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Biodegradable microfibers deliver the antitumor drug temozolomide to glioma C6 cells *in vitro*

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To develop effective implants for delivery of 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-as-tetrazine-8-carboxamide (temozolomide; TM) with low initial burst and less neurotoxicity, TM-loaded poly-propylene carbonate (PPC) fiber was fabricated by electrospinning. Some of the fiber sheets were then covered with alginate (ALG). Influences of several preparation parameters on drug delivery behavior were investigated. The micro-morphology of these fibers was studied using scanning electron microscopy and differential scanning calorimetry. *In vitro* release properties of two forms of samples were observed and their cytotoxicity against C6 glioma cells was assessed. Using strict preparation parameters, smooth and uniform fiber could only be obtained when the PPC concentration was 8 % by weight, at 20 cm and a voltage of 15 kV between the nozzle and the collection instrument. Fiber diameter was about 3 μm. The initial burst of drug-fiber sheets was reduced after the fiber sheets were covered with ALG. Cytotoxicity test results suggested that both forms of drug fibers inhibit the C6 glioma cells continuously; the pure drug-fiber sheets were strongly cytotoxic. We conclude that (a) electrospinning is a reliable fabrication method for M-loaded PPC fibers; and (b) an ALG coating reduces the initial burst of the fiber sheets.

1. Introduction

Malignant glioma is the most common type of primary brain tumor in adults (Jemal et al. 2007). Approximately 5 new cases per 100,000 in population are diagnosed each year. The current standard treatment for malignant glioma consists of surgical resection followed by radiation therapy and aggressive systemic chemotherapy (Ushio et al. 1984; Fine et al. 1993; Stewart 2002; Drappatz et al. 2010). However, the prognosis for patients with malignant glioma is relatively poor with a median survival of < 2 years (Azizi and Miyamoto 1998). While chemotherapy is a powerful therapeutic modality for other tumors, it is not greatly effective in treating glioma because of the blood–brain barrier. However, the FDA-approved Gliadel® wafer showed some success in improving survival of patients with brain tumors (Gururangan et al. 2001; Attenello et al. 2008; Westphal et al. 2003; Pan et al. 2008), suggesting that localized chemotherapy is a promising way to prolong survival (Gallia et al. 2005; Wang et al. 2002). Localized chemotherapy diffuses drugs directly into tumors, and can supply persistent treatment between the resection and radiotherapy and systemic chemotherapy.

Temozolomide (3, 4-dihydro-3-methyl-4-oxoimidazo[5, 1-*d*]-as-tetrazine-8-carboxamide; TM) is one of the most effective antineoplastic agents for malignant glial tumors (Reni et al. 2004; Galldiks et al. 2010; Trent et al. 2002; Peereboom et al. 2010). It is evident that local delivery of temozolomide by biodegradable polymers is superior to oral administration in a rodent glioma model (Brem et al. 2007; Kong et al. 2010). Now, polymeric implants delivering TM developed are either micro/nano-particles, compressed micro/nano-particle discs or wafers (Garside et al. 2007; Akbar et al. 2009). Unfortunately, due to high interstitial pressure in the brain, intratumoral injection of drug-loaded microparticles runs the risk of being expelled out of the target site, thus negating the advantages of localized delivery (Kumar Naraharisetti et al. 2007). The other disadvantage of using microparticles is the high initial burst because of the presence of the drug on the surface (Elkharraz et al. 2006); this will lead to undesired neurotoxicity.

Various techniques have been designed to prepare controlled drug delivery systems, including solvent casting/salt leaching, membrane lamination, phase separation, emulsion freezing/drying, and hydrogel freezing/drying (Xie and Wang 2005; Lin et al. 2005). Electrospinning is also an attractive approach for the fabrication of fibrous biomaterials, which could be used to make tissue scaffolds, implants, biosensors, membranes, and controlled drug delivery systems (Abraham et al. 2005). Electrospun mats have larger specific surface areas and smaller pore size for polymer degradation and drug diffusion (Smith and Ma 2004) compared to other controlled drug delivery systems.

Most work on electrospinning biodegradable polymers has focused on synthetic materials, notably PLA, PGA, PLGA,

Table 1: The situation at different parameter: ×, difficult to electrospin; √, could electrospin but lower and instability; √√, electrospin fast and seriate

w/w		The situation at different parameter			
3wt %	cm\ kV	10	15	20	25
	10	×	×	×	×
	15	×	×	×	×
	20	×	×	×	×
4wt %	cm\ kV	10	15	20	25
	10	×	×	×	×
	15	√	×	×	×
	20	√	√	×	×
8wt %	cm\ kV	10	15	20	25
	10	√√	×	×	×
	15	√√	√√	×	×
	20	√√	√√	√√	×

and PCL. However, these materials have some disadvantages, such as low hydrophilicity, and aseptic inflammation because of their acid degradation products (Agrawal and Athanasiou 1997). Poly-propylene carbonate (PPC) is a degradable material formed from copolymerization of propylene oxide and carbon dioxide, using an organic metal catalyst (Sonabend et al. 2008). Its thermal stability and mechanical properties have been widely studied, but there are few studies of its use as a biomaterial or possibilities for submicron fibers (Du et al. 2004).

Our object was to find an effective way to deliver TM with low initial burst and less neurotoxicity. First, TM-loaded PPC fibers were fabricated by electrospinning; some fiber sheets were covered with ALG. After examining effects of preparation parameters, physical characteristics of these fibers were studied with scanning electron microscopy (SEM) and differential scanning calorimetry (DSC). Finally, we assessed release properties *in vitro* and cytotoxicity against two forms of C6 glioma cells.

2. Investigations, results and discussion

The distance between the nozzle and a grounded collection target was 10 cm, 15 cm, 20 cm or 25 cm. At each distance, the voltage between the nozzle and grounded collection target was changed to 10 kV, 15 kV or 20 kV. Table 1 suggests that the parameters of electrospinning with PPC-TM solution were strict. Electrospinning could only be performed normally when PPC was 8 % by weight, distance was 10–20 cm and voltage was 10–20 kV. In Fig. 1 SEM images suggest that electrospun fibers were formed with different shapes at different parameters. Only at 8 % by weight were the fibers were smooth and uniform. As shown in Fig. 2 the fiber diameter was around 3 μm.

One or two minimum diameters appeared when distance and voltage were changed. This phenomenon was determined by the process parameters of electrospinning. Two kinds of instability may accompany with the electrospun flow. One is axisymmetric instability, caused by the collaborative effect of both solution viscosity and the power of the surface charge density in tangent direction of the electric field. Another is nonaxisymmetrical instability, caused by fluctuation in fluid dipole and charge, from power in axial normal direction in the electric field, which can result in the wrap. These two kinds of instability can be transferred from each other in the manufacturing process, and may enlarge with electrospinning. The axisymmetric instability is known as tensional instability, and the nonaxisymmetric is known as flexural instability. In fibers produced by electrospinning, tensional instability could diminish fiber uniformity, while

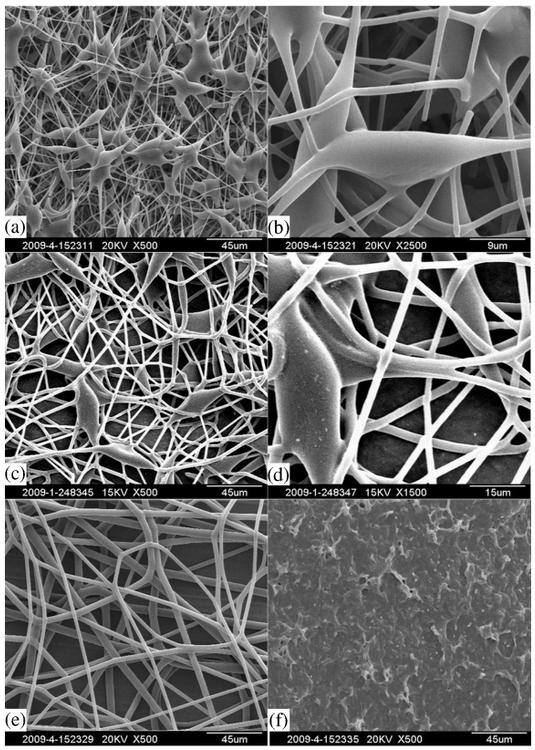

Fig. 1: SEM images of electrospun fiber. (a), (b): 8wt %, 15 cm, 15 kV; (c), (d): 4wt %

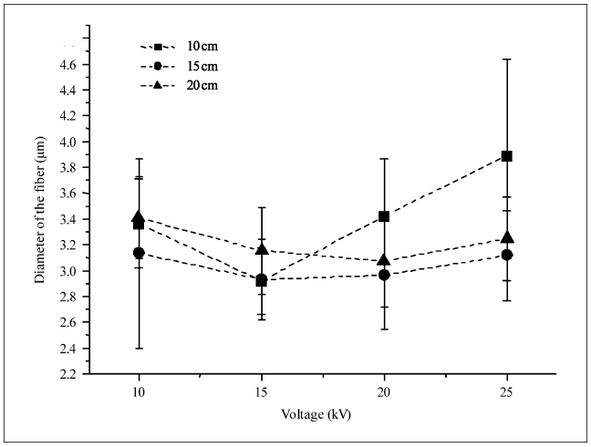

Fig. 2: The average diameter and the change with different voltage during the electrospining with PPC 8wt %. 15 %, 15 kV; (e): 8wt %, 20 cm, 15 kV; (f): the fiber covering ALG

whip movements caused by flexural instability could decrease flex instability in flow, thus enhancing fiber uniformity.

The DSC technique was used to characterize the physical status of TM in the fiber matrix after electrospinning. Pure TM shows an endothermic melting peak at 207.52 °C, but no peak was seen at temperatures of 100–250 °C for the fiber samples (Fig. 3). It is evident that the TM in the fiber was in an amorphous or disordered-crystalline phase of a molecular or a solid solution state in the matrix.

Table 2 shows that raising voltage and distance could enhance electrospinning and loading-rate stabilities. The EE data shows no drug loss in electrospinning—an advantage of this method over microparticles made by the emulsifying-solvent evaporation method.

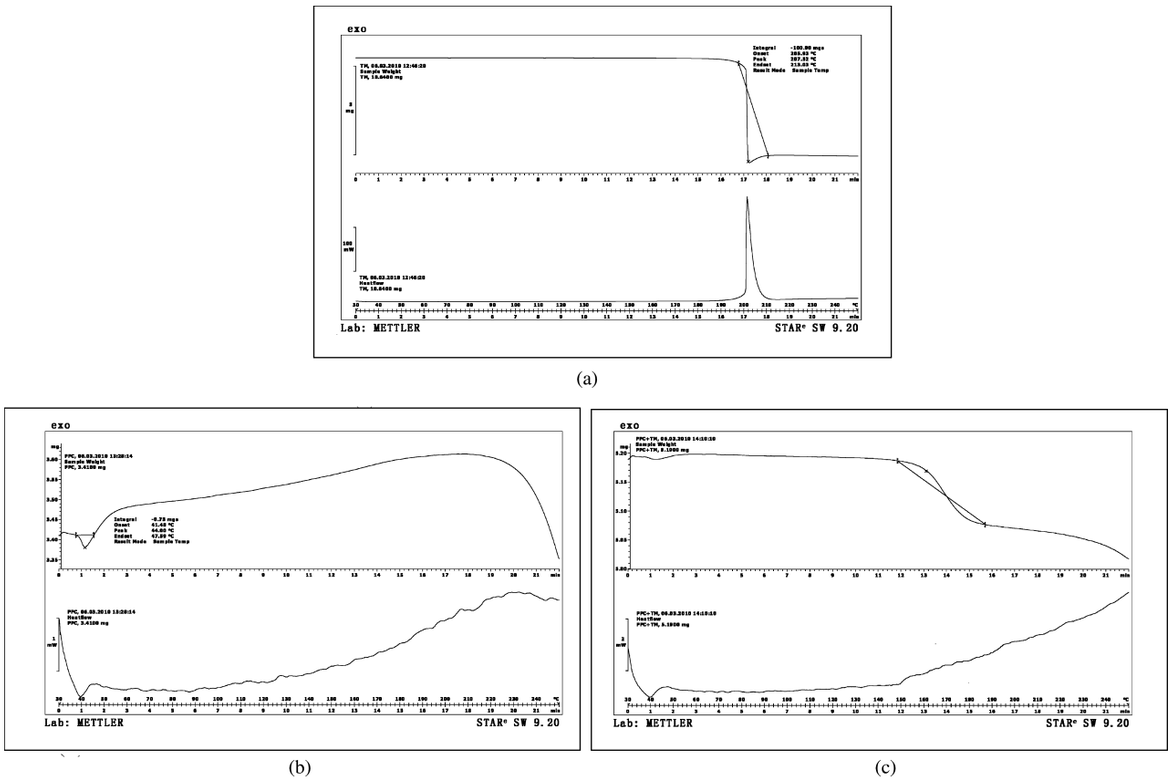

Fig. 3: DSC thermograms of TM, PPC, and TM-PPC fiber: (a) TM; (b) PPC; (c) TM-PPC fiber

The fiber sheets used for release study were formed with 8 % PPC concentrate by weight at 10 kV. One part of each fiber sheet was tested directly, and the other part was tested after being covered with ALG. Two kinds of fiber released TM for about 12 days (Fig. 4). The release rate of drug fiber without ALG was faster than that of the drug fiber with ALG; the former had an obvious burst in the first 3 days. The TM in the fiber had two compositions: TM embedded in the interior core of the fiber was released gradually along with degradation of material; TM on the surfaces of fibers, not coated by PPC gives an initial burst due to its release mechanism, which favors microparticles. In the 10 % loading drug fiber, the effect of fiber diameter on drug release was weak. The percentage of the initial burst and cumulative drug slightly increased as fiber diameter increased, possibly because small-diameter fiber underwent more elongation, allowing TM molecules to exist in the interior core of the fiber. After being covered with ALG, and TM need to permeate ALG layer to be released. The size of ALG micropores can be controlled by changing concentrations of ALF and cross linking agent in preparation, thus changing the release rate and prolonging TM release.

Table 2: Loading rate and encapsulation efficiency at different PPC concentration, voltage and distance

Condition	Distance (cm)	Loading (mg/g)	EE %
4wt %, 10kV	10	95.6	95.6 %
	20	96.3	96.3 %
8wt %,10kV	10	96.5	96.5 %
	15	97.4	97.4 %
	20	98.1	98.1 %
8wt %, 10kV, with ALG	15	71.2	96.2 %

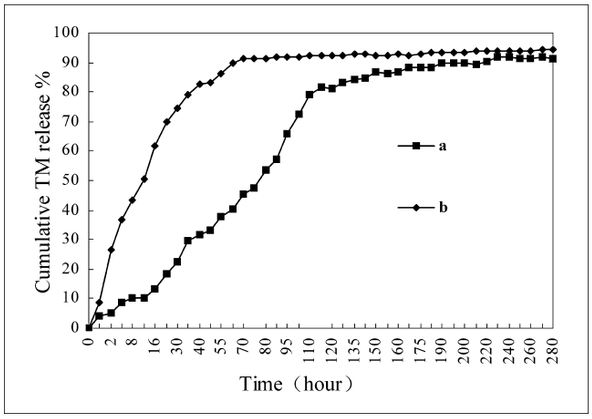

Fig. 4: TM release from two kinds of drug-fiber: (a), TM-PPC fiber sheets with ALG; (b), TM-PPC fiber sheets

This study was designed to show sustained cytotoxicity using degrading drug fibers. C6 glioma cells were used to quantify cytotoxicity of TM-PPC fiber and TM-PPC fiber with ALG. Blank PPC fiber was used as control. It was proved that TM powder was not persistently cytotoxic to the C6 cells (Zhang and Gao 2007). Fig. 5 shows that the PPC blank fiber was not cytotoxic to the C6 cells. Both kinds of drug-fiber sheets were significantly cytotoxic to the C6 cells, especially the TM-PPC fiber sheets. This is because that the TM-PPC fiber sheets released more drugs in the observing time due to the obvious initial burst, while TM-PPC fiber sheets with ALG covering released less drugs. Therefore, TM-PPC fiber sheets more easily induce neurotoxicity than TM-PPC fiber sheets with ALG covering.

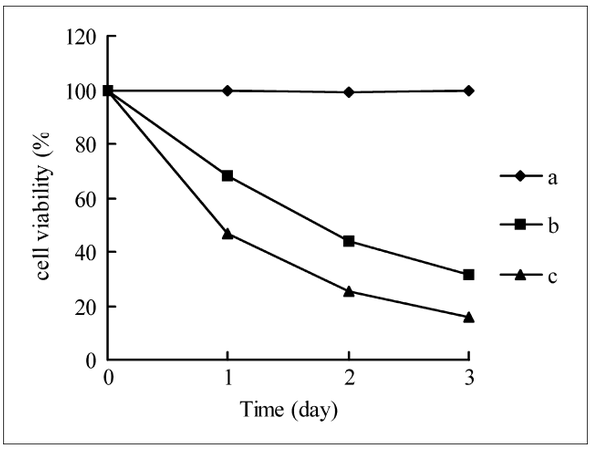

Fig. 5: Cell viability of glioma C6 cell treated with different groups: (a) blank; (b) TM-PPC fiber with ALG; (c) TM-PPC fiber

3. Experimental

3.1. Materials

PPC was purchased from Inner Mongolia Mengxi High-Tech Group Co. Ltd. TM was purchased from Kanglong Century Technology Development Co.Ltd. Wuhan, China. Sodium alginate (ALG), dichloromethane (DCM), dimethylformamide (DMF), acetonitrile (CAN) and calcium chloride were purchased from Beijing Chemical Reagent Company. All other materials and reagents used were of analytical grade.

3.2. TM-PPC fabricated by electrospinning

Purified PPC and TM at a 1:9 ratio were dissolved in the solvent acetonitrile, stirred for 6 h until dissolved. The polymer solution was placed in a 5-ml glass syringe, fitted with a blunt needle with a tip diameter of 0.9 mm, to which a high positive voltage of 10–20 kV was applied. The solution was pumped at a predetermined rate. Fibers were collected on an electrically grounded aluminum foil placed 10–20 cm below the needle tip. Before using, fiber sheets were dried under vacuum at room temperature for 24 h.

3.3. TM-PPC fiber sheets covered with alginate

Purified PPC and TM were exactly weighed and dissolved in the solvent acetonitrile under stirring for 6 hours until being dissolved. The drug-polymer solution was pumped at a predetermined rate using a syringe pump. The high voltage was 10 kV and the distance between the nozzle and the collection was 15 cm. The drug-fiber sheets obtained were soaked in the solution with 1 % sodium alginate until the solution permeated the sheets. The sheets were then soaked in the 1 % calcium chloride solution (pH 5) for 2 min, and dried at room temperature.

3.4. In vitro characterization

3.4.1. Fiber diameter, distribution and morphology

The size, distribution and surface morphology of the fibers were examined using a scanning electron microscope (S450, Hitachi, Japan) after gold coating.

3.4.2. Thermal analysis of the drug in the fiber matrices

Thermal analysis was performed using DSC. Fiber samples weighing 3–4 mg were hermetically sealed in a standard 40-μl aluminum crucible; an empty crucible was used as a reference. Thermal analysis was conducted over a temperature range of 25 °C–300 °C with a heating rate of 10 °C/min. The equipment was purged with N_2 at a rate of 30 ml/min; liquid nitrogen was used to reinstate the instrument to the starting temperature. Calibration of the instrument's temperature and enthalpy was done using pure indium. Characteristic peaks were obtained by analyzing pure TM and the fiber sheets.

3.4.3. Drug encapsulation efficiency (EE) and in vitro TM releasing study

To quantify encapsulation efficiency, fiber sheets were ground to powder, dissolved in DCM, and stirred for 2 h. The solution was filled with 0.1 mol/L Hcl and shaken thoroughly; TM was extracted from DCM. Residues were re-dissolved in 0.1 mol/L Hcl with 1 % EDTA until vanished. This solution mixed with the prior solution containing TM. The final solution was analyzed using a UV spectrophotometer. The loading rate and encapsulation efficiency were calculated according to the following equations:

$$\text{Loading rate} = W_m/W_t \times 100$$

$$\text{Encapsulation efficiency} = W_m/D_t \times 100,$$

where W_m is the amount of TM in the fibers, D_t is the amount of TM used for the preparation, and W_t is the weight of the fiber.
The *in vitro* TM release study was conducted by immersing fiber sheets into 20-ml PBS buffer containing 0.1 % Hcl in a 50-ml centrifuge tube, incubated at 37 °C and 100 rpm in a shaking water bath. At each specified time interval, the sample was picked out, washed with deionized water, and put into another prepared centrifuge tube. The solution was subjected to UV analysis at 334 nm. Standard TM concentration samples were used to produce calibration plots.

3.5. Cell culture

Glioma C6 cell lines were obtained from the laboratory of Shandong Provincial Hospital in Jinan, China. Cells were maintained in tissue culture in Dulbecco's minimum essential medium with 10 % fetal bovine serum, in atmosphere of 5 % CO_2, and 90 % relative humidity at 37 °C. The cells were grown to confluence, detached with 0.25 % trypsin in Dulbecco's phosphate-buffered saline, and re-suspended in medium.

3.6. Cytotoxicity test

The cytotoxicity assay was conducted by Cell Counting Kit-8(cck-8), which is better than MTT assay to quantify living cells (Tominaga et al. 1999). Cell viability was determined by a microplate reader. Four groups were set up, each group in triplicate. Cells were first transferred to a 96-well plate to ensure 1×10^4 cells per well. Overnight, the medium was changed with 200 μl medium. All samples were added at 0, 1, 2 days. One row was used as control without adding anything. Medium and fiber sheets were removed and changed with 100 μl medium. Cck-8 was added to ensure 10 μl per well. The plate was incubated for 1 h, then screened through a microplate reader. Cell viability was determined according to the following equation:

$$\text{Cell viability}\,(\%) = \text{Abs test cells}/\text{Abs control cells} \times 100.$$

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