In vitro and in vivo evaluation of taxol release from poly(lactic-co-glycolic acid) microspheres containing isopropyl myristate and degradation of the microspheres

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Abstract

This study describes the release and degradation profiles of taxol-loaded poly(lactic-co-glycolic acid) microspheres containing isopropyl myristate (IPM), namely, Taxol-IPM-PLGA-MS, in vitro and in vivo. Incorporation of IPM into the PLGA microspheres effectively increased the release of taxol from the microspheres in vitro. The molecular weight (Mol. Wt.) and copolymer ratio of lactic acid (LA) and glycolic acid (GA) displayed obvious influences on the characteristics of the microspheres and their in vitro release kinetics. A large number of micropores could be observed on the surface of the microspheres and the number of these pores tended to increase with the increment of Mol. Wt. and LA content of PLGA. This trend was in accordance with the rate of drug release from the microspheres in vitro. Moreover, the degradation of PLGA matrix in vitro was retarded by the incorporation of IPM to a less extent compared with its effect on the drug release. Therefore, the drug diffusion in the matrix was shown to be an important factor dominating the release rate, while the degradation rate of the polymer is a moderate factor in this case. Consistent with the in vitro results, the taxol concentrations in the lung after intravenous injection of the Taxol-IPM-PLGA-MS were 2–4 times higher over one week, as compared with those after injection of the microspheres without IPM. In conclusion, the Taxol-IPM-PLGA-MS may have great advantage in cancer chemotherapy after targeted delivery to a tumor site by chemo-embolization.

Keywords: Taxol; Poly(lactic-co-glycolic acid) microspheres; In vitro release; In vivo release; Biodegradation

1. Introduction

Taxol is a novel antineoplastic agent isolated from the bark of the Pacific yew tree, \textit{Taxus brevifolia} [1], with its novel mechanism of action as a promoter of tubulin assembly [2]. Phase I and II clinical studies have demonstrated the significant activity of taxol against a variety of solid tumors including breast cancer, advanced ovarian carcinoma, lung cancer, head and neck carcinomas, and acute leukemias [3,4]. Due to the poor solubility of taxol in water and many other acceptable pharmaceutical solvents, Cre-mophor EL (polyoxyethylated castor oil) is used to formulate taxol in the commercial injection. However, serious hypersensitivity reactions have been reported in certain individuals since the content of

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Cremophor EL used in the taxol formulation is significantly higher than that in any other marketed drug [5]. Therefore, some alternative dosage forms for taxol delivery have been developed to improve the solubility of taxol without the use of Cremophor EL, including liposomes [6,7], mixed micelles [8], parenteral emulsions [9], and cyclodextrin complexes [10].

The use of microspheres for chemo-embolization has considerable interests to enhance the therapeutic efficacy of anticancer agents and to reduce their systemic side effects; some satisfactory results in clinical trials have been obtained [11]. In order to maximize the efficacy of chemo-embolization using drug-incorporating microspheres, drug release from the microspheres should be properly controlled. Incorporation of isopropyl myristate (IPM) may change the structure of the microsphere matrix and form a composite matrix with many channels filled by IPM, through which the drug can diffuse out of the microspheres quickly, as reported for aclarubicin [12]. Moreover, the molecular weight (Mol. Wt.) and copolymer ratio of PLGA may have a strong effect on drug release patterns from the PLGA microspheres and degradation rate of the matrix, as observed for thyrotropin-releasing hormone (TRH) [13,14]. In our previous study [15], taxol-loaded poly(lactic-co-glycolic acid) microspheres containing IPM (namely, Taxol-IPM-PLGA-MS) have been prepared successfully and incorporation of IPM into the microspheres significantly increased the release rate of taxol in vitro. However, the effect of Mol. Wt. and copolymer ratio of PLGA on the drug release and degradation characteristics of Taxol-IPM-PLGA-MS remained to be elucidated. Therefore, the present study was conducted to examine the influence of these factors on the release properties of taxol from Taxol-IPM-PLGA-MS, as well as on the in vitro and in vivo degradation of the composite matrix.

2. Materials and methods

2.1. Materials

Taxol was a gift from Bristol-Myers Squibb Co. (Tokyo, Japan). Poly(D,L-lactic acid) (PLA with Mol. Wt. of 10 000), a series of poly(D,L-lactic-co-glycolic acid) (PLGA) listed in Table 1, isopropyl myristate (IPM), Cremophor® EL, phosphoric acid, dichloromethane (DCM), lithium lactate, glycolic acid sodium, and acetonitrile were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Gelatin and Tween 80 were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). n-Hexyl-p-hydroxy benzoate, an internal standard of taxol for HPLC assay, was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Lyophilized bovine serum albumin powder (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO., USA). Double distilled water (DDW) was used throughout the experiment.

2.2. Preparation of the taxol-loaded microspheres

A solvent evaporation technique was used to prepare the taxol-loaded microspheres [16]. The initial loading levels of taxol and IPM were fixed at

<table>
<thead>
<tr>
<th>Preparation</th>
<th>IPM level (w/v)</th>
<th>Characteristics of co-polymer</th>
<th>Average diameter (μm)</th>
<th>Drug trapping efficiency (%)</th>
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<tr>
<td></td>
<td></td>
<td>Mol. Wt. Molar ratio of LA / GA</td>
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<tr>
<td>A</td>
<td>30</td>
<td>5000 75/25</td>
<td>27.6</td>
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<td>B</td>
<td>30</td>
<td>10 000 75/25</td>
<td>30.1</td>
<td>95.5</td>
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<td>C</td>
<td>30</td>
<td>20 000 75/25</td>
<td>38.7</td>
<td>82.7</td>
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<tr>
<td>D</td>
<td>30</td>
<td>10 000 50/50</td>
<td>43.2</td>
<td>86.6</td>
</tr>
<tr>
<td>E</td>
<td>30</td>
<td>10 000 100/0</td>
<td>N.D.</td>
<td>28.6</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>10 000 75/25</td>
<td>29.1</td>
<td>97.6</td>
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</table>

N.D., not determined
* The weight ratio of IPM/PLGA
* Mean value of the duplicate preparations
5% (w/w) and 30% (w/w), respectively, unless otherwise mentioned. PLA or PLGA (50 mg), IPM (15 mg), and taxol powder (3.5 mg) were dissolved in 1.0 ml of DCM. The solution, after being cooled to 4° C to prevent the evaporation of DCM, was added in a dropwise manner to 50 ml of 4% (w/v) gelatin solution maintained at 25±1° C through a water bath and stirred at 600 rpm. To evaporate DCM, the stirring was continued for 1 h. Then 25 ml of DDW was added to dilute the gelatin solution and the microspheres were separated by centrifugation at 3000 rpm for 10 min. After removing the supernatant solution, the microspheres were collected by filtration through a cellulose nitrate membrane (pore diameter 1 μm, Toyo Roshi Kaisha Ltd., Tokyo, Japan), washed three times with water, and dried at room temperature under reduced pressure. The taxol microspheres containing no IPM were also formulated with the same method without the addition of IPM.

2.3. Characterization of the microspheres

2.3.1. Particle size determination

Microspheres were mounted on a slide glass and inspected under an optical microscope (IMT; Olympus, Tokyo, Japan) connected to a video camera (ICD-740; Olympus). The video signals were displayed on a computer, and 600 particles were measured for each batch of the microspheres using an image analysis software (Image 3.0, NIH, MD, USA).

2.3.2. Determination of taxol content in the microspheres

The taxol content in the PLGA microspheres was determined by HPLC according to the method described in our previous report [15]. The taxol-loaded microspheres (3 mg) were dissolved in 1 ml DCM, to which 5 ml of acetonitrile:2 mM phosphoric acid (50:50, v/v) was added, and the mixture was vortexed vigorously. After DCM was evaporated under a nitrogen stream, the solution was diluted with acetonitrile:2 mM phosphoric acid (50:50, v/v) and used for taxol analysis. The trapping efficiency of taxol was calculated as the ratio of actually measured drug content in the obtained microspheres to the loaded amount.

2.3.3. Scanning electron microscopy (SEM)

The shape and surface morphology of the obtained microspheres were examined with a scanning electron microscope (Hitachi S-4500, Tokyo, Japan) after gold-palladium coating of the microspheres using an ion-coater.

2.4. In vitro drug release test

The in vitro release profiles of taxol from the microspheres were examined by determining the residual drug amount in the microspheres [15], considering its strong adsorption to glassware or plastic tubes. Three mg of the taxol-loaded microspheres were suspended in 15 ml of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.1% (w/v) Tween 80 in a screw-capped glass tube. The tubes were incubated at 37°C and shaken horizontally at 80 rpm with a stroke distance of 6 cm. At given time intervals, three tubes for each microsphere formulation were withdrawn and microspheres were separated by centrifugation at 2000 rpm. After removing the supernatant, the microspheres were rinsed with DDW and lyophilized (Freeze Dryer, LABCONCO, MO, USA). The amount of residual taxol in the microspheres was determined by HPLC using the same procedure as described above.

2.5. In vivo release of taxol from the PLGA microspheres

Male ddY mice (8 weeks, 30–37 g) were obtained from Sankyo Laboratory Co., Ltd. (Toyama, Japan) and were allowed free access to standard rodent chow and water.

The in vivo release of taxol from PLGA microspheres prepared with or without IPM was examined as follows: The taxol-loaded microspheres (10 mg) were dispersed in 1 ml of saline containing 0.05% (w/v) of Tween 80 just before use and vortexed for better suspension of the microspheres. Two hundred μl of the suspension were administered by a single bolus injection through the jugular vein of mice (n=4) under light anesthesia with diethyl ether. At 2, 24, 72, and 168 h after administration of the microspheres, the lungs were dissected quickly from the mice after being sacrificed by exsanguination, weighed and homogenized in 1/15 M phosphoric acid.
acid solution at 1,400 rpm using a tissue homogenizer (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan). The homogenates were centrifuged at 3600 rpm for 15 min at 4°C and the supernatants were subjected to a solid-phase extraction procedure using Sep-Pak cartridge C18 columns (Waters Assoc., MA, USA) according to the method of Mase et al. [17]. The taxol content in the eluate was assayed by HPLC. The calibration curve used for the quantification of taxol was linear over the range of 0.1–5 μg/g in the lung with a correlation coefficient (r) of 0.999.

2.6. In vitro and in vivo degradation of the PLGA microspheres

The degradation of the placebo PLGA microspheres (no drug loaded) with or without the addition of IPM was assessed by the amount loss of the microspheres in vitro. The microspheres (5 mg) were suspended in 15 ml of PBS containing 0.1% (w/v) Tween 80 in a screw-capped glass tube and subjected to the same procedure as described for the in vitro release test. The residual quantity of the microspheres, after being separated by centrifugation and lyophilization, was assayed by HPLC according to the method of Kamei et al. [18] after hydrolysis of PLGA with KOH. Calibration curves used for the quantification of LA and GA were produced using lithium lactate and glycolic acid sodium, respectively. The percentage of PLGA amount remaining in each sample was calculated as the sum of GA (wt. %) and LA (wt. %) remaining in the microspheres.

In vivo degradation of the PLGA microspheres containing 30% (w/w) IPM was examined in the lung of mice. Two hundred μl of suspension of the microspheres (10 mg/ml) in saline containing 0.05% (w/v) of Tween 80 were administered through the jugular vein of mice (n=3). At given intervals, the lungs were excised and homogenized in 8 ml of acetone with a Polytron (Kinematica, GmbH, Lucern, Switzerland). The remaining amount of PLGA in the supernatant after centrifugation was assayed using the alkali hydrolysis method [18].

2.7. Statistical analysis

Significant differences between the observed data were assessed by Student’s t-test.

3. Results

3.1. Characterization of the microspheres

PLGA with different Mol. Wt. and molar ratio of LA/GA were used to formulate the microspheres (Preparations A–F) by a solvent evaporation technique with and without IPM. Characteristics of the copolymer, mean size, and drug trapping efficiency of the produced microspheres are summarized in Table 1. The particle size distribution curves and scanning electron micrographs of the obtained microspheres are shown in Fig. 1 and Fig. 2, respectively.

PLGA (LA/GA ratio, 75/25; Mol. Wt. 5000, 10 000, and 20 000) was employed for Preparations A, B, and C, respectively. The mean size of Preparation C was the largest (Table 1) and more particles dispersed in a larger size range (Fig. 1). With regard to drug trapping efficiency, the value for Preparation B was the highest. The microspheres produced in these preparations were spherical with a smooth surface. However, many micropores were observed on the microsphere surface and the number of the pores tended to increase as the Mol. Wt. of PLGA increased (Fig. 2 a–d).

PLGA (Mol. Wt., 10 000; LA/GA ratios of 50/50, 75/25, and 100/0) was employed for Preparations D, B, and E, respectively. The particle size of Preparation D was much larger than that of Preparation B (Table 1 and Fig. 1). A lot of the microspheres retained spherical shape with a coarse surface (Fig.
Fig. 2. Scanning electron micrographs showing the surface morphology of the taxol-loaded PLGA microspheres. (a) Preparation A; (b) Preparation B; (c) and (d) Preparation C; (e) Preparation D; (f) Preparation E; (g) Preparation F (see the details of these preparations in Table 1).
2e). In the case of Preparation E, a large number of micropores were observed on the external surface of the microspheres and some fragmentation occurred, possibly due to destruction of the microspheres during the preparation. In this case, particle size determination was not performed. The actual drug loading in Preparation E was the lowest among the microsphere formulations examined.

The microspheres formulated without IPM (Preparation F) showed a good sphericity and smooth surface, on which no pores and cracks were observed (Fig. 2g).

3.2. In vitro release of taxol from the microspheres

3.2.1. Effect of molecular weight on the drug release profiles

In vitro release profiles of taxol from Preparations A, B, and C are shown in Fig. 3. In all formulations, the release of taxol from the microspheres was nearly constant (i.e. zero-order kinetics) for two weeks and the drug release rate of Preparations A and C was significantly faster than that of Preparation B two weeks later \( p < 0.01 \). The microspheres of Preparation C exhibited a faster release in this period but the release slowed down significantly after two weeks; the accumulative amount of taxol released in three weeks from the microspheres was similar to that from Preparation B. In contrast, the drug release from Preparation A became faster after two weeks. In the case of Preparation B, the constant release of taxol from the microspheres was continued for three weeks.

3.2.2. Effect of LA/GA ratios on the drug release profiles

Fig. 4 shows the in vitro release profiles of taxol from Preparations B, D, and E. Taxol released more rapidly from the microspheres formulated with lower GA content. The drug release from Preparation D was significantly slower in comparison with that from Preparation B \( p < 0.01 \) at Days 7, 14, and 21. However, a large burst release of taxol was observed from Preparation E. Approximately 80% of the initial loaded amount of taxol was released within 2 days and thereafter the release became much slower.

3.2.3. Taxol release profiles in the lung of mice

The effect of IPM on the release profiles of taxol from the microspheres in vitro and in the lung in vivo are shown in Fig. 5a and b, respectively. When no IPM was incorporated, the release of taxol from the microspheres (Preparation F) in vitro was slow;

![Graph 3](image3.png)

**Fig. 3.** Effect of molecular weight of PLGA (LA/GA 75/25) on the in vitro release of taxol from the microspheres containing 30\% (w/w) of IPM. Mol. Wt. of PLGA: (●) 5000; (□) 10 000; (△) 20 000. Each point and bar represent the mean ± S.D. of three sample issued from the same batch of the microspheres. * \( p < 0.05 \) and ** \( p < 0.01 \), compared with the microspheres using PLGA with Mol. Wt. of 10 000.

![Graph 4](image4.png)

**Fig. 4.** Effect of copolymer ratio PLGA (Mol. Wt. 10 000) on the in vitro release of taxol from the microspheres containing 30\% (w/w) of IPM. LA/GA ratios: (○) 50/50; (□) 75/25; (△) 100/0. Each point and bar represent the mean ± S.D. of three sample. ** \( p < 0.01 \), compared with the microspheres using PLGA with LA/GA ratio of 75/25.
less than 14% of the initial loaded amount of taxol was released in three weeks. The addition of IPM increased significantly the drug release rate; approximately 24% of the initial loaded drug was released from the microspheres (Preparation B) after one week. Furthermore, the incorporation of IPM also changed significantly the time course of taxol release from the microspheres in the lung. The concentration of taxol in the lung was approximately 4 times higher at the initial phase and still 2.5 times higher after one week with Preparation B than that with Preparation F, indicating the accelerating effect of IPM on the release of taxol in vivo.

3.3. Degradation of the PLGA microspheres

Fig. 6a shows the amount changes of the PLGA microspheres containing 0%, 30%, and 50% (w/w) of IPM in vitro. The Mol. Wt. and LA/GA ratio of PLGA were fixed at 10 000 and 75/25, respectively. The degradation of PLGA matrix was retarded by the incorporation of IPM, but there was no significant difference in the degradation rate among the IPM levels tested.

The in vivo degradation experiment was conducted using the PLGA microspheres containing 30% (w/w) of IPM and the decrease of the PLGA
amount in the lung is illustrated in Fig. 6b. The degradation of the microspheres in vivo was significantly faster than that in vitro ($p<0.01$).

4. Discussion

PLGA has been used widely in the formulation of biodegradable microspheres containing pharmaceutical agents such as anticancer drugs, steroid hormones, and peptides [19–22]. The release kinetics of the drug-loaded PLGA microspheres have been studied widely and it has been demonstrated that drug diffusion in the matrix and degradation of the carrier play important roles in the release kinetics of the drug [23,24]. For hydrophilic drugs, the release rate is controlled by these dual effects and, in some cases, the drug diffusion process predominates when the drug release is much faster than the matrix erosion [13,23]. On the other hand, for microspheres containing hydrophobic drugs, a close correlation between the degradation of the polymer and the drug release has been reported [25]. The drug diffusion in this matrix might be much slower in this case.

In our preliminary experiment, incorporation of triacetin, an additive with a shorter carbon chain, to the PLGA microspheres showed no obvious influence on taxol release from the microspheres (data not shown). Since the incorporation of IPM significantly accelerated the release of a hydrophobic anticancer drug, aclorubicin, from PLA microspheres in vitro [12], we selected IPM as a release-accelerating agent for taxol from PLGA microspheres. Other additives known to accelerate hydrophobic drug release from microspheres, such as fatty acid esters with a longer-length alkyl chain (e.g. 12–16 carbons) ([12,26]), were not tested because of the high cost of taxol.

It has been reported that evaporation of DCM during preparation of PLGA microspheres forms occasionally some fine pores on the microsphere surface [21]. However, in this study, no pores nor cracks were noticed on the surface of the microspheres without IPM (i.e. Preparation F) as examined from Fig. 2g. The in vitro release of taxol from Preparation F turned out to be too slow (Fig. 5a). Thus, the diffusion of taxol in this matrix was thought to be very slow due to its poor solubility in water ($<0.01$ mg/ml), and the drug release may be dominated mainly by the degradation of the PLGA matrix, which is not so fast in vitro and has a lag phase as indicated in Fig. 6a. In contrast, some micropores were observed on the surface of the microspheres containing IPM (Fig. 2b), and the existence of IPM significantly increased the taxol release from these microspheres (Fig. 5a). It is supposed that there might exist many inner channels filled by IPM in the matrix. Because IPM is an oil phase in which taxol can be easily dissolved, the portion of taxol dispersed in the PLGA matrix might partition into the oil phase gradually, then diffuse out of the microspheres. Therefore, the accelerated release of taxol, a highly hydrophobic agent, from the microspheres containing IPM may be attributed mainly to the IPM-mediated drug diffusion through the pores and channels in the matrix. On the other hand, the degradation of the matrix will further accelerate the drug release from the microspheres [27] and should be recognized as another factor which affects the drug release kinetics. This drug release mechanism may illustrate the release profiles of taxol from the microspheres containing IPM displayed in Figs. 3 and 4.

During the initial stage of the release test, the degradation of the PLGA matrix is not evident and the drug release may be controlled mainly by its diffusion in the matrix. It seemed that formation of the micropores on the surface of the microspheres after incorporation of IPM aided drug diffusion in this study and the number of micropores increased with the increment of the Mol. Wt. and LA content of PLGA. It is known that polymers with high Mol. Wt. will precipitate quickly at the surface of droplet in oil-in-water emulsion during the preparation of microspheres [28]. Thus, the larger number of micropores on the particle surface of Preparation C may be ascribed to the faster precipitation of PLGA due to its higher Mol. Wt. Similarly, a quicker precipitation of PLA during the preparation process may account for a large number of micropores on the particle surface of Preparation E. Consequently, the release of taxol from Preparations C and E was faster and in the case of Preparation E, the burst effect occurred. However, existence of many micropores may be helpful for the release of IPM itself as well, which should be filled mainly in these pores [26]. After large amount of IPM was also released, the drug
diffusion in the matrix should be governed mainly by the degradation of the matrix and the drug release will slow down. This may be an explanation for the slow release of taxol after the burst release from Preparation E (Fig. 3). The accelerating effect of matrix degradation on drug release reinforces gradually with time. This effect is evident for Preparation A after two weeks, which were formulated with PLGA of lower Mol. Wt., and the drug release was accelerated (Fig. 3). The degradation of matrix gave no positive but negative effect on drug release from Preparation C (Fig. 3). This might be attributed to the faster loss of IPM from the matrix in two weeks due to the large number of micropores on the particle surface. In the case of Preparations B and D, the combination of the dual effects lead to a constant release of taxol from the matrix in three weeks.

Due to the good sphericity and suitable release rate in vitro, the microspheres of Preparation B were selected to further examine their drug release and degradation pattern in vivo. As displayed in Fig. 5b, there was a marked difference in the disappearance of taxol from the lung between the two formulations of microspheres (with and without IPM). In general, the rapid elevation of regional concentration of an anticancer agents over its therapeutic level and subsequent maintenance of the level should be the basis for effective chemotherapy of solid tumors. From this standpoint, the incorporation of IPM in the PLGA microspheres may show promise for the effective chemotherapy after targeted delivery to a tumor site by chemo-embolization. On the other hand, it should be noticed that tissue necrosis and cell degeneration in the normal lung may occur after the embolization of microspheres with this particular size due to their strong embolic effect.

Generally, microspheres with a diameter larger than 7 μm can be entrapped in the capillary bed of lung after i.v. administration. However, according to the particle size distribution curve shown in Fig. 1, it is clear that less than 10% of the microspheres (Preparation B) have a diameter lower than 7 μm on the basis of particle number. Therefore, only a small fraction of the microspheres may escape from the lung vasculature in this study. Moreover, the in vivo degradation data shown in Fig. 6B were expressed as the ratio of remaining amount of PLGA in the lung at time (t) to that at the initial time, which is a relative value. Therefore, the influence of the fraction of microspheres escaped from the lung on the in vivo degradation of the microspheres was supposed to be negligible.

In conclusion, the diffusion of taxol in the matrix through the micropores and channels formed by incorporation of IPM was shown to be the important factor dominating the release rate. Meanwhile, the degradation rate of the matrix affected the drug release kinetics of the microspheres to some extent. From the release of taxol from the IPM-incorporating microspheres and degradation of the matrix in the lung, it is suggested that targeted delivery (i.v. administration for the lung) of the Taxol-IPM-PLGA-MS (Preparation B) is advantageous for cancer treatment with higher efficacy and lower systemic side effects.

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References


