



Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model

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ABSTRACT

Inhaling corticosteroids, such as budesonide (BD), is the most common treatment for asthma. However, frequent steroid administration is associated with many side effects. We hypothesized that porous microparticles containing BD could provide an effective treatment method for asthma, as the sustained delivery of corticosteroid and a reduced number of doses could be achieved using porous polymeric microparticles. Porous microparticles were prepared from poly(lactic-co-glycolic acid) (PLGA) by a water-in-oil-in-water double emulsion method with ammonium bicarbonate as the porogen. Varying the porogen concentration controlled the morphology, particle size, and pore size of the PLGA microparticles, with particle size and pore size increasing as the porogen concentration increased. The BD loading efficiency in the porous PLGA microparticles was about 60%, and BD was released from the porous microparticles in a sustained manner for 24 h *in vitro*. Lung uptake efficiency of the porous PLGA microparticles in mice was significantly higher than that of non-porous PLGA microparticles. Budesonide-loaded porous PLGA microparticles were delivered to asthmatic mice, and the numbers of inflammatory cells in bronchoalveolar lavage (BAL) fluid and tissue sections were significantly reduced when the drug was administered every 3 days. We also found significantly reduced bronchial hyperresponsiveness of asthmatic mice after treatment with budesonide-loaded porous PLGA microparticles. This approach to controlling the porous structure of polymeric microparticles, as well as the release behavior of drugs from the microparticles, could have useful applications in the pulmonary delivery of many therapeutic drugs.

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1. Introduction

Asthma is a chronic airway disease caused by a complex interaction of environmental and genetic factors, which induces airway inflammation to cause wheezing, chest tightness and cough [1]. According to the latest World Health Organization estimates, about 300 million people suffer from asthma, and the prevalence of asthma has increased by 75% since 1994 [2]. The most common treatment method for asthma includes the use of controller medications such as corticosteroids, as they have excellent anti-inflammatory effects and efficiently inhibit a variety of inflammatory responses that evoke airway hyperresponsiveness. Corticosteroids, which can reduce the number of inflammatory cells and protect blood vessels from leaking fluid into the airway tissues, can be administered in three ways, including inhalation via a metered dose inhaler (MDI) or dry powder inhaler, oral administration of a

tablet, and injection of a liquid form [3]. However, despite the excellent therapeutic effects of these treatments, their use has been limited due to systemic side effects such as adrenocortical suppression, Cushing's syndrome and osteoporosis [4,5].

Budesonide (BD) is a glucocorticoid with high anti-inflammatory activity and low systemic effects due to high receptor affinity, airway selectivity, and rapid diversion [6–8]. This drug is a potential candidate for the treatment of asthma, allergic rhinitis, and inflammatory bowel disease through inhalation. In addition, it has been known to prevent lung tumors [9]. Inhaled BD is widely used for the management of asthma with a daily dose of 160–640 µg based on severity. However long-term use, as well as frequent BD administration, may generate many unwanted side effects such as growth suppression, skin thinning, reduced bone mineral density, and cataract [10].

Pulmonary drug delivery is an alternative for the systemic delivery of therapeutic drugs, as it has many advantages over oral delivery and injection [11,12]. For example, when a drug is administered via a pulmonary route, absorption of the drug is very effective due to high solute permeability and the large surface area of the lungs. It has been reported that many drugs such as insulin, calcitonin, lysozyme, and

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deslorelin can be delivered via a pulmonary route [13,14]. Recently, dry powder inhaler (DPI) technology was found to be efficient for pulmonary drug delivery, but it requires a thorough characterization of the drug powder, including the determination of particle size, density, morphology, and surface area [15].

Polymeric micro- or nanoparticles have been frequently used as a pulmonary delivery vehicle for therapeutic drugs due to their many advantages, including the controlled release of encapsulated drugs and easy administration in a minimally invasive manner. However, there are difficulties associated with the use of small microparticles (1–5 μm in diameter) and nanoparticles for effective pulmonary drug delivery because these particles are susceptible to alveolar macrophage removal and mucociliary clearance in the lungs. In contrast, large microparticles (10–20 μm in diameter) with a low density (i.e., high porosity) are suitable for deep lung delivery, as they are protected from alveolar macrophage clearance due to their large size [16]. Poly(D,L-lactide-co-glycolide) (PLGA) has been widely used for fabrication of particulate systems [17–20], including porous microparticles [21–23]. However, few studies have reported the mechanism of porous microparticles containing glucocorticoid as a long acting agent for asthma treatment when delivered via a pulmonary route.

In this study, porous PLGA microparticles containing BD were prepared by a water-in-oil-in-water (w/o/w) double emulsion method using ammonium bicarbonate as the porogen. Characteristics of the porous PLGA microparticles, including particle size, pore size, density, porosity, drug loading efficiency, and *in vitro* release behavior, were investigated. In addition, the therapeutic efficacy of the BD-loaded porous PLGA particles was tested in a murine asthma model.

2. Materials and methods

2.1. Materials

Resomer® RG 504 H (MW 30,000, 0.45–0.60 dl/g, lactide:glycolide = 50:50) was purchased from Boehringer Ingelheim. Budesonide (BD) methacholine, ammonium bicarbonate, poly(vinyl alcohol) (PVA, MW 27,000–32,000), citric acid monohydrate, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Methylene chloride was purchased from J.T. Baker. Phosphate-buffered saline (PBS) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco. 1,1'-Diocadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) was purchased from Invitrogen.

2.2. Preparation of budesonide-loaded porous PLGA microparticles

Budesonide (BD)-loaded porous PLGA microparticles were prepared using a water-in-oil-in-water (w/o/w) double emulsion method [24]. Briefly, PLGA (500 mg) and BD (20 mg) were dissolved in 5 ml methylene chloride. Ammonium bicarbonate solution (1.5% or 3%; 1 ml) was added to the polymer solution, and the mixture was sonicated with a probe-type sonicator (Branson Digital Sonifier®) for 12 s at 70 W. The sonicated mixture was added to 25 ml 4% PVA solution and then homogenized at a rate of 10,000 rpm for one min (Ultra-Turrax® T25 basic, IKA®-Werke). The solution was then poured into a 0.4% PVA solution (400 ml) and stirred for 4 h to remove the organic solvent. The particles were collected by centrifugation at 10,000 rpm for 10 min, washed five times with distilled water, and lyophilized for 4 days. Non-porous PLGA microparticles were also prepared as a control using the same procedure as the porous PLGA microparticles, only without the addition of the ammonium bicarbonate solution.

2.3. Characterization of budesonide-loaded porous PLGA microparticles

The morphology and size of BD-loaded porous PLGA microparticles were examined by scanning electron microscopy (S-4800 UHR

FE-SEM, Hitachi) and Image-Pro Plus Software (Media Cybernetics). A suspension of microparticles in distilled water was mounted onto aluminum holders at room temperature, dried overnight, and then coated with platinum under vacuum. The images were captured and analyzed using Image-Pro Plus Software to calculate the mean diameter and standard deviation of the microparticles. The density and porosity of microparticles were determined by high-pressure mercury intrusion porosimeter (Micromeritics Instrument Co), and used for calculation of the mass median aerodynamic diameter (MMAD) of porous particles [25].

2.4. Drug loading efficiency

To determine the loading content of BD in porous particles, the particles were immersed in a methanol solution for 24 h, followed by sonication using a probe-type sonicator for 20 s at 70 W. The extract was filtered through a 0.45- μm syringe filter (Millipore), and the concentration of BD was determined by high performance liquid chromatography (HPLC) equipped with a Waters 1525 pump, a Waters 2487 dual λ absorbance detector, and a C₁₈ Waters symmetry® column (mobile phase = water/alcohol (57/43, v/v), flow rate = 1 ml/min).

2.5. *In vitro* drug release

A suspension of BD-loaded PLGA microparticles in DPBS was placed into a dialysis bag (molecular weight cut-off: 3500, Spectrum Laboratories) and *in vitro* release behavior of BD from PLGA microparticles in DPBS was monitored at 37 °C [26]. All of the release media were removed and replaced with fresh media, and the BD concentration was determined by HPLC.

2.6. *In vivo* studies

2.6.1. Lung uptake efficiency of porous PLGA microparticles

Female BALB/c mice weighing between 19 and 22 g were anesthetized with an intraperitoneal injection of rompun (6.25 mg/kg) and ketamine (43.75 mg/kg) ($n = 5$). Dil-loaded porous PLGA microparticles were administered to the mouse lung using a Microspayer® Aerosolizer attached to a high-pressure syringe (Penn-Century). To confirm pulmonary deposition, both tissue sections and the whole lung were observed with fluorescence microscopy (Nikon) and an Image Station 4000MM (Kodak), respectively. Non-porous PLGA particles were also used as a control. To quantify the lung uptake efficiency of the Dil-loaded porous PLGA microparticles, the mouse lung was ground into a fine powder and dispersed in DMSO. After 24 h, the fluorescence intensity was measured using a spectrophotometer (SpectraMAX, Molecular Devices). All procedures were in compliance with the Hanyang University Guidelines for the care and use of laboratory animals.

2.6.2. Murine asthma model

Female BALB/c mice weighing between 19 and 22 g were used for the induction of asthma ($n = 10$). To induce asthma, each mouse was sensitized intraperitoneally with 75 μg ovalbumin (OVA, Sigma) and 2 mg aluminum hydroxide (Sigma) dissolved in 200 μl PBS on days 0 and 7, as this model is widely used and well-characterized with features similar to those in human allergic asthma [27]. The mice were challenged with BD solution, BD-loaded non-porous PLGA microparticles, or BD-loaded porous PLGA microparticles using a Microspayer® Aerosolizer every 3 days (total amount of BD = 0.24 mg/kg). In addition, 50 μg ovalbumin per mouse was administered intranasally on days 13 to 22 in order to maintain sensitivity. On day 24, the mice were sacrificed, an incision was made at the level of the esophagus, and the lung lobes were isolated along with the trachea. All procedures were in compliance with the Hanyang University Guidelines for the care and use of laboratory animals.

2.6.3. Isolation of bronchoalveolar lavage fluid

To obtain bronchoalveolar lavage (BAL) fluid, 1 ml PBS was perfused through the trachea using a 24-gauge catheter, and BAL fluid was collected. The lungs were then reperfed with the recovered fluid. To obtain inflammatory cells, the fluid was centrifuged at 3,000 rpm for 10 min. The number of total inflammatory cells was counted using a hemacytometer, and specific inflammatory cells were also quantified using a cytospin (Thermo Shandon). The number of macrophages (Mac), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym) was counted under optical microscopy (Olympus).

2.6.4. Lung histology

After recovery of BAL fluid, the lungs were inflated with 3 ml PBS. Lung tissue was excised from the whole lung, fixed with 10% formalin, and embedded in paraffin. Serial paraffin sections (3 μ m) were cut and stained with hematoxylin and eosin (H&E). The captured images were analyzed using Image J software (NIH) to determine thickness of the airway walls.

2.6.5. Methacholine bronchial provocation test (MBPT)

The airway hyperresponsiveness (AHR) was assessed using non-invasive whole-body plethysmography (OCP-3000, Allmedicus). A spontaneously breathing mouse was placed into the main chamber of a plethysmograph, and pressure differences between main chamber and reference chamber were recorded. Airway reactivity was expressed as enhanced pause (Penh), which is linearly correlated with airway resistance, impedance, and intrapleural pressure, and is derived from the following equation:

$$\text{Penh} = \text{PEF} / \text{PIF} \times (\text{Te} - 1)$$

where PEF is the peak expiratory flow, PIF is the peak inspiratory flow, and Te is the expiration time. Mice were exposed to nebulized PBS for 3 min to establish baseline values, and this was followed by increasing nebulized methacholine concentrations (6.25–50 mg/ml). After each nebulization, readings were taken and averaged for 3 min.

2.7. Statistical analysis

All data are presented as the mean $\pm$ standard deviation. Statistical analysis was performed using a Student's *t*-test. Values of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 were considered statistically significant.

3. Results and discussion

3.1. Characteristics of budesonide-loaded porous PLGA microparticles

Budesonide-loaded porous PLGA microparticles were prepared using a water-in-oil-in-water (w/o/w) double emulsion method with ammonium bicarbonate as a gas foaming agent. We first investigated how the morphology, particle size, and pore size of BD-loaded porous PLGA microparticles were regulated by varying the ammonium bicarbonate concentration (Table 1 and Fig. 1). Both non-porous and porous PLGA microparticles were spherical in shape. The mean diameter of the non-porous PLGA microparticles was $6.4 \pm 2.3 \mu\text{m}$. The porous PLGA microparticles prepared with 1.5% and 3% ammonium bicarbonate were $8.2 \pm 1.5 \mu\text{m}$ and $9.2 \pm 2.5 \mu\text{m}$, respectively. The pore size and mean diameter of the PLGA microparticles increased with an increase in an ammonium bicarbonate concentration. The particle size is critical for lung deposition as nanoparticles and small microparticles (e.g., 1–5 μm in diameter) can be rapidly cleared by lung macrophages [28]. For this reason, porous microparticles with an approximate 10 μm diameter were chosen and used in this study. The pore size of PLGA microparticles increased with an increase in ammonium bicarbonate concentration; however, the use

Table 1

Characteristics of budesonide-loaded porous PLGA microparticles (*n* = 3).

Sample code	PM0	PM1	PM2
Porogen concentration (% w/v)	0	1.5	3.0
Particle size (μm)	6.4 ± 2.3	8.2 ± 1.5	9.2 ± 2.5
Pore size (μm)	– ^a	0.7 ± 0.3	1.5 ± 0.4
Density (g/ml)	0.98	0.12	0.07
Porosity (%)	– ^a	96.3	99.6
MMAD ^b (μm)	6.4 ± 2.3	2.8 ± 0.5	2.5 ± 0.7
Budesonide loading efficiency (%)	67.0 ± 0.1	59.0 ± 0.1	56.0 ± 2.0

^a Non-porous microparticles.

^b Mass median aerodynamic diameter.

of more than 4% ammonium bicarbonate inhibited the formation of porous microparticles, which was likely due to the generation of excess gas bubbles (data not shown). We confirmed that the size of

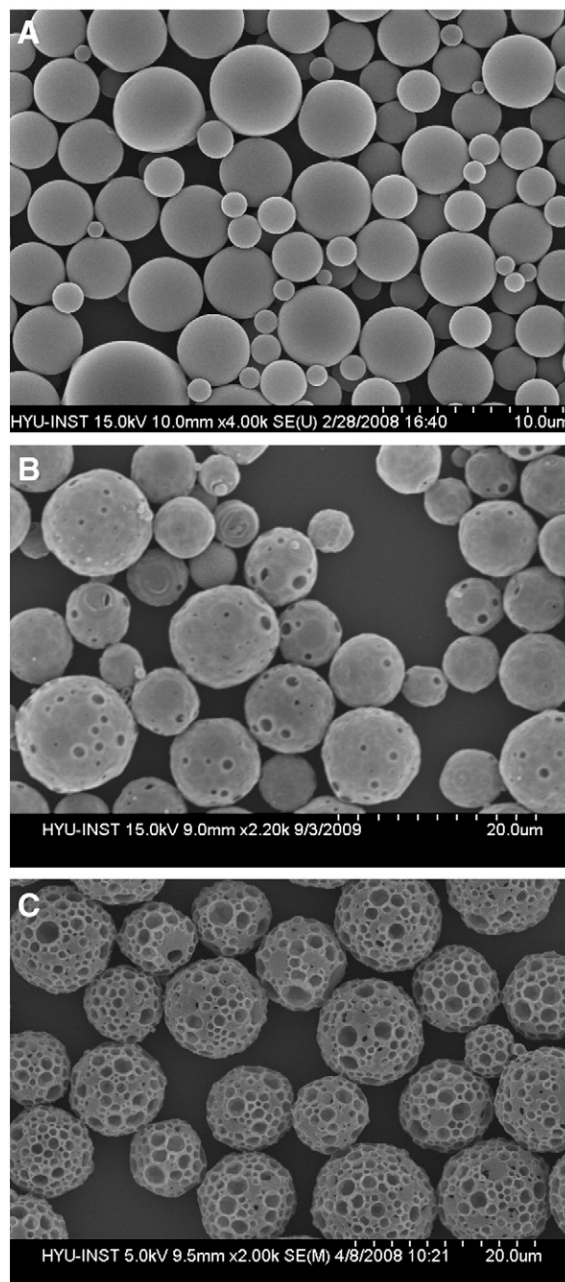


Fig. 1. Morphology of budesonide-loaded PLGA microparticles prepared with (A) 0% (PM0), (B) 1.5% (PM1), and (C) 3.0% (PM2) ammonium bicarbonate solution.

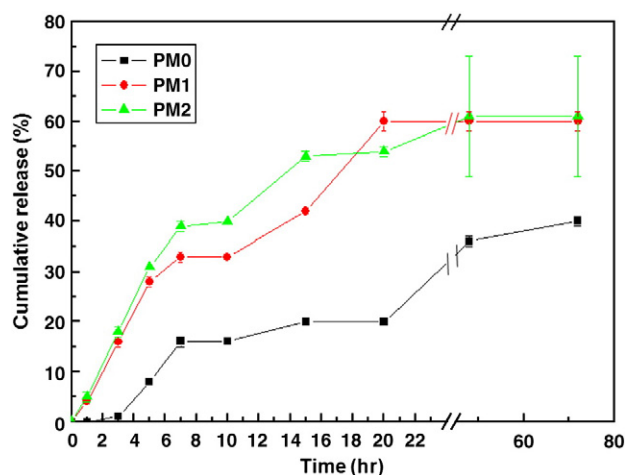


Fig. 2. Cumulative release of budesonide from PLGA microparticles prepared with 0% (PM0), 1.5% (PM1), and 3.0% (PM2) ammonium bicarbonate solution ($n = 4$).

the BD-loaded porous PLGA microparticles, as well as their pore sizes, increased as the concentration of ammonium bicarbonate increased, consistent with previous studies reporting the preparation of porous structures using this gas foaming method [29–32]. However, porous PLGA microparticles had the smaller MMAD than that of non-porous microparticles. The MMAD has been regarded as a key factor for the design of pulmonary drug delivery systems, including many polymeric microparticles [33–35].

3.2. *In vitro* release of budesonide from porous PLGA microparticles

We next determined the loading efficiency of BD in PLGA microparticles fabricated at different porogen concentrations. As listed in Table 1, the loading efficiency of non-porous PLGA microparticles was 67%, whereas that of porous PLGA microparticles was approximately 60%. In the case of non-porous PLGA microparticles, the drug loading efficiency was about 10% higher than that of the porous PLGA microparticles, indicating a loss of BD while developing the porous structure of the microparticles. Fig. 2 shows the *in vitro* release profiles of BD from PLGA microparticles depending on porogen concentrations. It has been reported that BD entrapped in poly(methacrylic acid-*g*-ethylene glycol) microparticles [7] and PLGA microparticles [9] can be released *in vitro* within 24 h with large initial burst, even though BD has poor solubility in water (21.5 $\mu\text{g}/\text{ml}$ under constant motion). However, we observed the sustained release of BD from porous PLGA microparticles during 24 h. The release rate of BD from porous particles was faster than that from the non-porous PLGA microparticles, which was expected as BD can easily escape from porous PLGA microparticles through the pores. No significant difference, however, in the release behavior of BD from PM1 and PM2 was observed likely due to high porosity of the microparticles (>96%).

3.3. *In vivo* lung uptake of porous PLGA microparticles

Images of mouse lungs taken after intratracheal delivery of DiI-loaded, porous PLGA microparticles are shown in Fig. 3. Pulmonary deposition of the porous PLGA microparticles was substantially enhanced compared to that of the non-porous PLGA microparticles, indicating that porous microparticles are more effective at pulmonary delivery than are non-porous microparticles. In addition, a uniform distribution of porous PLGA microparticles in the lung tissues was observed (Fig. 4). The extent of lung deposition of porous PLGA microparticles was dependent on the porous structure of microparticles used in this study; the bigger is the pore size, the greater is

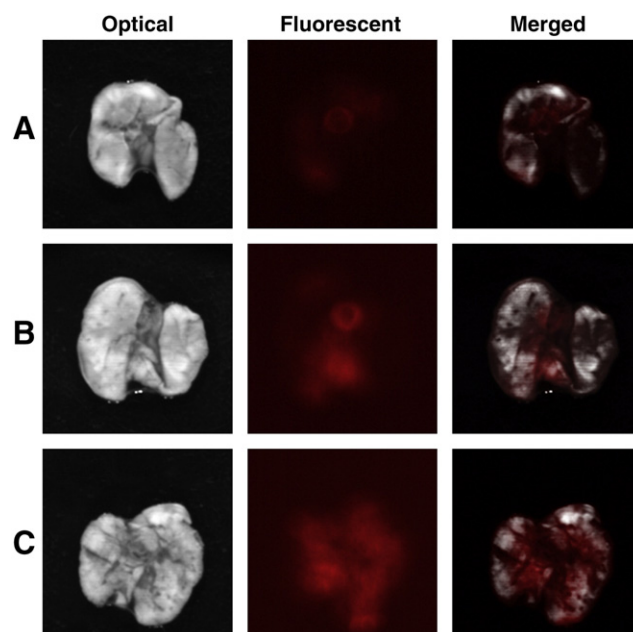


Fig. 3. Images of DiI-loaded PLGA microparticles prepared with (A) 0%, (B) 1.5%, and (C) 3.0% ammonium bicarbonate solution in the murine lung.

the number of particles found in the lung. The lung uptake efficiency of PLGA microparticles with various pore sizes was determined and is shown in Fig. 5. Uptake efficiency increased with an increase of the pore size in the range tested in this study. Porous microparticles have many advantages for pulmonary drug delivery because they can be

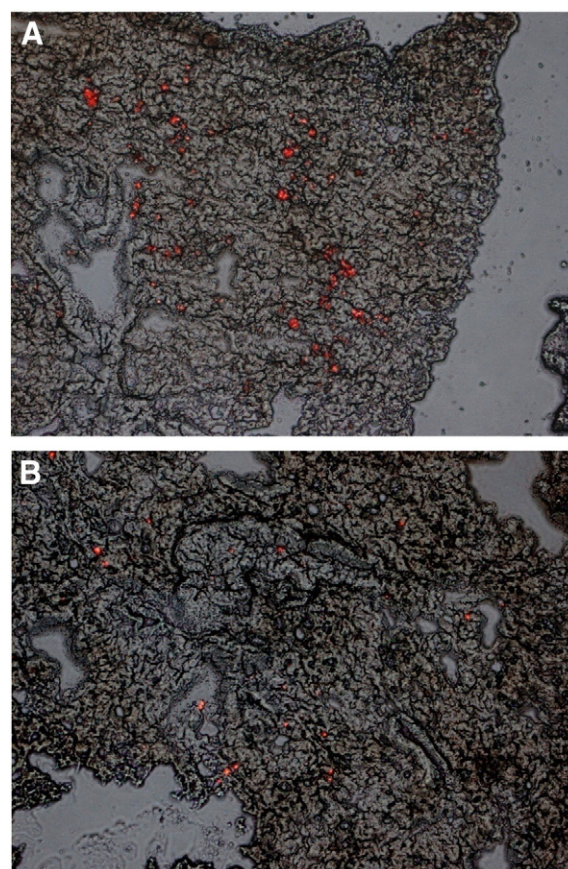


Fig. 4. Fluorescence images of DiI-loaded (A) porous and (B) non-porous PLGA microparticles in murine lung tissue sections.

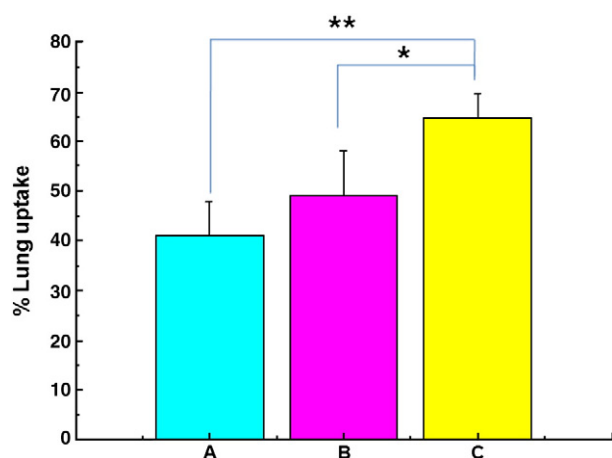


Fig. 5. The *in vivo* lung uptake efficiency of Dil-loaded PLGA microparticles prepared with (A) 0%, (B) 1.5%, and (C) 3.0% ammonium bicarbonate solution (* $P < 0.05$, ** $P < 0.01$).

delivered to the deep lungs due to their low density, they can be aerosolized more efficiently, and they can reduce deposition loss before entry into the airway [13,34,35]. We also confirmed that our porous PLGA microparticles were much more effective than non-porous PLGA microparticles for the purpose of pulmonary drug delivery.

3.4. Therapeutic efficacy in a murine asthma model

We next evaluated the therapeutic efficacy of BD-loaded porous PLGA microparticles in a murine asthma model. We induced asthma in mice using ovalbumin (OVA) sensitization and challenge, and the asthmatic mice were then treated with BD-loaded porous PLGA microparticles. The representative characteristic of asthma is an increase of inflammatory cells such as macrophages, eosinophils, neutrophils and lymphocytes. Therefore, we counted the number of inflammatory cells in the BAL fluid obtained from the mouse lung (Fig. 6). The total number of inflammatory cells in mice with asthma was 12.3×10^5 cells/ml, which was substantially higher than that of control mice (no asthma). This number was significantly reduced by treatment with BD-loaded porous PLGA microparticles (1.6×10^5

cells/ml), compared to those treated with BD only (4.4×10^5 cells/ml) and those treated with non-porous PLGA microparticles (3.7×10^5 cells/ml), all of which were given the same dose of BD. In specific, the number of macrophages and eosinophils, the most representative inflammatory cells often found in asthma, was substantially lowered in mice treated with BD-loaded porous PLGA microparticles, indicating that BD-loaded porous PLGA microparticles were effective in reducing the number of inflammatory cells *in vivo* when they were administered into the mice every 3 days. The number of neutrophils in the BAL fluid was also significantly reduced by the use of BD-loaded porous PLGA microparticles, compared to other treatments. This might be attributed to the high lung deposition efficiency of porous particles and the controlled release of BD from the particles compared to those of the groups treated with either BD only or with BD-loaded non-porous particles. Although PLGA used in this study is not degradable in a few days in the body, no significant accumulation in the lung was observed after repeated inhalation.

The results of the histological analysis of lung tissue sections are shown in Fig. 7. Severe peribronchial inflammation with macrophages, lymphocytes, and neutrophils as well as a thickening of the airway wall were observed in asthmatic mice. Surprisingly, delivery of BD-loaded porous PLGA microparticles into the mice showed a significant reduction in peribronchial infiltration of inflammatory cells compared to those of the other experimental groups. In addition, thickening of the airway wall barely increased when the mice were treated with BD-loaded porous PLGA microparticles, compared to BD only and BD-loaded non-porous microparticles (Fig. 8).

We next measured responses to inhaled methacholine in mice and used increasing values of enhanced pause (Penh) as an index of airway obstruction, as inhaled methacholine results in contraction of

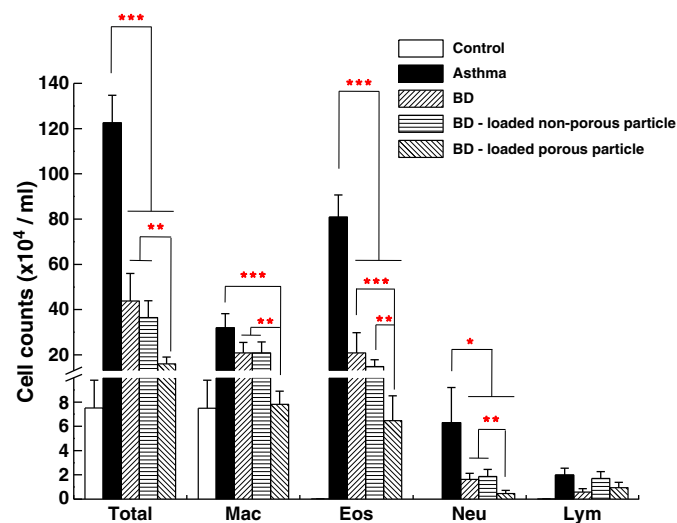


Fig. 6. Therapeutic effect of porous PLGA microparticles on airway inflammation in OVA-induced mouse asthma model. The number of inflammatory cells, including macrophages (Mac), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym) in the BAL fluid after treatment ($n = 10$).

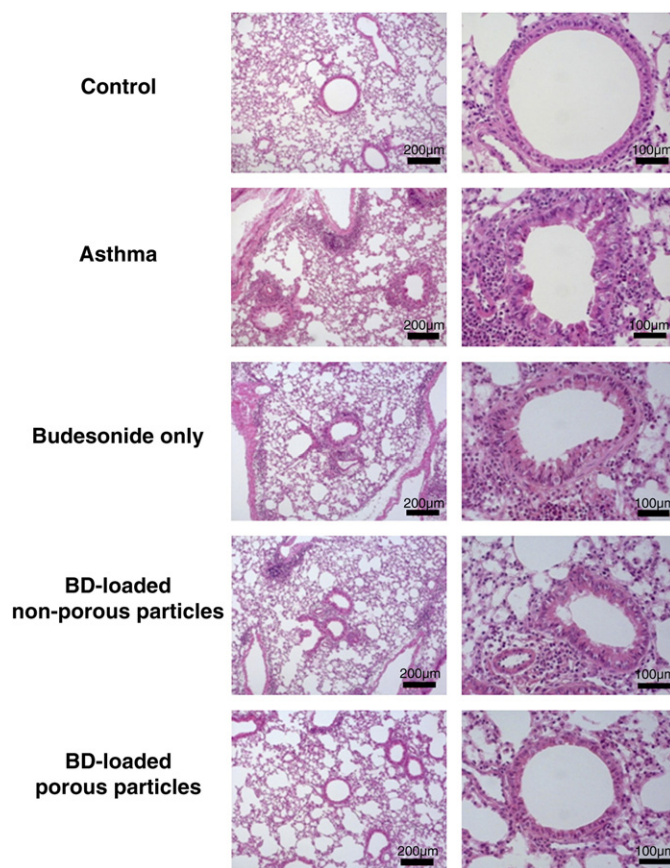


Fig. 7. Histological examination of lung tissue. Lung sections were obtained from PBS- or OVA-challenged mice treated with BD, BD-loaded non-porous particles or BD-loaded porous particles. Sections were stained with H&E for the analysis of tissue morphology and the level of inflammation.

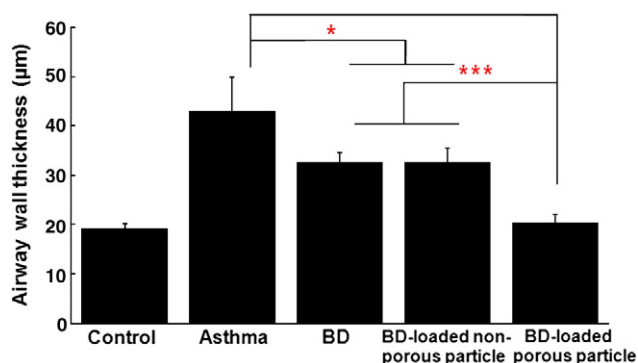


Fig. 8. Assessment of airway wall thickness.

airway smooth muscle [36]. All the OVA-challenged mice showed increased bronchial hyperresponsiveness as the methacholine concentration increased. However, responses in mice treated with BD-loaded porous microparticles were significantly reduced as the methacholine concentration increased (Fig. 9). This finding clearly indicates that administration of BD-loaded porous microparticles was useful to diminish bronchial hyperresponsiveness of asthmatic mice.

4. Conclusions

We demonstrated that porous PLGA microparticles could be useful for pulmonary delivery of BD for the treatment of asthma. We prepared BD-loaded porous PLGA microparticles using a double emulsion method with ammonium bicarbonate as the porogen. Varying the porogen concentration controlled the porosity of the PLGA microparticles. The sustained release of BD from porous PLGA microparticles was achieved *in vitro*, and the lung uptake efficiency of porous PLGA microparticles was higher than that of non-porous PLGA microparticles. In addition, delivery of BD-loaded porous PLGA microparticles into OVA-induced asthmatic mice showed a significant reduction in the number of inflammatory cells such as macrophages and eosinophils in the BAL fluid as well as in lung tissue sections compared with other groups treated with BD only and BD-loaded non-porous PLGA microparticles. In addition, a use of BD-loaded

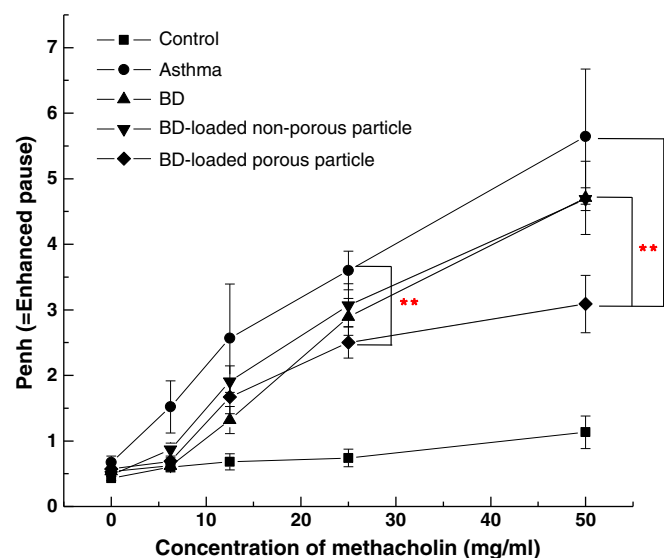


Fig. 9. Changes in Penh for asthmatic mice treated with BD only (▲), BD-loaded non-porous microparticles (▼), and BD-loaded porous microparticles (◆).

porous microparticles significantly reduced bronchial hyperresponsiveness of asthmatic mice. This approach to controlling the porous structure of microparticles and the drug release behavior from porous microparticles could be useful for the design and tailoring of novel pulmonary drug delivery systems.

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