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Efficient encapsulation of a water-soluble corticosteroid in biodegradable nanoparticles

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ABSTRACT

Solid nanoparticles consisting of biodegradable polymers have emerged as a promising carrier for various drugs, but unfortunately the encapsulation of drugs remains challenging. In this study, a technique for encapsulation of water-soluble drugs in solid nanoparticles was developed. Nanoparticles were prepared from a blend of biodegradable polymers, including poly(lactic acid) (PLA) and poly(lactic/glycolic acid) (PLGA), and monomethoxypolyethyleneglycol–polylactide block copolymer by an oil-in-water solvent diffusion method. Betamethasone sodium phosphate (BP) was not encapsulated by the nanoparticles due to its hydrophilicity, but it was effectively encapsulated in the presence of appropriate amounts of zinc and diethanolamine. It was found that BP formed an ionic complex with zinc at a certain pH range obtained by addition of diethanolamine. Furthermore, a carboxyl group located at the end of PLA/PLGA was shown to be essential for encapsulation of BP in nanoparticles, and the molar ratio among BP, zinc, and carboxyl groups in various nanoparticles was almost constant. These results strongly suggested that the encapsulation was promoted by zinc creating an ionic bridge between a carboxyl group on PLA/PLGA and a phosphate group on BP. This technique for entrapment of water-soluble drugs in solid biodegradable nanoparticles may expand the use of nanoparticles for various therapeutic applications.

1. Introduction

Great efforts have been made to develop colloidal carriers for pharmaceutical agents, including liposomes, solid nanoparticles, polymeric micelles, and lipid emulsions, in order to expand the utility of drugs for various clinical applications (Moghimi et al., 2005). However, lipid emulsions and liposomes cannot generally retain drugs *in vivo* for a long period (Igarashi et al., 1992; Lian and Ho, 2001). On the other hand, solid particles seem to be more promising carriers for the stable retention of drugs (Jain, 2000). Solid particles with a high level of drug retention would be expected to have the following advantages in terms of pharmacokinetics and pharmacodynamic. First, stable retention of drugs by the solid particles could prevent inactivation *in vivo*. Second, particles formed from biodegradable polymers such as PLGA and PLA may be used to achieve a long-term therapeutic effect by slow release of drugs along with degradation of the polymers (Okada and Toguchi, 1995). Third, the biodistribution of drugs could be controlled by such particles and specific delivery to target sites should be able to enhance therapeutic efficacy while reducing side effects. In general, colloidal particles that are administered systemically are taken up by the mononuclear phagocyte system (MPS), resulting in accumulation in the liver and spleen (Moghimi et al., 2001). On the other hand, the pioneering work by Langer's group showed that polymeric micelles formed from monomethoxypolyethyleneglycol–polylactide (PEG–PLA) block copolymers could remain in the circulation for a prolonged period because the steric barrier of PEG chains on surface reduces interaction with opsonins and cells of the MPS (Gref et al., 1994). Furthermore, these long-circulating nanoparticles show preferential accumulation in tumors and at sites of inflammation due to the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). In order to take advantage of these properties, it is necessary to establish a method for preparing nanoparticles with a high drug content and good retention. In addition, the nanoparticle production system should reduce the quantity of particles required for administration of a sufficient amount of drug *in vivo* as well as decreasing drug wastage during manufacture (Govender et al., 1999).

A large number of poorly water-soluble drugs are widely used in clinical practice. These drugs are generally incorporated by solid microparticles/nanoparticles through hydrophobic interactions, using methods such as dialysis, oil-in-water emulsion/diffusion, and salting-out. The extent of entrapment and retention of such drugs by the particles is considerably influenced by the physicochemical properties of both the drug and the hydrophobic core (Yamamoto et al., 2007; Letchford et al., 2008).

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Water-soluble drugs are also commonly used for various therapeutic applications, but it is generally difficult to encapsulate water-soluble drugs in solid particles (Astete and Sabliov, 2006). Chemical modification of these drugs, such as esterification, may increase their encapsulation efficiency, but may also decrease bioactivity. Some researchers have encapsulated water-soluble drugs (including peptides and proteins) in PLA/PLGA particles by the water-in-oil-in-water and solid-in-oil-in-water solvent evaporation methods, but these particles have a diameter in the micrometer range (Okada and Toguchi, 1995; Takada et al., 2003; Homayoun et al., 2003). To obtain nanoparticles of a suitable size for intravenous administration, the major obstacles to overcome include the complicated preparation process and a low drug encapsulation efficiency. Despite these problems, development of solid nanoparticles encapsulating water-soluble drugs could contribute to expanding the availability of such drugs.

We previously reported that PLA/PLGA nanoparticles (less than 200 nm in diameter), which encapsulated water-soluble betamethasone 21-disodium phosphate (BP) with a high efficiency, could be successfully prepared by an oil-in-water solvent diffusion method in the presence of zinc (Ishihara et al., 2005; Higaki et al., 2005). Our results showed that zinc increased the encapsulation of BP in nanoparticles by forming a complex with BP. This enhancement of the encapsulation was thought to be induced by a hydrophobic interaction between PLA/PLGA and water-insoluble zinc-BP complexes. It was also suggested that the zinc-BP complexes interacted with PLGA molecules because the molar ratio of zinc to PLGA in the nanoparticles was almost constant. However, the detailed mechanism involved was still unclear.

It was also reported that nanoparticles prepared from a mixture of PEG–PLA block copolymers and PLA/PLGA showed a prolonged retention time *in vivo* (Beletsi et al., 2005), resulting in accumulation of the nanoparticles at sites of disease (Ishihara et al., 2008), while BP-encapsulated nanoparticles without PEG chains were rapidly eliminated by the MPS (Ishihara et al., 2005). Therefore, the present study was performed to develop long-circulating nanoparticles with a high BP content by clarifying the mechanism of BP encapsulation.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic/glycolic acid) (PLGA) with a lactic/glycolic acid ratio of 50/50 and poly(D,L-lactic acid) (PLA) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), and were purified by precipitation in water to remove free acids prior to use. PLA was also synthesized by ring-opening polymerization of D,L-lactide (Purac America, IL). D,L-Lactide, 1-octanol, and stannous octoate were dissolved in DMF and let stand for 6 h at 130 °C. The resulting polymer was precipitated twice in an excess of 2-propanol and then in an excess of water, after which polymers were obtained by freeze-drying. The molecular weight of the polymers was determined by gel permeation chromatography on a KF803L column (Shodex, Japan) with an RI detector, using THF as the mobile phase. The instrument was calibrated with monodispersed polystyrene standards (Tosoh Co., Tokyo, Japan). PEG–PLA block copolymer was synthesized by ring-opening polymerization of D,L-lactide in the presence of monomethoxy-PEG (Mw 5600; NOF Co., Tokyo, Japan) according to the reported method (Pişkin et al., 1995; Riley et al., 2001). The composition and molecular weight of the block copolymers were evaluated by ^{1}H NMR and gel permeation chromatography as mentioned above. PEG–PLA with an average Mw of 15,000 was used in this study. BP and betamethasone 21-acetate (BA) were purchased from Sigma Chemical Co. (MO, USA). Hydrocortisone 21-succinate (HS), hydrocortisone (H), hydrocortisone 17-butyrate 21-propionate (HBP), betamethasone (B), betamethasone 17-valerate (BV), and betamethasone 17,21-dipropionate (BDP) were purchased from Wako Pure Chemical Industries Ltd. Other chemicals were also purchased from Wako. Partition coefficients ($\log P_{OW}$) for BP, B, H and HS were obtained from the literature (Flynn, 1990; Lucangioli et al., 2003) and those for the other drugs were measured by HPLC with a ZORBAX Eclipse XDB-C18 column (Agilent) and a UV/vis detector, using acetonitrile/phosphate buffer (20 mM, pH 7) (v/v = 70/30) as the mobile phase, according to the OECD Guideline for Testing of Chemicals.

2.2. Determination of carboxyl groups in the polymers

The carboxyl group content of the polymers was determined fluorometrically as follows. After 9-anthryldiazomethane (Funakoshi Co. Ltd.; Tokyo, Japan) was dissolved in acetone (10 mg/ml), the resulting solution was diluted with acetonitrile to 0.4 mg/ml. Acetonitrile solutions of each polymer (5 mg/ml) were mixed with a 5-fold excess volume of the 9-anthryldiazomethane solution. After incubation for 1 h at room temperature, conjugation of 9-anthryldiazomethane with the polymer was assessed by gel permeation chromatography with a KF803L column and a fluorescence detector (excitation at 365 nm/emission at 412 nm), using THF as the mobile phase. Myristic acid was used as a standard to calibrate the carboxyl group content.

2.3. Interaction of zinc with BP

Zinc chloride ($ZnCl_2$, 45 mM), BP (13 mM), and various amounts of diethanolamine (DEA) were mixed in a 22.5 mM aqueous solution of HCl. After incubation for 30 min at 25 °C, the pH of the resulting solutions/suspensions was monitored and centrifugation was done at 16,000 × *g* for 10 min to precipitate insoluble BP. An aliquot of the supernatant (50 μl) was mixed with 1000 μl of an aqueous solution of EDTA (50 mM, pH 7.2), and the BP content was determined by HPLC as mentioned above. Mixtures of various amounts of zinc chloride and BP (13 mM) were also analyzed similarly at a fixed pH of 6, which was adjusted by addition of DEA. In addition, the formation of complexes between zinc and BP was analyzed in acetone/water (97.5/2.5, v/v, %).

2.4. Preparation of nanoparticles

Nanoparticles were prepared by the oil-in-water solvent diffusion method. First, 7.2 mg of block copolymers, 42.8 mg of PLA/PLGA, and 10 mg of the drug were dissolved in 1500 μl of acetone in the presence or absence of DEA and zinc. Zinc chloride was dissolved in a 0.5 M aqueous solution of HCl at a concentration of 1 M and the resulting solution was added to the acetone solutions of polymers and drugs. The mixture thus obtained was allowed to stand for 10 min at room temperature. Then the mixture was added dropwise to 25 ml of distilled water (stirred at 1000 rpm) at the rate of 48 ml/h using a 26 G needle. During diffusion of the organic solvent into the water, nanoparticles formed rapidly along with solidification of PLA/PLGA and the block copolymers. Finally, 125 μl of a 200 mg/ml aqueous solution of Tween 80 was added to the resulting nanoparticle suspension to enhance stable dispersion of the nanoparticles. The particle size was determined by the dynamic light scatter method (Zetasizer Nano ZS, Malnern, UK).

2.5. Encapsulation of drugs in nanoparticles

The encapsulation efficiency of the nanoparticles for each drug was defined as the ratio of the drug weight to the total weight

of PLA/PLGA in nanoparticles. In order to solubilize free (unencapsulated) drugs, sodium dodecyl sulfate (SDS) was added to the nanoparticle suspension at a final concentration of 10 mg/ml. After agitation, the suspension was centrifuged at 20,000 × *g* for 15 min. The nanoparticle precipitate was washed by centrifugation with water twice and then dissolved in acetonitrile. The drug content was determined by HPLC on a ZORBAX Eclipse XDB-C18 column (Agilent) with a UV/vis detector. The total PLA/PLGA content was calculated from the amount of lactic acid/glycolic acid produced by hydrolysis of PLA/PLGA and PEG–PLA in an aqueous solution of sodium hydroxide, as reported previously (Kamei et al., 1992; Ishihara et al., 2008). The zinc content of the nanoparticles was determined by using a sequential plasma spectrometer (ICPS-8000, Simadzu Co., Kyoto, Japan) after hydrolysis of the nanoparticles.

3. Results and discussion

PLA and PLGA with different molecular weights were purchased and purified by precipitation in water to remove free acids. Because these polymers were obtained by condensation polymerization of lactic acid/glycolic acid, they theoretically had a carboxyl group at the end of the polymer chain. The molecular weights of the polymers were determined by gel permeation chromatography and the carboxyl group content of each polymer was determined by using 9-anthryldiazomethane, which binds to carboxyl groups without a catalyst (Table 1). Although the molecular weight (Mn) of PLA/PLGA calculated by carboxyl group detection was always lower than that determined by gel permeation chromatography, the carboxyl group content decreased along with an increase of the molecular weight determined by gel chromatography, indicating that carboxyl groups existed at the ends of the polymers. PLA (cPLA) was also synthesized by ring-opening polymerization of D,L-lactide with 1-octanol as an initiator. In this case, the carboxyl group content was markedly lower, indicating that many carboxyl groups at the polymer ends were capped by octanol.

Nanoparticles were prepared from a mixture of PLA/PLGA and PEG–PLA block copolymers with various drugs by the solvent diffusion method. Because block copolymers have a surfactant effect, the blending ratio of these polymers influences the size of the resulting nanoparticles (Dong and Feng, 2006). Thus, to minimize the effect of size on encapsulation efficiency, nanoparticles were prepared from a mixture with a constant blending ratio in the present study and the resulting particles had a diameter ranging from 120 to 150 nm. The encapsulation efficiency of the nanoparticles depended significantly on the drugs tested (Fig. 1A).

Table 1
Characteristics of PLA and PLGA

Code[a]	Mw[b]	Mn[b]	Mw/Mn[b]	COOH[c] (μmol/g)	Mn[c]
PLA6k	6170	3300	1.87	394	2540
PLA10k	10050	4610	2.18	298	3350
PLA13k	13150	5230	2.51	240	4170
PLA16k	16440	6120	2.69	201	4980
PLGA5k	4620	3020	1.53	479	2090
PLGA6k	5820	3290	1.77	440	2280
PLGA7k	7310	4250	1.72	366	2730
PLGA9k	8870	4640	1.91	288	3470
PLGA16k	15590	6590	2.37	209	4790
cPLA5k	5260	3920	1.34	21	47460
cPLA11k	11200	5970	1.88	32	31670
cPLA16k	15740	7310	2.15	38	26330

[a] PLA and PLGA with a lactic/glycolic acid ratio of 50/50 were purchased from Wako. cPLA was synthesized from D,L-lactide with 1-octanol.

[b] Molecular weights were determined by gel permeation chromatography.

[c] The carboxyl group content of the polymers was determined fluorometrically using 9-anthryldiazomethane and Mn was calculated.

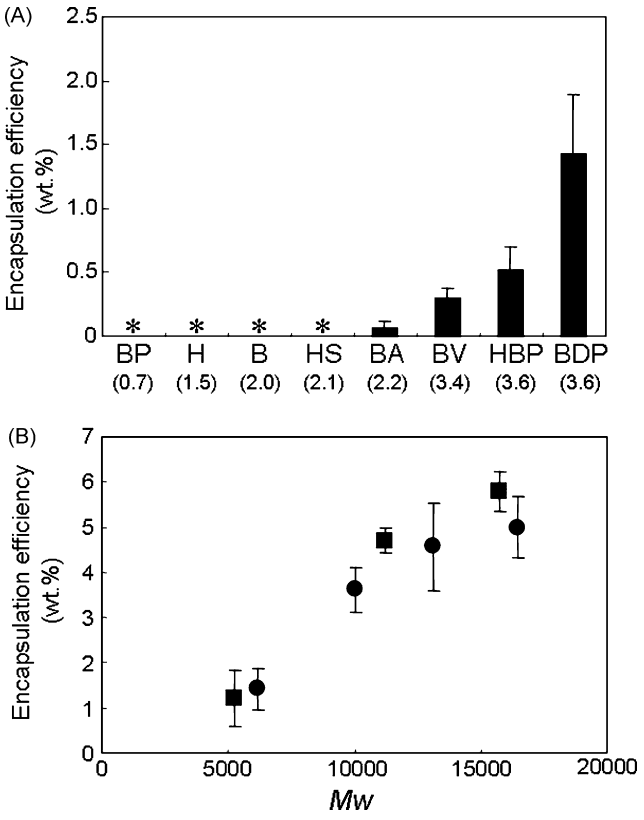

Fig. 1. Encapsulation of drugs in nanoparticles. (A) Encapsulation of various corticosteroids in nanoparticles. Nanoparticles were prepared with 42.8 mg of *PLA6k*, 7.2 mg of PEG–PLA, and 10 mg of each drug (BP: betamethasone sodium phosphate, H: hydrocortisone, B: betamethasone, HS: hydrocortisone succinate, BA: betamethasone acetate, BV: betamethasone valerate, HBP: hydrocortisone butyrate propionate, BDP: betamethasone dipropionate) by the solvent diffusion method, as described in Section 2. The partition coefficient ($\log P_{OW}$) of each drug is shown in parentheses. Each data point represents the mean ± S.D. of three independent experiments. Asterisk indicates that no drug was detected. (B) Encapsulation of BDP in nanoparticles prepared from PLA with different molecular weights. Nanoparticles were prepared with 42.8 mg of the PLA (*circle*) or cPLA (*square*) shown in Table 1, 7.2 mg of PEG–PLA, and 10 mg of BDP. Each data point represents the mean ± S.D. of three independent experiments.

As can be seen from the partition coefficient ($\log P_{OW}$) values of each drug, there was an increase of encapsulation along with an increase of drug hydrophobicity. This result suggested that the drugs were physically encapsulated in the nanoparticles through hydrophobic interaction with the polymers. However, the encapsulation efficiency differed among BV, HBP, and BDP, although these drugs had similar $\log P_{OW}$ values. When solubilization of hydrophobic drugs in micelles is attempted, it has been reported that the extent of solubilization is influenced by the physicochemical properties of the solubilizers and the drugs used (Rangel-Yagui et al., 2005). The drugs we tested had different physicochemical properties with regard to the molecular weight, conformation, polarity, crystallinity, and melting point, in addition to their hydrophobicity. Thus, although it is certain that hydrophobicity is the main determinant of drug encapsulation in nanoparticles, other properties of drugs may partly influence the encapsulation efficiency. Encapsulation of BDP was also investigated using PLA/cPLA of different molecular weights, and an increase of encapsulation was found along with an increase in the molecular weight of both polymers (Fig. 1B). In the present oil-in-water solvent diffusion method, nanoparticles are formed as solidification of PLA/PLGA and the block copolymers occurs during diffusion of the organic solvent (acetone) into the surrounding water. Therefore, these results sug-

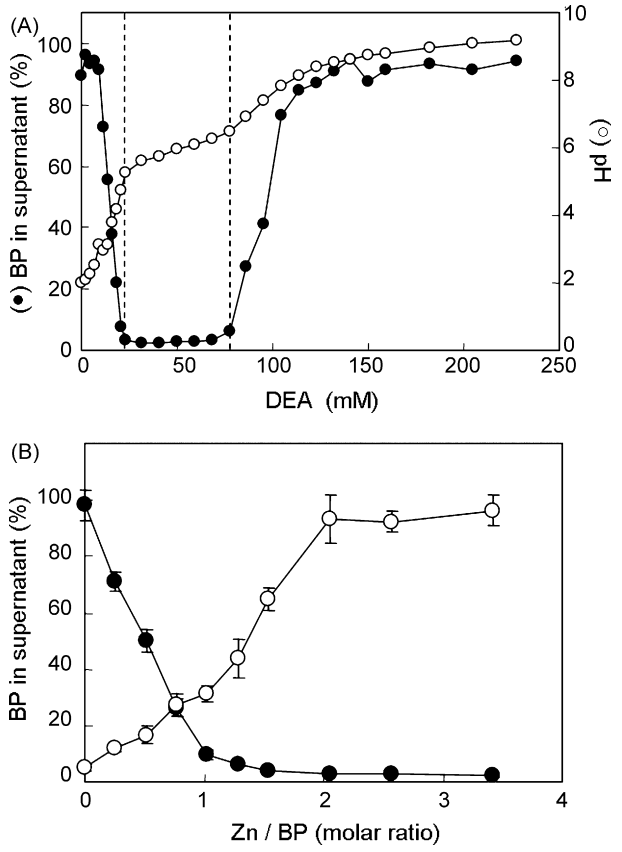

Fig. 2. Solubility of BP in the presence of zinc. (A) Zinc chloride (45 mM), BP (13 mM) and various amount of DEA were mixed in a 22.5 mM aqueous solution of HCl and the pH (*open circle*) was monitored. After centrifugation at 16,000 × *g*, the BP content (*filled circle*) in the supernatant was determined by HPLC. (B) Various amounts of zinc chloride and BP (13 mM) were added to water (*filled circle*) or to an acetone/water (97.5/2.5, v/v, %) solution (*open circle*). The pH of each mixture was adjusted to 6 by the addition of DEA. After centrifugation at 16,000 × *g*, the BP content in the supernatant determined by HPLC. Each data point represents the mean ± S.D. of three independent experiments.

gest that the greater hydrophobicity of PLA with a high molecular weight induced more rapid solidification.

Fig. 2A shows the solubility of BP in aqueous solutions of zinc chloride with different pH values obtained by addition of DEA. At a pH between 5.3 and 6.5, the residual BP content of the supernatant after centrifugation was very low. On the other hand, no precipitate of BP was formed in the absence of zinc at any pH value (data not shown). These results indicated that the formation of a water-insoluble complex between BP and zinc in the pH range. At lower and higher pH values, most BP remained in the supernatant and did not form complexes, probably due to protonation of a phosphate group on BP and neutralization of the positive charge of zinc, respectively. Formation of complexes was also investigated by changing the zinc content at a constant pH (pH 6 adjusted by DEA) (Fig. 2B). BP formed a complex with an equimolar amount of zinc in aqueous solution. Addition of zinc enhanced the solubilization of BP in acetone, while BP alone hardly dissolved in acetone, indicating that the complex with zinc was even formed in acetone. Furthermore, a two-fold molar excess of zinc was required to solubilize BP completely in acetone, while equimolar zinc formed a water-insoluble complex with BP in aqueous solution, suggesting that complexes with different compositions were formed in aqueous solution and acetone.

Formation of water-insoluble complexes of BP was expected to lead to efficient incorporation in solid particles as shown in Fig. 1, so

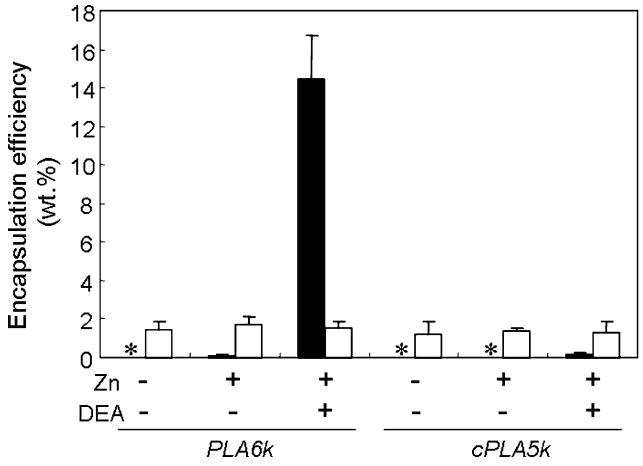

Fig. 3. Effect of zinc and DEA on encapsulation of drugs in nanoparticles. Nanoparticles were prepared with 42.8 mg of PLA (*PLA6k* or *cPLA5k*), 7.2 mg of PEG–PLA, and 10 mg of BP (*filled bars*) or BDP (*open bars*) in the presence or absence of zinc and DEA. Zinc chloride and DEA were added to the acetone solution of polymers and drug at concentrations of 45 mM and 63 mM, respectively. The concentration of BP in the acetone solution corresponds to 13 mM. Each data point represents the mean ± S.D. of three independent experiments. Asterisk indicates that no drug was detected.

nanoparticles were prepared from PLA, PEG–PLA block copolymer, and BP in the presence of zinc and DEA. Because the results shown in Fig. 2 indicated that the pH affected the formation of complexes between BP and zinc, various types of nanoparticles were prepared by altering the ratios of the additives in order to optimize the conditions for efficient encapsulation and clarify the mechanism involved. Initially, encapsulation of BP (or BDP) by nanoparticles prepared in the presence or absence of zinc and DEA was examined (Fig. 3). In the absence of both zinc and DEA, BP was not incorporated into *PLA6k* nanoparticles, probably due to its rapid diffusion out of the organic phase into the aqueous phase during the process of nanoparticle formation. Addition of zinc alone could not enhance the incorporation of BP. On the other hand, a marked increase in the encapsulation of BP by *PLA6k* nanoparticles was observed in the presence of both zinc and DEA, and encapsulation was dependent on the amount of DEA added (Fig. 4). Addition of more DEA led to a marked increase in the encapsulation of BP as well as the formation of complexes between BP and zinc (Fig. 2A). This suggested that BP was incorporated in the nanoparticles by

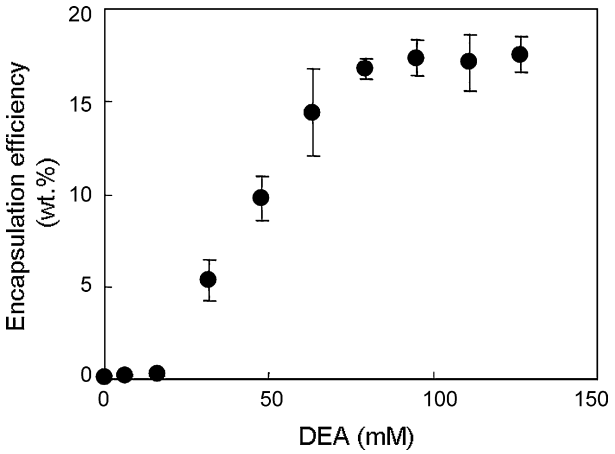

Fig. 4. Effect of DEA on encapsulation of BP in nanoparticles. Nanoparticles were prepared with 42.8 mg of *PLA6k*, 7.2 mg of PEG–PLA, and 10 mg of BP in the presence of zinc and DEA. The concentration of zinc chloride in acetone was fixed at 45 mM. Each data point represents the mean ± S.D. of three independent experiments.

the formation of complexes with zinc along with neutralization of the acidic environment derived from zinc chloride by the buffering effect of DEA. However, maximum encapsulation of BP was observed at over 80 mM DEA (Fig. 4), while the zinc complexes formed in the range from 23 to 77 mM DEA (Fig. 2A). This difference may have occurred because excess DEA is necessary to neutralize the acidic environment induced by PLA with a terminal carboxyl group when incorporating BP into nanoparticles. In the case of *cPLA5k*, which had almost the same molecular weight as *PLA6k* and an octanol-capped end, BP was hardly encapsulated by the nanoparticles even in the presence of zinc and DEA (Fig. 3). In addition, BDP was encapsulated by both *PLA6k* and *cPLA5k* nanoparticles independently of the presence of zinc and DEA. In a previous report, we proposed that a hydrophobic interaction between zinc-BP complexes and PLA/PLGA enhanced the encapsulation of BP (Ishihara et al., 2005). However, the results of the present study indicated that a hydrophobic interaction was not a determinant for the encapsulation of BP and suggested a role of carboxyl groups of PLA instead.

Next, the influence of the molecular weight of the polymer on encapsulation was investigated. The encapsulation efficiency of BP decreased along with the molecular weight of PLA/PLGA (Fig. 5A). On the other hand, little BP was encapsulated by nanoparticles prepared from cPLA of any molecular weight. The content of carboxyl groups in the polymers composing each type of nanoparticle was proportional to the amount of BP encapsulated (Fig. 5B), indicating that the carboxyl group was a determinant of BP encapsulation. The amount of zinc encapsulated in the nanoparticles also increased along with an increase of BP encapsulation (Fig. 5C). The molar ratio among BP, zinc, and carboxyl groups in each type of PLA/PLGA nanoparticle was almost constant and was calculated as 1:1.6 (±S.D. 0.14):1.4 (±S.D. 0.14). Also, the ratio of positive charges derived from zinc (Zn^{2+}) to negative charges derived from BP (PO_4^{2-}) and carboxyl groups (COO^-) was calculated as 0.94. These results strongly suggested that zinc played a role as an ionic bridge between a phosphate group of BP and a carboxyl group of the polymer, resulting in the neutralization of net charges in the nanoparticle (Fig. 6). The composition of the complex formed among BP, zinc, and PLA/PLGA in the nanoparticles is still unclear. However, the relative molar ratio of each component (BP:zinc:PLA/PLGA = 1:1.6:1.4) in the nanoparticles suggested that complexes with different compositions were formed. A mixture of an equimolar amount of complexes formed at molar ratios of 1:2:2 and 2:3:2 may be the most likely candidate, because the mixture is estimated to be formed at a molar ratio of 1:1.67:1.33 and neutral.

The advantages of this technique for encapsulating water-soluble drugs also include the simple purification of nanoparticles, in addition to enhanced drug entrapment. During the process of manufacturing nanoparticles, it is necessary to purify drug-encapsulated nanoparticles from a mixture containing free (unencapsulated) drug. In the case of hydrophobic drugs, nanoparticles are purified using methods such as membrane/gel filtration, centrifugation, ultrafiltration, and dialysis. Depending on the drug, however, these methods may be insufficient for purification because some hydrophobic drugs form self-assembled colloids in aqueous media and adsorb to the nanoparticles (Zweers et al., 2006). Consequently, entrapment efficiency may be overestimated and burst drug release (detachment) may actually occur when such nanoparticles are administered *in vivo*. In the present study, SDS was used as a solubilizer to dissolve free hydrophobic drug and dissociate any adsorbed drug, and the nanoparticles were pelleted by high-speed centrifugation for purification and subsequent determination of the drug content. It was confirmed that all drugs used in this study dissolved in the aqueous solution of SDS, and no drug precipitate was obtained after centrifugation. Although this method

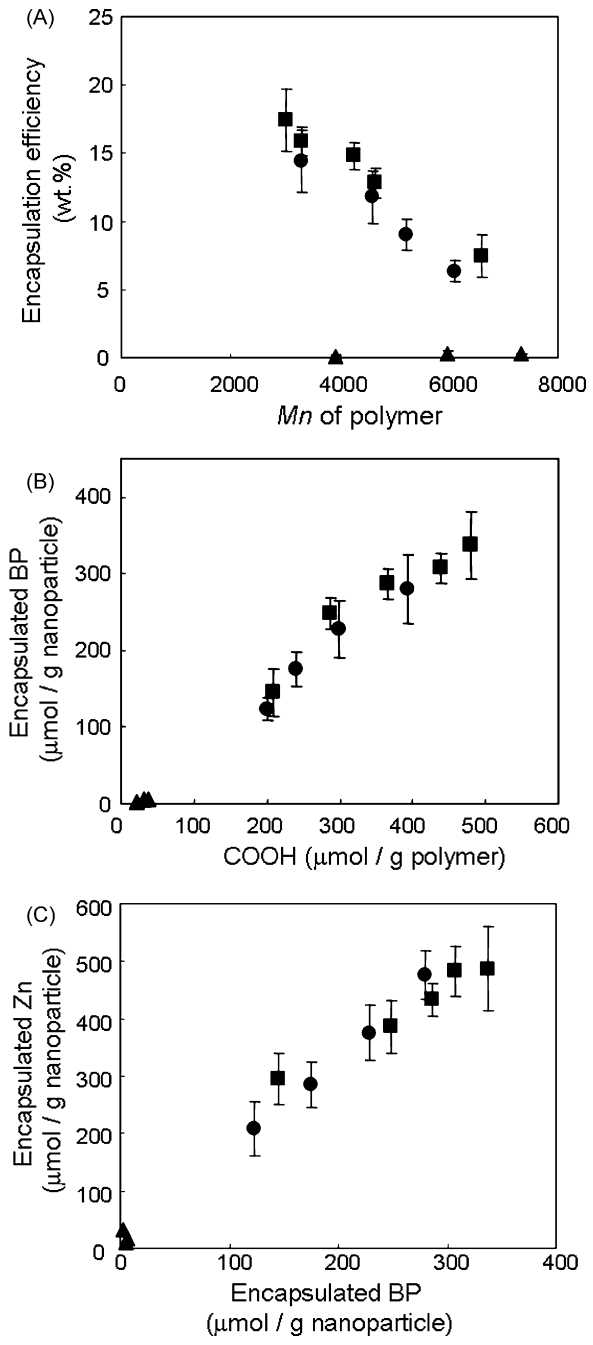

Fig. 5. Encapsulation of BP in nanoparticles prepared from various polymers. (A) Effect of the molecular weight (Mn) of the polymers on encapsulation of BP in nanoparticles. The nanoparticles were prepared with 42.8 mg of the various polymers (*square*; PLA, *circle*; PLGA, *triangle*; cPLA) shown in Table 1, 7.2 mg of PEG–PLA, and 10 mg of BP in the presence of 45 mM zinc and 63 mM DEA. (B) Relation of the carboxyl group content of the polymer to the BP content of nanoparticles. (C) Relation of the BP content to the zinc content of nanoparticles. Each data point represents the mean ± S.D. of three independent experiments.

was useful for determination of the amount of encapsulated drug, it was not adequate for recovery of purified nanoparticles because the particles in the pellet could not be redispersed in aqueous media due to denaturation by the strong centrifugal force. With small nanoparticles <100 nm in size, it is difficult to separate the particles from any surfactants that are used because these generally form micelles in aqueous media. On the other hand, it is not necessary to use a surfactant for purification with the present technique

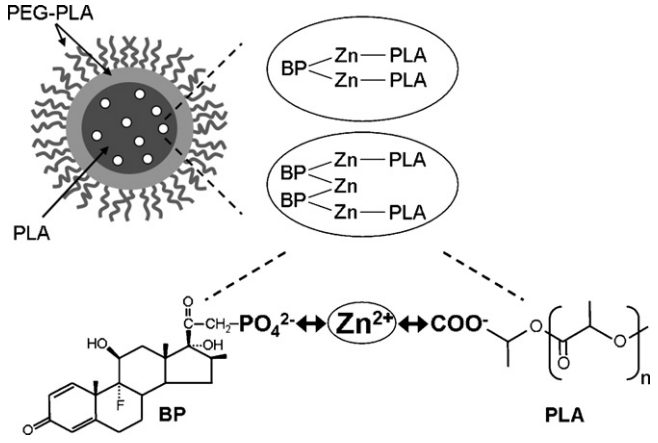

Fig. 6. Diagram of a PLA nanoparticle encapsulating BP.

for encapsulation of water-soluble drugs. Unencapsulated zinc-BP complexes readily released BP after the addition of an excess of zinc chelating agent while zinc-BP complexes encapsulated in the nanoparticles were unaffected. The amount of BP encapsulated in nanoparticles treated with citric acid and SDS was almost equal, indicating sufficient purification of the particles by the chelating agent. Purified nanoparticles could be successfully recovered by addition of citric acid and ultrafiltration, even when the particles were prepared on a large scale (data not shown).

The present technique could also be applied for other drugs, in addition to BP. Our recent study showed that prostaglandin E_1, which has a carboxyl group, is less hydrophobic, and shows diverse and strong biologic activities, could be efficiently encapsulated inside nanoparticles in the presence of iron and DEA by using a similar method (Ishihara et al., 2008). Thus, this technique may be applied to other water-soluble drugs, although it is limited to those with a functional group such as a phosphate group or a carboxyl group.

Our previous study showed that BP was stably retained in PLA/PLGA nanoparticles without any burst release in diluted serum, and was only released gradually from the particles (Ishihara et al., 2005). Furthermore, prostaglandin E_1-containing nanoparticles prepared from a mixture of PLA and PEG–PLA showed a prolonged circulation time *in vivo*. Thus, the BP-encapsulated nanoparticles prepared in the present study are expected to have potential for use as a novel anti-inflammatory agent with a long duration of action on sites of inflammation *in vivo*.

4. Conclusions

Nanoparticles were prepared from PLA, PEG–PLA block copolymer, and BP in the presence of zinc and DEA using the oil-in-water solvent diffusion method. BP could be more efficiently encapsulated in the nanoparticles compared with hydrophobic betamethasone derivatives. A water-insoluble complex was formed between zinc and BP at a specified pH adjusted by addition of DEA. The carboxyl group content of PLA/PLGA was also found to be a determinant of the encapsulation of BP in the nanoparticles. Zinc played a role as an ionic bridge between the carboxyl group of PLA/PLGA and the phosphate group of BP, resulting in a high level of encapsulation of BP in the nanoparticles. This technique for encapsulation of water-soluble drugs in solid biodegradable nanoparticles may open up various opportunities for new clinical applications.

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