Preparation and Characterization of Poly(lactic-co-glycolic acid) Microspheres for Targeted Delivery of a Novel Anticancer Agent, Taxol

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Received April 25, 1996; accepted June 20, 1996

This study describes the preparation and characterization of poly(lactic-co-glycolic acid) microspheres containing a novel anticancer agent, taxol (namely, Taxol-PLGA-MS). A solvent evaporation technique was utilized to prepare Taxol-PLGA-MS. The trapping efficiency of taxol in the microspheres was greater than 90% and reproducible. The *in vitro* release rate of taxol from the microspheres was very low, and less than 15% of the initial amount of taxol was released in three weeks, irrespective of the drug loading level. When a chemical additive, isopropyl myristate (IPM), was introduced at the level of 30% (w/w), the release of taxol increased significantly; approximately 70% of the initial amount of taxol was released at a nearly constant rate for three weeks. Elevation of the loaded IPM level to 50% (w/w) produced a more rapid release of the drug. Scanning electron microscopy showed that Taxol-PLGA-MS were spherical with a smooth surface. More than half (55—65%) of the microspheres had a diameter of 20—45 μm. Incorporation of IPM had no significant influence on the particle size, surface morphology, or degradation behavior of the microspheres. It was strongly suggested that the release of taxol from the microspheres was dominated mainly by the drug diffusion in the matrix. As evaluated from the particle size, drug content, and *in vitro* release property, IPM-containing Taxol-PLGA-MS may be suitable for chemoembolization therapy of cancer diseases.

Key words taxol; poly(lactic-co-glycolic acid) microsphere; controlled release; solvent evaporation technique; chemoembolization

Taxol is a novel antineoplastic agent isolated from the bark of the Pacific yew tree, *Taxus brevifolia*.[1] Its unique mechanism of action is related to its ability to promote microtubule assembly and inhibit cell-cycle replication in the late G_2 or M phases of the cell cycle.[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, the formulation of taxol used widely in clinical trials consists of dehydrated ethanol and cremophor EL (polyoxyethylated castor oil) at a volume ratio of 50:50. The content of cremophor EL used in the taxol formulation is significantly higher than that in any other marketed drug, and the vehicle has been demonstrated to cause serious hypersensitivity reaction in certain individuals.[5,6] Thus, some alternative dosage forms for taxol delivery have been developed to improve the solubility of taxol without the use of cremophor EL, including liposomes,[7-10] mixed micelles,[11] parenteral emulsions,[12,13] and cyclodextrin complexes.[14] Although some of the dosage forms can solubilize sufficient quantities of taxol and have shown improved antitumor effects in animal models, problems such as the *in vitro* stability of the liposomes and dose-limiting toxicity due to some vehicles (*e.g.*, triacetin in emulsion formulation and cyclodextrin) have also been noticed.[12,14]

The use of microspheres for the targeted delivery of anticancer agents has considerable interest regarding enhancing therapeutic efficacy and reducing systemic side effects, by which some satisfactory results in clinical trials have been obtained.[15,16] Although preliminary research on the development of taxol-loaded microspheres for targeted delivery has been reported recently,[17,18] the formulated microspheres were not necessarily adequate for cancer chemotherapy since the *in vitro* release of taxol from the microspheres turned out to be too slow. The degradation of the polymer matrix of the microspheres is expected to accelerate the drug release in a later period and may overcome the problem of the reduced release over time. Thus, in the present study, novel taxol-loaded microspheres were produced using a lactic-co-glycolic acid copolymer with a lower molecular weight from which we expect the faster release of taxol. Moreover, an additive, isopropyl myristate (IPM), was incorporated into the microspheres to further enhance the release rate of taxol. The characteristics of the microspheres were examined under various conditions.

Materials and Methods

Materials Taxol was a gift from Bristol-Myers Squibb Co. (Tokyo, Japan). Poly(lactic-co-glycolic acid) (average molecular weight, 10000; lactic acid/glycolic acid, 75:25), isopropyl myristate, phosphoric acid, dichloromethane and acetonitrile (HPLC grade) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Gelatin and Tween 80 were of reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Double distilled water was used throughout in the experiment.

Preparation of PLGA Microspheres Containing Taxol A solvent evaporation technique was used to prepare taxol-loaded poly(lactic-co-glycolic acid) microspheres (namely, Taxol-PLGA-MS).[19] PLGA (50 mg) and a specified amount of taxol were dissolved in 1 ml of dichloromethane (DCM). The solution, after being cooled to 4 °C, was loaded into a 2-ml glass syringe to which a 26-gauge needle was attached, and then 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 by a magnetic stirrer (Yamato M-41, Tokyo, Japan). To evaporate DCM, the stirring was continued for 1 h. Then, 25 ml of distilled water 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,

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Toyo Roshi Kaisha, Ltd., Tokyo, Japan), washed three times with water, and dried at room temperature under reduced pressure. The obtained microspheres were kept in a desiccator at room temperature before use.

For the IPM-containing microspheres, a designated amount of IPM, taxol, and PLGA were dissolved in 1 ml of DCM, then the microspheres were produced according to the method described above. The amount of PLGA used in every formulation was fixed at 50 mg. Moreover, placebo microspheres (without taxol) were prepared by the same method.

Determination of Taxol Content in the Microspheres The taxol content in the PLGA microspheres was determined according to the method of Burt et *al.*[18] with some modification. The taxol-loaded microspheres (3 mg) were dissolved in 1 ml DCM, 5 ml of acetonitrile: 2 mM phosphoric acid (50 : 50, v/v) was added, and the mixture was vortexed vigorously for 1 min by a vortex mixer. A nitrogen stream was introduced to evaporate DCM at 37 °C until a clear solution was obtained. Then the solution was diluted to 25 ml with acetonitrile: 2 mM phosphoric acid (50 : 50, v/v), and the taxol content in the samples was measured by HPLC. For HPLC analysis, a reverse-phase Inertsil ODS-5 column (150 × 4.6 mm i.d., pore size 5 μm, GL Science, Tokyo, Japan) was used, and the column temperature was controlled at 40 °C with a column oven (CTO-6A, Shimadzu, Kyoto, Japan). The mobile phase, which consisted of a mixture of acetonitrile and 2 mM phosphoric acid (50 : 50), was delivered at a flow rate of 1.5 ml/min with a pump (LC-6AD, Shimadzu, Japan). A 20 μl aliquot of the samples was injected with an autoinjector (SIL-9A, Shimadzu, Japan) and column effluent was detected at 227 nm with a UV detector (SPD-6A, Shimadzu, Japan). The area of each eluted peak was integrated with an integrator (C-R6A, Shimadzu, Japan) and used for taxol quantitation.

To determine the recovery efficiency of the above extraction procedure, designated amounts of taxol (12.5—150 μg) and placebo PLGA microspheres (3 mg) were dissolved with 1 ml DCM and subjected to the same procedure. The trapping efficiency of taxol into the PLGA microspheres was calculated as the ratio of drug weight in the produced microspheres to that used for the preparation.

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, Japan). The video signals were displayed on a computer, and 300 particles were measured for each sample, implemented by image analysis software (Image 3.0, N.I.H, U.S.A.). Three samples were used for the size determination for each batch of the microspheres. The size distribution of the microspheres was obtained on a number basis and the average diameter was calculated as follows[20]:

$$\text{the average volume diameter } (d_v) = \sqrt[3]{\frac{\sum_{n=1}^{i} \Delta n_i \bar{d}_i^3}{\sum_{n=1}^{i} \Delta n_i}}$$

where Δn_i is the particle number dispersed in a size increment of 5 μm, and $\bar{d}_i$ is the median value of the size range established by the increment.

Adsorption of Taxol to Containers The adsorption test of taxol in 15 ml glass tubes and 15 ml unsiliconized polypropylene tubes was performed at room temperature. A series of taxol stock solutions in methanol was prepared, and 0.1 ml of the solution was added to 10 ml of 10 mM phosphate-buffered saline (PBS, pH 7.4) and vortexed for 30 s. The final concentrations of taxol were 100, 500, 1000, and 5000 ng/ml. Aliquots of the solution (100 μl) were taken 10 min and 24 h after the experiment and injected into the HPLC directly for taxol analysis. As a control (no adsorption), serial standard solutions of taxol in methanol (100, 500, 1000, and 5000 ng/ml) were also analyzed by HPLC.

***In Vitro* Drug Release Test** The release of taxol from the microspheres was examined using the following two methods.

Method 1: For the determination of taxol released in the outer medium, Taxol-PLGA-MS (10 mg) was suspended in 10 ml of PBS containing 0.1% (w/v) Tween 80 in a screw-capped tube. The tubes were placed in a shaker bath maintained at 37 °C and shaken horizontally at 80 rpm. At given time intervals, the tubes were centrifuged at 2000 rpm for 5 min and the supernatants kept until analysis. Then, the precipitated microspheres were resuspended in 10 ml of fresh release medium and placed back in the shaker bath. Taxol in the release medium was extracted with DCM, evaporated to dryness under a stream of nitrogen, reconstituted in 1 ml of the mobile phase, and analyzed by HPLC.

Method 2: For the determination of residual drug amount in the microspheres, 3 mg of taxol-loaded microspheres were suspended in 15 ml of the release medium in a screw-capped tube, and the tubes were incubated at 37 °C and shaken horizontally. At given time intervals, three tubes for each microsphere formulation were withdrawn and centrifuged at 2000 rpm for 5 min. The supernatant was removed and the precipitated microspheres were resuspended with 10 ml of distilled water and centrifuged again. After removing the supernatant, the microspheres were lyophilized (Freeze dryer, Lab Conc., U.S.A.). The amount of residual taxol in the microspheres was determined by HPLC.

Scanning Electron Microscopy (SEM) The microspheres were sampled at days 0, 7, 14, and 21 during the *in vitro* release test, separated by centrifugation, and lyophilized. The shape and surface morphology of the microspheres were examined with a scanning electron microscope (Hitachi S-4500, Kyoto, Japan). Microspheres were coated with gold-palladium using an ion-coater and examined under the microscope at 5.0 kV.

Statistical Analysis Significant differences between the observed data were assessed by Student's *t*-test.

Results

Size, Drug Content, and Trapping Efficiency of the Microspheres Taxol-loaded PLGA microspheres were successfully produced by a solvent evaporation technique. The composition of the materials used to formulate the microspheres in this study, the mean size, and drug trapping efficiency are summarized in Table 1. Preparations A, C, and E were formulated without IPM at the drug loading levels of 1%, 2%, and 5% (w/w), respectively. The loading levels of taxol showed no influence on the size of the microspheres. When IPM was incorporated at its levels of 30% (w/w) (preparations B, D, and F) and 50% (preparation G), there was no significant influence on the formation of microspheres (*i.e.*, the shape, diameters and particle size distribution of the microspheres). The size distribution histogram of PLGA microspheres containing 5% (w/w) of taxol is shown in Fig. 1. The number of microspheres dispersed in 20—45 μm was changed over the range of 55—65%, while the values on the basis of particle weight were constantly higher than 60%.

The trapping efficiency of the microspheres with taxol was greater than 90% for all drug loading levels, and the

Table 1. Composition of Materials Used in the Preparation and Characterization of Taxol-Loaded PLGA Microspheres

Preparation	Drug level[a] % (w/w)	Additive	Additive/PLGA % (w/w)	Average diameter (mean ± S.D. μm)	Drug trapping efficiency (%)
A	1.0	No	0	27.3 ± 1.5	98.8
B	1.0	IPM	30	27.8 ± 1.6	92.8
C	2.0	No	0	25.6 ± 2.5	94.6
D	2.0	IPM	30	29.5 ± 1.7	95.3
E	5.0	No	0	28.0 ± 1.0	98.8
F	5.0	IPM	30	29.3 ± 1.4	97.9
G	5.0	IPM	50	29.2 ± 1.9	96.9

a) The weight ratio, drug/(drug + additive + PLGA). The amount of PLGA was fixed at 50 mg.

values were not influenced by the incorporation of IPM. The calibration curve used for the quantification of taxol content in PLGA microspheres was linear over the range of 200—6000 ng/ml with a correlation coefficient of $r^2 > 0.999$. The recovery of taxol in the extraction procedure was 96.6±2.3%, 97.1±0.6%, and 97.2±0.6% (mean±S.D., $n=4$) at drug concentrations of 500, 3000, and 6000 ng/ml, respectively.

Adsorption of Taxol to Containers As shown in Table 2, 10 min after the preparation of the taxol solution in the glass tubes, approximately 25—50% declines in taxol concentration were observed, irrespective of the drug concentrations, and these changes were irregular after 24 h. In the case of taxol solution in unsiliconized tubes, the declines were more conspicuous.

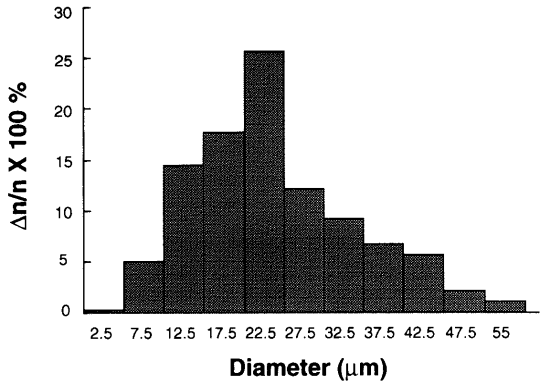

Fig. 1. Particle Size Distribution Histogram of PLGA Microspheres Containing 5% (w/w) of Taxol

Each area of the column represents the particle size distribution on a number basis. Δn is the number of particles in a size increment of 5 μm; n is the total number of particles.

Table 2. Declines in Taxol Concentrations in PBS (pH 7.4) in Glass and Unsiliconized Polypropylene Tubes

Taxol conc. (ng/ml)	Container	Taxol remaining (%)[a]	
		10 min	24 h
100	Glass tube	51.3± 6.0	56.9± 6.1
500	Glass tube	53.6± 4.0	46.6±12.1
1000	Glass tube	76.2±11.4	59.9± 4.0
5000	Glass tube	58.2± 4.7	30.8±10.3
100	Polypropylene tube	36.8± 2.5	N.D.[b]

a) Mean±S.D. of three samples. *b*) No sample peak was detected.

***In Vitro* Release Test Using Method 1** As shown in Fig. 2, for PLGA microspheres containing 1% and 2% of taxol, the cumulative drug amount in the outer medium after 2 weeks was 2.37% and 2.88% of the initial amount, respectively; the coefficient of variation of the determination ranged from 10% to 50%.

***In Vitro* Release Test Using Method 2** *In vitro* release profiles of taxol from PLGA microspheres at the drug loading levels of 1%, 2%, and 5% (w/w) were shown in Fig. 3A—C, respectively. The release of taxol assessed by method 2 was shown to be significantly higher than that by method 1 (*i.e.*, 2—3% with method 1 *vs.* 9—11% with method 2 after 2 weeks) ($p<0.01$). In the case of the microspheres without IPM, the release of taxol was very slow, and less than 5% of the initially loaded amounts of taxol were released in the first week. Though the release was slightly faster in the latter period of the release test, approximately 85% of initially loaded taxol still remained in the microspheres at all drug loading levels after three weeks. On the other hand, when IPM was incorporated, a significant increase ($p<0.01$) in the release rate of

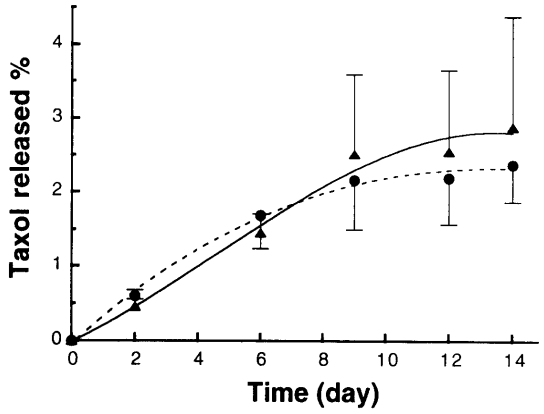

Fig. 2. *In Vitro* Release Profiles of Taxol from PLGA Microspheres in PBS (pH 7.4) at 37 °C Using Method 1

Drug loading levels are 1% (w/w) (●) and 2% (w/w) (▲). Solid and dotted lines show the fitted curves according to polynomial equations.

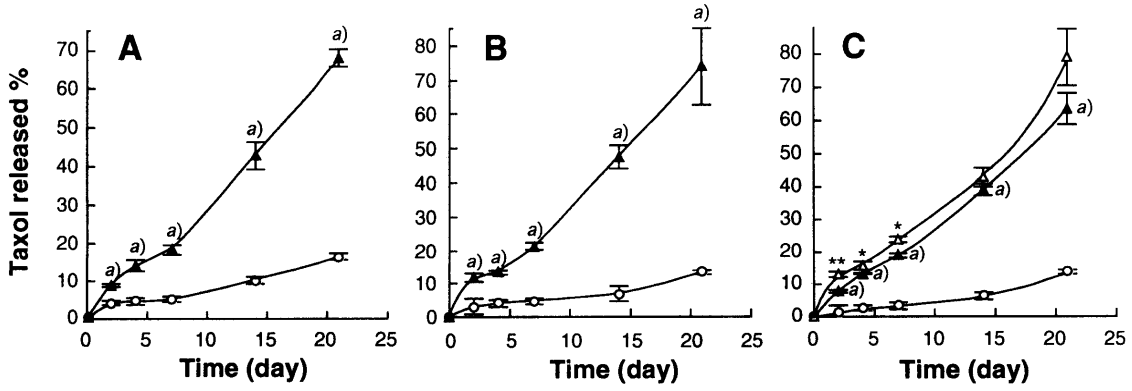

Fig. 3. *In Vitro* Release Profiles of Taxol from PLGA Microspheres without (○) and with 30% (w/w) (▲) or 50% (w/w) (△) of IPM in PBS (pH 7.4) at 37 °C

Taxol content in the microspheres: A, 1% (w/w); B, 2% (w/w); C, 5% (w/w). Each point and bar represent the mean±S.D. of three samples. Solid lines show the fitted curves according to polynomial equations. *a*) Significantly different from the microspheres without IPM ($p<0.01$). $*p<0.05$, $**p<0.01$, significantly different from the microspheres containing 30% (w/w) of IPM.

taxol from the microspheres was observed. Approximately 8—10% of the drug was released in 2 d, and more than 20% of taxol was released within the next week. The release rate of taxol was nearly constant from the IPM-incorporating microspheres, which is not common for drug release from microspheres, and approximately 70% of the drug was released after three weeks. When the loading level of IPM was increased from 30% (w/w) to 50% (w/w), a more rapid release of taxol from the microspheres was observed, and this increment was significant in the initial period (Fig. 3C, $p<0.01$ at day 2; $p<0.05$ at days 4 and 7).

As observed in Fig. 3A—C, the release patterns of taxol from the microspheres incorporating 30% (w/w) of IPM were almost linear and were similar among the drug loading levels, from 1% to 5%, with a correlation coefficient of $r^2>0.99$. Thus, the release of taxol from the microspheres was not affected by the initial drug loading levels.

Surface Morphology of the Microspheres The electron micrographs of Taxol-PLGA-MS without and with the incorporation of IPM at different stages of the *in vitro* release test are shown in Figs. 4 and 5, respectively. The microspheres containing 5% (w/w) taxol before the release test were spherical, with a smooth surface on which no pores or cracks were found (Fig. 4A). There was no evidence to indicate the existence of taxol crystals on the surface of the microspheres. The incorporation of 30% IPM had no influence on the shape of the microspheres but some pores were observed (Fig. 5A). A similar morphology was observed for Taxol-PLGA-MS which incorporated 50% (w/w) IPM (result not shown).

After the microspheres were subjected to an *in vitro* release test for 1—2 weeks, the compact surface and spherical shape could still be observed for most Taxol-PLGA-MS, but matrix degradation began to develop in this stage, as viewed from the emergence of some sponge mass (Figs. 4B and 4C). Remarkable degradation was observed in approximately 30—40% of the microspheres 3 weeks later, accompanied by changes of their shape, the formation of a porous surface, and a fused mass composed of an erosion matrix of the microspheres (Fig. 4D). A similar degradation pattern was also observed for IPM-incorporating microspheres (Fig. 5B—D).

Discussion

Generally, microspheres are mainly characterized with respect to their particle size, drug-loading, and release profile.[21] It has been shown that the antitumor effect of cisplatin assessed by the suppression of tumor growth in an animal model tended to be higher when microspheres with a relatively smaller size (20—37 μm) were used.[22] Thus, in the present study, the taxol-loaded microspheres with a smaller size were produced and characterized. The continuous release of an anticancer agent from microspheres for a week to a month is usually required for the maintenance of an effective concentration of the

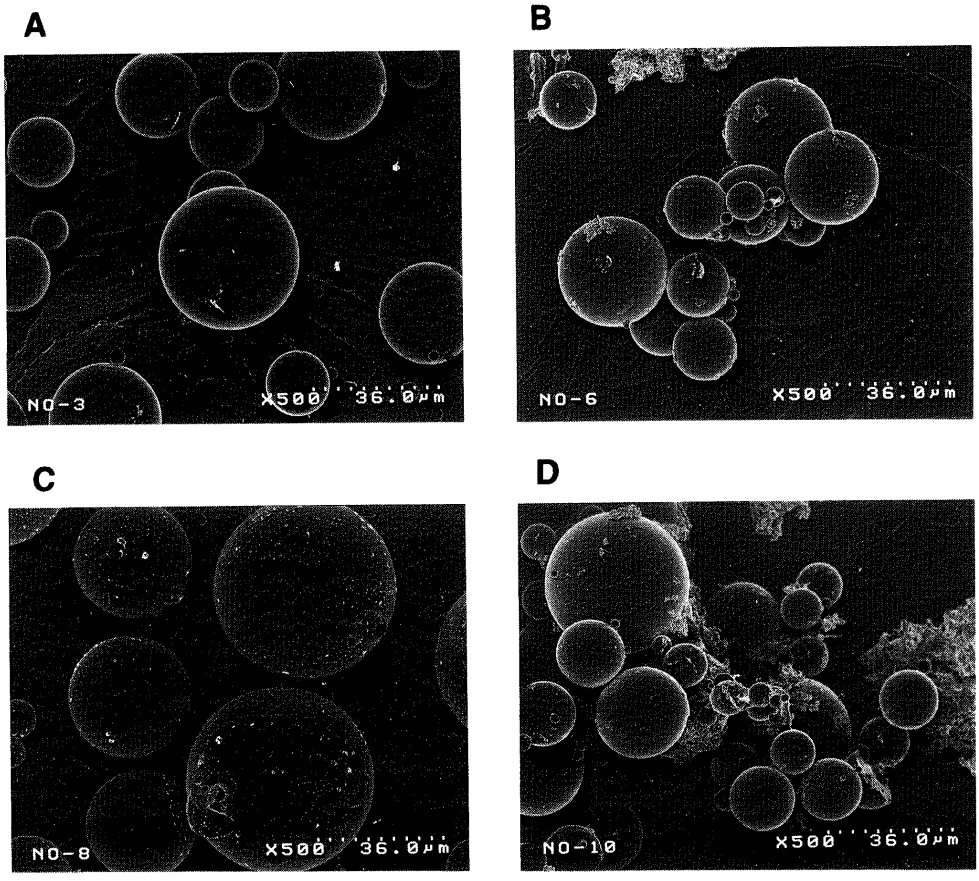

Fig. 4. Scanning Electron Micrographs of Taxol-PLGA-MS Showing Their External Surfaces at Different Stages of the *in Vitro* Release Test

The samples were observed before the release test (A), and after 1 week (B), 2 weeks (C) and 3 weeks (D). The taxol loading level was 5% (w/w).

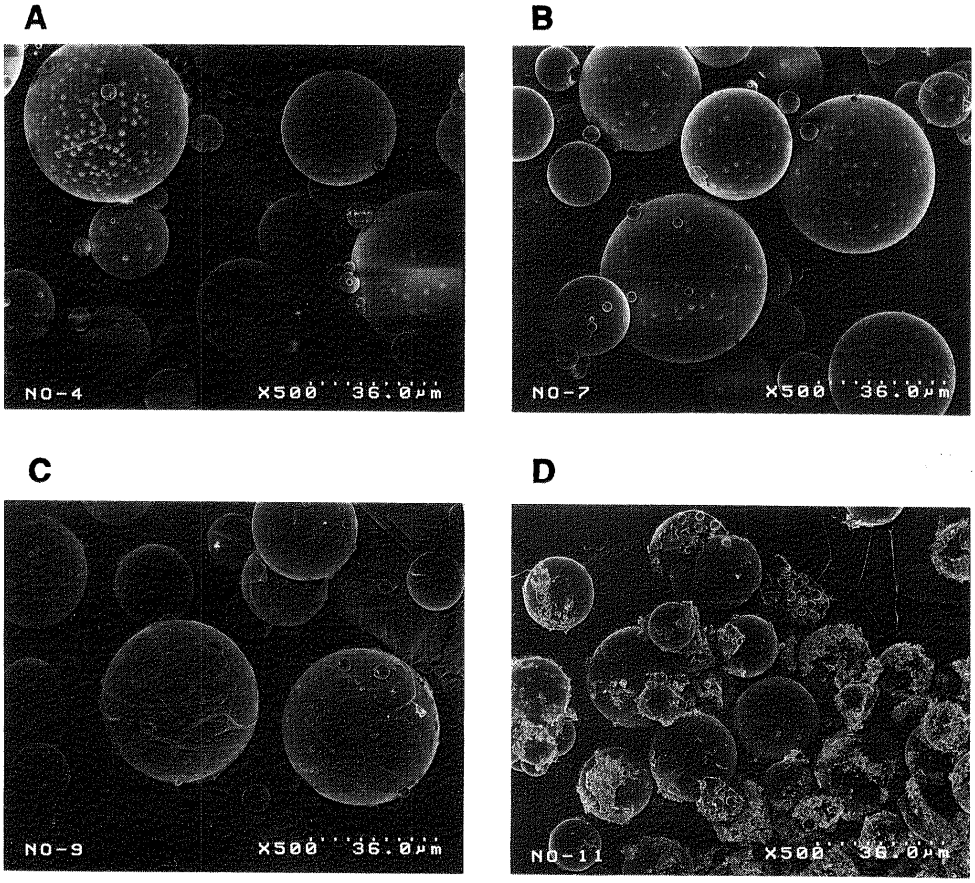

Fig. 5. Scanning Electron Micrographs of IPM-containing Taxol-PLGA-MS Showing Their External Surfaces at Different Stages of the *in Vitro* Release Test

The samples were observed before the release test (A), and after 1 week (B), 2 weeks (C) and 3 weeks (D). The loading levels of taxol and IPM were 5% and 30% (w/w), respectively.

drug after administration.[23] The diffusion of drugs from the microsphere matrix, which is dependent upon the characteristics of the drug and polymer material,[24] plays an important role in the release kinetics of the drug. A relatively slower release of taxol from microspheres formulated with poly(ε-carpolactone)[17] or a blend of ethylene-vinyl acetate and poly(*d,l*-lactic acid)[18] may be attributed to the slow diffusion of taxol due to its poor aqueous solubility in the high-molecular-weight polymer matrix. Therefore, in this study, IPM, a fatty acid ester with a long alkyl chain, was incorporated to increase the drug diffusion in microspheres. On the other hand, it is known that the drug release from microspheres of a low-molecular-weight polymer is faster, and that matrix degradation will further accelerate the drug diffusion.[25,26] This led us to select PLGA with a low molecular weight to formulate taxol into microspheres in this study.

The *in vitro* release of a drug from microspheres is usually evaluated by measuring the amount of drug released into the outer medium, that is, method 1 in this study. However, this method may not be applicable to taxol, because of the significant binding of the drug to plastic and glass containers, as presented in Table 2. This is consistent with a recent report by Song *et al.*,[27] in which the taxol concentration decreased to 60—80% of the initial level in 5 min and continued to decrease to a plateau. Moreover, the coefficient of variation of the determination ranged from 10—50% with method 1, reflecting the large variability of drug adsorption into the container (Fig. 2). Therefore, the remaining amount of taxol in the microspheres was determined using method 2 in order to evaluate its release behavior in a reproducible and quantitative manner.

A drug release profile from PLGA microspheres can usually be divided into the following three phases: 1) rapid drug release from the surface, 2) relatively slow release before the significant degradation of a polymer matrix occurs, and 3) increased release accompanied by the erosion of microspheres.[25] These three phases were also observed in the release profiles of Taxol-PLGA-MS, but in the case of the IPM-incorporating microspheres, the difference among the three phases was not clear (Fig. 3). The incorporated IPM was thought to be dispersed in the PLGA matrix, and taxol might be dissolved in both the PLGA matrix and IPM. It is likely that the small pores on the surface of the microspheres are filled with IPM, through which taxol can diffuse into the release medium relatively quickly. From the facts that IPM itself was released very little from the microspheres[28,29] and that IPM is an oil phase in which taxol can be easily dissolved, the portion of taxol dispersed in the PLGA polymer matrix might partition into the additive phase gradually, then diffuse out of the microspheres. Moreover, since the degradation profile was not influenced by

the incorporation of IPM, it was strongly suggested that the release of taxol from the microspheres was dominated mainly by drug diffusion in the matrix.

We employed 1—5% taxol loading levels for the preparation of PLGA microspheres. Indeed, a higher drug content is advantageous for the chemoembolization therapy of cancer. However, the high cost of taxol hampered the evaluation of taxol release at higher drug loading levels, such as higher than 20% (w/w), and thus prevented evaluation of the enhancing effect of IPM on them. Nevertheless, as suggested from the present experimental results, the release rate of taxol at higher drug loading levels might be controlled and modulated by adjusting the ratio of IPM and PLGA. On the other hand, it has been reported that the degradation of microspheres produced with PLGA (M.W. 10000) shows matrix degradation with a half-life of 20 d on a weight basis.[25] The chemoembolization with PLGA microspheres (M.W. 7000) has been shown to effectively blockade the blood supply of a tumor for a sufficiently long period.[30] Thus, IPM-incorporating microspheres may be suitable for the chemoembolization therapy of malignant tumors.

In conclusion, taxol-loaded PLGA microspheres were successfully produced by the solvent evaporation technique. Incorporation of IPM into the microspheres significantly increased the release rate of taxol *in vitro*, but had no significant influence on the surface morphology and degradation behavior of the microspheres. The microspheres formulated in this study may be suitable for the targeted delivery of taxol in cancer chemotherapy.

Acknowledgments This work was partly supported by the Shimabara Science Promotion Foundation awarded to H.S. One of the authors (Y.M.W.) wishes to thank the Nagai Memorial Foundation for financial support. The authors thank Mr. Masahiko Kawahara for his excellent technical assistance in producing the scanning electron micrographs of the microspheres.

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