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RESEARCH PAPER

Albumin-Coated Porous Hollow Poly(Lactic-co-Glycolic Acid) Microparticles Bound with Palmityl-Acylated Exendin-4 as a Long-Acting Inhalation Delivery System for the Treatment of Diabetes

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ABSTRACT

Purpose To study the development of porous poly(lactic-co-glycolic acid) microparticles (PLGA MPs) coated initially with albumin and then with palmityl-acylated exendin-4 (Pal-Ex4) as an inhalation system for treating diabetes.

Methods Porous PLGA MPs were prepared by w/o/w double emulsification using hydroxypropyl-β-cyclodextrin and poly(ethylene-*alt*-maleic anhydride). Albumin was covalently attached to the MPs using EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide); Pal-Ex4 was then bound on the albumin surface. Albumin-binding degree and aerosolization efficiency were investigated. Deposition of the MPs after insufflations into the lungs of ICR mice was observed by image monitoring, and pulmonary hypoglycemic efficacies were examined in db/db mice. Cytotoxicity and histopathology induced by MPs were examined in Calu-3 and A549 cells and in the lungs of db/db mice, respectively.

Results Albumin-coating and Pal-Ex4-binding to porous MP were performed with acceptable efficiencies. Pal-Ex4-bound albumin-coated MPs (Pal-Ex4/HSA-PLGA MP) were of high porosity and had appropriate aerodynamic sizes. Furthermore, this MP was efficiently deposited throughout mouse lungs, and exhibited a prolonged hypoglycemia and no significant lung tissue damage in db/db mice.

Conclusion Pal-Ex4/HSA-PLGA MP demonstrated many meaningful pharmaceutical advantages for the treatment of diabetes, in terms of aerosolization efficiency, drug loading, sustained drug-release, and hypoglycemic duration *in vivo*.

KEY WORDS albumin-binding · albumin-coating · inhalation · palmityl-acylated exendin-4 · porous microspheres

INTRODUCTION

The great majority of peptide and protein drugs are still administered by injection and, thus, cause pain-associated problems (1,2). Hence, non-invasive delivery systems for such drugs are required to improve patient compliance (3), and, especially, the pulmonary route has prominent

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physiological advantages over other non-parenteral routes due to the huge surface area available (~75 m^2 in adults) and the low thickness of alveolar epithelium (approx. 0.1~0.2 μm) (4–8). Actually, the intrapulmonary bioavailabilities of some peptides and proteins have been reported to be 10 to 200 times greater than those offered by other non-invasive routes (5). For this reason, the lung route is considered to be the most efficient and effective for delivering peptides and proteins non-invasively and systemically (6,7).

Polymeric microparticles (MP) have been used as long-acting inhalation delivery systems because they can provide a sustained-release effect (9–12). However, heavy, compact MPs with densities >~1.0 g/cm^3 and particle sizes of >5 μm seldom reach the alveolar region, and microparticles of <1~2 μm are likely to be engulfed by macrophages after being deposited in alveoli (13,14). Accordingly, light, large porous MPs with densities of <0.4 g/cm^3, aerodynamic diameters of 1~5 μm, and large geometric diameters of ~30 μm have been suggested to improve deposition in alveoli and retard phagocytosis (13–15). These porous polymeric MPs can be fabricated using extractable porogens (16), osmogens (10–12), and gas-foaming salts (17,18).

However, these MPs are so highly porous that they are short of polymer matrix frames to incorporate drugs; moreover, if their internal polymer networks are too delicate, incorporated drugs may be very rapidly released, as previously reported (11,16). On the other hand, aerosolization efficiency is also a key consideration, and poly(lactic-co-glycolic acid) (PLGA) MPs tend to agglomerate due to the extreme hydrophobicity of PLGA and their particle sizes.

The inhalation delivery of anti-diabetic protein drugs, which are otherwise injected, give a significant clinical advantage to diabetic patients in the view of compliance, and this is why another insulin inhalation product (AFREZZA™, MannKind Corp.) has tried to get FDA approval (19) despite the failure of the first-approved inhaled insulin formulation (Exubera®, Pfizer). Furthermore, the inhalable polymeric microparticles provide an additional therapeutic merit of extended bioactivity *in vivo* due to the sustained drug-release property. In actuality, dry powders of PLGA/cyclodextrin large porous particles containing insulin have shown meaningful *in vitro* aerodynamic behavior and *in vivo* anti-diabetic effect via lung routes in terms of prolonged hypoglycemia (20,21).

In this study, we describe porous PLGA MP covalently surface-coated with human serum albumin (HSA) and then non-covalently bound with palmityl-acylated exendin-4 (Pal-Ex4). Exendin-4, a potent glucagon-like peptide-1 (GLP-1) agonist, is a 39 amino acid peptide, and it has many therapeutic merits for the treatment of diabetes, i.e., glucose-dependent insulin release, β-cell proliferation, and appetite suppression (22,23). We hypothesized that an albumin coating would improve the aerosolization efficiency of porous PLGA MPs, that Pal-Ex4 would bind well to surface-coated albumin, and that Pal-Ex4 would survive even longer *in vivo* by binding to circulating albumin after release from the surfaces of MPs. Accordingly, highly porous PLGA MPs were prepared using the osmotic porogen hydroxypropyl-β-cyclodextrin (HPβCD), and their particle sizes, morphologies, and aerosolization efficiencies were examined. In addition, the *in vivo* deposition and hypoglycemic efficacy of these albumin-coated MPs were evaluated after the intrapulmonary administration using a dry powder insufflator in a type 2 diabetic rodent model.

MATERIALS AND METHODS

Materials

Exendin-4 and N-hydroxysuccinimidyl-activated palmitic acid (PAL-NHS) were purchased from the American Peptide Company (Sunnyvale, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Poly(d,l-lactic-co-glycolic acid) (PLGA) (Mw: 10,000; lactic acid : glycolic acid = 50:50) was purchased from Wako Pure Chemical (Tokyo). Polyvinyl alcohol (PVA, Mw: 30,000~70,000 Da) and human serum albumin were purchased from Sigma-Aldrich. Poly(ethylene-*alt*-maleic anhydride) (PEMA, Mw: 400,000; 1 : 1) was purchased from Polysciences, Inc. (Warrington, PA), and Cy5.5 NHS ester dye from GE Healthcare (Piscataway, NJ). All other reagents, unless otherwise specified, were obtained from Sigma-Aldrich.

Experimental Animals

Male type 2 diabetic C57BL/6 *db/db* mice (4–5 weeks old) were purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejon, South Korea), and male ICR mice weighing (4 weeks old) were purchased from the Hanlim Experimental Animal Laboratory (Seoul). Animals were cared for in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 80–23, revised in 1996). Animals were housed in groups of 6–8 under a 12-h light/dark cycle (lights on 6 a.m.), water and food were allowed *ad libitum*, and animals were acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Pusan National University.

Preparation Optimization of Porous PLGA MP

Porous PLGA MPs were prepared using a w/o/w double emulsion-solvent evaporation technique using hydroxypropyl-β-cyclodextrin (HPβCD; Sigma-Aldrich) as an osmotic poro-

gen, using a modification of a previously described procedure (10–12). Briefly, HPβCD (10, 20 or 30 mg) and sodium chloride (20 mg) were dissolved in 0.5 ml of deionized water (DW), poured into 3 ml of a PLGA solution (lactic acid: glycolic acid (50:50); 300 mg) in methylene chloride, and then sonicated in ice bath using a Sonics Vibra-Cell Ultrasonic Processor (Sonics & Materials Inc. Newtown, CT, USA) for 30 s at an amplitude of 15%. The primary emulsion obtained was then emulsified in 100 ml of an ice-cold 0.1 M sodium chloride solution (pH 7.0) containing 0.5% (*w/v*) of PEMA for 2 min at 3,000 rpm using a Silverson Laboratory Mixer (model L4RT) with a 3/4-inch head (Silverson Machines, Inc. East Longmeadow, MA, USA). The resultant emulsion was allowed to evaporate under gentle magnetic stirring (300 rpm) under an air current at 40°C for 5 h. To produce plain porous MPs (non-coated), the hardened MPs were harvested by centrifugation, washed three times with DW and freeze-dried. Mean particle sizes of the MPs produced were determined in a water suspension using a laser diffraction particle size analyzer (Mastersizer, Malvern Instruments, USA).

Preparation of HSA-Coated Porous PLGA MP (HSA-PLGA MP)

The surfaces of porous PLGA MPs were coated by HSA using a modification of a previously described procedure (24,25). Briefly, after the hardening process mentioned above, MPs were harvested by centrifugation, washed three times with DW, and then suspended in 30 ml of 0.1 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0) containing 0.02% Tween 20, 300 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide•HCl (EDC), and 150 mg sulfo-N-hydroxsuccinimide (Sulfo-NHS) for 12 h. MPs were then washed twice with DW and harvested in 30 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.02% Tween 20 and 150 mg HSA. The conjugation reaction between HSA and the carboxylate groups of PEMA bound to the surfaces of PLGA MPs was allowed to continue for 12 h. Finally, the HSA-PLGA MPs were washed twice with DW and lyophilized. Especially for the *in vivo* experiment samples, the HSA-PLGA MPs prepared were wet-sieved very slowly using a 20 μm sieve (Nonaka Rikaki Co., Ltd., Japan) to remove some aggregates.

Morphologies of PLGA MPs by Scanning Electron Microscopy

The surface morphologies of PLGA MPs were investigated by scanning electron microscopy (SEM, Hitachi S3500N, Japan). Dry MP specimens were attached to specimen stubs using double-sided tape and then sputter-coated with gold-palladium in an argon atmosphere using a Hummer I sputter coater (Anatech Ltd. St. Alexandria, VA, USA). Average MP sizes and pore diameters were determined by observing at least 30 particles of each type. In particular, some microparticles (20 mg) were chopped with a razor blade at least 30 times to reveal their internal morphologies.

Preparation of Palmityl-Acylated Exendin-4 (Pal-Ex4)

Palmityl-acylated exendin-4 (Pal-Ex4) was prepared using a modification of a previously described procedure (26–28). Briefly, 7.5 mg of exendin-4 was mixed with 1.0 mg of PAL-NHS (molar ratio 1:1.5) in 5 ml of a 0.3% triethylamine/dimethylsulfoxide and allowed to react at room temperature for 1 h. The reaction mixture was subjected to RP-HPLC (reversed-phase high-performance liquid chromatography) on a LiChrospher 100 RP-18 column (250× 4.0 mm, 5 μm, Merck, Germany) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 ml/min using solvent A (0.1% trifluoroacetic acid (TFA) in DW) and solvent B (0.1% TFA in acetonitrile) using the following gradient profile: 30–50% of B for 10 min and 50–90% of B for 20 min. Eluates were monitored at 215 nm, and the fraction corresponding to Pal-Ex4 was collected, dried under nitrogen, and stored in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 4°C until needed. The final concentration of Pal-Ex4 was always maintained below 30 μg/ml to prevent micelle formation.

Confocal Laser Scanning Microscopy (CLSM) Visualization of PLGA MPs

Degree of modification of PLGA MPs by HSA and the binding degree of Pal-Ex4 onto surface-bound HSA were observed. First, a 10 mg sample of MPs prepared using PEMA was activated with EDC in 1 ml of 0.1 M MES buffer (pH 6.0) as described above, and then fluoresceinamine (10 mg, Sigma-Aldrich) was added. Excess fluoresceinamine in supernatant was removed by centrifugation. These MPs were compared with plain MPs prepared using PVA in terms of surface fluorescence intensity. Second, a sample (10 mg) of HSA-PLGA MPs was then treated with fluorescein-NHS (5 mg, Pierce, Rockford, IL) in 1 ml of 0.1 M phosphate buffer (pH 7.5), and residual fluorescein-NHS was also removed by centrifugation. Third, to determine the binding degree of Pal-Ex4 onto surface-coated albumin, a sample (2 mg) of Pal-Ex4 was added to two molar equivalents of rhodamine-NHS (Pierce) in 50 mM of phosphate buffer (1 ml, pH 7.0) and maintained for 3 h at room temperature. The reaction mixture was then dialyzed for 24 h *versus* 50 mM PBS (pH 7.4) using a dialysis kit (Mw cutoff = 3,500, Gene Bio-Application Ltd., Israel). The concentration of rhodamine-tagged Pal-Ex4 was measured using a BCA protein assay kit (Pierce,

Rockford, IL). Briefly, an aliquot (10 ml) of rhodamine-tagged Pal-Ex4 (~25 μg/ml) was added to 2 ml of HSA-PLGA MP (5 mg) previously hydrated in 50 mM PB (pH 7.4) containing 0.02% Tween 20 for 12 h at room temperature, and supernatant was removed by centrifugation. The fluorescence images of individual PLGA MPs were visualized by CLSM (Carl Zeiss Meta LSM510, Germany).

Binding and Release of Pal-Ex4 in HSA-PLGA MP

Aliquots (50 ml) of Pal-Ex4 (25 μg/ml) were added to HSA-PLGA MP (25 mg), which had been previously hydrated and dispersed in 10 mM PBS (pH 7.4) at room temperature, and agitated using a rotator gently for 12 h. Supernatants were then harvested and concentrated 200 fold using a Centricon-10 concentrator (Millipore, Beverly, MA, USA). The concentrations of final solutions were measured using a BCA protein assay kit (Pierce, Rockford, IL), and Pal-Ex4 amount bound to 1 mg of HSA-PLGA MP were calculated. Using a similar procedure, rhodamine-tagged Pal-Ex4 was allowed to adsorb onto the surfaces of HSA-PLGA MP for 12 h. Pellets were then collected by centrifugation, washed thoroughly three times with DW, and lyophilized. Separately, rhodamine-tagged Pal-Ex4 -bound HSA-PLGA MPs (5 mg) were suspended in 1 ml of PBS (pH 7.4) containing 0.02% (*v*/*v*) Tween 20, and gently shaken at 37°C. At predetermined times, supernatants were carefully collected, and fluorescence intensities were measured at excitation and emission wavelengths of 544 and 576 nm, respectively. Cumulative amounts released were expressed as percentages of initial loadings. All samples were prepared and analyzed in triplicate, and the fluorescence images of HSA-PLGA MPs at 1, 2, 3, 5, and 7 days after incubation were also visualized by CLSM.

Cytotoxicity Evaluation of HSA-PLGA MP or Non-coated PLGA MP

The cytotoxicity of HSA-PLGA MP or PLGA MP was evaluated in human lung adenocarcinoma epithelial cell lines such as Calu-3 and A549 (Korea Cell Line Bank, Seoul, Korea) by using a modification of a previously described MTT assay procedure (29,30). Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% (*v*/*v*) fetal bovine serum (FBS, Gibco) containing 1% penicillin/streptomycin (Gibco), and kept in an incubator set to 37°C, 5% CO_2 and 95% humidity. Cells were seeded in 96-well plates at 2×10^4 cells/well, pre-incubated for 24 h, and further incubated with suspended MPs (final concentration: 1 mg/ml) at predetermined times (12, 24, 48 h). After the MTT treatment, cell viability percentages (%) for respective microparticles group *vs.* control were calculated, and the inhibitory concentrations (IC_{50}) of each group were determined. Data are presented as means ± SDs of at least six independent determinations.

Aerosolization of HSA-PLGA MP

Images of the aerosolization of HSA-PLGA MP were captured at 0.04 s intervals after actuation using a digital video-camera. The aerosolization efficiencies of HSA-PLGA MP and of non-coated PLGA MP were examined by measuring amounts of particles delivered by dry powder insufflators after two actuations. The air volume used by insufflators during a single actuation was set at 1.0 ml. Data are presented as means ± SDs of at least six independent determinations.

Pulmonary Administration of PLGA MPs Into Mice

The pulmonary administration of various MPs was accomplished using a modification of a previously described procedure (31,32). In brief, male ICR or db/db mice were anesthetized with a single intraperitoneal (i.p.) injection of tiletamine (20 mg/kg). Freeze-dried PLGA MPs (~3 mg) were directly insufflated into the lungs using an insufflator device (DP-4M) and an air pump (AP-1) (Penn-Century, Inc., Philadelphia, PA). Visualization of the tracheal opening was achieved using an otoscope (Heine Mini3000, Germany).

Evaluation of the Deposition of HSA-PLGA MP in Lungs

To observe the lung deposition of HSA-PLGA MP, a sample (~3 mg) of HSA-PLGA MP surface-modified with cy5.5 dye was insufflated into the lungs of ICR mice, which were sacrificed at 0.5 and 3 h after administration—entire lung lobes were excised with tracheas. Excised lungs were visualized using Image Station 4000 MM (Kodak) (at an emission wavelength of 700 nm).

Hypoglycemic Efficacies of Insufflated Pal-Ex4-Bound HSA-PLGA MP in Non-fasted db/db Mice

To evaluate pulmonary hypoglycemic efficacies, Pal-Ex4-bound HSA-PLGA MP (Pal-Ex4/HSA-PLGA MP, ~3 mg) (approx. 120 μg of Pal-Ex4/mouse) was administered by insufflation to male *db/db* mice ($n=6\sim8$/group, 7 weeks old) previously anesthetized by tiletamine (20 mg/kg, i.p.). PLGA MP without Pal-Ex4 was also administered as a control. Mice were kept under non-fasting conditions with free access of water and food until the end of experiment, as previously described (33,34). A drop of blood was drawn from a tail vein of each mouse at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, and 120 h after the administrations, and

blood glucose levels (BGL) were determined using a one-touch blood glucose meter (ACCU-CHEK® Sensor, Roche Diagnostics Corp., USA).

Histology of Lung Tissues of db/db Mice Administered PLGA MPs

The histology of lung tissues of db/db mice treated with PLGA MPs was assessed by using a modification of a previously described procedure (32). At 14 days after the inhalation of Pal-Ex4/HSA-PLGA MP or blank HSA-PLGA MP, the lungs of db/db mice in each group were harvested after sacrifice. The lung specimens were obtained through a series of procedures, i.e. formalin/paraffin treatment, cross-sliced sections, and hematoxylin and eosin (H&E) staining, and further examined by light microscopy. The histopathology of the lung tissues was briefly investigated and compared with that of a MP-untreated db/db mouse with the same week age.

Data Analysis

Data are presented as means ± SDs. Statistical significances were determined using the Student's *t*-test, and *p*-values of <0.05 were considered statistically significant.

RESULTS

Characterization of HSA-PLGA MP

PLGA MPs were prepared using a combination of HPβCD and sodium chloride as osmogens. As shown in Fig. 1a, b, and c, although the amount (10, 20, 30 mg) of HPβCD did not appear to affect the degree of porosity significantly, some irregular large pores were observed in PLGA MPs using more than 20 mg of HPβCD added. However, overall the microparticles seemed to be suitable for inhalation experiments. In particular, they were almost hollow, and this hollowness increased with the amount of HPβCD used (Fig. 1d, e). HSA-PLGA MPs were also highly porous and were similar sizes as the parent PLGA MPs (Fig. 1f; HSA-PLGA MP mean particle size 17.2 ± 2.1 μm). However, separately, when using the albumin-coating protocol mentioned above, the amount of albumin on the surface of 1 mg of PLGA MPs was determined to be 220 ± 23 μg ($n=3$).

CLSM Visualization of HSA-PLGA MP

As shown in Fig. 2a and a′, high fluorescence intensities were obtained by conjugating fluoresceinamine to PLGA

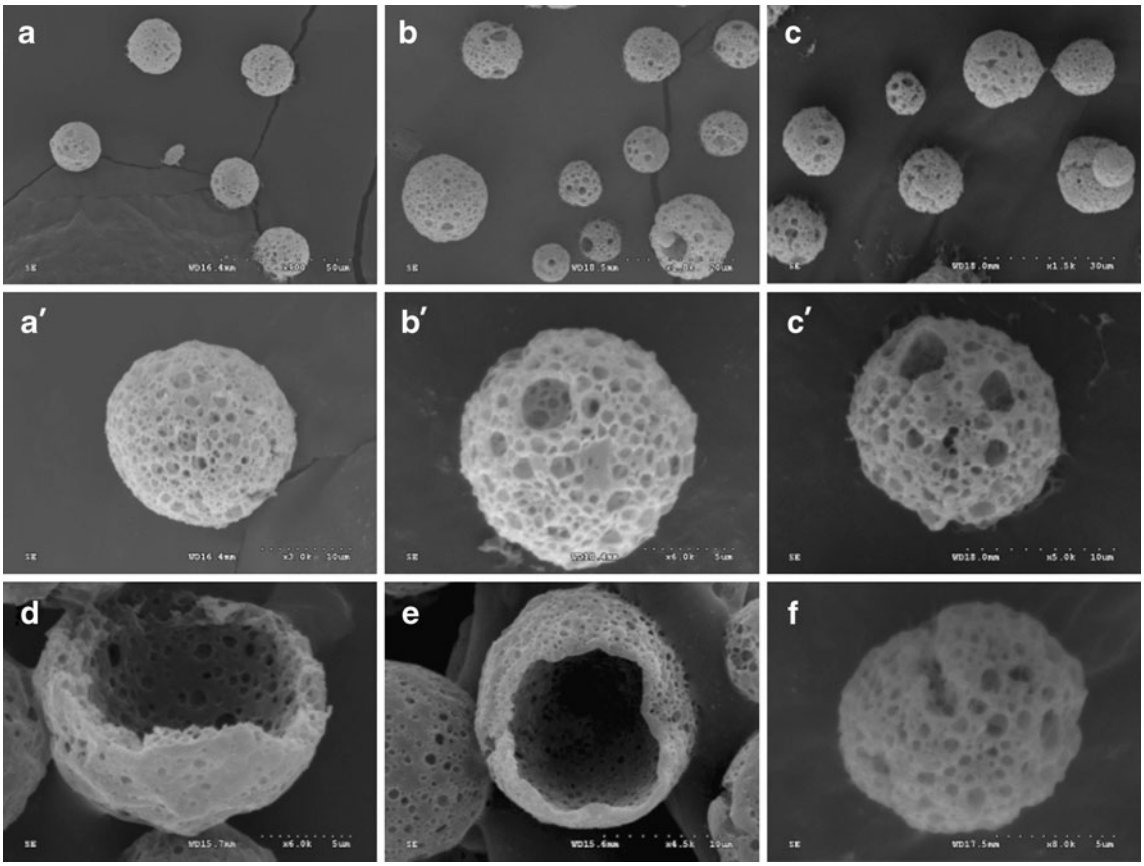

Fig. 1 Morphology of porous PLGA MPs by scanning electron microscopy (*SEM*). **a**, **a′** HPβCD 10 mg/NaCl 20 mg, **b**, **b′** HPβCD 20 mg/NaCl 20 mg, **c**, **c′** HPβCD 10 mg/NaCl 20 mg, **d** cross-section of (**b**); **e** cross-section of (**c**); **f** HSA-PLGA MP (HPβCD 20 mg/NaCl 20 mg).

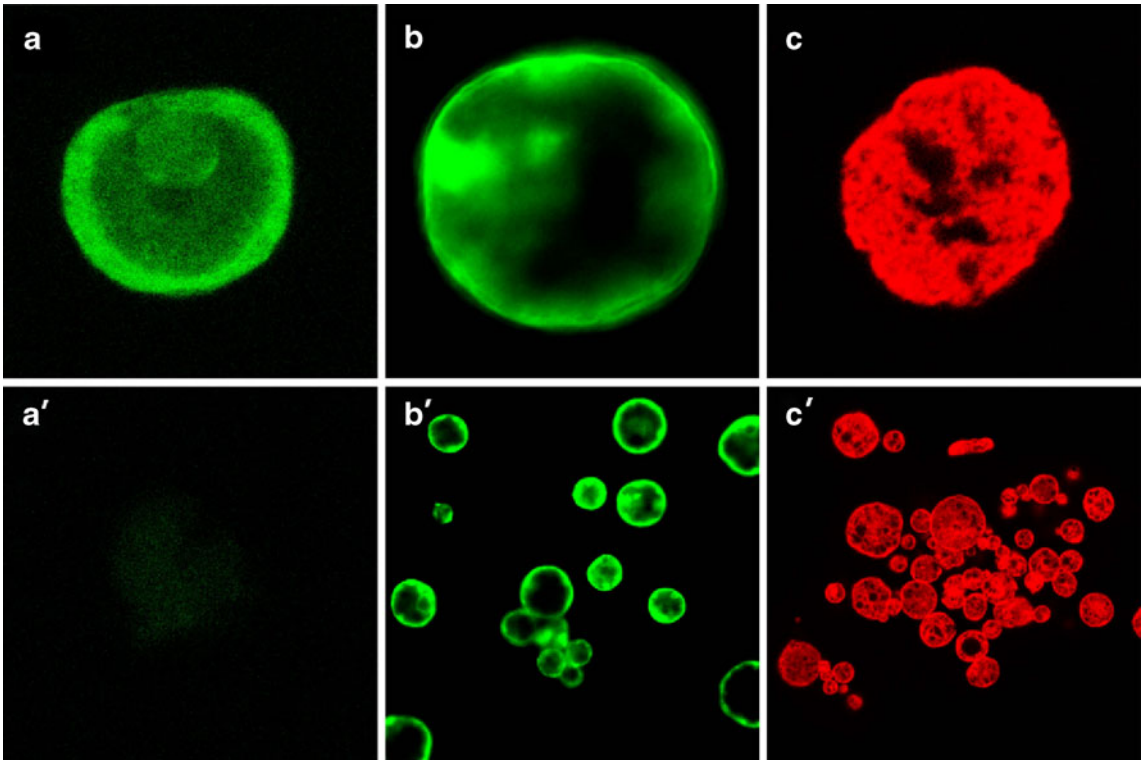

Fig. 2 Confocal laser scanning microscopy (*CLSM*) of various porous PLGA MPs. **a** fluoresceinamine-modified PLGA MP prepared using PEMA; **a′** fluoresceinamine-modified PLGA MP prepared using PVA; **b**, **b′** HSA-PLGA MP reacted with fluorescein-NHS; **c**, **c′** albumin-coated MP bound with rhodamine-tagged Pal-Ex4.

MPs prepared using PEMA after EDC activation, whereas those of PLGA MPs prepared using PVA were negligible. Strong green fluorescence was shown by HSA-PLGA MPs after they were incubated with fluorescein-NHS (Fig. 2b and b′). Furthermore, as shown in Fig. 2c and c′, rhodamine-tagged Pal-Ex4 was found to efficiently bind to the surfaces of HSA-PLGA MPs.

Binding and Release of Pal-Ex4 from HSA-PLGA MP

BCA assay results showed that *ca.* 38.1 μg of Pal-Ex4 bound to 1 mg of HSA-PLGA MPs. As shown in Fig. 3, Pal-Ex4 was slowly released from Pal-Ex4/HSA-PLGA MPs in 10 mM PBS (pH 7.4). Pal-Ex4 release was fairly linear over the first 5 days without a marked initial burst. In

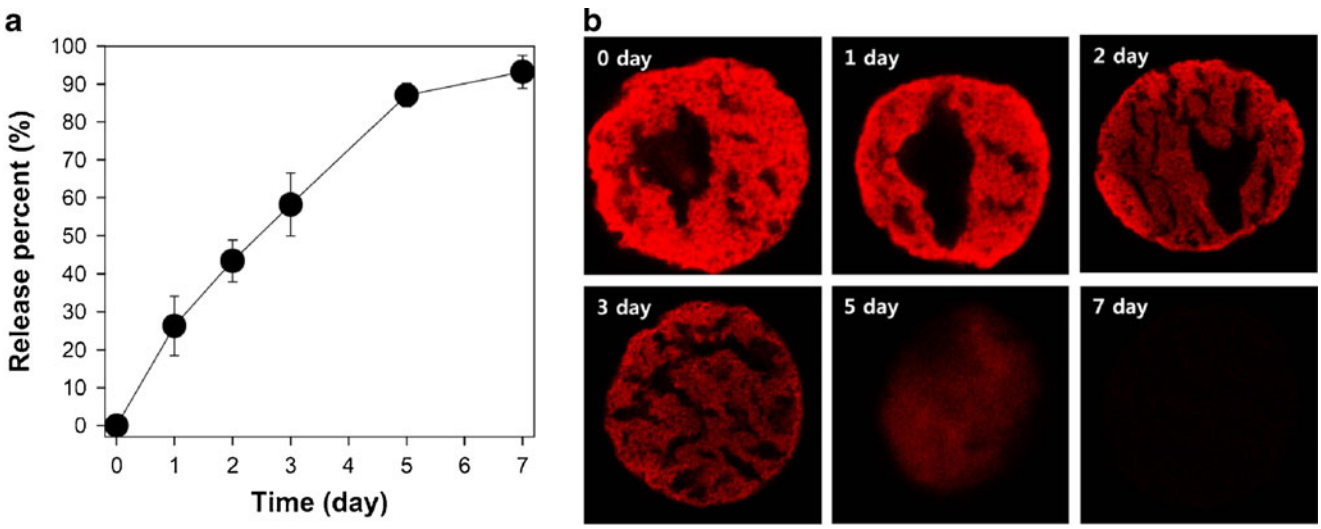

Fig. 3 Release characteristics of Pal-Ex4 from HSA-PLGA MP. **a** Release profile; **b** CLSM images of Pal-Ex4/HSA-PLGA MP.

fact, only ~26% of the Pal-Ex4 was transferred to supernatant for 1 day after incubation (Fig. 3a). Confocal microscopy also confirmed this release pattern, indicating that Pal-Ex4 was still tightly bound to the surface albumin until ~2 days and then was lost over the subsequently 5 days (Fig. 3b).

Aerosolization of HSA-PLGA MP

As shown in Fig. 4a, the initial aerosolization of HSA-PLGA MP was maintained for ~0.12 s after actuation, and aerosol particles showed good mobility. Furthermore, the aerosolization efficiency of HSA-PLGA MP was 1.6-fold greater than that of non-coated PLGA MP, showing the aerosolization amount corresponding to two consecutive actuations (3.3±0.3 mg *vs.* 2.1±0.5 mg, respectively; $p<0.007$) (Fig. 4b).

Lung Deposition of Insufflated HSA-PLGA MP

Lung deposition images were obtained at 0.5 and 3 h after administering HSA-PLGA MP to ICR mice. Fig. 5a, b, c, and d show various images of RGB, red, optical, and merged with red and optical. At 0.5 h after administration, most HSA-PLGA MPs appeared to be reasonably well deposited, especially in upper lung lobes, and at 3 h they were totally dispersed throughout entire lung lobes including alveoli.

Evaluation of Pulmonary Hypoglycemic Efficacy of Pal-Ex4/HSA-PLGA MP in Non-fasted db/db Mice

The hypoglycemic efficacy of Pal-Ex4/HSA-PLGA MP (120 μg of Pal-Ex4/mouse) was examined in non-fasted type 2 diabetic db/db mice. As shown in Fig. 6, the lowest glucose level (150.7±60.8 mg/dl) achieved by Pa-Ex4/HSA-PLGA MPs was significantly lower than that of the control (413.0±81.5 mg/dl, $p<0.001$) and the mean baseline value (445.5±67.3 mg/dl, $p<0.0001$). Also, the hypoglycemia induced by Pal-Ex4/HSA-PLGA MP remained significant until 5 days after administration (331.4±61.8 mg/dl), whereas the blood glucose levels (425.3±64.0 mg/dl) of Ex4/HSA-PLGA MP-treated mice were nearly recovered to their initial levels (485.3±59.1 mg/dl) at 2 days after administration (Fig. 6).

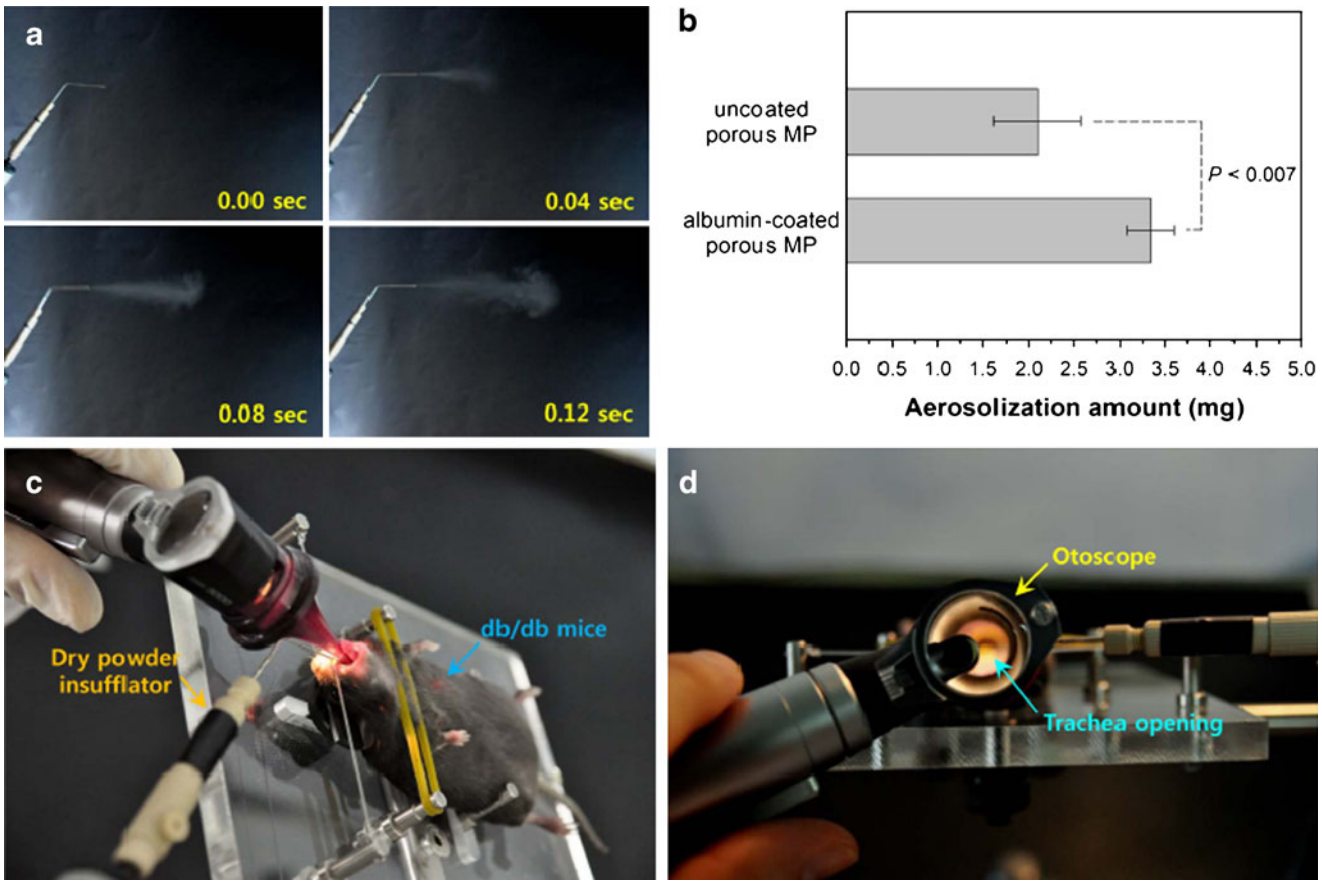

Fig. 4 **a** Aerosolization of albumin-coated porous PLGA MPs using a dry powder insufflator after the actuation. **b** Aerosolization efficiencies of HSA-PLGA MP and non-coated PLGA MP when using a dry powder insufflator. **c** Photograph of the administration of HSA-PLGA MP to db/db mice using a dry powder insufflator and an otoscope; **d** Magnified photograph of the tracheal opening of a db/db mouse.

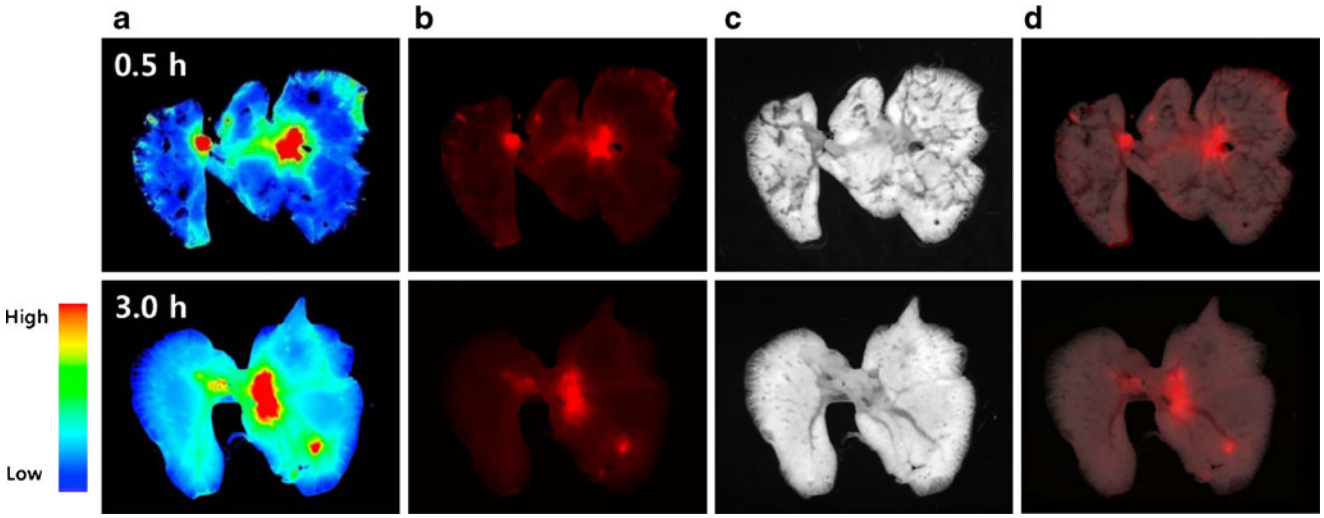

Fig. 5 Monitoring of lung deposition of HSA-PLGA MP in ICR mice (upper line 0.5 h and lower line 3 h post-administration). **a** RGB spectrum; **b** red; **c** optical; **d** merged with red/optical.

Cytotoxicity of PLGA MPs in Human Lung Epithelial Cells

The acute cytotoxicity of PLGA MPs on Calu-3 and A549 cells was evaluated. As shown in Fig. 7A, HSA-PLGA MPs exhibited relatively low cytotoxicity (97.4±5.5% and 90.6± 7.4% cell viability) to both cells over 48 h. Nevertheless, HSA-PLGA MPs appeared to have slight greater cytotoxicity in A549 cells than in Calu-3 cells. Although there is no statistical significance in cell viabilities caused by two MP groups, the average cytotoxicities by HSA-PLGA MP were obviously lower than those by non-coated PLGA MP in both cells.

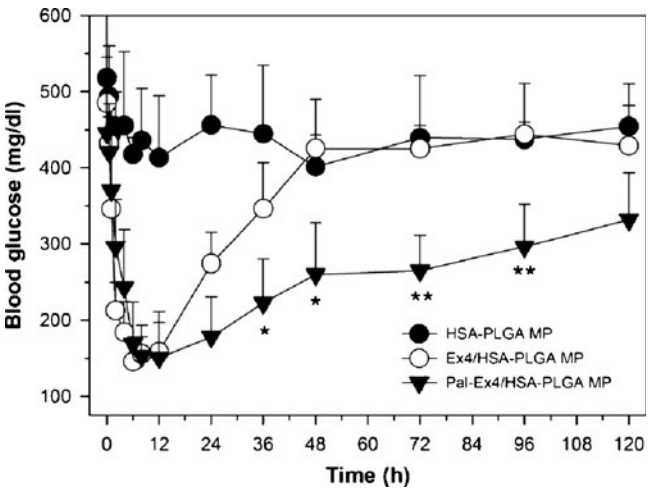

Fig. 6 Pulmonary hypoglycemic efficacies after the administration of Pal-Ex4/HSA-PLGA MP in non-fasted type 2 diabetic db/db mice. Data are means ± SDs. Significances were determined using the Student's *t*-test (*$p < 0.01$ over non-coated PLGA MP; **$p < 0.007$ over non-coated PLGA MP).

Histological Evaluation of Lung Tissues in db/db Mice Administered PLGA MPs

At 14 days after the pulmonary administrations of porous PLGA MPs to db/db mice, the histology of lung tissues and their tissue integrity were briefly checked by H&E staining. As shown in Fig. 7B, the lung tissue specimens treated with HSA-PLGA MP and Pal-Ex4/HSA-PLGA MP did not show significant histological difference *vs.* control in terms of tissue necrosis.

DISCUSSION

Unlike compact microparticles, porous microparticles are believed to offer a means of producing efficient inhalation delivery systems, which are capable of achieving three major goals, namely, alveolar deposition, phagocytosis avoidance, and sustained action. However, despite these benefits, the shortcomings associated with these pores are lower levels of encapsulation and rapid drug release. In particular, osmogens cause severe leaching of drugs through the micro-channels in microparticles (17). Also, initial drug release from porous microparticles is so great that some proteins, such as bovine serum albumin and human growth hormone, have shown >90% release in 12 h (11,16). In this regard, we sought to develop an alternative system that binds the active principle at the particle surface using a well-known albumin-binding concept. Palmityl-acylated exendin-4 (Pal-Ex4) was selected as an anti-diabetic model peptide to explore this developmental concept.

Cyclodextrins, such as sulfobutylether and hydroxy-propyl derivative, have been used to create pores during

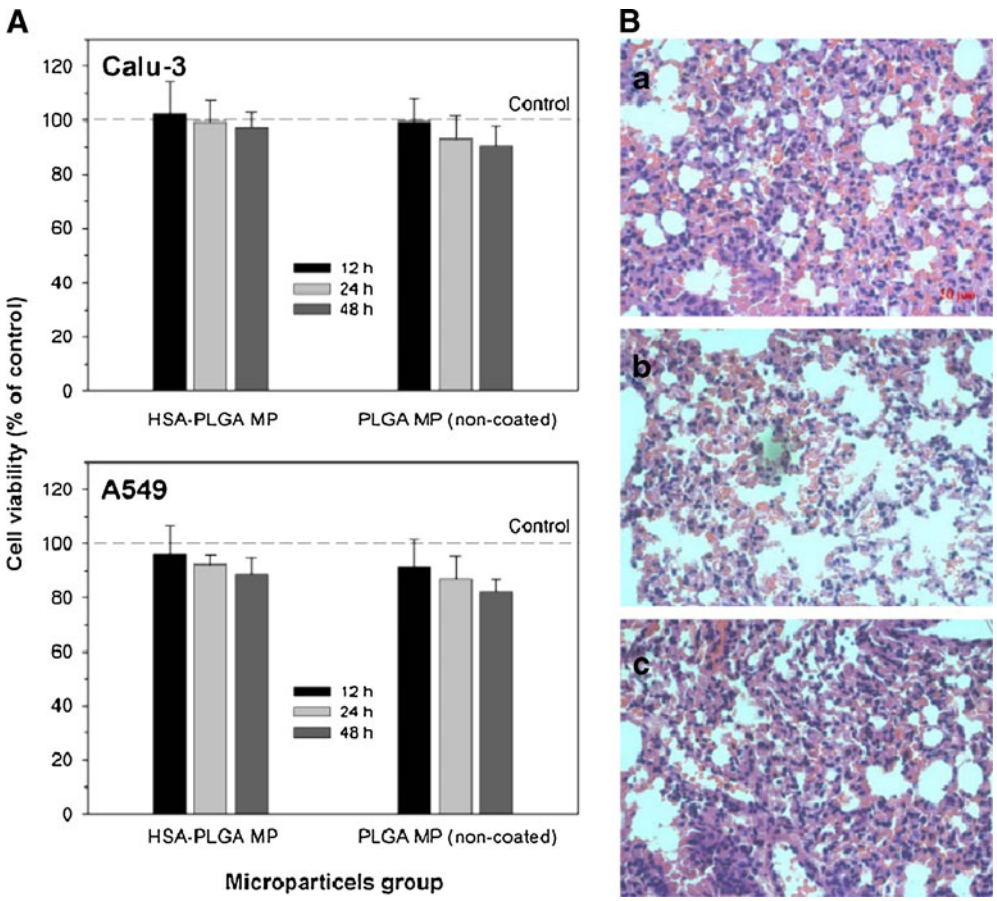

Fig. 7 **A** Effect of HSA-PLGA MP or non-coated PLGA MP (1 mg/ml each) on the viability of Calu-3 and A549 human lung epithelial cells. **B** Histology of lung tissues at 14 days after the pulmonary administration of PLGA MPs: *a*, control untreated; *b*, blank HSA-PLGA MP; *c*, Pal-Ex4/HSA-PLGA MP.

the fabrication of microparticles by utilizing their osmotic properties (10–12,21). In particular, hydroxypropyl-β-cyclodextrin (HPβCD) was reported to be a good porogen and stabilizer when used with proteins. In the present study, the amount of HPβCD (10~30 mg) was significantly higher than previously reported (11,21), and sodium chloride (20 mg) was also added to the primary emulsion to maximize the osmotic pressure. As a consequence, the PLGA MPs prepared were hollow with highly porous surfaces, like the hemi-shells previously reported (35) (Fig. 1). Actually, the mass median aerodynamic diameter (MMAD, density-based) of the Pal-Ex4/HSA-PLGA MP product formulated finally was determined to be much low at 3.2±0.3 μm as compared with its actual diameter (D_{50}: 17.2±2.1 μm) (Table I).

To achieve covalent albumin-coating on the surfaces of PLGA MPs, poly(ethylene-*alt*-maleic anhydride) (PEMA) was chosen as an emulsifying agent. Unlike polyvinyl alcohol (PVA), PEMA possesses a great number of carboxylate groups, which are able to induce the substitution of amines in proteins via EDC activation (Fig. 8). Although uncapped PLGA has carboxylate groups at one end, the number of carboxylate groups on the surface of MP is limited. In fact, the carboxylate modification efficiency of microsphere surfaces fabricated using PEMA

Table I Preparation Conditions and Characterization of Pal-Ex4/HSA-PLGA MP

	Polymer	Porogens		Solvents	Albumin-coating[b]	Drug loading[c]	Particle size [MMAD[d]]
	PLGA (lactic acid : glycolic acid) = 50:50 (Mw: 10,000)	HPβCD	NaCl	DP:CP ratio[a]			
Pal-Ex4/HSA-PLGA MP	300 mg	20 mg	20 mg	3.5%	220±23 μg	~38.1 μg	17.2±2.1 μm [3.2±0.3 μm]

[a] Volume ratio of dispersed phase (water/methylene chloride) : continuous phase (water)

[b] Covalently conjugated HSA amount (μg) per PLGA MP (mg)

[c] Bound Pal-Ex4 amount (μg) per HSA-PLGA MP (mg)

[d] Mass median aerodynamic diameter

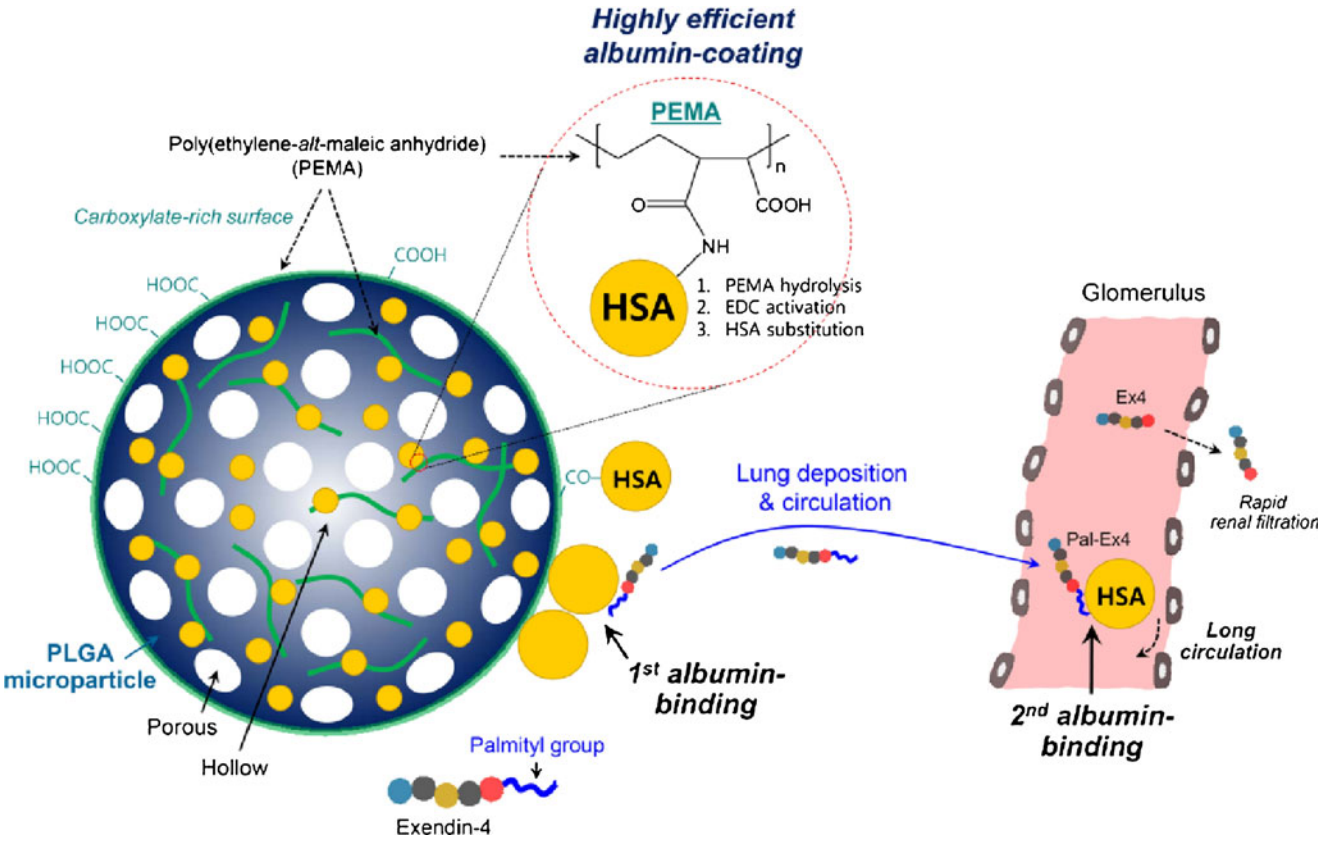

Fig. 8 An illustration for the proposed concept underlying the design of the albumin-coated porous PLGA MPs bound with Pal-Ex4.

has been reported to be >15 times than that of microspheres prepared using PVA (24). Due to this sufficiency of carboxylate groups from PEMA on microparticle surfaces, albumin efficiently reacted with MP surfaces.

Particle interactions also inhibit the delivery of porous microparticles to the lungs, and in the case of dry powders, particle aerosolization is greatly hampered by aggregation, which markedly reduces its mobility to the lungs (36–38), because particle separation is the most important measure of effective aerosol generation (39). Microparticles made of PLGA are prone to aggregate when emulsified despite the use of surface-active agents because PLGA is extremely hydrophobic. To prevent particle aggregation and improve particle aerosolization, many different type of carriers particles, such as sugars (e.g. lactose, mannitol, trehalose), lipids (e.g. DPPC, cholesterol), amino acids (e.g. leucine), surfactants (e.g. bile salts), and proteins (e.g. albumin), have been used (35). However, in the present study, an albumin-coating was favored because it decreases exposure of the PLGA backbone and causes inter-particulate electric repulsion due to its substantial negative charge. Our results demonstrate that the aerosolization of HSA-PLGA MPs was ~1.6-fold better than that of non-coated PLGA MPs (Fig. 4B). Furthermore, albumin has an additional merit that it is safe and, in particular, does not accumulate when administered chronically (39).

Because of the pharmaceutical benefits for inhalation, HSA-PLGA MPs were successfully delivered to mouse lungs. However, unlike our expectation, lung images did not show perfect direct deposition in alveoli (Fig. 5), which was attributed to the conditions of mice during administration, because mice were anaesthetized supine and breathing was weak. Perfect inhalation is reported to be troublesome to untrained adult humans. Accordingly, under this experimental condition, the microparticles seemed to be initially deposited in the central lung region by impaction, and then gradually moved into the lower part of the lungs due to sedimentation and Brownian diffusion, as reported previously (7,32).

Human serum albumin has five fatty acid binding sites with binding constants in the range 10^7–10^8 (L/mol) (40), which enables the tight binding of some fatty acid-modified peptides to circulating albumin *in vivo* (26,27). Fatty acid-conjugated peptide derivatives, such as a myristic acid-insulin (Levemir®, Novo Nordisk) and a palmitic acid-glucagon-like peptide-1 analogue (Victoza®, Novo Nordisk), based on this binding of fatty acids by albumin, have been recently approved by FDA. These products have

extended therapeutic durations *in vivo*, as compared with their parent peptides. Furthermore, exendin-4 derivatives modified with fatty acids (e.g., myristic/palmitic acid) or cholic acids have been documented to have protracted hypoglycemic efficacies in fasted and non-fasted db/db mice (26,27). In this study, palmityl acylated exendin-4 was designed to bind to two different albumins. First, the binding to surface-coated albumin produced acceptable Pal-Ex4 loading (>3.8%) and sustained Pal-Ex4 release for 7 days (Fig. 3), and second, the binding to circulating plasma albumin extended the hypoglycemic efficacy of Pal-Ex4 (~5 days) (Fig. 6). Likewise, such dual albumin-bindings might result in the long hypoglycemia by Pal-Ex4/HSA-PLGA MP in the pulmonary route (Fig. 8).

Safety of inhaled particles is one of the most mandatory consideration factors in the pulmonary delivery systems because these particles may bring some harmful results to lungs in terms of inflammatory or immune response and toxicity (41–43). Therefore, the cytotoxicity and histological damage induced by PLGA MPs were examined *in vitro* and *in vivo*. Despite the relatively high concentration (30), HSA-PLGA MP was found to have little cytotoxicity to Calu-3 and A549 lung epithelial cells, and moreover, the histological change of lung tissues of db/db mice administered HSA-PLGA MP or Pal-Ex4/HSA-PLGA MP seemed insignificant when compared with the control group. Consequently, although such investigations belong to a basic toxicity test, our particles are believed to be quite safe, to a certain degree, on the basis of these toxicity results.

CONCLUSION

In this study, we described a new prototype of albumin-coated PLGA MP with surface-bound palmityl acylated exendin-4 (Pal-Ex4/HSA-PLGA MP). This MP was found to be hollow as well as porous due to the combination use of two osmotic porogens such as HPβCD and sodium chloride. The porosity and hollowness of the MP resulted in the relatively small aerodynamic size (~3.2 μm, MMAD) appropriate for aerosolization and insufflations into the lungs of mice, despite its large geometric diameter (~17.2 μm). The use of PEMA, instead of the conventional emulsifier PVA, also provided the MP with carboxylate-rich surface, which favors covalent albumin-modification, and this coating significantly increased the aerosolization efficiency, probably by reducing the inter-particulate attraction between the PLGA MPs. On the other hand, the use of palmityl acylated exendin-4 is believed to bind to two albumin types on the microparticle surfaces and in circulating plasma, which provided acceptable drug-loading and sustained drug-release *in vitro* and also resulted in much extended hypoglycemic duration in type 2 diabetic rodents. Accordingly, we consider that the described inhalation delivery system using this albumin-coated PLGA MP is a promising candidate treatment for type 2 diabetes.

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