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Preparation and characterization of a nanoparticulate formulation composed of PEG-PLA and PLA as anti-inflammatory agents

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ABSTRACT

We have prepared polymeric nanoparticles using a blend of poly(lactic acid) and monomethoxypolyethyleneglycol(PEG)-polylactide block copolymer along with betamethasone disodium phosphate (BP). Nanoparticles have been screened for anti-inflammatory activity using experimental rat models of inflammation. In the present study, we examined the degradation of nanoparticles in vitro during incubation. We found that the nanoparticles lost the PEG chains present on their surfaces within a few days, and subsequently gradually released BP. Furthermore, we found that these nanoparticles preferentially accumulated in the inflammatory lesion in adjuvant arthritis rat models, and that the amount of BP gradually depleted from the lesion over 14 days. These results suggested the mechanism underlying the anti-inflammatory effect of the nanoparticles in vivo: the initial accumulation of BP in the lesion due to the enhanced permeability and retention effect, the subsequent internalization in inflammatory macrophages due to the loss of PEG, and the release of BP in cells during the hydrolysis of polymers. The nanoparticles were successfully prepared on a large-scale and stably stored in the form of a freeze-dried formulation for at least 69 weeks below 25 °C. These results suggest that the nanoparticles can be used as an anti-inflammatory pharmaceutical formulation in a clinical setting.

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1. Introduction

Great efforts have been made to develop colloidal carriers such as liposomes, solid nanoparticles, polymeric micelles, and lipid emulsions so that they can be used as pharmaceutical agents for expanding the utility of drugs in a clinical setting (Yih and Al-Fandi, 2006; Peer et al., 2007). Since the last 2 decades, studies have particularly focused on the stealth-type carriers (Moghimi et al., 2001). Polyethyleneglycol (PEG) has been widely used for modifying the surface of the stealth-type carriers because the PEG chains on the surfaces of these carriers act as a steric barrier and reduce interaction with opsonins and cells of the mononuclear phagocyte system; as a result, the carriers remain in the blood circulation for a prolonged duration. These stealth-type (long-circulating) carriers show preferential accumulation in tumors and sites of inflammation because of the enhanced permeability and retention (EPR) effect (Maeda et al., 2000).

In our previous report, we described the preparation of polymeric nanoparticles from a blend of poly(D,L-lactic acid-co-glycolic acid)/poly(D,L-lactic acid) (PLGA/PLA) homopolymers and PEG-poly(D,L-lactide-co-glycolide)/poly(D,L-lactide) (PEG-PLGA/PLA) block copolymers (Ishihara et al., 2009a,b). Although the efficient encapsulation of drugs in these solid nanoparticles remains a challenging task, water-soluble betamethasone disodium 21-phosphate (BP) could be efficiently encapsulated in the nanoparticles by a unique technique involving the use of zinc ions (Ishihara et al., 2009a). Furthermore, by controlling the blend ratio and the compositions or molecular weights of the polymers, various types of nanoparticles with different drug loading capacities and diameters and PEG chains of different lengths or densities at the surfaces of the nanoparticles could be easily prepared (Ishihara et al., 2009b). It has been found that these parameters significantly influence the stealthiness of the nanoparticles and the release of BP in vitro. It is assumed that the therapeutic efficiency of these nanoparticles in vivo is influenced by multiple complex factors such as the systemic distribution, local distribution, and release behavior of BP. Therefore, nanoparticles with the highest anti-inflammatory activity were finally screened using experimental rat models of inflammation (Ishihara et al., 2009c). The nanoparticles (diameter, approximately 120 nm) that were composed of the PLA (Mw: 6170) homopolymer and a block copolymer of PEG (Mw: 5580) and PLA (Mw: 9430) (PEG content in the polymer blend: 10 wt.%) exhibited the highest anti-inflammatory activity.

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In this study, we examined the release profiles of each component of the nanoparticles in vitro and the residence period of the nanoparticles in the inflammatory lesion in vivo. It is necessary to expand the scale of preparation and standardize the protocol for the industrial manufacture of these nanoparticles for the use of these nanoparticles as pharmaceutical agents. Hence, we prepared the nanoparticles on a large-scale and the ingredients in the resulting formulation were evaluated. In addition, we assessed the long-term stability of freeze-dried nanoparticles during storage at various temperatures.

2. Materials and methods

2.1. Materials

PLA was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The molecular weight of the polymer was determined by gel permeation chromatography as reported before (Ishihara et al., 2009a). PLA with an average Mw of 6170 was used in this study. PEG-PLA was synthesized by ring-opening polymerization of D,L-lactide (Purac America, IL) in the presence of monomethoxy-PEG (Mw: 5580; NOF Co., Tokyo, Japan) (Riley et al., 2001). The composition and molecular weight of the block copolymers were evaluated by ^{1}H NMR and gel permeation chromatography (Ishihara et al., 2009a). PEG-PLA with an average Mw of 15,010 was used in this study. PEG-PLA with a terminal carboxyl group was similarly synthesized using alpha-hydroxy-omega-carboxy PEG (OH-PEG-COOH, Mw: 3640) (Laysan Bio, Arab, AL). The resulting COOH-PEG-PLA was labeled with 4-(aminomethyl)fluorescein (Wako) by condensation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 4-(dimethylamino)-pyridine in DMSO. The average molecular weight of resulting fluorescein-PEG-PLA was calculated as 15,700 (Mw) by gel permeation chromatography. Spectral analysis showed that the polymer contained 39 nmol of fluorescein in 1 mg. BP and diethanolamine (DEA) were purchased from Sigma–Aldrich (St. Louis, MO). Zinc chloride and polysorbate 80 (Tween 80) were purchased from Wako. A time-resolved fluoroimmunoassay (TR-FIA) kit for betamethasone was supplied by Shionogi & Co., Ltd. (Osaka, Japan). Cy7-dodecylamine conjugate was synthesized by mixing Cy7 mono-N-hydroxy succinimide ester (GE Healthcare, Chalfont St. Giles, UK), dodecylamine and 4-(dimethylamino)-pyridine in DMSO and purified by HPLC (Ishihara et al., 2008, 2009c).

2.2. Preparation of nanoparticles

In this study, nanoparticles with the highest anti-inflammatory activity were prepared by the oil-in-water solvent diffusion method as reported previously (Ishihara et al., 2009b,c). In brief, a mixture of 7.8 mg PEG-PLA and 42.2 mg PLA was dissolved in 1 ml of acetone. To this solution, 500 μl of an acetone solution of DEA (15 mg/ml), followed by 68 μl of an aqueous solution of zinc chloride (1 M; pH 1.9), and then 28 μl of an aqueous solution of BP (350 mg/ml) were added; the mixture was then allowed to stand for 30 min at room temperature. To 25 ml of distilled water stirred at 1000 rpm, the mixture was added dropwise at the rate of 48 ml/h using a 26G needle. A combination of 1 ml of 0.5 M citrate (pH 7.0) aqueous solution and 125 μl of 200 mg/ml polysorbate 80 aqueous solution was immediately added. The nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50, Millipore, Bedford, MA). Finally, the nanoparticles were sterilized by filtration through a 0.2-μm re-generated cellulose membrane (Minisart RC, Sartorius AG, Goettingen, Germany). Nanoparticles with dyes (fluorescein and Cy7) were similarly prepared by mixing 1 mg of fluorescein-PEG-PLA and Cy7-dodecylamine conjugate, respectively (Ishihara et al., 2009c).

The preparation of the nanoparticles was scaled up 800-fold in a similar manner. The mixture of 6.2 g PEG-PLA, 33.8 g PLA and 6 g DEA was dissolved in 1200 ml of acetone. To this acetone solution, 53 ml of 1 M zinc chloride aqueous solution (pH 1.9), and BP aqueous solution (6 g in 17 ml water) were added in order. After 30 min, the solution was added to 20 l of distilled water stirred at 1000 rpm with magnetic stirrer, at the rate of 40 l/h through a glass tube with 6.4 mm internal diameter. For the large-scale production of nanoparticles, the rate of acetone addition and the rate of water stirring were optimized, as mentioned above. Next, 1 l of 0.4% polysorbate 80 in 200 mM citrate buffer (pH 7.0) was added to the resulting nanoparticles suspension. The nanoparticles were purified from unencapsulated BP using polyethersulfone ultrafiltration slice cassettes (MWCO: 300 kDa, Sartorius) and then concentrated. The concentrated suspension (700 ml) was sterilized by filtration through a 0.2-μm filter. Finally, the nanoparticle suspension and the aqueous sucrose solution (270 mg/ml) were mixed to attain a final sucrose concentration of 90 mg/ml. The suspension in each vial was frozen at −50 °C for 4 h on the shelf and freeze-dried through primary step (shelf temperature −20 °C, chamber pressure 25 mT, 50 h) and following secondary step (shelf temperature 20 °C, chamber pressure 0 mT, 10 h) using Dura-Top™ and Dura-Stop™ freeze-dryer system (FTS Systems, Inc., Stone Ridge, NY). The vials were plugged with nitrogen under reduced pressure and stored under various temperatures (4, 25, and 37 °C). The recovery efficiency and the loading efficiency were calculated as follows.

$$\text{Recovery efficiency}\,(\%) = \frac{\text{total amount in obtained nanoparticles}}{\text{amount of feed}}$$

$$\text{Loading efficiency}\,(\text{wt.\%}) = \frac{\text{amount of BP in nanoparticles}}{\text{amount of PLA in nanoparticles}}$$

2.3. Ingredients of a formulation

Seven milliliters of water was added to a vial after lyophilization. The resulting suspension was agitated, after which the volume of the suspension was exactly adjusted at 10 ml by addition of water using a messflask. This suspension was used for each analysis as follows.

2.3.1. BP

The nanoparticles suspension (200 μl) was added to 600 μl of acetonitrile and the solution was agitated to dissolve PLA and PEG-PLA completely. After the addition of 1200 μl of EDTA aqueous solution (50 mM, pH 7.0) to chelate zinc, the BP content in the solution was determined by HPLC (Ishihara et al., 2009a). The content of unencapsulated BP in the suspension was also determined as follows. The nanoparticles suspension was mixed with equal volume of EDTA aqueous solution (50 mM, pH 7.0). After centrifugation of the solution in the filter cap of Ultrafree-MC centrifugal filter unit (Ultrafree-MC with PL-30 membrane, Millipore) at 5000 × g for 30 min, the BP content in the filtrate was determined by HPLC. The residual BP in the nanoparticles was calculated as the difference between total BP and unencapsulated BP.

2.3.2. Acetone and metals

The nanoparticles suspension (1 ml) was added to 1 ml of 6 M sodium hydroxide aqueous solution. After incubation for 3 h at room temperature, 1 ml of 8 M hydrochloric acid aqueous solution was added on ice bath. This acidic solution was used for gas chromatography analysis using GC-2014 (Shimadzu Co., Kyoto, Japan) and Rtx-624 column to determine the acetone content in a vial. The acidic solution was also used for the determination of metals. The zinc and stannous content of the solution was evaluated by Induc-

tively Coupled Plasma Atomic Emission Spectroscopy (ICPS-8000, Shimadzu) (Wendt and Fassel, 1965).

2.3.3. DEA

The nanoparticles suspension (200 μl) was added to 600 μl of acetonitrile. After the addition of 1200 μl of water, the solution was centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was diluted with 2-fold volume of water, after which DEA in the solution was determined by ion chromatography using TSKgel SuperIC-Cation Column (Tosoh Co., Tokyo, Japan).

2.3.4. Citric acid

The nanoparticles suspension (1000 μl) was centrifuged at 39,000 × *g* for 30 min at 4 °C. The supernatant was diluted with 99-fold volume of water, after which citric acid in the solution was determined by ion chromatography using TSKgel OApak-A column (Tosoh).

2.3.5. Sucrose

The nanoparticles suspension (100 μl) in the filter cap of Ultrafree-MC centrifugal filter unit was centrifuged at 5000 × *g* for 30 min at 4 °C. The filtrate was diluted with 2-fold volume of water and then with 9-fold volume of acetonitrile. The mixture was analyzed by HPLC using Asahipak NH2P-50 4E column (Showa Denko K.K. Kawasaki, Japan) and a RI detector, with water/acetonitrile (25/75 v/v) as the mobile phase.

2.3.6. Lactic acid

The nanoparticles suspension was mixed with an equal volume of 4 M sodium hydroxide aqueous solution. After incubation of the suspension for 18 h at 50 °C, 9-fold volume of 340 mM phosphoric acid aqueous solution was added. The content of lactic acid was determined by HPLC using Inertsil ODS-2 column (GL Sciences Inc., Tokyo, Japan) and an UV/vis detector, with 20 mM phosphoric acid aqueous solution as the mobile phase.

2.3.7. Polysorbate 80

In the case of polysorbate 80, 3 ml of methanol-d_4 instead of water was added to a vial. The suspension was allowed at −30 °C for 1 h and then centrifuged at 20,000 × *g* for 3 min at 0 °C. The resulting supernatant was incubated at 50 °C for 2 h and then centrifuged at 20,000 × *g* for 3 min at room temperature. The supernatant was diluted with equal volume of methanol-d_4 containing of 2 mg/ml benzene, after which the solution was analyzed by ^{1}H NMR using JNM-ECP 600 (Jeol Datum Ltd., Tokyo, Japan). Based on the peak area of an internal standard (benzene), the concentration of polysorbate 80 was calculated from peak area in 0.9 ppm derived from oleate.

2.4. Size of nanoparticles

The particle size was determined by the dynamic light scatter method (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). The nanoparticles suspension was diluted with 4-fold volume of water, after which the size was measured. Also, NIST traceable particle size standard (STADEX 100 nm, 123 nm, 144 nm, JSR Co., Tokyo, Japan) was measured. The size of the nanoparticles was adjusted based on those of the standard particles.

2.5. Release behavior of component of nanoparticles

The nanoparticles with fluorescein were dispersed in the phosphate-buffered saline (PBS) at a BP concentration of 50 μg/ml. After incubation at 37 °C at specified times, the suspension (100 μl) was centrifuged at 39,000 × *g* for 30 min. The precipitate was washed with water by centrifugation and then freeze-dried. The content of lactic acid and BP in the precipitate was determined as reported (Ishihara et al., 2008, 2009a). After dissolution of the dried precipitate in DMF, the fluorescein content was fluorometrically determined using a fluorescence detector (RF-5300PC, Shimadzu) at excitation wavelength 525 nm and emission wavelength 546 nm.

2.6. Animal experiments

Accumulation of the nanoparticles encapsulating fluorescence dye in inflammatory lesion was evaluated by in vivo imaging. Arthritis was induced in Lewis rats (7 weeks old male, weighing 200–250 g, obtained from SLC (Shizuoka, Japan)) by injecting 50 μl of incomplete Freund's adjuvant solution (DIFCO, Detroit, MI) containing 6 mg/ml of *Mycobacterium butyricum* into the subplantar region of the left hind paw (Ishihara et al., 2009c). Five hundred microliters of Cy7-dodecylamine aqueous solution (2.6 μg/ml) or the suspension of nanoparticles encapsulating Cy7-dodecylamine conjugate (Cy7-dodecylamine: 2.6 μg/ml) was intravenously administrated in adjuvant arthritis rats on 14 days after administration of the adjuvant. After 1 day, fluorescence images of left hind paw were observed by explore Optix in vivo fluorescence imaging system (GE Healthcare).

The BP content in the left hind paw was also determined. The nanoparticles with BP (40 μg as BP/500 μl of saline) were intravenously administrated in adjuvant arthritis rats on 14 days after administration of the adjuvant. At specified times, the hind paw was amputated and frozen. The paw was incised using a knife and incubated for 18 h at 37 °C in Hanks' balanced salt solution including 0.05% type 1 collagenase, 0.05% elastase, 0.05% trypsin, 16 U/ml dispase, 0.02% hyaluronidase, 1 mM sodium calcium and 1 mM sodium magnesium. After repeated freeze–thawing (three times), 3-fold volume of acetonitrile and 5.5-fold volume of water were added in this order. The suspension was filtrated through 0.45-μm filter, after which the filtrate was dried using a vacuum concentrator. The dried residue was incubated with alkaline phosphatase (2.7 units/ml, from calf intestine; Toyobo, Tokyo, Japan) and the resulting betamethasone was extracted by ethyl acetate. After dry up of ethyl acetate, betamethasone in the residue was determined by TR-FIA immunoassay. The BP content was shown as weight of detected betamethasone per weight of wet amputated paw.

All animal experiments were performed in accordance with the Animal Experiment Guidelines of the Jikei University School of Medicine.

3. Results and discussion

The nanoparticles were prepared using the PEG-PLA block polymer containing fluorescein at one terminal of the PEG chain. The residual content of BP, lactic acid, and fluorescein in the nanoparticles was determined during incubation in PBS at 37 °C. As shown in Fig. 1A, a large amount of fluorescein was rapidly released from the nanoparticles (residual fluorescein at 4 days, 31%), while BP and lactic acid were gradually released. In a previous study, we analyzed the internalization of various nanoparticles in macrophage-like cells (RAW264.7) after preincubation of the nanoparticles (Ishihara et al., 2009b). The internalization of the nanoparticles (same as described in Fig. 1A) accelerated after a 3-day preincubation period. The results of the previous study along with those of the present study indicate that the release of PEG from the surface of the nanoparticles enhances the affinity of nanoparticles for cells. In addition, the extent of internalization did not depend on the length of the PLA/PLGA segments in the block copolymers but on the composition of PLA/PLGA. This suggests that the release of PEG from the nanoparticles was triggered by the hydrolysis of the PLA/PLGA segment and not by the dissociation of the block copolymers (Ishihara et al., 2009b). In this study, fluorescein released from the nanopar-

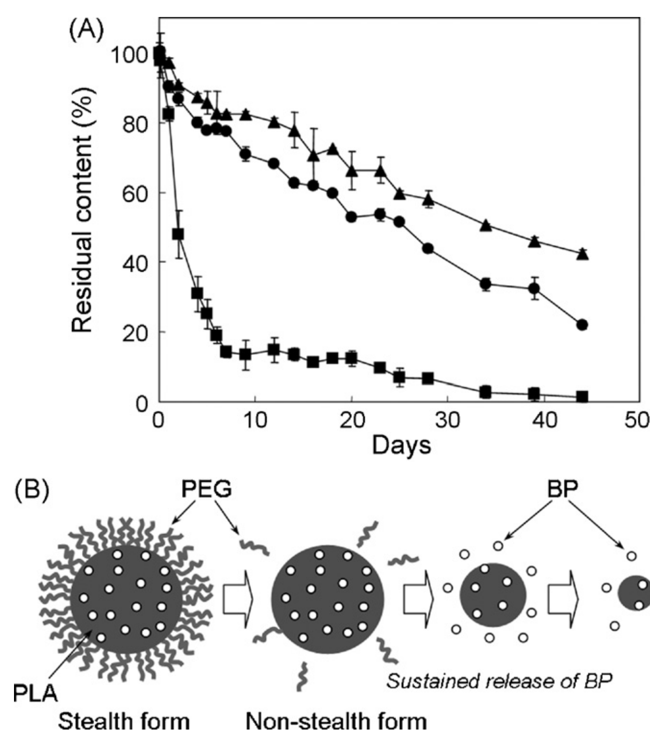

Fig. 1. Degradation of nanoparticles during incubation. (A) Release behavior of each component of the nanoparticles. The amount of BP (circle), lactic acid (triangle), and fluorescein (square) in the nanoparticles was determined during the incubation of nanoparticles in PBS at 37 °C. Each data point represents the mean ± standard deviation (SD) of 3 independent experiments. (B) Schematic illustration of the degradation of nanoparticles during incubation.

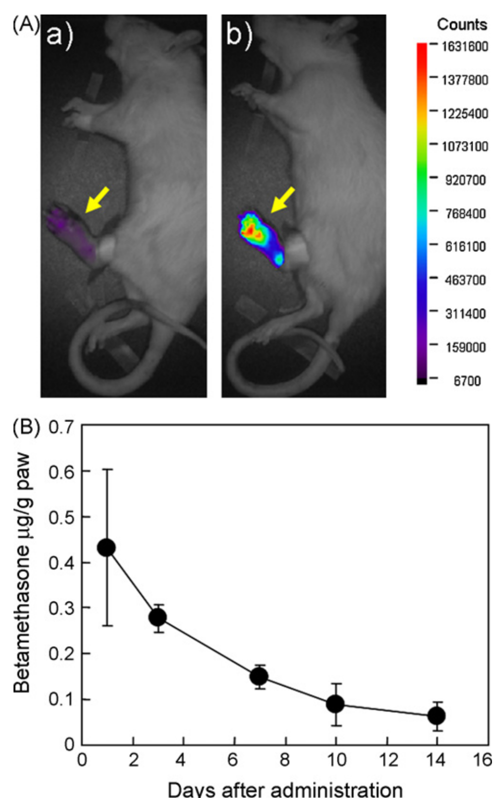

Fig. 2. Accumulation of nanoparticles in the inflammatory lesion in an adjuvant arthritis rat model. (A) Accumulation of (a) Cy7-dodecylamine or (b) Cy7-dodecylamine conjugate-encapsulated nanoparticles on the first day after intravenous administration. The left hind paw of rats was observed using the explore Optix in vivo fluorescence imaging system. The color bar shows the fluorescence intensity (count) of Cy7. (B) The betamethasone content in the inflammatory lesion (left hind paw) was determined as described in Section 2.6 after the administration of the nanoparticles to adjuvant arthritis rat models. Each data point represents the mean ± SD of 3 rats.

ticles into the bulk solution was determined by a fluorometric analysis. In addition, gel permeation chromatography was performed; it revealed that the molecules with fluorescein in the bulk solution had approximately the same molecular weight as a PEG segment, indicating the production of fluorescein-PEG-(lactic acid) [or oligo(lactic acid)] as a result of the hydrolysis of the PLA segment. After alkali-hydrolysis of the nanoparticles, we measured the residual lactic acid content and found that the content gradually decreases during the period of incubation. In addition, the residual BP content corresponded to the residual lactic acid content. This observation suggested that BP is uniformly distributed in the nanoparticles and is released because of surface erosion during PLA hydrolysis (Fig. 1B). The residual content of BP, lactic acid, and fluorescein was measured when the nanoparticles were incubated for 44 days at 4 °C and was found to be 96.1 ± 2.4%, 95.7 ± 1.4%, and 99.9 ± 0.7%, respectively, indicating that the degradation of nanoparticles is greatly inhibited at low temperatures.

A Cy7-dodecylamine conjugate that was used as a labeling material for in vivo imaging was encapsulated in the nanoparticles. Imaging analysis demonstrated preferential accumulation of the nanoparticles in the target lesion (i.e., the left hind paw) (Fig. 2A); this preferential accumulation is probably attributable to the EPR effect. At 1 day after the administration of the nanoparticles, the amount of BP in the lesion was considerably high (Fig. 2B), while after administration of BP alone, the amount of BP was below the detection limit (below 0.04 μg/g tissue). Generally, it is believed that because of the EPR effect, the prolonged residence of carriers in the blood leads to higher accumulation in the lesions. However, higher accumulation does not necessarily correspond to higher therapeutic activity because the local distribution of the carriers in the lesion after circulation remains unknown (Romberg et al., 2008). In a previous study on animals, we found that the nanoparticles with the longest blood half-life did not have the highest anti-inflammatory activity (Ishihara et al., 2009c). The carriers may show stealth properties in the lesion. Such carriers probably have a low affinity for cells and may reenter the blood circulation or a lymph vessel during prolonged residence. On the other hand, depending on the drugs used, it is necessary to determine whether drugs should be released in the intracellular space or the extracellular spaces. In some studies, PEG was formulated such that after its accumulation in the tumor, it would be released from the carriers in response to stimuli such as change in pH and enzymatic reaction, and would thus result in higher therapeutic activity (Mishra et al., 2004; Ambegia et al., 2005; Hatakeyama et al., 2007). Therefore, the stealthiness of the nanoparticles should be controlled in a time-dependent manner to achieve high therapeutic efficiency.

As observed in Fig. 2B, the BP content gradually decreased and was detected even at 14 days after administration. Although we have no apparent evidence to explain this phenomenon, the results obtained in vitro provide a potent hypothesis. The nanoparticles initially possess abundant PEG chains on their surfaces but these are lost within a few days. During this time, most of the BP still remains in the nanoparticles and subsequently is gradually released as shown in Fig. 1A. These results indicated that the accumulation of nanoparticles in the lesion (inflamed paw) was due to the EPR effect and that the internalization of the nanoparticles in inflammatory macrophages was induced by the loss of PEG from the surfaces of the nanoparticles. Thereafter, BP may be gradually released in the cells during PLA hydrolysis. However, the period of residence (approximately 14 days) of BP in the lesion was shorter than that of the release of BP from the nanoparticles in vitro (over 44 days)

Table 1
Ingredients of the freeze-dried formulation.

	Content/vial
BP (mg)	5.6
Betamethasone (μg)	5.6
Acetone (μg)	340
Zinc (μg)	1130
Stannum (μg)	3.0
Diethanolamine (μg)	190
Citric acid (μg)	440
Polysorbate 80 (mg)	3.7
Sucrose (mg)	430
PLA (mg)	40.6

as shown in Figs. 1A and 2A. In a previous report, we showed that during in vitro incubation of cells for 8 days, approximately 80% of the BP in nanoparticles internalized in cells was released from the cells (Ishihara et al., 2005). These results strongly suggested that the release of BP from cells was accelerated because of hydrolysis in an acidic environment such as in endosomes or lysosomes. The release rates of drugs from carriers also significantly influence therapeutic activity.

Stealth nanoparticles with a maximum therapeutic potential should be designed by taking the following two factors into consideration: (i) the time-controlled distribution of drugs in the body or tissue and (ii) the time-controlled release of drugs. The nanoparticles presently used have two time switches: one controls the stealth property, which is observed during the association or dissociation of PEG, and another controls the period of BP release. This simple technique based on the hydrolysis of biodegradable polymers will greatly contribute to the design and optimization of nanoparticles for various applications in a clinical setting.

Next, we attempted to execute the preparation of the nanoparticles on a large-scale in order to evaluate manufacture on an industrial scale. Nanoparticles with the same properties as those that are prepared on a small-scale could be successfully prepared on an 800-fold scale. They were sterilized by filtration using a 0.2-μm membrane filter and freeze-dried in the presence of sucrose. The nanoparticles in the freeze-dried formulation could be uniformly dispersed in water and had the same diameter as nanoparticles that were not subjected to freeze-drying because the sucrose acted as a lyoprotectant and prevented the aggregation of nanoparticles (Ishihara et al., 2005). In this manufacturing process, the recovery efficiency of BP and PLA (i.e., the total amount of PLA in the mixture of PEG-PLA and PLA) was 24.1 wt.% and 26.3 wt.%, respectively. In order to reduce the cost, we need to increase the recovery efficiency of the nanoparticles, because a large amount of the cost depends on that of PEG-PLA and PLA. The loading efficiency of BP in the nanoparticles was 13.8 wt.%. Although it would be ideal to increase the loading efficiency and thus decrease the amount of additives, higher loading may affect the properties of the nanoparticles. In principle, it may be easy to achieve further scale-up because homogenizers or emulsifiers are not used in this method.

The additives used in the manufacture of nanoparticulate formulations seem to be acceptable for clinical use. A number of Food and Drug Administration (FDA)-approved products available in the market contain PLA and PLGA for use as excipients to achieve sustained release of the bioactive molecules (Chaubal, 2002). In addition, block polymers containing PEG and PLA/PLGA also seem to be safe for use because many animal experiments have shown that these polymers are biocompatible and have low immunogenicity and little toxicity (Shive and Anderson, 1997; Plard and Bazile, 1999). Some ongoing clinical trials will, in the near future, clarify the safety of these polymers. In addition to BP, extremely small amounts of betamethasone were also detected by HPLC in the freeze-dried nanoparticles (Table 1). This may be explained by the possible production of betamethasone because of the hydrolysis of BP, which occurred during the process of nanoparticle preparation. It is possible to reduce the residual content of acetone to 340 μg per vial by ultrafiltration and freeze-drying (Table 1). According to the Guidelines for Residual Solvents (International Conference on Harmonisation [ICH] Harmonised Tripartite Guidelines), acetone is classified into class 3, and the level of permitted daily exposure (PDE) is limited to <50 mg per day. Hence, the residual content of acetone in the formulation was sufficiently low. Zinc is one of the vital minerals. The level of residual zinc also seems acceptable because zinc chloride solution has been used as an additive in intravenous solutions for total parenteral nutrition (Zinc Chloride Injection, Hospira Inc., Lake Forest, IL). Stannous octoate was used as a catalyst for the polymerization of PLA/PLGA. Since this compound is accepted by the FDA as a food additive (Kim et al., 1992) and the level of stannum in the formulation was extremely low (3 μg per vial, Table 1), the residual stannum content is acceptable as discussed elsewhere (Gunatillake and Adhikari, 2003). Further, other materials used in the formulation that are listed in Table 1 have already been used as additives in a clinical setting.

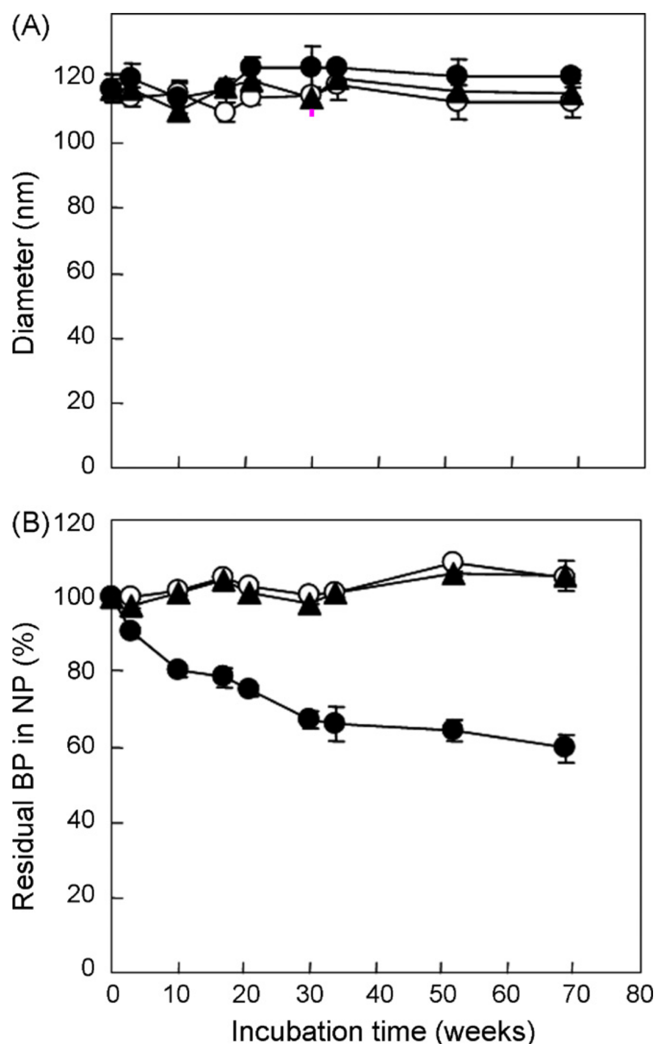

Fig. 3. Storage stability of the freeze-dried nanoparticles at various temperatures (open circle, 4 °C; closed triangle, 25 °C; and closed circle, 37 °C). (A) Diameters of the nanoparticles after resuspension in water. (B) Residual BP content in the nanoparticles. Each data point represents the mean ± SD of 3 independent experiments.

We evaluated the stability of freeze-dried nanoparticles during storage at various temperatures. The diameters of the nanoparticles were constant even after resuspension in water (Fig. 3A) and

no chemical changes in BP were observed on varying the temperature (data not shown). On the other hand, we did not observe leakage (release) of BP from the freeze-dried nanoparticles during 69 weeks of incubation at 4 °C and 25 °C, while BP was released during incubation at 37 °C (Fig. 3B). This temperature-dependent release may be attributable to the thermodynamic stability of the polymers (PLA and PEG-PLA) used in the nanoparticle preparation. In general, the glass transition temperature (T_g) of a polymer is the temperature at which the polymer changes from the glassy state to the rubbery state. In the rubbery state, the diffusion of the drug from the nanoparticles is easier because of the high mobility of the polymer chains (Wischke and Schwendeman, 2008). On the basis of this finding, we can conclude that this formulation can be stably stored at low temperatures (below 25 °C) for at least 69 weeks.

4. Conclusions

Polymeric nanoparticles formed using a blend of PLA and PEG-PLA with BP were prepared by an oil-in-water solvent diffusion method. The results of the present and previous studies have strongly suggested the behavior of the nanoparticles in vivo: initial accumulation in the lesion, internalization in inflammatory macrophages, and gradual release of BP in cells. Furthermore, the nanoparticles were successfully prepared on a large-scale, and freeze-dried nanoparticles were stably stored for at least 69 weeks below 25 °C. The present results indicate that this nanoparticulate formulation can be used in a clinical setting.

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