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Preparation of a copoly (dl-lactic/glycolic acid)-zinc oxide complex and its utilization to microcapsules containing recombinant human growth hormone

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Abstract

A procedure to prepare a complex of copoly (dl-lactic/glycolic acid) and zinc oxide (PLGA-zinc oxide complex) was developed. Out of sparingly water-soluble zinc compounds, zinc oxide was most remarkably soluble in a PLGA/dichloromethane solution and the dissolution rates became faster as the water contents in the PLGA/dichloromethane solutions increased. Since the solubility of zinc oxide was saturated at approximately 0.5-fold molar ratio to PLGA and water was generated with dissolution of zinc oxide in the PLGA/dichloromethane solutions, it is suggested that zinc oxide interacts with the terminal carboxyl group of PLGA. In addition, the glass-transition temperature of a solid material obtained by vacuum-drying the PLGA/dichloromethane solution dissolving zinc oxide became higher as the zinc content increased, suggesting that the formation of a PLGA-zinc oxide complex. Microcapsules were prepared with the PLGA-zinc oxide complex using recombinant human growth hormone (rhGH) in order to evaluate an effect of the complex on protein release and stability of protein in the microcapsules. Released rhGH amount from the microcapsules prepared with the PLGA-zinc oxide complex after subcutaneous administration in rats was significantly larger than that from microcapsules prepared with PLGA alone, indicating that rhGH molecules in the microcapsules was stabilized by the PLGA-zinc oxide complex.

Keywords: Copoly (dl-lactic/glycolic acid) (PLGA); Zinc oxide; Complex; Water content; Recombinant human growth hormone (rhGH); Stabilization in microcapsules

1. Introduction

The application of biodegradable polymers to prepare microcapsules which release peptides over 1 month has been investigated in the last two decades, and some successes were reported (Ogawa

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et al., 1988a,b,c; Okada et al., 1989). However, such delivery systems could not be adapted to proteins-delivery because of their extreme susceptibility. The first critical problem to apply biodegradable polymers to protein formulations is a deterioration reaction during fabrication of the microcapsules (Tabata et al., 1993), and the second one is denaturation after hydration of the microcapsules (Lu and Park, 1995a,b). To overcome those problems, several additives such as oligosaccharides (Cleland and Jones, 1996) and surfactants (Morita et al., 2000) were incorporated into the microcapsules to stabilize proteins. Since some proteins such as recombinant human growth hormone (rhGH) are known to form a complex with zinc ions (Cunningham et al., 1991; Berg and Shi, 1996) and their thermostability is improved (Maa et al., 1998), protein molecules in the microcapsules could be stabilized if zinc is supplied into the microcapsules.

Recently, an insulin-containing microcapsules prepared with a zinc salt of copoly (dl-lactic/glycolic acid) (PLGA) obtained by a water-in-oil-in-water (W/O/W) emulsion solvent evaporation method using an aqueous zinc acetate solution as an inner water phase and a PLGA/dichloromethane solution as an oil phase was reported (Okamoto et al., 1997). Those microcapsules reduced an initial burst and sustained serum insulin levels for a long time when administered to rats. In this system, a zinc ion is supplied from the zinc salt of PLGA and insulin might form a hexamer with zinc ions in the microcapsules (Derewenda et al., 1989). On the other hand, it was found that zinc oxide dissolved in a PLGA/dichloromethane solution and the generated complex of PLGA and zinc oxide (PLGA-zinc oxide complex) was useful for preparation of protein-containing microcapsules (Yamagata et al., 1998). Though the PLGA-zinc oxide complex is more convenient for preparation of the microcapsules than the zinc salt of PLGA, the dissolving mechanism of zinc oxide is not sufficiently studied yet.

In this study, the factors which influence the solubility of zinc oxide in a PLGA/dichloromethane solution and the dissolving kinetics were examined. Then, characteristics of the PLGA-zinc oxide complex obtained by drying the PLGA/dichloromethane solution dissolving zinc oxide were elucidated. RhGH-containing microcapsules were prepared using the PLGA-zinc oxide complex and the release properties of the microcapsules were compared with microcapsules prepared using PLGA alone in an in-vivo release study.

2. Materials and methods

2.1. Materials

Two sorts of PLGA with lactic/glycolic acid ratio of 50/50 and number average molecular weight (Mn) of 2150 and 3890, respectively (weight average molecular weight (Mw) of 5850 and 12 800) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Zinc oxide with particle size of 0.02 μm and dehydrated dichloromethane (water content is less than equal 30 ppm) were obtained from Wako Pure Chemical. Zinc hydroxide and basic zinc carbonate were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). (RhGH) was produced by a genetic engineering technology at Takeda Chemical Industries, Ltd. (Osaka, Japan) (Nishimura et al., 1998; Suenaga et al., 1999). Polyvinyl alcohol (PVA) (Gosenol® EG-40) was obtained from Nippon Synthetic Chemical Industries, Co., Ltd. (Osaka Japan). The water used during the study was ultra pure grade from a Milli-Q plus® system (Millipore, Paris, France). Other chemicals were of reagent grade. Male Sprague–Dawley rats were purchased from Clea Japan, Inc. (Tokyo, Japan).

2.2. Methods

2.2.1. Solubility of sparingly water-soluble zinc compounds

In this study, we measured turbidity of PLGA/dichloromethane suspensions containing zinc compounds to represent dissolution rates of the compounds in stead of measuring zinc concentrations in the PLGA/dichloromethane solutions because of simplicity of the procedures. In addition, the turbidity measurement enabled a continuous observation of the dissolution process. The

turbidity of zinc oxide, zinc hydroxide and basic zinc carbonate in the PLGA/dichloromethane solution was measured as follows. Each compound (0.125, 0.25 and 0.5 molar ratio against PLGA) was added to a PLGA (Mn = 2150)/dichloromethane solution (1.0 g/2.5 ml) and shaken at 25 °C for a week with a reciprocal shaker (SR-I, Taitec Corporation, Saitama, Japan). Turbidity of each suspension was represented by the absorbance at 600 nm measured with a spectrophotometer (DU 7400, Beckman Instruments, Inc., Fullerton, CA).

2.2.2. Preparation of a PLGA-zinc oxide complex

Zinc oxide was added to a PLGA (Mn = 3890)/dichloromethane solution (1.0 g/2.5 ml) at various molar ratios against PLGA and shaken on the reciprocal shaker at 25 °C for 3 days. The turbidity of each suspension was measured by the same method described above. Insoluble components in the suspensions were separated by filtering with Omnipre® (Nihon Millipore Ltd., Yonezawa, Japan) and water contents in the filtrates were measured by coulometoric Karl Fisher methods with AQ-6 (Hiranuma Sangyo Co., Ltd., Ibaragi, Japan). The residual filtrates were vacuum-dried and glass-transition temperature (Tg) of the obtained PLGA-zinc oxide complex was measured with a differential scanning calorimeter (DSC 7, Perkin–Elmer, Norwalk, CT) at an ascending rate of 10 °C/min. The zinc content in the obtained complex was measured with an atomic photospectrometer (Z-8000, Hitachi, Ltd., Tokyo, Japan).

2.2.3. Effects of water content on zinc oxide dissolving kinetics

Water-saturated dichloromethane was obtained by shaking 25 ml of dehydrated dichloromethane and 25 ml of water at 25 °C overnight. PLGA (Mn = 3890) /dichloromethane solutions (3.5 g/7 ml) with different water content were prepared by mixing the water-saturated dichloromethane and the dehydrated dichloromethane at various ratios. The water content in each solution was measured by the coulometric Karl Fisher method using 1 ml of the solutions. Five milligram of zinc oxide was added to 6 ml of the solutions and shaken at 25 °C and the turbidity of each suspension was measured by the same method described above.

2.2.4. Preparation of microcapsules containing rhGH

A freeze-dried powder of rhGH was obtained by freezing 100 ml of an aqueous rhGH solution in an 1 liter-round-bottom-glass container using dry ice/acetone, followed by drying the ice with a vacuum dryer (DF-01H, ULVAC Japan, Ltd., Kanagawa, Japan). Microcapsules were prepared by a solid-in-oil-in-water (S/O/W) emulsion solvent evaporation method using the freeze-dried powder of rhGH as follows. A PLGA-zinc oxide complex/dichloromethane solution was obtained by adding 12.5 mg of zinc oxide to a PLGA (Mn = 3890)/dichloromethane solution (2.36 g/5.0 ml), followed by shaking overnight at 25 °C with the reciprocal shaker. One hundred twenty-five milligram of the freeze-dried powder of rhGH was added to the PLGA-zinc oxide complex/dichloromethane solution and the powder was micronized with a sonicator (Model G112SP1T, Laboratory supplies Co., Inc., Kickville, NY). The obtained suspension was emulsified in 800 ml of 0.1% PVA aqueous solution (18 °C) using a mixer (T.K.Autohomomixer®, Tokushukika Kogyo Co., Ltd., Osaka, Japan) at 6000 rpm. The emulsions were hardened by mixing with a propeller (Heidon® 3000 H, Shinto Scientific, Co., Ltd., Tokyo, Japan) for 3 h and the microcapsules were collected by centrifugation, then freeze-dried. Microcapsules without zinc oxide were prepared using 100 mg of the rhGH powder and a PLGA alone/dichloromethane solution (1.9 g/2.5 ml) by the same method described above.

The shape and surface characteristics of the dried microcapsules were examined with a scanning electron microscope (SEM) (model ABT-60, Topcon Co. Ltd., Tokyo). The mean particle size of the microcapsules was determined with a Coulter® Multisizer II (Coulter Electronics, Co., Ltd., Bedfordshire, UK).

2.2.5. Determination of rhGH content and zinc content in the microcapsules

RhGH contents in the microcapsules were measured by high performance liquid clomatogra-

phy (HPLC) as follows. Microcapsules (4 mg) were added to a mixture of acetonitrile (0.3 ml) and an aqueous solution (0.7 ml) containing 0.05% trifluoro acetic acid (TFA) and 0.02% bovine serum albumin (BSA) to extract rhGH. After vortex-mixing, the dispersion was centrifuged and rhGH was extracted to the supernatant. RhGH in the supernatant was determined by HPLC (Shimadzu LC-6A model, Kyoto, Japan) with an ultra violet detector as follows: column, TSK gel Phenyl-5PW (Tosoh Co., Ltd., Kanagawa, Japan); column temperature, 40 °C; mobile phase, a 34% acetonitrile aqueous solution containing 0.05% TFA; acetonitrile gradient, 2%/min; flow rate, 0.8 ml/min; wavelength, 215 nm. A zinc content in the microcapsules was determined with the atomic absorption spectrophotometer as mentioned above.

2.2.6. *In vivo release study*

Release profiles of rhGH from the microcapsules were evaluated in vivo using an immunosuppressed rat model. The immuno-suppressed rats were obtained by subcutaneous injection of tacrolimus (Fujisawa Pharmaceutical, Co., Ltd., Osaka, Japan) at doses of 0.4 mg per animal (3 days before microcapsule-administration), and followed by injecting 0.2 mg per animal of tacrolimus twice a week. The microcapsules were dispersed in a vehicle (an aqueous solution containing 5% mannitol, 0.5% sodium carboxymethyl cellulose (Hercules Inc., Hopewell, CA), 0.1% Tween 80 (Bio Rad Laboratories, Hercules, CA)) at concentration of 8 mg/ml as rhGH and 0.75 ml of the dispersion was injected subcutaneously at the nape of the rat (6 weeks of age) under ether anesthesia. At predetermined time, 0.5 ml of blood was withdrawn through the tail vein under ether anesthesia. The serums were stored at −40 °C until the assay of rhGH concentration. The remaining microcapsules were excised from the injection site of the rats on day 21 after sacrificed with ether. The rhGH content in the excised microcapsules was determined as follows. Six millilitre of acetoritrile was added to the excised microcapsules and the suspension was homogenized with Polytron (Kinematica, Switzerland) at 20 000 rpm for 20 s. Then, 7 ml of phosphate-buffered saline (PBS) (5 mM, pH 8.0) containing 0.2% BSA was added and the suspension was homogenized again at 20 000 rpm for 10 s and finally 7 ml of PBS containing 0.2% BSA was added. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was filtered with Omnipore® with 0.5 μm pore size. The samples were stored at −40 °C until the assay of their rhGH content.

RhGH concentration in the serum and the extracted samples obtained above were measured using a commercially available immunoradiometric assay kit (Ab bead HGH'Eiken', Eiken Chemical Co., Ltd., Tokyo, Japan).

2.2.7. *Statistical analysis*

Statistical analysis was performed by applying *t*-test of Statistical Analysis System (SAS). All results were expressed as the mean value ± standard error.

3. Results

3.1. *Solubility of various zinc compounds in a PLGA/dichloromethane solution*

The absorbance at 600 nm of each PLGA/dichloramethane suspension containing zinc oxide, zinc hydroxide and basic zinc carbonate are shown in Table 1, indicating the solubility of those zinc compounds in the PLGA/dichloromethane solution after 1 week shaking at 25 °C. Zinc oxide dissolved up to 0.5 molar ratio to PLGA. Zinc hydroxide dissolved at low molar ratios to PLGA, however, did not dissolve completely at 0.5 molar ratio. In contrast, basic zinc carbonate was spar-

Table 1
Solubility of sparingly water-soluble zinc compounds in PLGA/dichloromethane solutions

Added amount	Absorbance at 600nm		
(molar ratio to PLGA)	ZnO	$Zn(OH)_2$	Basic $ZnCO_3$
0.125	0.002	0.007	1.285
0.25	0.000	0.075	2.290
0.5	0.002	1.463	2.974

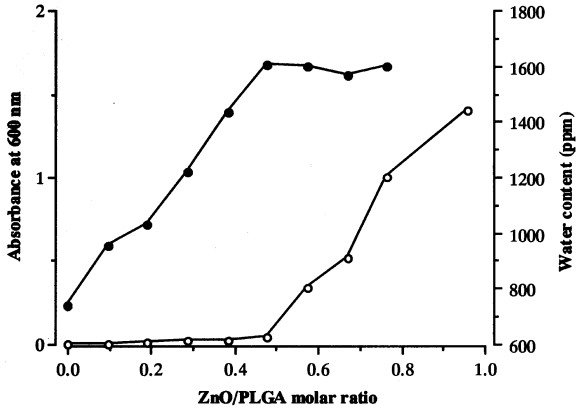

Fig. 1. Relationship between solubilization of zinc oxide and increase of water content in the PLGA/dichloromethane solutions as a function of added amount of zinc oxide. Solubility is represented as absorbance at 600 nm (open circles) and water content (solid circles) is measured by coulometric Karl Fischer methods.

ingly soluble in the PLGA/dichloromethane solution.

3.2. Solubilization of zinc oxide in a PLGA/dichloromethane solution and complex formation

Since the turbidity of the PLGA/dichloromethane suspension containing zinc oxide increased with more addition of zinc oxide from 0.5 molar ratio to PLGA, solubility of zinc oxide in the PLGA/dichloromethane solution was likely to be saturated at 0.5 molar ratio to PLGA (Fig. 1). Water contents in the PLGA/dichloromethane solution increased linearly until zinc oxide/PLGA molar ratio reached 0.5 and were saturated with more addition of zinc oxide. Zinc contents in the PLGA-zinc oxide complexes obtained by vacuum-drying the solutions increased linearly until zinc oxide/PLGA molar ratio reached 0.5 (Fig. 2). In proportion to the zinc contents, Tg of the PLGA-zinc oxide complexes increased. Zinc oxide dissolved in a first order manner in the PLGA/dichloromethane solutions with different water contents (Fig. 3). The dissolution rates increased correlating with the water contents in the PLGA/dichloromethane solutions. Logarithms of the dissolution rate constants obtained from the slopes of four linear regression lines in Fig. 3 were linearly correlated with the water contents in the PLGA/dichloromethane suspensions (Fig. 4).

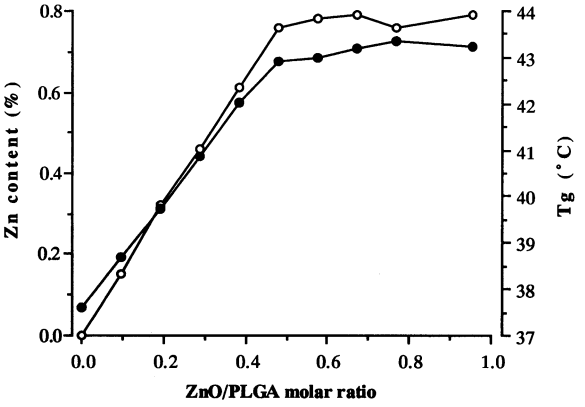

Fig. 2. Relationship between zinc content (open circles) and Tg (solid circles) of solid materials after vacuum-drying the PLGA/dichloromethane solution as a function of the added amount of zinc oxide.

3.3. Characteristics of the microcapsules containing rhGH

The SEM observation revealed that the shape of the microcapsule prepared using the PLGA-zinc oxide complex and the freeze-dried rhGH powder

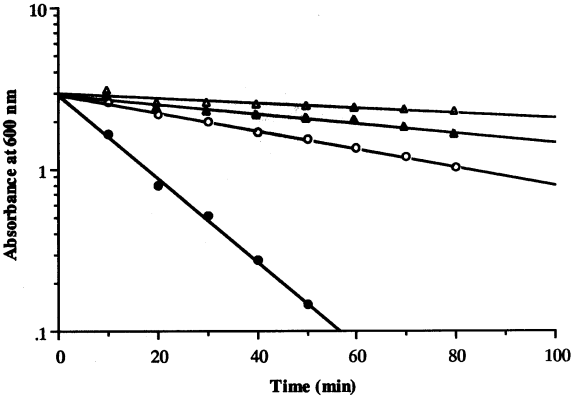

Fig. 3. Effects of water contents in PLGA/dichloromethane solutions on kinetic profiles of zinc oxide solubilization. Open triangles, 440 ppm; solid triangles, 820 ppm; open circles, 1100 ppm; solid circles, 1700 ppm. Solid lines represent least squares linear regression lines of the data points. The obtained equations: $y = 2.9 \times 10^{-0.0015x}$, 440 ppm; $y = 2.9 \times 10^{-0.0029x}$, 820 ppm; $y = 2.9 \times 10^{-0.0056x}$, 1100 ppm; $y = 2.9 \times 10^{-0.0258x}$, 1700 ppm ($x$ means time and y means absorbance at 600 nm).

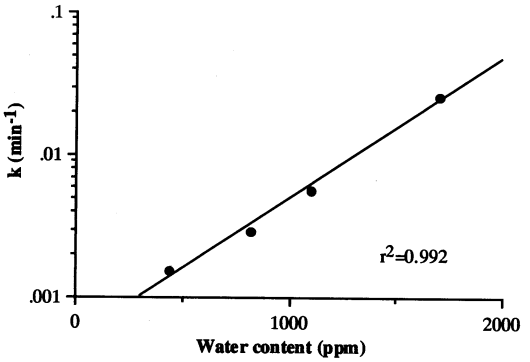

Fig. 4. Relationship between water contents in the PLGA/dichloromethane solutions and kinetic rate constants of zinc oxide solubilization. A solid line represents a least squares linear regression line of the data points.

was spherical with a smooth surface (Fig. 5). The mean particle size of the microcapsules prepared using the PLGA-zinc oxide complex was 34.4 μm. The entrapment of rhGH in the microcapsules prepared using the PLGA-zinc oxide complex and PLGA alone was 91.4 and 99.0%, respectively. A zinc content in the microcapsules prepared using the PLGA-zinc oxide complex was 0.34%.

3.4. Release profiles of rhGH from the microcapsules

After the injection of the microcapsules prepared with the PLGA-zinc oxide complex (6 mg rhGH per animal), serum rhGH concentration in rats was initially increased to approximately 2000 ng/ml, followed by a constant concentration more than 10 ng/ml for 11 days (Fig. 6). The initial serum rhGH concentration after the injection of the microcapsules prepared with PLGA alone was comparable to that after the injection of the microcapsules prepared with PLGA-zinc oxide complex. On the contrary, the following serum levels were lower than those after the injection of the microcapsules prepared with the PLGA-zinc oxide complex. AUC of the microcapsules prepared with the PLGA-zinc oxide complex was significantly larger than that of the microcapsules prepared with PLGA alone (Fig. 7). RhGH amounts remaining on day 21 after the injection of the microcapsules prepared with the PLGA-zinc oxide complex and the PLGA alone were 0.16 and 0.18% of the injected rhGH amounts, respectively.

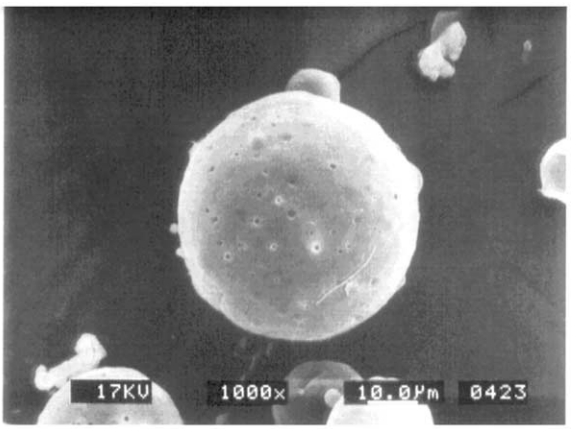

Fig. 5. A scanning electron micrograph of the microcapsule prepared with the freeze-dried rhGH powder and the PLGA-zinc oxide complex.

4. Discussion

Wada et al. reported that metal salts of PLGA which terminal carboxyl groups interact with metal ions could be obtained by immersing PLGA in an aqueous solution of metal salts (sodium and calcium) (1991). In addition, it was reported that a zinc salt of PLGA could be obtained by a W/O/W emulsion solvent evaporation method using an aqueous zinc acetate solu-

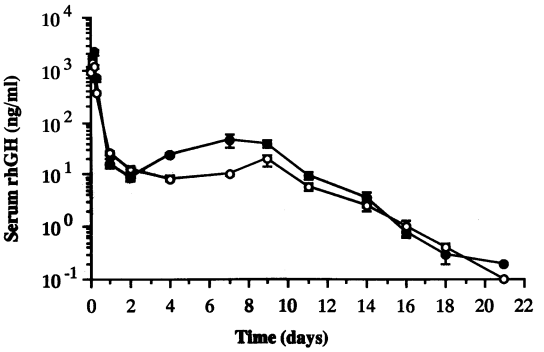

Fig. 6. In-vivo release profiles of rhGH from microcapsules prepared by the S/O/W solvent evaporation method using the PLGA-zinc oxide complex (solid circles) and PLGA alone (open circles) in rats (6 mg rhGH per animal). Each symbol represents the mean and bar represents the S.E. ($n = 4$).

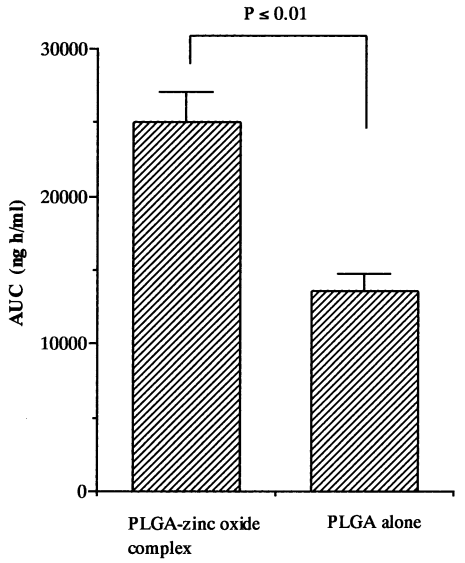

Fig. 7. AUC calculated form the data shown in Fig. 6. Each column represents the mean and bar represents the S.E. ($n = 4$).

tion as an inner water phase, a PLGA/dichloromethane solution as an oil phase and an aqueous 0.1% PVA solution as an outer water phase (Okamoto et al., 1997). These results indicate that free carboxyl groups of PLGA electrically interact with metal cations. Water-soluble metal cations were used to prepare the metal salts of PLGA in those methods. In this study, zinc oxide was found to be most soluble in a PLGA/dichloromethane solution out of water-sparingly soluble zinc compounds **(Table 1). Since the solubility was saturated at 0.5-fold molar ratio of zinc oxide against PLGA, it was suggested that terminal carboxyl groups of two PLGA molecules interacted with one zinc oxide molecule (Fig. 1). Zinc contents in PLGA-zinc oxide complexes obtained by drying the PLGA/dichloromethane solution dissolving zinc oxide increased in proportion to the added zinc oxide and was saturated at 0.5-fold molar ratio of zinc oxide against PLGA (Fig. 2). In addition, Tg of the PLGA-zinc oxide complexes also became higher as the added zinc oxide increased (Fig. 2). These results indicate that the terminal carboxyl group of PLGA and zinc oxide molecularly interact each other in the obtained PLGA-zinc oxide complex. Increase of Tg was also reported in the metal salts of PLGA in which the terminal carboxyl groups of PLGA interact with metal ions (Wada et al., 1991).

The dissolving kinetic analysis revealed that the dissolving rate of zinc oxide in the PLGA/dichloromethane solution increased in proportion to the water contents in the PLGA/dichloromethane solution (Fig. 3). The water in the PLGA/dichloromethane solution accelerated the dissolving rate constant in an exponential manner (Fig. 4). Zaks et al. reported that a reaction rate of an enzyme powder dispersed in an organic solvent exponentially increased as the water content in the organic solvent increased (Zaks and Klibanov, 1984; Zaks and Russell, 1988). Their results are consistent with our data and it is suggested that the increase of the water content in an organic solvent which is a rate limiting factor of reaction accelerates the dissolving reaction of zinc oxide. One possible hypothesis of the dissolving mechanism of zinc oxide in the PLGA/dichloromethane solution is as follows. Firstly, zinc oxide dissolves in water existing in the PLGA/dichloromethane solution and becomes a Zn^{2+} ion, then electrically interacts with the terminal carboxyl groups of PLGA and produces water as a result of this reaction. The increase of the water content in the PLGA/dichloromethane solution supports this hypothesis (Fig. 1). However, we could not distinguish whether there is zinc oxide or a zinc ion in the PLGA-zinc oxide complex obtained by drying the PLGA/dichloromethane solution dissolving zinc oxide.

Recently developed cryogenic spray drying process using a zinc-complexed rhGH enabled the microsphere preparation that stably deliver rhGH in vivo (Johnson et al., 1996, 1997; Bartus et al., 1998). Denaturation of proteins in the microspheres prepared with PLGA alone after hydration is reported due to low pH environment (Lu and Park, 1995a,b). In the microspheres prepared by the cryogenic spray drying process, rhGH is likely to be stabilized with zinc because rhGH forms a dimer with zinc (Cunningham et al., 1991) and zinc increases thermal stability of rhGH (Maa et al., 1998). In our study, it was clarified that the microcapsules prepared using the PLGA-zinc oxide complex also stably delivered rhGH compared with the microcapsules prepared using

PLGA alone because the AUC after the injection of the microcapsules prepared with the PLGA-zinc oxide complex was significantly larger than that after the injection of the microcapsules prepared with PLGA alone though the remaining amounts of rhGH in both microcapsules on day 21 were almost 0 (Figs. 6 and 7). These results suggest that the PLGA-zinc oxide complex supplies zinc to rhGH in the microcapsules after the hydration and the stability of rhGH increases during the release period. Though both the microspheres prepared by the cryogenic spray drying process and the microcapsules prepared by the solvent evaporation method using the PLGA-zinc oxide complex stabilize rhGH with zinc, the shapes of them are different, i.e. the microspheres have a rugged surface (Johnson et al., 1997) whereas the microcapsules have a smooth surface (Fig. 5). The reason of that difference is likely to be due to the difference in fabrication processes (spraying in the air or emulsifying in the water) and/or materials used (PLGA solution containing separately dispersed zinc carbonate particles or the PLGA-zinc oxide complex/dichloromethane solution). The mean particle size of the microspheres is larger than that of the microcapsules, suggesting that the injection of the microcapsules will be easier.

A zinc salt of PLGA was also reported to be used as a reservoir of zinc and increase stability of insulin encapsulated in the microcapsules (Okamoto et al., 1997). The fabrication process for the preparation of the microcapsule using the PLGA-zinc oxide complex is more convenient compared with the microcapsules using the zinc salt of PLGA, indicating that the PLGA-zinc oxide is a superior material for preparing microcapsules containing proteins. An application of the PLGA-zinc oxide complex for preparation of microcapsules containing other proteins is interesting.

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