Preparation of Injectable Paclitaxel Sustained Release Microspheres by Spray Drying for Inhibition of Glioma *In Vitro*

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ABSTRACT: The major aim of this work was to prepare injectable paclitaxel-loaded poly(D,L-lactide) microspheres for the inhibition of brain glioma. Paclitaxel-loaded PLA microspheres were prepared by spray drying method employing ethyl acetate as solvent. And the microspheres were characterized by scanning electron microscopy (SEM) for the morphology and differential scanning calorimetry for thermal analysis. The encapsulation efficiency (EE) and *in vitro* release profiles of paclitaxel-loaded microspheres were determined by using ultraviolet spectrophotometer. The results showed that the microspheres possess a narrow size distribution with the average diameter of 4.6 μm. The surface of the microspheres was smooth, and the paclitaxel dispersed in microspheres in amorphous state. The solvent residue was 0.03%, and the EE reaches ~ 90%. The microspheres exhibited a sustained release behavior, and the release period last for at least three months, depending on the EE of the microspheres. The γ irradiation sterilization had little effect on the EE and drug release *in vitro*. Compared with the commercial formulation, the sustained release microsphere showed a stronger inhibition on the tumor cells, suggesting the potential application of long-term delivery of paclitaxel-loaded PLA microspheres in clinic tumor therapy. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1534–1539, 2010

Key words: paclitaxel; polylactic acid; spray drying; injectable microshperes; glioma

INTRODUCTION

Injectable drug loaded PLA microspheres have gained increasing attention and application in clinic tumor therapy because of the well biodegradability, biocompatibility of the polymer and the local sustain drug release behavior of the microspheres, which can achieve high drug concentration in tumor location and decreasing systemic toxicity significantly.[1–3]

Injectable drug-loaded PLA microspheres are usually produced by solvent evaporation and phase separation.[4] However, the microspheres prepared by phase separation are easy to agglomerate, and the organic solvent used in the process is hard to be removed.[5] While the solvent evaporation requires complex steps and is hard to achieve industrial production.[6] Compared with the former two methods, spray drying is a simpler method for scaling up, which is beneficial to industrial production of formulations.

Paclitaxel, a natural extract of the bark of pacific yew tree, is an effective antineoplastic chemotherapy drug. It is a well mitotic inhibitor which can promote the formation of microtubule and then stabilize it, thus inhibiting mitosis, leading to the death of the cancer cells.[7] Paclitaxel has been widely applied to treat various cancers, especially ovarian,[8] breast, and nonsmall cell lung cancer.[9] However, its high insolubility in water and most pharmaceutical solvents limit the development of paclitaxel formulations.[10] One method to overcome this problem is to use Cremophor® EL(polyoxyethylated castor oil) and ethanol as adjuvants. The product named Taxol® has been applied in clinic to treat various cancers. But its applications was always limited by the side effects, such as short-term stability upon dilution with aqueous medium,[11] incompatibility with certain infusion sets,[12] serious CrEL-associated side effects[13] and the emergence of paclitaxel-resistant phenotypes mediated by the Pglycoprotein (P-gp) efflux pump.[14] The purpose of present study was to develop one kind of dosage constituted by biodegrable microspheres which suitable for the steric injection to treat

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tumor locally, so the side effects of paclitaxel can be dramatically minimized. To fulfill this purpose, we prepared paclitaxel-loaded PLA microspheres by spray drying method, choosing ethyl acetate as solvent.

MATERIALS AND METHODS

Materials

Paclitaxel was obtained form Tianfeng Biotechnology, Shenyang, China, poly(D,L-lactide) (M_n 30,000, polydispersity index = 1.64) was purchased from Medical Instrument Institute, Shandong, China, acetonitrile, ethyl acetate and dichloromethane were supplied by Chemical reagent, Tianjin, China. All of the reagents are analytically pure.

Preparation of microspheres

Preparation of microspheres by spray drying method

The preparation of paclitaxel-loaded PLA microspheres was carried out by spray drying process (Spray-dryer L-117, Laiheng, Beijing). Briefly, 2 g of poly(D,L-lactide) and different amount of paclitaxel was dissolved in 50 mL of ethyl acetate. The ratio (paclitaxel: PLA) was varied from 5 to 10%. Then the resultant solutions were sprayed through the nozzle of the spray dryer. Assay conditions were: inlet air temperature 40°C ± 2°C, outlet temperature 40°C ± 2°C, spray flow 0.45 m^3/min, pump speed 1.6 L/h, and compressed spray air flow (represented as the volume of the air input) 10 L/min. Microspheres were collected from the spray dryer cyclone separator, and then stored in a desiccator until use.

Preparation of microspheres by emulsion solvent evaporation method

For comparison, paclitaxel-loaded PLA microspheres were also prepared by emulsion solvent evaporation. Briefly, a certain quality of paclitaxel and PLA were dissolved in 4 mL of ethyl acetate. Then the solution was added drop by drop into the aqueous solution containing PVA and emulsifier under ultrasonic action. After well dispersed, the organic solvent in the emulsion was volatilized by rotary evaporation. The microspheres were obtained after centrifugal separation.

Characterization of microspheres

Morphology studies of paclitaxel-loaded microsphers

The morphology of the microspheres was observed by SEM (model S-2250n, Hitachi, Japan). Samples were mounted on glass stub by double-sided tape and coated with gold under argon atmosphere.

Drug load rate and encapsulation efficiency (EE)

About 3 mg of paclitaxel-loaded microspheres were dissolved in 1 mL DCM and after well dissolved, 2 mL mixture solution (the ration of acetonitrile : deionized water was 1 : 1) was added. Then a nitrogen stream was introduced to evaporate DCM at room temperature. After that, the solution was centrifuged at 12,000 rpm for 20 min. The supernatant was analyzed by ultraviolet spectrophotometer at 227 nm.

Differential scanning calorimetry (DSC) analysis

The physicochemical status of the paclitaxel in the microspheres was measured by differential scanning calorimetry (DSC) analysis. 3 mg of microspheres was sealed in standard aluminum pans with lids. The samples were purged with pure dry nitrogen at a flow rate of 50 mL/min. The heat flow was recorded from −20 to 80°C at the rate of 10°C/min.

Determination of solvent residue

Gas chromatography with mass spectrometer detector (GC-MSD) was used to determine the content of residual ethyl acetate in the microspheres. The temperature was 80°C for 40 min and DMF was used as the solvent.

γ rays sterilization

Paclitaxel-loaded microspheres were placed in vials and sealed under an argon atmosphere. Then the samples were irradiated with a ^{60}Co source. The dose was 25 kGy in order to achieve effective sterilization. The process was performed in the presence of dry ice to maintain a low temperature during the irradiation process (−78.5°C), preventing undesired thermal effects.

In vitro release of paclitaxel from paclitaxel/PLA microspheres

In vitro release of paclitaxel was carried out as follows: 30 mg of microspheres were suspended in 30 mL of phosphate buffer (0.01 *M*, pH = 7.4) and then incubated in shaking water bath under constant shaking rate (72 rpm) at 37°C to mimic physiological conditions. At appropriate time intervals, the solution was removed and replaced with fresh buffer. Then the paclitaxel content of the solution was extracted by adding 2 mL of DCM. Then DCM was evaporated under a stream of nitrogen. After that,

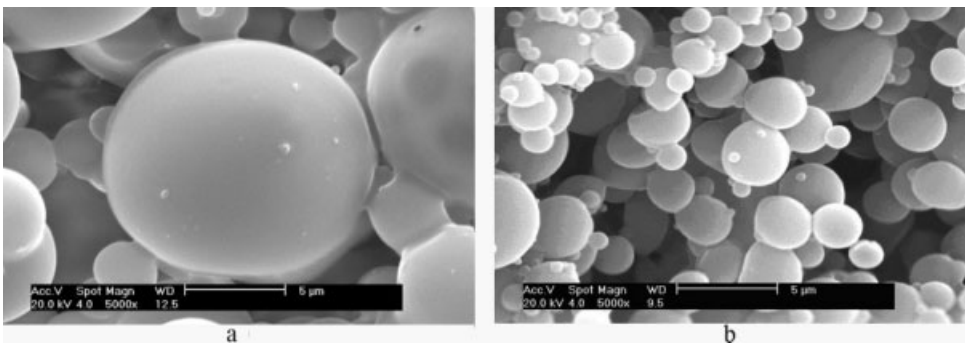

Figure 1 Surface morphology of paclitaxel-loaded PLA microspheres. (a) microspheres prepared by solvent evaporation, (b) microspheres prepared by spray drying (The theoretical encapsulation ratio = 10%.).

2 mL mixture solution (the ration of acetonitrile : deionized water is 1 : 1) was added into the remaining solution. The absorbance of the solution was identified by ultraviolet spectrophotometer at 227 nm. Then the paclitaxel content was obtained by referring to the standard curve.

Degradation of PLA microspheres

PLA microspheres (30 mg) was suspended in 30 mL of phosphate buffer (0.01 *M*, pH = 7.4) and then incubated in shaking table under constant shaking rate (72 rpm) at 37°C. At appropriate time intervals, some of the microspheres were taken out and centrifuged. The obtained microspheres were then frozen dried and the molecular weight was analyzed by gel permeation chromatography (GPC). Tetrahydrofuran was used as the mobile phase. Columns were calibrated using poly(methyl methacrylate) standards (Polymer Lab). Samples were dissolved in tetrahydrofuran and then microfiltered before the measurement.

In vitro cell viability and proliferation

The MTT assay was performed to measure the cell proliferation using a U251 human glioma cell line. U251 glioma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells (1×10^5) were plated in 60 mm cell culture dishes and grown overnight at 37°C with 5% CO_2 and 95% air until they were 50–80% confluent. Then the cells were trypensized with 0.125% (w/v) trypsin/EDTA (pH 8.0) and plated into each well of a 96 well plates. The cells were incubated with the paclitaxel-loaded microspheres suspension or commercial paclitaxel formulation at concentrations 1 μg/mL for 24, 48, 72, 96, and 120 h. At designated time intervals, the medium was removed and the wells were washed with PBS for two times. Culture medium (90 mL) and 10 mL of MTT (5 mg/mL in PBS) were added to the wells and the cells were incubated at 37°C for additional 4 hr, then the reaction was stopped by lysing the cell with 200 μL of DMSO for 5 min and quantification measurements (optical density) were obtained at wavelength of 570 nm, and expressed as normalized percentage. All assays were performed in triplicate.

RESULTS AND DISCUSSION

Microspheres preparation and characterization

Generally, drug-loaded microshperes can be developed by two methods, i.e., solvent evaporation[15] and spray drying.[16] In present study, paclitaxel loaded PLA microspheres were prepared by spray drying method, employing ethyl acetate as solvent considering their low toxicity (ICH, class 3), and for the comparison, the microspheres were also prepared employing emulsion solvent evaporation. Both of the microspheres prepared by two methods presented spherical shape and smooth surface. But the size of the microspheres prepared by spray drying method was smaller than those by solvent evaporation (Fig. 1).

The theoretical encapsulation ratio (T.E.R.), actual encapsulation ratio (A.E.R.) and encapsulation ratio (EE) were listed in Table I. Compared with the microspheres prepared by solvent evaporation, the microspheres prepared by spray drying method had much higher EE and A.E.R. This can be attributed to the one-step process of the spray drying with little loss of the drug. Based on the analysis above, it can be concluded that spray drying method could be better than solvent evaporation in the preparation of drug-loaded microspheres.

DSC analysis

The DSC technique is a useful tool to analyze the thermal activity of the drug-loaded microspheres. It can provide qualitative and quantitative information about the physicochemical status of the drug in the

TABLE I
Preparation and Encapsulation of the Microspheres

Sample	T.E.R. (%)	Before γ irradiation		After γ irradiation	
		A.E.R. (%)	EE (%)	A.E.R. (%)	EE (%)
10% PAC/PLA (spray drying)	10.0	9.28 ± 0.38	92.8	8.10 ± 0.16	81.0
5% PAC/PLA (spray drying)	5.0	4.85 ± 0.28	97.0	4.68 ± 0.20	93.6
10% PAC/PLA (solvent evaporation)	10.0	3.98 ± 0.18	39.8	3.77 ± 0.24	37.7
20% PAC/PLA (solvent evaporation)	20.0	4.53 ± 0.31	22.7	4.21 ± 0.50	21.0

microspheres, which was reported to be involved in the endothermic or exothermic process.[17,18] The DSC thermograms of blank PLA microspheres, paclitaxel-loaded microspheres (T.E.R., 5% and 10%) were shown in Figure 2. It can be seen that the addition of paclitaxel had little effect on the glass transition temperature (T_g) of PLA. The absence of melting endotherm peak of paclitaxel also indicated that the paclitaxel dispersed in the microspheres in amorphous or disordered-crystalline phase.[16]

Determination of residual solvent

Residual solvents in pharmaceutical preparations, especially injectable formulations *in vivo*, are a growing concern due to the cell toxicity associated with these residual.[19–21] Although dichloromethane is a class 2 solvent according to the ICH classification, and its administration should be limited (PDE 6 mg/day; concentration limit 600 ppm), however, most of the articles used DCM as the solvent to prepare microspheres, whether in solvent evaporation or spray drying method.[22] To obtain microspheres with low solvent residue, postprocessing such as drying in higher temperature or vacuo storage were needed.[23] In present study, GC-MSD analysis was carried out to detect the residual solvent in the spray dried microspheres, the results indicated a low residual of ethyl acetate content 0.03%, which was significantly lower than the limit for application *in vivo*. The results showed that the spray drying method is qualified in the in the preparation of microspheres with low residual solvent.

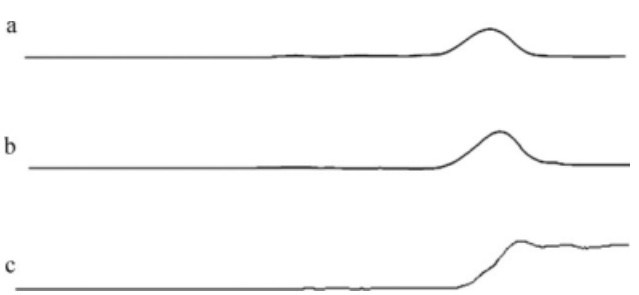

Figure 2 DSC thermograms of paclitaxel-loaded microspheres [T.E.R., (a) 5%, (b) 10%, (c) blank microspheres] (the order was a, b, c from up to down).

Degradation of PLA and drug release *in vitro*

There are three primary mechanisms by which the loaded drug can be released from the microspheres carrier, i.e., diffusion, degradation, and swelling followed by diffusion.[24] To determine the release mechanism of the microspheres, we analyzed the degradation behavior of microspheres carriers by gel permeation chromatography and the results were correlated with the release profiles (Fig. 3). At the first three days, the degradation of PLA microspheres was obvious and the molecular weight was decreased from 34 kD to 27 kD, correspondingly, ~ 20% paclitaxel were released for all the types of the paclitaxel-loaded microspheres, irrespective of the initial drug content. After that, the degradation of microspheres changed to be steady, and the drug release exhibited a zero order kinetics approximately. No significant channel were found in the surface of the microspheres from the SEM images (Fig. 4) after 60 days drug release and the microspheres were still represented spherical structure, indicating that the drug release in our study was controlled by diffusion.

The effect of the A.E.R on the release rate *in vitro* was studied. Wang et al. and Gang Ruan reported that with the increase of drug loading rate, the

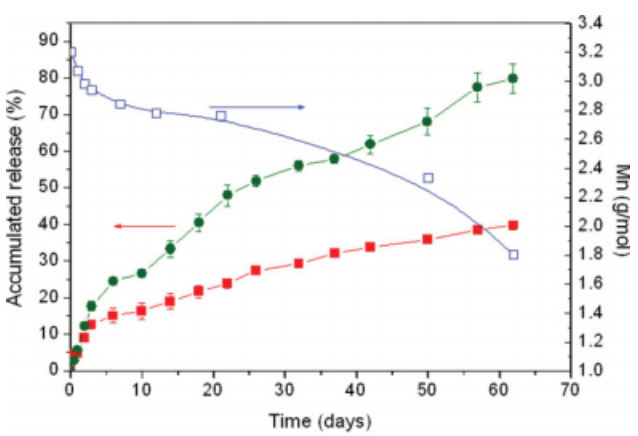

Figure 3 The degradation profile of PLA blank microspheres and the *in vitro* release profiles of paclitaxel/PLA microspheres. (□) The degradation profile; T.E.R., (●) 10%; (■) 5%. [Color figure can viewed in the online issue, which is available at www.interscience.wiley.com.]

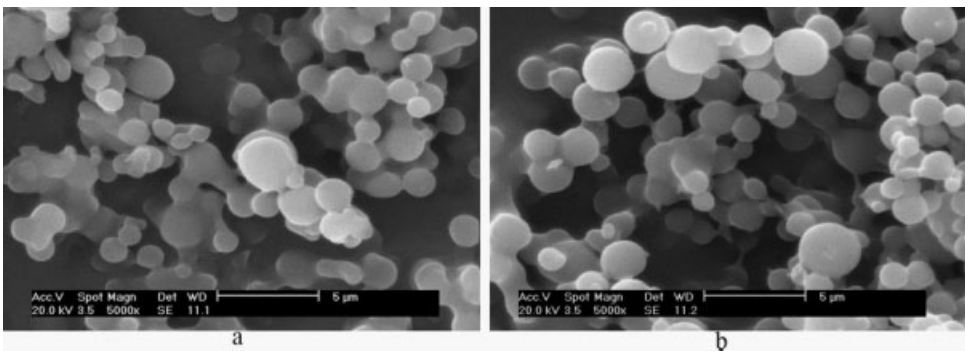

Figure 4 Surface morphology of paclitaxel-loaded PLA microspheres after 60 days *in vitro* release [T.E.R., (a) 5%, (b) 10%].

release rate decreased,[16,25] while Liggins reported an opposite trend.[26] Figure 3 showed the *in vitro* release curves of 5 and 10% paclitaxel-loaded mcirospheres, which obviously showed that higher drug loading resulted in faster drug release. This can be attributed to that with the similar degradation of PLA, higher drug content can resulted in the bigger difference of drug concentration between in and out of the microspheres, which can accelerated the drug release.

γ irradiation sterilization

Several literatures have reported that γ irradiation did not have effects on the drug loading values.[27–29] However, from Table I, it can be seen that the EE of the sterilized microspheres was lower than those nonsterilized microspheres. The loss of the paclitaxel after irradiation may be explained by that paclitaxel is sensitive to irradiation.[30] However, although the sterilization decrease the drug loading rate in the microspheres, it seemed have little influence on the drug release behavior of microspheres *in vitro*. As shown in Figure 5, the release patterns of the irradiated and nonirradiated microspheres appeared to be similar, which accord with Ana Fern'andez-Carballido' report.[31]

Inhibition of glioma tumor *in vitro*

The cytotoxic activity of paclitaxel and paclitaxel-loaded PLA microspheres was evaluated by assessing cell viability by the MTT assay. As can be seen in Figure 6, a marked reduction in cell viability was observed when tumor cells were incubated with 1g/mL paclitaxel for 24 h at 37°C. At this concentration, the cell growth was almost totally inhibited after 72 h of incubation. The cell viability, however, increased slightly for the 96 h incubation times tested. Paclitaxel loaded-microspheres showed a different behavior. The cytotoxicity increased with the increasing times of incubation. After 120 h of incubation with this formulation, a reduction of ~ 70% in cell viability was detected for all samples tested. No significant cytotoxic activity was found for the blank

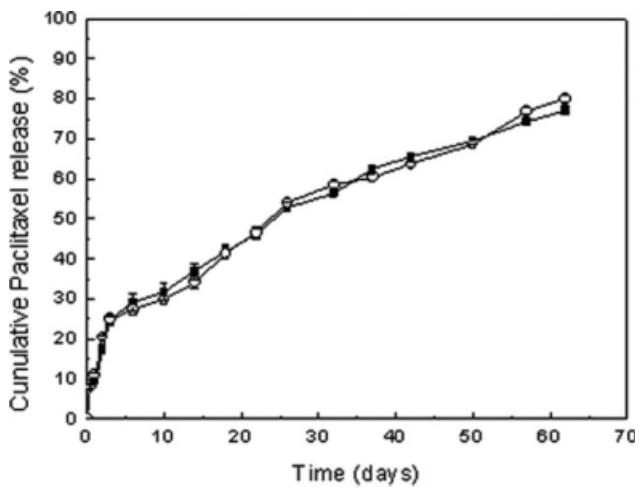

Figure 5 The influence of g irradiation on the drug release profiles *in vitro*. (○) γ irradiation (■) without γ irradiation.

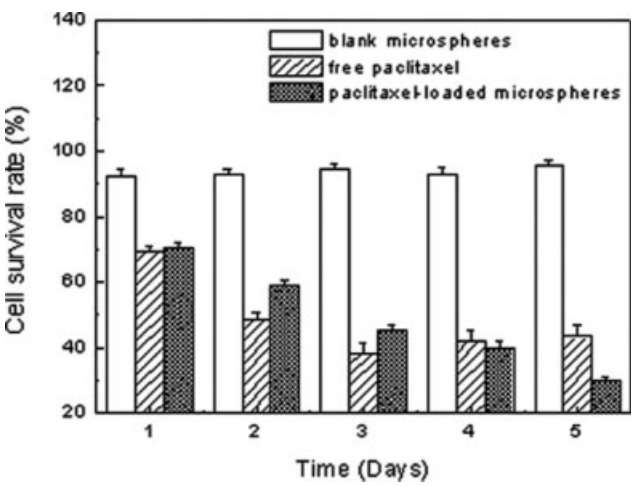

Figure 6 The cytotoxity effect of free paclitaxel and paclitaxel-loaded microspheres.

microshperes, suggesting the well biocompatibility of the microspheres.

CONCLUSIONS

Paclitaxel-loaded PLA microparticles were fabricated with the spray drying method employing ethyl acetate as solvent. The drug-loaded microspheres prepared presented spherical shape and smooth surface, and the solvent remained in the microshperes less than 0.03%. The paclitaxel trapped in microspheres existed in amorphous state, and the γ irradiation sterilization decrease the EE slightly but have little influence on the release profile of the paclitaxel from microspheres. The release was controlled mainly by diffusion mechanism. The paclitaxel-loaded microspheres had higher antitumor activity than free drug. So, we believe that spray drying method is superior to solvent evaporation in the preparation of microspheres and the injectable paclitaxel-loaded microspheres fabricated by spray drying method may be useful in the application of therapy of cancers.

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