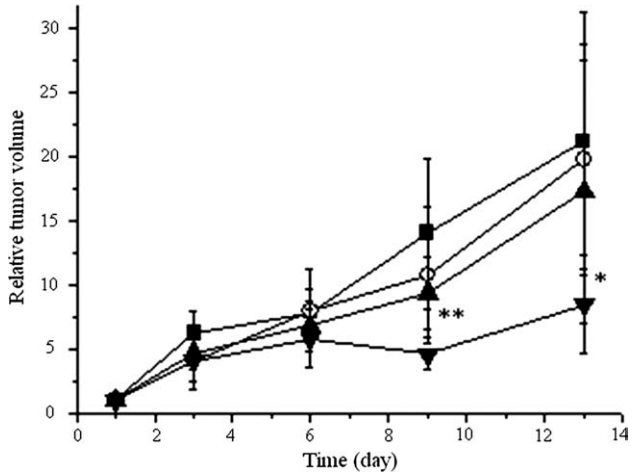

Figure 4 The curve of tumor volume plotted to growth time. (■) Control, (○) Injection i.v., (▲) Injection *in situ*, (▼) Medicated fiber mats. The relative volumes were obtained by dividing actual cancer volumes by that (46 ± 2 mm^3) on Day 1. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with injection *in situ* group ($n = 8$).

In experiments, solid tumors formed in mice after seven days of inoculation. When the tumor volume reached about 46 ± 2 mm^3, the treatments started; and the day when treatments started was designated as day one. On Days 1, 3, 6, 9 and 13, the tumor volumes of each mouse were measured, respectively.

Table I showed the detailed tumor volumes. The results showed that the tumor growth of medicated fiber mats group was significantly lower than that of other groups, but no tumor atrophy was observed. To make tumor growth in each group clear, the relative tumor volumes were calculated, and the curve was plotted, as shown in Figure 4. The relative tumor volumes on early stages were not distinguishable between the groups, but those on Days 9 and 13 had statistically significant differences ($P < 0.01$ and $P < 0.05$, respectively). The relative tumor volume exhibited following order: medicated fiber mats group < injection *in situ* group < injection i.v. group < control group, implying that medicated fiber mats group was significantly superior to other groups. This was attributed to a local high dose of Dox to kill tumors resulted from the sustained release of Dox from medicated fiber mats, similar to the case in section 3.3.

Since Dox kills tumors by interfere with DNA synthesis in nucleus to induce apoptosis, the tumor apoptosis should be investigated. On Day 14, all mice were sacrificed to remove their tumors. These tumors were used in detection of Fas protein and FCM analysis (section Tumor volume measurement and FCM analysis).

Detection of Fas protein

Fas (also CD95), an apoptosis-promoting protein, is expressed in many tumor tissues although its

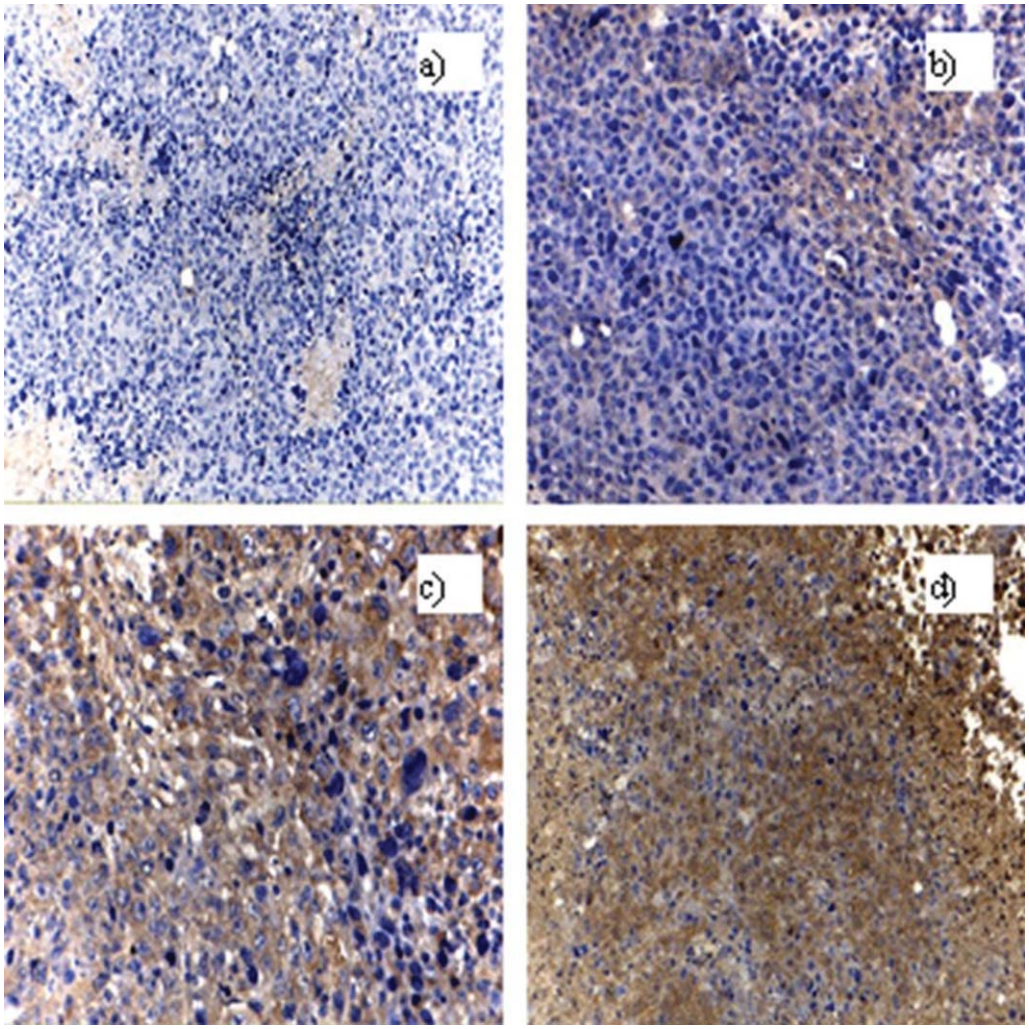

Figure 5 Immunohistochemistry assay on fas protein. The nucleus was stained into blue color and the cytoplasma into brown color in apoptosis cells. (a) Control; (b) injection i.v.; (c) injection *in situ*; (d) medicated fiber mats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

heterogeneous expression varied very much,[19,20] and its over-expression implies that apoptosis has occurred.

In this study, Fas was taken as the target to measure the Dox-facilitating apoptosis via immunohistological method as Kit's instruction. The cellular nucleus was stained into blue color, the plasma and membrane of Fas positive cells into brown color. As Figure 5 shown, the medicated fiber mats group exhibited the least blue and the brownest color than other groups, namely the degree of Fas expression was the highest. It indicated the implant of medicated fiber mats facilitated H22 apoptosis greatly. The H22 apoptosis originated from the interaction between nucleus and medicated fiber mats-released Dox.

The percentage of apoptosis (brown cells) in Figure 5 was counted for a quantitative compare. The results showed the apoptotic rate of medicated fiber mats group was 12 ± 2%, significantly higher ($P < 0.01$, $n = 6$) than other groups (injection *in situ* 7 ± 1%, injection i.v. 6 ± 1% and control 1 ± 1%), indicating medicated fiber mats had great potentials to facilitate H22 apoptosis. The effective outcomes were attributed to the sustained release of Dox from medicated fiber mats.

FCM analysis

Cell cycle includes G_0, G_1, S, G_2, and M phases. G_0 phase is a dormancy stage out of cell cycle, cells in G_1 phase synthesize mRNA, rRNA, tRNA and ribosome and cells in S phase synthesize DNA. G_2 phase is the mitosis prophase and M phase is the mitotic phase. Dox can interfere with the growth of cells by intercalating into the DNA strands, inhibiting further DNA and RNA biosynthesis, and eventually causing cell death.[4–6] This interference does not shorten cell cycle, but reduces cell amounts in cell cycle and

TABLE II
The Percentage of Apoptotic Cells (Hypodiploids) and the Cell Cycle Distribution for H22 Cells (Mean ± SD, $n = 8$)

Groups	Cell cycles (%)			Apoptosis (%)
	G0-G1	S	G2-M	
Medicated fiber mats	74.62** ± 11.91	21.42** ± 9.25	3.96* ± 2.23	24.21** ± 5.05
Injection *in situ*	47.02$^{\#}$ ± 3.11	46.38 ± 8.09	6.60$^{\#\#}$ ± 3.60	15.22$^{\#}$ ± 2.58
Injection i.v.	43.24$^{\#}$ ± 4.57	50.57 ± 12.84	6.19$^{\#\#}$ ± 2.52	14.48$^{\#}$ ± 1.56
Control	37.34 ± 1.2	48.22 ± 7.24	14.44 ± 2.58	10.77 ± 0.55

** $P < 0.01$, * $P < 0.05$ compared with injection *in situ* group, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared with control group.

accumulates block cells in a certain phase to cease cell cycle.[21]

In this study, FCM of H22 cells was performed to test the possible changes in the cell cycle distribution and the percentage of apoptosis (hypodiploids) in response to the treatment, and the results were as shown in Table II. Because apoptosis can activate endogenous DNA endonuclease to incise genome, there was a hypodiploid peak (called apoptotic peak) before G_1 phase in apoptotic cells. Usually, tumors consume a large amount of nutriments to support their vigorous growth. Some parts of solid tumors would therefore be dystrophy due to their distempered vascular supply and even be apoptotic. Thus the apoptotic peak also appeared in control.

Compared with control, significant apoptotic peaks were observed for the cells treated with medicated fiber mats ($P < 0.01$), injection *in situ* ($P < 0.05$) and injection i.v. ($P < 0.05$). Among them, the apoptotic peak of the cells treated with medicated fiber mats was the strongest ($P < 0.01$). Because the area of apoptotic peak can directly reflect the number of apoptotic cells, the medicated fiber mats showed the most effective antitumor activity *in vivo*. The cell cycle distribution of control group was 37.34 ± 1.2%, 48.22 ± 7.27%, and 14.44 ± 2.58% in the G0/G1, S and G2/M phases, respectively. The cells treated with medicated fiber mats, injection *in situ* and injection i.v. 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. Among them, most H22 cells treated with medicated fiber mats accumulated in G0/G1 phase (74.62 ± 11.91%), and would inhibit their progression through the cell cycle, i.e., cell apoptosis was induced, implying medicated fiber mats showed the strongest anticancer activity; meanwhile, the cell apoptosis rate reached 24.21 ± 5.05%, and the percentages of the cells in G_0/G_1, S, and G_2/M phases were 74.62 ± 11.91%, 21.42 ± 9.25%, and 3.96 ± 2.23%, respectively. The results showed a marked increase in cell growth inhibition and apoptosis against H22 cells *in vivo* when the cells were treated with medicated fiber mats.

In animal experiments, tumors were inhibited, but there was no atrophy or fade. The reason may be: (1) the same dose of Dox 0.1 mg (3.3 mg per kilogram of body weight) was administrated to each test group in experiments. It is convenient for compare among groups, but the total dose is low. Especially for medicated fiber mats group, fiber mats release Dox slowly, insufficient to kill vigorous tumor cells; (2) on tumor-bearing animal models, solid tumors were in vigorous growth, there are therefore lots of cancer cells in number; while, the quantities of Dox molecules are less. However, in postoperation local chemotherapy, residual tumors are rarely, thus the same dose of Dox could inhibit cancer cells better. This explanation would be verified in further experiments.

CONCLUSIONS

Doxorubicin-loaded PEG-PLA electrospun fiber mats (medicated fiber mats) had the properties of loading doxorubicin hydrochloride (Dox) and sustained release of Dox with the degradation of fiber matrix, and retained apparently in a long term both *in vitro* cytotoxicity and *in vivo* anticancer activities by local implantation. Medicated fiber mats are therefore suitable for a local therapy device.

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