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Temozolomide/PLGA microparticles and antitumor activity against Glioma C6 cancer cells *in vitro*

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Abstract

The purpose of the present study was to develop implantable poly(D,L-lactide-*co*-glycolide) (PLGA) microparticles for continuous delivery of intact 3,4-dihydro-3-methyl-4-oxoimidazo[5,1-*d*]-as-tetrazine-8-carboxamide (temozolomide, TM) for about a 1-month period and to evaluate its cytotoxicity against Glioma C6 cancer cells. The emulsifying-solvent evaporation process has been used to form TM-loaded PLGA microparticles. The influences of several preparation parameters, such as initial drug loading, polymer concentration, and stirring rate were investigated. Scanning electron microscopy (SEM) showed that such microparticles had a smooth surface and a spherical geometry, i.e. microspheres. The differential scanning calorimetry (DSC) and powder X-ray diffraction (XRD) results indicated that TM trapped in the microparticles existed in an amorphous or disordered-crystalline status in the polymer matrix. The release profiles of TM from microparticles resulted in biphasic patterns. After an initial burst, a continuous drug release was observed for up to 1 month. Finally, a cytotoxicity test was performed using Glioma C6 cancer cells to investigate the cytotoxicity of TM delivered from PLGA microparticles. It has been found that the cytotoxicity of TM to Glioma C6 cancer cells is enhanced when TM is delivered from PLGA polymeric carrier and, PLGA only did not affect the growth of the cells. Meanwhile, the cytotoxic activity of TM powder disappeared within 12 h.

Keywords: Temozolomide (TM); Poly(D,L-lactide-*co*-glycolide) (PLGA); Emulsifying-solvent evaporation; Microparticles; Cytotoxicity

1. Introduction

A major factor limiting intracranial therapeutic levels of systemically administered chemotherapeutic agents is the physiologic barriers of the brain (Gallia et al., 2005). The presence of the blood–brain barrier (BBB) restricts permeability of certain drug molecules within the brain and prevents diffusion of these agents into the brain tumor (Kornblith and Walker, 1988). 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 tumor, partially due to its ability to cross the BBB (Reni and Mason, 2004). However, TM must be administered in high systemic doses to achieve therapeutic brain levels due to its short half-life of about 1.8 h in plasma (Baker et al., 1999). Furthermore, prolonged systemic administration is associated with some side effects such as nausea, vomiting, fatigue and headache.

Recent reports have shown that polymeric devices implanted into the brain can release locally neuroactive substances for extended periods of time (Menei et al., 1996, 1997). In this manner, the brain implantation of polymeric devices has been achieved in men for the treatment of malignant cerebral tumors (Brem et al., 1995). Poly(D,L-lactide-*co*-glycolide) (PLGA) is a well-known biodegradable polymer, which has long history of safe use in pharmaceutical and medical applications (Hutchison and Furr, 1990). Furthermore, the study have demonstrated the biocompatibility and biodegradability of blank poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres implanted in the brain tissue (Emerich et al., 1999; Menei et al., 1993).

The present research report describes the *in vitro* release of TM from PLGA-based microparticles fabricated using the emulsifying-solvent evaporation method. The influences of several preparation parameters, such as initial drug loading, polymer concentration, and stirring rate were investigated. The physical characteristics of TM-loaded PLGA microparticles were studied using scanning electron microscopy (SEM), powder X-ray diffraction (XRD), and differential scanning

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calorimetry (DSC). The *in vitro* antitumor activity of TM released from the microparticles was assessed against Glioma C6 cancer cells in comparison with that of TM powder.

2. Materials and methods

2.1. Materials

PLGA (75:25 mole ratio of lactide to glycolide) having molecular weight of 20,000 g/mol were purchased from Shandong Medical Equipment Research Institute (China). TM was supplied by Friend Pharmaceutical Co. Ltd. (Beijing, China). Polyvinyl alcohol (PVA) (88% hydrolyzed, Beijing, China) was used as the emulsifying agent. The methylene chloride (CH_2Cl_2) was used without further purification (Shanghai, China). All other chemicals were reagent grade. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France).

2.2. Microparticles preparation

TM-loaded PLGA microparticles were prepared using emulsifying-solvent evaporation method. Microparticles were performed according to the following procedure: PLGA (0.2 g) was dissolved at room temperature in a known volume of CH_2Cl_2. A definite amount of TM (10–80 mg) was then dispersed in the resulting solution. This obtained organic phase was added under an agitation of 600 rpm for 5 min, to 80 ml of aqueous phase containing 2% (w/v) PVA (saturated with TM beforehand). The emulsion was continuously stirred at room temperature until the organic solvent evaporated or diffused from the microparticles completely. The formed microparticles were washed twice with 100 ml of deionized water, recovered by filtration and then freeze-dried.

Two main process parameters were investigated: the polymer/solvent ratio (w/v, %) (5, 5.71, 6.67, 8, 10, 13.33) and the stirring rate of the emulsion (400, 600, 800 rpm).

2.3. Characterization of microparticles

TM-loaded PLGA microparticles were observed by scanning electron microscopy (SEM, model S-2250N, Hitachi, Japan) in order to examine the morphology and size of the microparticles. Samples for SEM were mounted on metal stub double-sided tape and coated with platinum for 30 s under argon atmosphere using plasma sputter (SC 500K, EMscope, UK). The microparticles physical status characterization was performed by jointly measuring the differential scanning calorimetry (DSC) and X-ray diffractometry (XRD) patterns. To carry out DSC tests, 4 mg of sample was sealed in standard aluminum pans with lids. The sample was purged with pure dry nitrogen at a flow rate of 50 ml/min. The temperature ramp speed was set at 10 °C/min from 0 to 250 °C. Indium was used as the standard reference material to calibrate the temperature and energy scales of the DSC instrument. Crystalline state of TM was analyzed by powder XRD (D/MAX-III B, Rigaku, Japan). A Ni filter at 45 kV and 40 mA generated the radiation. The samples were placed in a quartz sample holder and scanned from 0 to 80° at a scanning rate of 10°/min.

2.4. Determination of TM content in microparticles

EE is defined as the percentage of the actual mass of drug encapsulated in the polymeric carrier relative to the initial amount of drug loaded. In the determination of the EE, microparticles were accurately weighed and dissolved in a certain volume of acetonitrile via sonication till complete solubility. The drug content of each sample was then analyzed using UV-spectrophotometer at λ_{max} 327 nm.

The encapsulation efficiency (EE) was calculated from the following equation (Seong et al., 2003):

$$\mathrm{EE}\,(\%) = \frac{D_\mathrm{m} \times 100}{D_\mathrm{t}}$$

where D_t is the amount of TM used for the preparation and D_m is the amount of TM in the freeze-dried microparticles.

2.5. In vitro release study

The *in vitro* release of TM loaded microparticles was measured in phosphate buffered saline (PBS, pH 7.4) in triplicate at temperature of 37 °C. Fifteen milligrams of TM loaded microparticles were suspended in 10 ml of PBS in a screw capped tubes, which were placed in an orbital shaker bath maintained at 37 °C and shaken horizontally at 60 min^{-1}. At specific time following incubation samples were taken out and centrifuged at 4000 rpm for 5 min. The supernatant was withdrawn and the residue was dissolved in acetonitrile, UV analysis can then be done as previously described. Due to the unstability of TM in the release test condition the amount of TM released into PBS was calculated by the amount of TM remained in the microparticles after specific release test period.

2.6. Cell culture

Glioma C6 cell lines were obtained from Institute of Biochemistry and Cell Biology, SIBS, CAS and cultured using methods as described by Freshney (Freshney, 1983). Briefly, cells (10^4 cells/ml) were grown and routinely maintained at 37 °C in 75 cm^2 culture flasks, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin in atmosphere of 5% CO_2, and 90% relative humidity. The cells were harvested with trypsinethylenediaminetetraacetic acid. Medium was changed every other day.

2.7. Cytotoxicity test

The cytotoxicity assay was conducted by using MTT assay, which is commonly used to quantify the living cells which are still metabolizing (Rongy et al., 2005). The cell viability was determined by a microplate reader. Cells were transferred to 96-well plate first to ensure 1×10^4 cells per well. Medium was changed every other day until 80% confluence was reached. Then the medium was changed with 100 μl

Table 1
Influence of stirring rate on the microparticles size

Stirring rate (rpm)	Microparticles size (μm)
400	71.7
600	62.6
800	54.3

medium with TM-loaded microparticles of different concentrations and PLGA only microparticles. The plate was incubated for 1, 2 and 3 days. One row of 96-well plates was used as control without adding microparticles. At different intervals, suspension was removed and the wells were washed thrice using PBS. Ten microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 90 μl medium were then added to the wells. After incubation for around 3–4 h, solution was removed, leaving the precipitate. One hundred microliters of DMSO was then added to the wells before the plate was observed using microplate reader. Cell viability was determined by the following equation:

$$\text{cell viability}\,(\%) = \left(\frac{\text{Abs test cells}}{\text{Abs control cells}}\right) \times 100$$

where Abs test cells and Abs control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (non-treated), respectively (Jingwei and Chi-Hwa, 2005). All experiments were performed in triplicate.

3. Results and discussion

3.1. Microparticle preparation

TM-loaded PLGA microparticles were prepared using emulsifying-solvent evaporation method. In the process, aqueous phase containing 2% (w/v) PVA was saturated with TM beforehand to improve the encapsulation efficiency of TM. It was found that when aqueous phase was not saturated with TM, TM in the organic phase was almost diffused into the aqueous phase and the encapsulation efficiency was only about 30%. The encapsulation efficiency was significantly improved when aqueous phase was saturated with TM. Table 1 shows the effect of stirring rate on the microparticles size, then the stirring rate being set at 600 rpm. Table 2 shows the influence of polymer concentration on the microparticles size. It was noticeable that the size

Table 2
Influence of the polymer concentration on the microparticles size (stirring rate 600 rpm)

PLGA concentration (%)	Microparticles size (μm)
5	55.2
5.71	59.8
6.67	62.6
8	65.1
10	68.3
13.33	73.6

Table 3
Influence of the initial TM amount on drug content, encapsulation efficiency and microparticles size (polymer concentration 6.67%)

TM (mg)	Encapsulation efficiency (%)	Microparticles size (μm)
10	81.23	61.5
20	84.69	62.6
40	80.44	64.9
60	73.15	63.3
80	62.37	62.2

of the microparticles was significantly influenced by the polymer concentration in the dispersed phase during preparation. An increase in the polymer concentration resulted in a larger particle diameter. This might be explained by a greater probability of fusion of semi-formed particles when they ran into each other in the medium. In addition, increasing the concentration of dissolved polymer also increased the viscosity of the organic phase, which might prevent an optimal shearing of the emulsion when agitated. Similar results have been previously reported (Sturesson et al., 1993 and Jeffery et al., 1991). The adjustment of the previous process conditions was done without the presence of TM.

In a last series of experiments, TM was introduced in increasing amounts in the organic phase. Table 3 reports the encapsulation data obtained along with the mean sizes of the particles. The microparticles size was not affected by the presence of the drug.

EE does not vary much with increased amount of TM from 10 to 40 mg, however, it decreased to 73.15 and 62.37% when the amount of TM increased to 60 and 80 mg, respectively. It may caused by the low solubility of TM in CH_2Cl_2 and weak miscibility with PLGA. Even the aqueous phase saturated with TM, TM in the organic phase remains to diffused into the aqueous phase and then the crystalline of TM formed in the aqueous phase. TM continuously diffused outwards till the solubility limit was attained in the organic phase, then the system reachs stabilization. So when the drug loading reachs to a certainty amount, EE decreased as drug loading increased.

SEM observations showed that microparticles were spherical and exhibited a very smooth surface (Fig. 1). There was no evidence of pore formation.

3.2. DSC and XRD analysis

The DSC technique can provide qualitative and quantitative information about the physicochemical status of the drug in the microparticles, which was reported to be involved in the endothermic or exothermic process (Dubernet, 1995). The related thermal transitions include melting, recrystallization, decomposition, out-gassing, or a change in heat capacity. DSC is useful to monitor different samples of the same material to assess their similarities or differences or the effects of additives on the thermal properties of a material. Using the DSC analysis of drug, polymer materials and produced microparticles, the nature of the drug inside the polymer matrix can be assessed, which may emerge in solid solution, metastable molecular

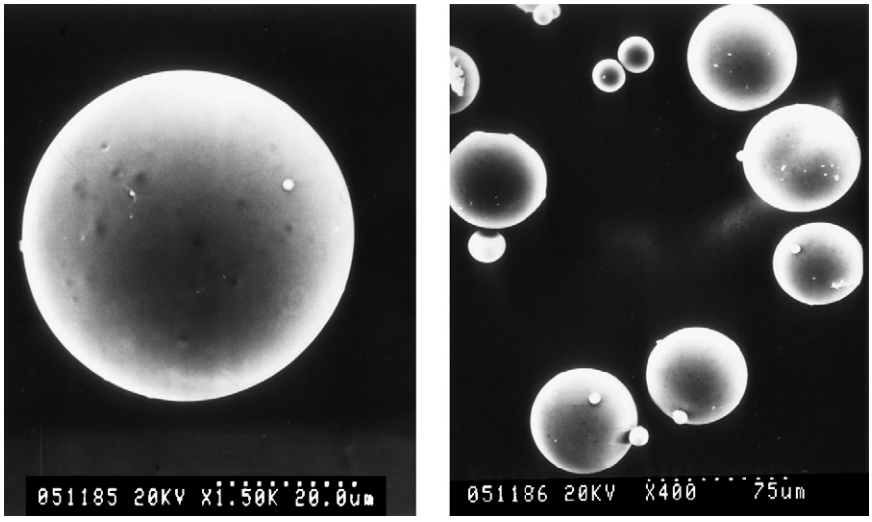

Fig. 1. Scanning electron micrograph of TM-loaded PLGA microparticles (TM amount 20 mg).

dispersion or crystallization (Dubernet, 1995) and may display relevant properties during *in vitro* release. In the present work, the DSC thermograms of pure TM, pure PLGA materials and TM-loaded microparticles were detected and shown in Fig. 2. The drug loading of the samples in DSC experiment is about 10%. The pure TM shows an endothermic peak of melting at 212.11 °C. There was no peak observed at the temperature range of 100–250 °C for the samples. The DSC study did

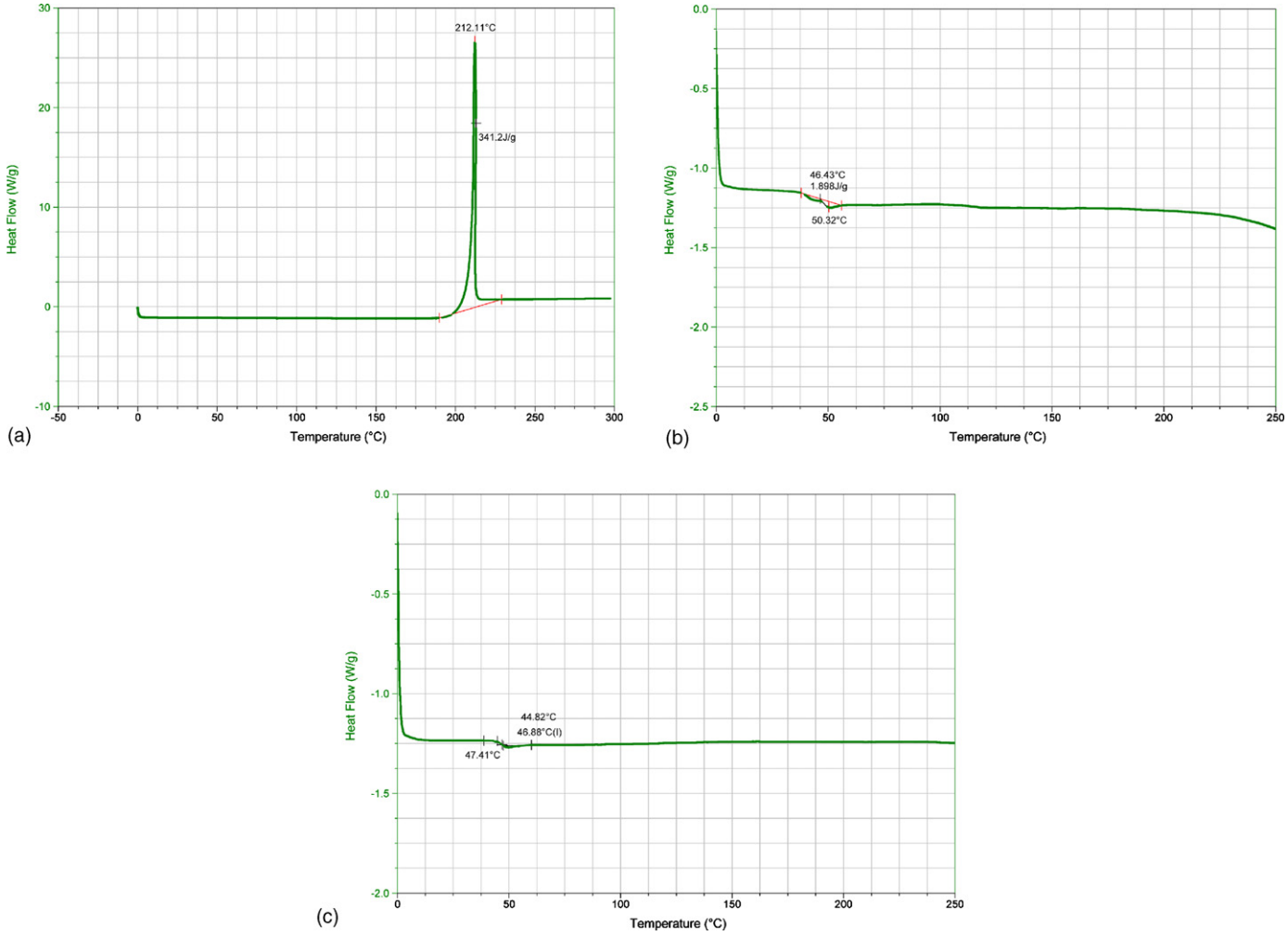

Fig. 2. DSC thermograms of TM, PLGA, and TM-loaded PLGA microparticles: (a) TM; (b) PLGA; (c) TM-loaded microparticles.

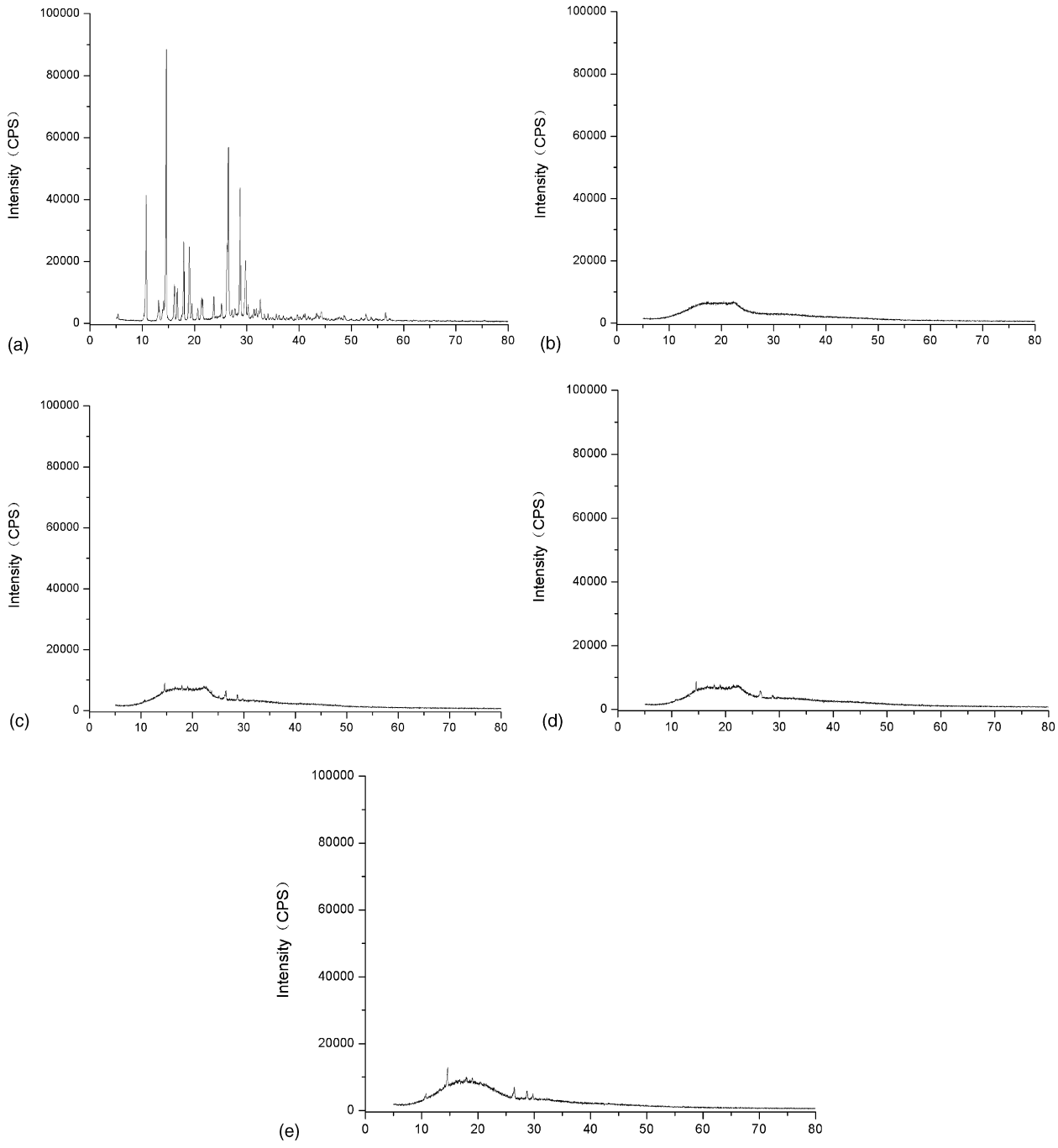

Fig. 3. XRD patterns of TM, PLGA, and TM-loaded PLGA microparticles: (a) TM; (b) PLGA; (c) TM-loaded microparticles (TM amount 20 mg); (d) TM-loaded microparticles (TM amount 40 mg); (e) TM-loaded microparticles (TM amount 60 mg).

not detect any crystalline drug material in the microparticles samples. It could thus be concluded that the TM formulated in the samples was in an amorphous or disordered-crystalline phase of a molecular dispersion or a solid solution state in the polymer matrix after fabrication. The X-ray patterns of pure TM, pure PLGA materials and TM loaded microparticles are shown in Fig. 3. It was observed that characteristic peak of TM almost could not be found in microparticles. This indicated that no crystalline drug was detected, in accordance with DSC results of TM-loaded PLGA microparticles.

3.3. In vitro release study

The release profiles of TM from TM-loaded PLGA microparticles are shown in Fig. 4. The release rate of TM from PLGA microparticles increased with the increase of TM loading amount and the period of 100% TM release was almost 35 days. It showed an initial burst in the first day. Initial burst also increased with the increase of TM loading amount. The reason of initial burst could be due to diffusional release of drug particles on the surface of microparticles and a higher drug-loading

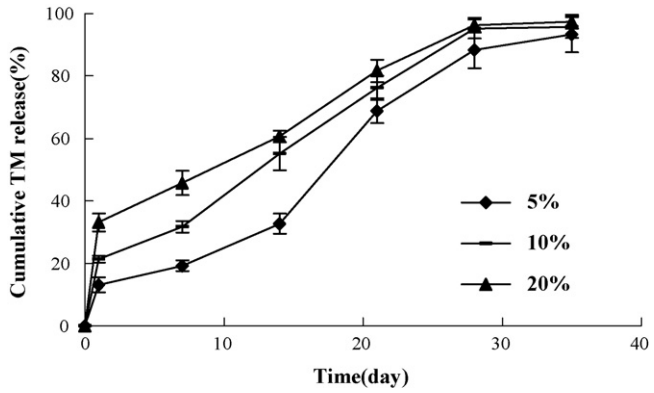

Fig. 4. Effect of the drug loading ratio on the release profiles of TM from PLGA microparticles.

amount resulted in a high amount drug particles on the surface of microparticles (Bodmeier and Chen, 1989). The release rate and pattern of drug from PLGA matrix is mainly dependent not only on diffusion of drug through the matrix but also on the degradation of PLGA (Schwendeman et al., 1996). Therefore, drug loading amount, molecular weight, and monomer ratio of copolymer are the major factors affecting the drug release rate and pattern. A period of sustained release estimated from approximately 1–35 days while release amount increased continuously. This release pattern is mainly dependent on the diffusion of the drug through the polymer matrix that has many channels due to the polymer degradation after the water uptake.

3.4. Cytotoxicity of TM-loaded PLGA microparticles

In this cytotoxicity test, we selected the 10% TM loading microparticles, because, in the previous *in vitro* releasing test, its releasing pattern was satisfied with our purpose. The cytotoxicities of the TM-loaded microparticles were evaluated by assessing cell viability using the MTT assay on glioma C6 cell lines. To facilitate the basis for comparison, cells were incubated with concentrations of microparticles that contained the same amount of drug as that of free TM sample with TM concentration of 5, 10 and 20 μg/ml, respectively. The cytotoxicities of the TM-loaded microparticles and PLGA only microparticles are shown in Fig. 5. In the case of PLGA only microparticles

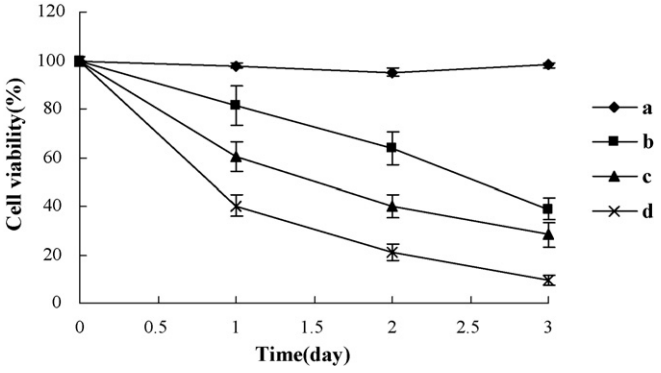

Fig. 5. Cell viability of glioma C6 cells treated with microparticle samples at the TM concentration of (b) 5 μg/ml, (c) 10 μg/ml, (d) 20 μg/ml and PLGA only (a).

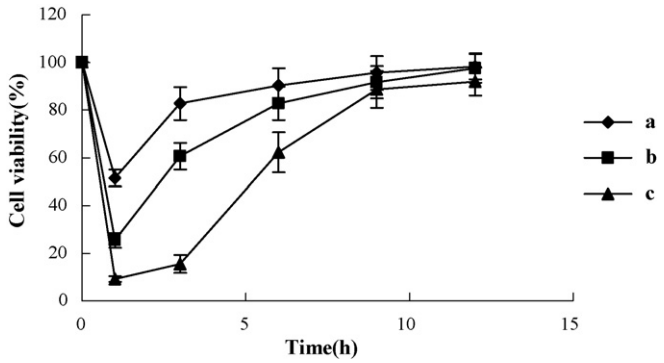

Fig. 6. Cytotoxicity of TM to the glioma C6 cells in vitro. The TM powder were dissolved in cell culture medium at various concentrations. (a) 5 μg/ml; (b) 10 μg/ml; (c) 20 μg/ml.

(without TM), the samples did not reveal cytotoxicity to the C6 cells. In the case of TM-loaded microparticles, all the samples inhibited the cell growth for all the time course tested. In the case of free of TM powder (Fig. 6), all samples of different TM concentrations did not reveal cytotoxicity to the C6 cells after 12 h. This observation shows that the TM-loaded microparticles have higher cytotoxicity than TM because of the sustained release properties shown in Fig. 4.

4. Conclusions

TM-loaded PLGA microparticles were prepared by emulsifying-solvent evaporation method in reproducible manner. Several preparation parameters, such as initial drug loading, polymer concentration, and stirring rate played a predominant role in the preparation. Microparticles had spherical shape, i.e. microspheres. From SEM, X-ray and DSC results, it appeared that TM trapped in the microparticles existed in an amorphous or disordered-crystalline status in the polymer matrix. The release profiles of TM from microparticles resulted in biphasic patterns. The release rate of TM from PLGA microparticles increased with the increase of TM loading amount and the release of TM was almost finished at 35 days. The results of cytotoxicity test showed that the cytotoxicity of TM to Glioma C6 cells could be enhanced when TM is delivered from polymeric device. However, despite the results obtained, due to cell culture limitation, long-term reproductive survival of tumor clonogens can only be determined using further in vivo animal tests.

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