



Highly porous large poly(lactic-co-glycolic acid) microspheres adsorbed with palmityl-acylated exendin-4 as a long-acting inhalation system for treating diabetes

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ABSTRACT

A porous large poly(lactic-co-glycolic acid) (PLGA) microspheres (MS) adsorbed with palmityl-acylated exendin-4 (Ex4-C₁₆) was devised as an inhalation delivery system. The porous MS was prepared by a single o/w emulsification/solvent evaporation method using extractable Pluronic F68/F127, and its fabrication and formulation conditions were carefully optimized. Results show that the prepared MS was in the appropriate size range for inhalation and contained many surfaces and internal pores meaning low aerodynamic density. Ex4-C₁₆ was more efficiently adsorbed onto porous PLGA MSs than native exendin-4, and an approximately 5% loading of Ex4-C₁₆ onto this porous MS (RG504H) was achieved. This optimized porous MS was found to be efficiently deposited throughout the entire lungs of mice including alveoli region. Furthermore, this porous MS adsorbed with Ex4-C₁₆ (approx. 100 µg/mouse) displayed much protracted hypoglycemic efficacy in non-fasted type 2 diabetic db/db mice. Porous PLGA MS with adsorbed Ex4-C₁₆ showed the dual-advantages of (i) sustained release and acceptable drug-loading due to strong hydrophobic interaction and (ii) longer *in vivo* pulmonary hypoglycemic duration due to albumin-binding by the palmityl group. We consider that this new prototype of porous PLGA MS has considerable pharmaceutical potential as a type 2 anti-diabetic inhalation treatment.

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1. Introduction

Pulmonary delivery by inhalation is a practical alternative for the systemic delivery of therapeutic peptides or proteins that must otherwise be injected, due to its feasibility and high bioavailability [1,2]. These advantages are attributed to the unique physiological features of the lung, i.e., its large surface area [3], thin alveolar epithelium, elevated blood flow, and the avoidance of a first hepatic pass [4]. Despite the failure case of Exubera[®] (Pfizer), the pulmonary route is still the most attractive because it offers non-invasiveness and incomparable bioavailability as compared with other routes, such as, the oral, rectal, buccal, and nasal routes [1,5].

Porous particles are viewed as effective tools especially for the inhalation of peptide and protein drugs. In general, therapeutic dry

powders have been made with mass densities of $\sim 1 \pm 0.5 \text{ g/cm}^3$ and mean geometric diameters of 1–5 µm [6]. However, particles as heavy as $\sim 1.0 \text{ g/cm}^3$ are likely to deposit in inhalation devices and in the upper airways, and small particles of 1–2 µm are vigorously cleared by macrophages [1,6,7], and both of these processes seriously reduce bioavailability. Hence, based on the mass median aerodynamic diameter (MMAD) concept, large but light-porous particles with a low mass density of $< 0.4 \text{ g/cm}^3$, an aerodynamic diameter of 1–5 µm, and a large geometric diameter of 5–30 µm have been proposed to circumvent the pharmaceutical challenges presented by alveoli deposition and phagocytosis escapement [8–12].

Such porous polymeric microparticles are prepared using several unique pore-forming agents (porogens). Osmogens (e.g. salts or cyclodextrins) distributed in the internal phase create pores due to osmotic pressure differences between the internal and external phases [9–11]. Extractable porogens (e.g. Pluronics and fatty acid salts) make pores by the time difference between PLGA hardening and the extraction of porogens from an oil-phase using

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water [13–15]. In addition, effervescent agents (e.g. ammonium bicarbonate) can form many gas bubbles, and hence, pores [12,16].

However, on account of the original problem of pore-existence on its surface, porous microspheres are compelled to have quite considerable empty volume. Therefore, it has much smaller inner rooms of PLGA matrix to incorporate drugs. In addition, incorporated drugs are likely to be released rapidly because the PLGA matrix frame is thin, which attenuates the sustained-release effect required. On the other hand, peptides have short circulating half-lives *in vivo*, and thus, display limited therapeutic durations after absorption through alveoli. Exendin-4 is a potent glucagon-like peptide-1 (GLP-1) agonist. Although this peptide has many therapeutic benefits for diabetes, such as glucose-dependent insulin release, β -cell proliferation, and appetite suppression etc., it is known to have a relatively short half-life (2–4 h) *in vivo* [17–20].

In this study, we sought to develop a porous PLGA microparticle type for anti-diabetic inhalation with the dual-advantages of (i) sustained release and (ii) extended half-life *in vivo*. For this purpose, palmityl-acylated exendin-4 (Ex4-C₁₆) was used. We hypothesized that the sixteen carbons of palmitic acid would aid strong Ex4-adsorption onto the hydrophobic surfaces of porous PLGA microspheres, and that Ex4-C₁₆ would survive much longer *in vivo* by binding to human serum albumin (HSA) after entering the systemic circulation. Highly porous PLGA microparticles were prepared using a Pluronic F68/127 mixture, and their sizes and morphologies were investigated. The adsorption/release profile and *in vivo* deposition/efficacy of Ex4-C₁₆ after intrapulmonary administration using dry powder insufflator were also evaluated.

2. Materials and methods

2.1. Materials

Exendin-4 and N-hydroxysuccinimide-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: approx. 10,000–43,000 Da) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA, Mw: 30,000–70,000 Da) was purchased from Sigma–Aldrich. Pluronic F68 or F127 was donated by BASF (Seoul). Cy5.5 NHS ester dye was purchased from GE Healthcare (Piscataway, NJ). All other reagents, unless otherwise specified, were obtained from Sigma–Aldrich.

2.2. Experimental animals

Type 2 diabetic C57BL/6 db/db mice (male, 4–5 weeks old) were purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Male ICR mice weighing 20–22 g were purchased from the Hanlim Experimental Animal Laboratory (Seoul, Korea). 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.), allowed food and water *ad libitum*, and acclimatized for 2 weeks. This study was approved by the Ethical Committee on Animal Experimentation at Pusan National University.

2.3. Preparation and optimization of porous PLGA microspheres

Porous PLGA microspheres (MS) were prepared using Pluronic F68 or F127, as extractable porogens, using a modification of a previously described procedure [14,15]. Briefly, PLGA (lactic acid: glycolic acid = 50:50, 300 mg) and Pluronic F68 or F127 (700 mg) were dissolved in 3–5 ml dichloromethane 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 polymer solution obtained was then emulsified in 100 ml of ice-cold deionized water (DW) containing 0.5% (w/v) of PVA for 2 min at 2000–6000 rpm using a Silverson Laboratory Mixer (model L4RT) with a 3/4-inch head (Silverson Machines, Inc. East Longmeadow, MA, USA). The resultant emulsion solution was further allowed to evaporate the solvent under gentle magnetic stirring under an air current at predetermined temperatures for 6 h. The solidified MS was then harvested by centrifugation, washed three times with D.W., and freeze-dried. The mean particle size of porous PLGA MS was determined in a water suspension using a laser diffraction particle size analyzer (Mastersizer, Malvern Instruments, USA). Especially for the *in vivo* samples, the porous PLGA MS prepared was wet-sieved very slowly using a 20 μ m sieve (Nonaka Rikaki Co., Ltd., Japan) in order to remove some possible big particles or physical aggregates.

2.4. Preparation and characterization of palmityl-acylated exendin-4s (Ex4-C₁₆)

Palmityl-acylated exendin-4 (Ex4-C₁₆) was prepared using a modification of a previously described procedure [21–24]. Briefly, a 10 mg of exendin-4 was mixed with 1.3 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 reversed-phase high-performance liquid chromatography (RP-HPLC) on a LiChrospher 100 RP-18 column (250 × 4.0 mm, 5 μ m, 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). The following gradient profile was used: 30–50% B for 10 min and 50–90% B for 20 min. Eluates were monitored at 215 nm, and the fraction corresponding to Ex4-C₁₆ was collected, dried under nitrogen, and stored in 10 mM phosphate buffer saline (PBS, pH 7.4) or 10 mM acetate buffer (pH 4.0) at 4 °C until needed. Molecule sizes of Ex4-C₁₆ were measured by using a Zetasizer Nano-S90 (Malvern Instruments, USA) with a He–Ne laser beam at 633 nm, and a fixed scattering angle of 90°.

2.5. Scanning electron microscopy morphology of porous PLGA MS

The surface morphology of porous MS was investigated by scanning electron microscopy (SEM, Hitachi S3500N, Japan). Dry MS was attached to specimen stubs using double-side tape and sputter-coated with gold–palladium in an argon atmosphere using a Hummer I sputter coater (Anatech Ltd. St. Alexandria, VA, USA). Average MS sizes and pore diameters were determined by observing at least 30 particles.

2.6. Confocal laser scanning microscopy (CLSM) visualization of Ex4-C₁₆-adsorbed porous PLGA MS

The adsorption of Ex4-C₁₆ onto porous PLGA MS was observed by CLSM after fluorescent derivatization. A portion (1 mg) of Ex4 or Ex4-C₁₆ was added to 2 M equivalents of fluorescein or rhodamine-NHS (Pierce, Rockford, IL, USA) in 50 mM of phosphate buffer (1 ml, pH 7.0), and left for 3 h at room temperature. Mixtures were dialyzed for 24 h versus 50 mM PBS (pH 7.4) or 50 mM acetate buffer (pH 4.0) using a dialysis kit (Mw cutoff = 3500, Gene Bio-Application Ltd., Israel). Concentrations of fluorescent-tagged Ex4 derivatives were measured using a BCA protein assay kit (Pierce, Rockford, IL). Separately, 0.6 ml aliquots of fluorescent-tagged Ex4 derivatives (30 μ g/ml) were added to 0.4 ml of porous MS (1 mg) previously hydrated in 50 mM PB (pH 7.4) or 50 mM acetate buffer (pH 4.0) for 6 h at room temperature. After removing free fluorescent agents by washing three times with each buffer, fluorescence images of porous MS were visualized by CLSM (Carl Zeiss Meta LSM510, Germany).

2.7. Adsorption monitoring of Ex4-C₁₆ onto porous PLGA MS

Aliquots (0.6 ml) of Ex4 or Ex4-C₁₆ (each 30 μ g/ml) were added to 0.4 ml of porous MS (~1.5 mg) prepared with PLGAs of different Mw and hydrated in 50 mM PB (pH 7.4) or 50 mM acetate buffer (pH 4.0) for 4 h at room temperature. Pellets were obtained by centrifugation at 10,000 rpm for 5 min, and the supernatant concentrations were measured using a BCA protein assay kit. Amounts adsorbed were calculated by subtracting amounts in supernatant from initial amounts.

2.8. Monitoring of Ex4-C₁₆ release from porous PLGA MS

Aliquots (50 ml) of rhodamine-derivatized Ex4 or Ex4-C₁₆ (30 μ g/ml) were added to porous PLGA MS (30 mg, RG504H) previously hydrated in 50 mM acetate buffer (pH 4.0) at room temperature. After 6 h, pellets were then collected by centrifugation, washed three times with D.W., and freeze-dried. Ex4-C₁₆-adsorbed porous MS (5 mg) was then 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 the fluorescence intensities were measured at excitation and emission wavelengths of 544 and 576 nm, respectively. The cumulative release amount was expressed as a percent vs. initial loading amount at each time point. All samples were prepared and analyzed in triplicate.

2.9. Pulmonary administration of porous PLGA MS into mice

The pulmonary delivery of porous PLGA MS was performed using a modification of a previously described procedure [25,26]. In brief, male ICR or db/db mice were anesthetized with a single intraperitoneal (i.p.) injection of tiletamine (20 mg/kg). Especially, tiletamine was used at a minimum dose to induce short anesthesia adequate for the pulmonary administration. Freeze-dried porous PLGA MS (approx. 2 mg) was directly administered into the lungs via trachea of mice using an assembly of insufflator device (DP-4M) and air pump (AP-1) (Penn-Century, Inc., Philadelphia, PA). The visualization of the tracheal opening is obtained with the otoscope set (Heine Mini3000, Germany) attached to the mouse speculum.

2.10. Evaluation of lung deposition of porous PLGA MS

To evaluate the lung deposition, a portion (~2 mg) of porous PLGA MS surface-modified with cy5.5 dye was administered into the lungs of ICR mice. The mice were sacrificed at 0.5, 3, 6 h after the administration, and their entire lung lobes were excised along with the trachea. The excised lungs were visualized by Image Station 4000 MM (Kodak) (emission wavelength: 700 nm).

2.11. Hypoglycemic efficacy evaluation after the pulmonary administration of porous PLGA MS adsorbed with Ex4-C₁₆ in non-fasted type 2 diabetic db/db mice

To evaluate the pulmonary hypoglycemic efficacy, a portion (~2 mg) of porous PLGA MSs adsorbed with Ex4 or Ex4-C₁₆ (approx. 100 µg/mouse each) was administered into the lungs of male db/db mice weighing 30–32 g (*n* = 9/group), which were previously anesthetized with an i.p. injection of tiletamine (20 mg/kg). Blank porous PLGA MS (~2 mg) was also administered to check the normal blood glucose level. As previously described [27–29], mice were kept under non-fasting conditions with free access of water and food until the end of experiment. In addition, 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, 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). Hypoglycemic efficacies, which are expressed as total hypoglycemic degrees (HD_{total}; % vs. control group), were calculated using: $[(AUC_{\text{control, 0-120 h}} - AUC_{\text{test, 0-120 h}}) / AUC_{\text{control, 0-120 h}}] \times 100$.

2.12. Histological evaluation of lung tissues of db/db mice

At 2 weeks after the pulmonary administrations of porous PLGA MS adsorbed with Ex4-C₁₆ or blank porous PLGA MS, the lungs of db/db mice in each group were harvested after sacrifice. The lungs were then fixed with 10% formalin and blocked by paraffin. Cross-sliced sections of the lungs were stained with hematoxylin and eosin (H&E), and further examined by light microscopy. The histopathology of the lung tissues was briefly observed and compared with that of a PLGA MS-untreated db/db mouse with same week age.

2.13. Data analysis

Data are presented as means ± SDs. Statistical significances were determined using the One-Way ANOVA test. *p*-values of <0.05 were considered statistically significant.

3. Results

3.1. Preparation and characterization of porous PLGA MS

Porous PLGA MS was prepared using a single o/w emulsification/solvent evaporation method using F68 or F127 as porogen, varying DP/CP ratio, homogenization speed, and evaporation temperature. Porous PLGA MSs were found to have many surface pores (Fig. 1B and C), versus non-porous PLGA MS (Fig. 1A). In addition, cross-sectional SEM images revealed the internal network structure of interconnecting pores (Fig. 1D). As shown in Fig. 2A-1 and A-2, F68 was found to form smaller pores than F127. Their combined use resulted in the required porosity of 0.5–2 µm and a relatively smaller particle size of ~18 µm (Fig. 2A-3), versus their individual uses. Increasing DP/CP ratio did not markedly affect pore size, but a 3% formulation was found to have soft surface (Fig. 2B). Increasing homogenization speed caused a gradual decrease in particle size, and the particle size was ~12 µm at 6000 rpm (Fig. 2C). As shown in Fig. 2D, the optimum evaporation temperature was 25–40 °C; porous particles produced by evaporation at 50 °C were large (>40 µm) and had a rough surface.

3.2. Characterization of Ex4-C₁₆

As shown in Fig. 3A, the hydrophobicity of Ex4 seemed to significantly increase after palmitic acid (C₁₆) conjugation, and the retention time of Ex4-C₁₆ was 9.22 min as compared with 5.28 min for Ex4. The molecule size of Ex4-C₁₆ increased with increasing its concentration (Fig. 3B). At concentrations below 30 µg/mL, Ex4-C₁₆ particles were ~4 nm, but increased to >100 nm at concentrations >40 µg/mL, and reached ~200 nm at 100 µg/ml. Also, the size distribution became much broader at high concentrations, suggesting micelle formation (Fig. 3C).

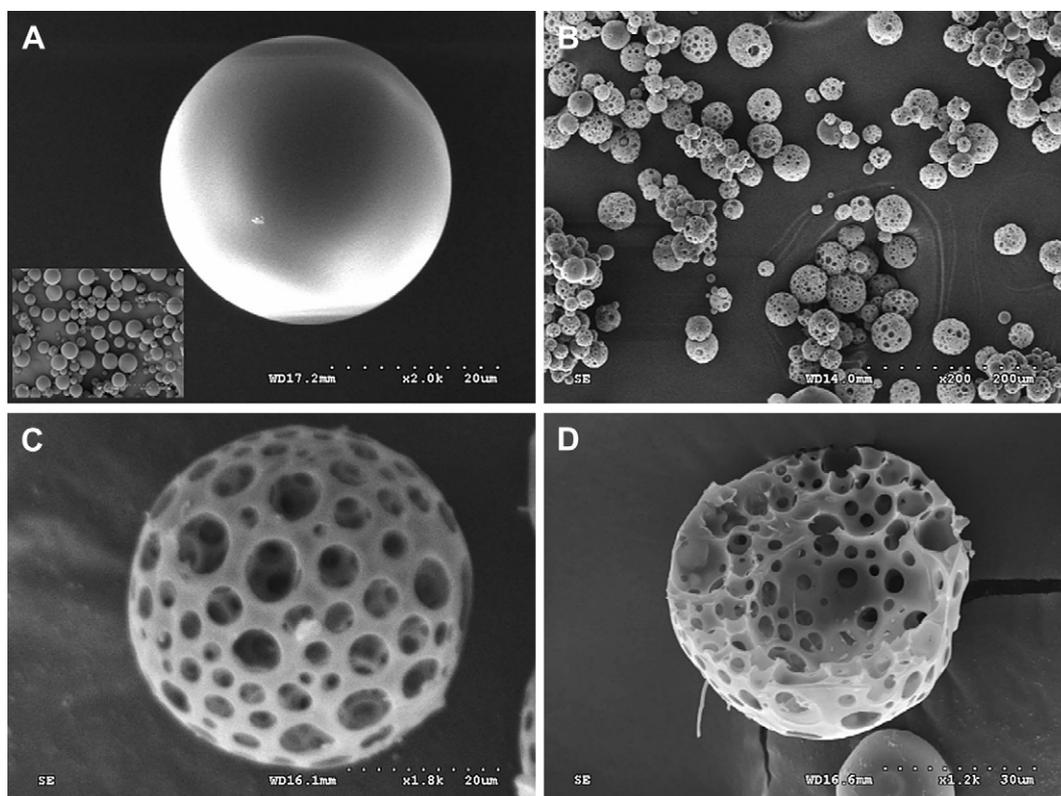


Fig. 1. Morphology of porous PLGA MS observed by scanning electron microscopy (SEM). (A) Surface of non-porous PLGA MS, (B) porous PLGA MS, (C) surface and (D) cross-section of porous PLGA MS.

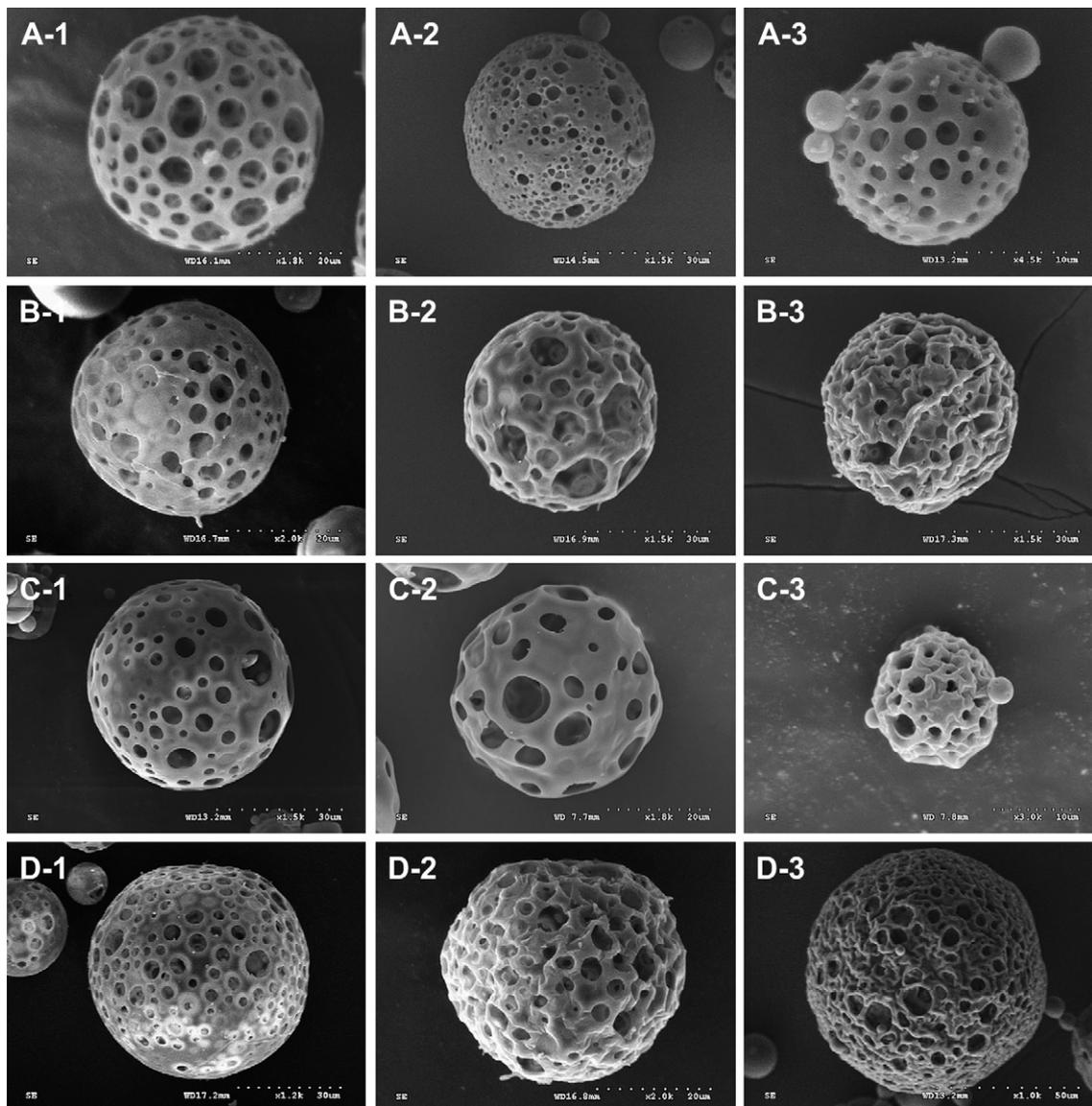


Fig. 2. Morphology of porous PLGA MS prepared under different conditions. (A) porogen type – A-1 F127, A-2 F68, A-3 F127/F68; (B) DP/CP ratio – B-1 3%, B-2 4%, B-3 5%; (C) homogenization speed – C-1 2000 rpm, C-2 3000 rpm, C-3 6000 rpm; (D) evaporation temperature – D-1 25 °C, D-2 40 °C, and D-3 50 °C.

3.3. CLSM images of Ex4-C₁₆ adsorption onto porous PLGA MSs

The adsorption of fluorescein or rhodamine-tagged Ex4-C₁₆ onto porous PLGA MS was visualized by CLSM, and compared with that of native Ex4. As shown in Fig. 4A-a/4A-d and 4A-a', whereas rhodamine-Ex4 seemed to be hardly adsorbed onto the RG504H porous MS at pH 4.0, rhodamine-Ex4-C₁₆ was efficiently adsorbed onto this MS. Particularly, unlike Ex4, rhodamine-Ex4-C₁₆ was also adsorbed at pH 7.0 (Fig. 4A-b and 4A-b'). In addition, even at the RG502H porous MS with lower hydrophobicity than RG504H porous MS, fluorescein-Ex4-C₁₆ appeared to be highly adsorbed, showing strong green fluorescence at pH 7.0, whereas Ex4-treated RG502H porous MS did not (Fig. 4A-c/A-d' and A-c').

3.4. Ex4-C₁₆ adsorption onto porous PLGA MSs

Fig. 4B-a demonstrates that increasing the Mw of PLGA from RG502H to RG504H resulted in a gradual increase in the adsorption of Ex4-C₁₆ onto porous MS, irrespective of medium pH. In

particular, the adsorption of Ex4-C₁₆ at pH 4.0 was significantly greater than at pH 7.0 ($p < 0.01$), and 100.8 ± 0.2 and $75.8 \pm 1.1\%$ of Ex4-C₁₆ was adsorbed onto porous RG504H MS at pH values of 4.0 and 7.0, respectively (Fig. 4B-b). The adsorption degree of Ex4-C₁₆ onto porous RG504H MS was much greater than that onto the non-porous RG504H MS at pH 4.0 ($7.1 \pm 2.9\%$, $p < 0.0001$). Furthermore, adsorption amounts of Ex4-C₁₆ at pH 4.0 were significantly greater than those of native Ex4 in every case. As shown in Fig. 4B-b, the adsorption of Ex4-C₁₆ was 6.7-fold greater than that of Ex4 on porous RG504H MS at pH 4.0 (100.8 ± 0.2 vs. $15.3 \pm 2.1\%$).

3.5. Ex4-C₁₆ release from porous PLGA MSs

Adsorbed Ex4-C₁₆ was slowly released from porous PLGA MS (RG504H) in 10 mM PBS (pH 7.4), when compared with native exendin-4 (Fig. 4B-c). The initial burst release of Ex4-C₁₆ at 6 h was found to be only $31.2 \pm 4.2\%$, whereas almost all native Ex4 ($94.5 \pm 3.2\%$) from porous PLGA MS was released to the

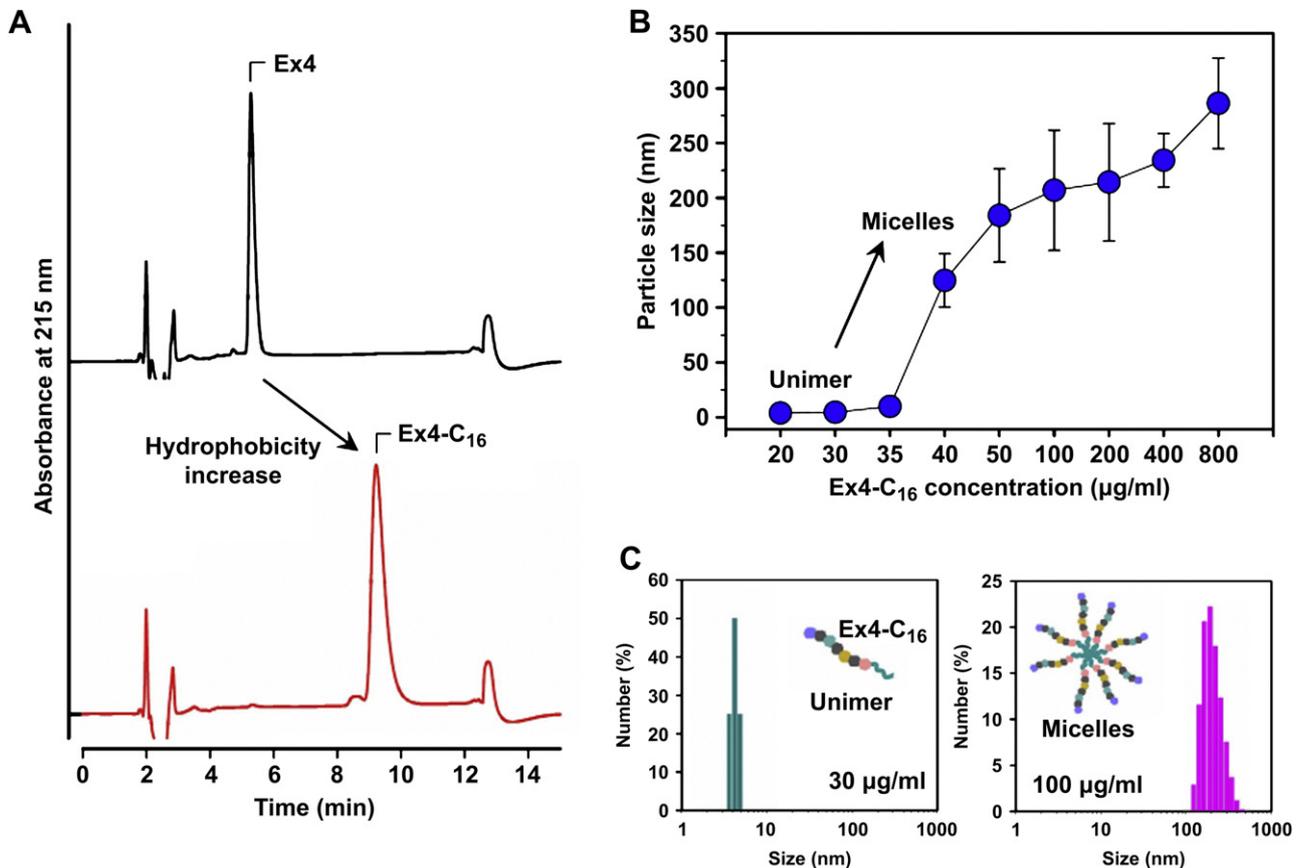


Fig. 3. Characterization of Ex4-C₁₆. (A) Reversed-phase HPLC chromatograms of Ex4 and Ex4-C₁₆, (B) molecule sizes of Ex4-C₁₆ produced at different concentrations, (C) molecular size histograms of Ex4-C₁₆ at 30 and 100 µg/ml.

supernatant. Furthermore, the release of Ex4-C₁₆ appeared to gradually increase until 5 days.

3.6. Lung deposition monitoring of inhaled porous PLGA MS

The images for lung deposition were visualized at the time points of 0.5, 3, 6 h after pulmonary administrating porous PLGA MS adsorbed with Ex4-C₁₆ into ICR mice. As shown in Fig. 5A–D, the lung images were represented as spectra of RGB, red, optical or optical merged with red. At 0.5 h after the administration, most porous PLGA MS seems to be located in the middle part of respiratory duct. However, porous PLGA MS gradually spread into the lower part of the lungs, and the porous PLGA MS were totally deposited throughout the entire lung lobes including alveoli at 6 h.

3.7. Evaluation of the pulmonary hypoglycemic efficacy of porous PLGA MS adsorbed with Ex4-C₁₆ in non-fasted db/db mice

The hypoglycemic efficacy induced by the porous PLGA MS adsorbed with Ex4-C₁₆ (100 µg/mouse), which was administered into lungs, was examined in non-fasted type 2 diabetic db/db mice. As shown in Fig. 6B, the bottom glucose level (155.6 ± 41.9 mg/dl) achieved by the porous PLGA MS (Ex4-C₁₆) was significantly lower than those of control (406.0 ± 67.9 mg/dl, $p < 0.005$) and its initial level (466.4 ± 72.6 mg/dl, $p < 0.001$). Its total hypoglycemic degree (HD_{total}: 43.4 ± 6.7%) was much greater than that of porous PLGA MS (Ex4) (HD_{total}: 15.9 ± 4.0%, $p < 0.01$). Also, the hypoglycemia induced by porous PLGA MS (Ex4-C₁₆) significantly continued until 5 days after the administration, showing a still lower blood glucose

level (290.3 ± 98.0 mg/dl) than the other groups at 5 days, whereas the hypoglycemia by porous PLGA MS (Ex4) was negligible from 2 days (Fig. 6).

3.8. Histological evaluation of lung tissues in db/db mice administered porous PLGA MS adsorbed with Ex4-C₁₆

At 2 weeks after the pulmonary administrations of porous PLGA MSs to db/db mice, the histology of lung tissues and their tissue integrity were briefly examined by H&E staining. As shown in Fig. 6C, the lung tissue specimens treated with porous PLGA MSs (both with and without Ex4-C₁₆) were not significantly different with that of control lung tissues.

4. Discussion

Porous MSs are considered to be an effective means of delivering an intrapulmonary drug because they are light enough to aerosolize and penetrate deeply into the lungs, and also because their large sizes prevent phagocytosis by lung macrophages. However, the drug loading is lower and drug release is apt to be faster than for plain non-porous MSs. Particularly, when osmogens or extractable porogens are used, considerable amount of drug is leached out through the large channels formed when porogens are extracted [8–10,15]. In this respect, a different type of porous MS with an adsorbed not an incorporated fatty acylated drug was achieved to solve these problems. Fatty acylation of peptides is believed to induce drug adsorption onto porous MS via hydrophobic interactions and to extend circulation half-life by promoting HSA-binding.

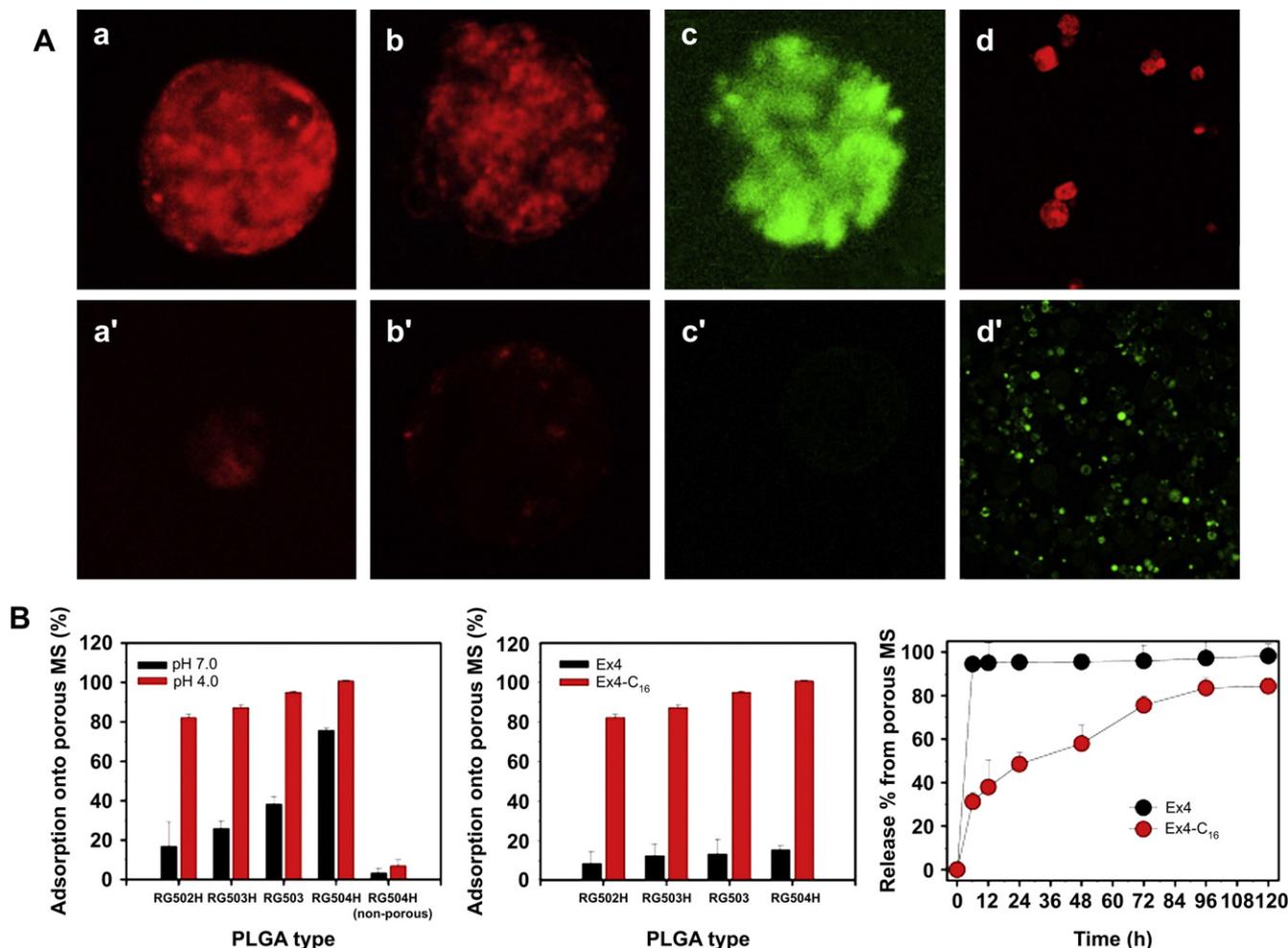


Fig. 4. (A) Confocal laser scanning microscopy of Ex4-C₁₆-adsorbed porous PLGA MS (RG504H): a, rhodamine-Ex4-C₁₆ (pH 4.0); a' rhodamine-Ex4 (pH 4.0); b' rhodamine-Ex4-C₁₆ (pH 7.0); b', rhodamine-Ex4 (pH 7.0); c' fluorescein-Ex4-C₁₆ (pH 7.0, RG502H); c', fluorescein-Ex4 (pH 7.0, RG502H); d' rhodamine-Ex4-C₁₆ (pH 4.0); d', fluorescein-Ex4-C₁₆ (pH 7.0). (B) Adsorption and release characteristics of Ex4-C₁₆ in porous PLGA MS: a, effect of pH on the adsorption of Ex4-C₁₆ onto porous PLGA MS; b, comparison of adsorptions of Ex4 and Ex4-C₁₆ at pH 4.0; c, release profiles of Ex4 and Ex4-C₁₆ from porous PLGA MS.

In the present study, exendin-4 was selected as an anti-diabetic model peptide to explore this hypothesis.

Of the various well-established porogens, Pluronics were used to make pores in PLGA MSs, because they are known to have appropriate HLB values and high water extractabilities [14,15]. Pluronic F68 and F127 both made PLGA MS highly porous, but F68 appeared to make smaller pores than F127, possibly due to its small molecular weight (Mw: 8400 vs. 12,600, respectively). Disperse phase (DP)/continuous phase (CP) ratios in the range 3–5% did not make a significant difference to the porosity in our system. Other preparative factors like homogenization speed and temperature produced some morphological changes. As shown in Table 1, the final conditions used were: a 1:1 porogen formulation of F68 and F127, a 3% DP/CP ratio, a 3000 rpm of homogenization speed, and an evaporation temperature of 40 °C. The actual diameters and calculated median aerodynamic diameters of the porous PLGA MS (density-based) prepared using these conditions were in the ranges of 16.2 ± 2.3 and 4.3 ± 0.5 μm , respectively. These values were considered appropriate for inhalation, on the basis of our and other groups' previous reports [6–10].

PLGA is hydrophobic, and thus, hardly soluble in water, and has a free carboxylic acid at one terminal, if not capped [15]. Therefore, this polymer is likely to accommodate hydrophobic moieties or positive charged molecules. Furthermore, it is known that

electrostatic and hydrophobic interactions and hydrogen bonding play critical roles in protein adsorption [13]. On the other hand, exendin-4 carries a substantial negative charge at neutral pH because it has a pI value of 4.7, and does not have a critical hydrophobic center on its surface. Accordingly, we expected that exendin-4 would show a negligible tendency to interact with the surface of PLGA MSs. Unlike native exendin-4, Ex4-C₁₆ efficiently adsorbed onto the surface of porous PLGA MS, presumably because of the presence of the hydrophobic palmitic entity. This adsorption was achieved well even at the porous PLGA MS made of RG502H, of which hydrophobicity is the lowest due to its low Mw. Furthermore, the finding that the adsorption of Ex4-C₁₆ at pH 4.0 was greater than at pH 7.0 indicated that charge interactions are also associated with adsorption, because Ex4 becomes charge-neutral at pH 4.0. Also, the overall adsorption of Ex4-C₁₆ onto porous MS was about ~10 times greater than its adsorption onto non-porous MS, probably because its surface area is greater.

However, at times the palmitic carbon chain of Ex4-C₁₆ interfered with its adsorption onto PLGA MS because it forms micelles, which hide these carbons into the core and thus reduces its adsorption at >40 $\mu\text{g}/\text{ml}$ (Fig. 3), as we reported previously [21]. Accordingly, the adsorption process was maintained at <30 $\mu\text{g}/\text{ml}$ of Ex4-C₁₆ to prevent the micelle formation, and then the porous PLGA MS adsorbed with Ex4-C₁₆ was finally lyophilized for further

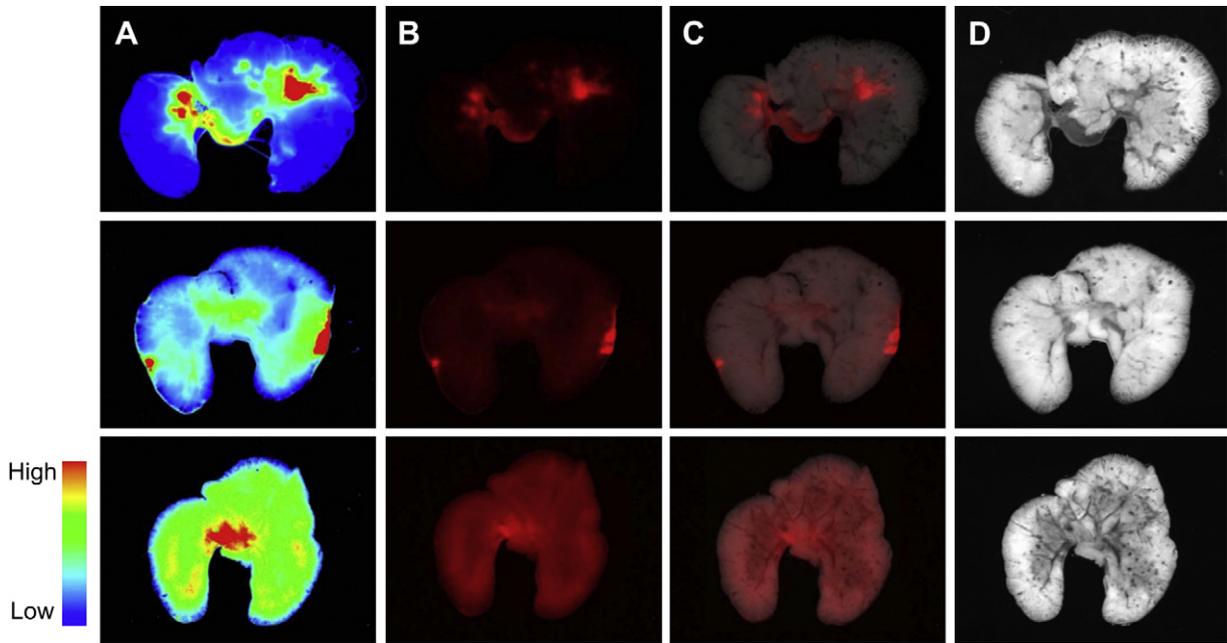


Fig. 5. Monitoring of lung deposition of porous PLGA MS in ICR mice (upper line – 0.5 h, middle – 3 h; lower – 6 h). (A) RGB spectra (B) red spectra (C) merged with red/optical (D) optical.

experiments. Under this carefully optimized condition, the actual loading of Ex4-C₁₆ increased to ~5%, which is comparable to the encapsulation efficiency of peptides or proteins into non-porous PLGA MS (Table 1).

The release pattern of adsorbed Ex4-C₁₆ from porous PLGA MS (RG504H) was found to be acceptable (Fig. 4B-c). Although Ex4-C₁₆

was not incorporated into the MSs, but rather adsorbed onto the surfaces of MSs, the initial burst was below ~32%, and continuous release was maintained until ~5 days. We attribute this to the tight adsorption of the palmityl group of Ex4-C₁₆ by the hydrophobic regions on the surfaces of porous PLGA MS. In contrast, native exendin-4 adsorbed onto negative PLGA MS surface by only very

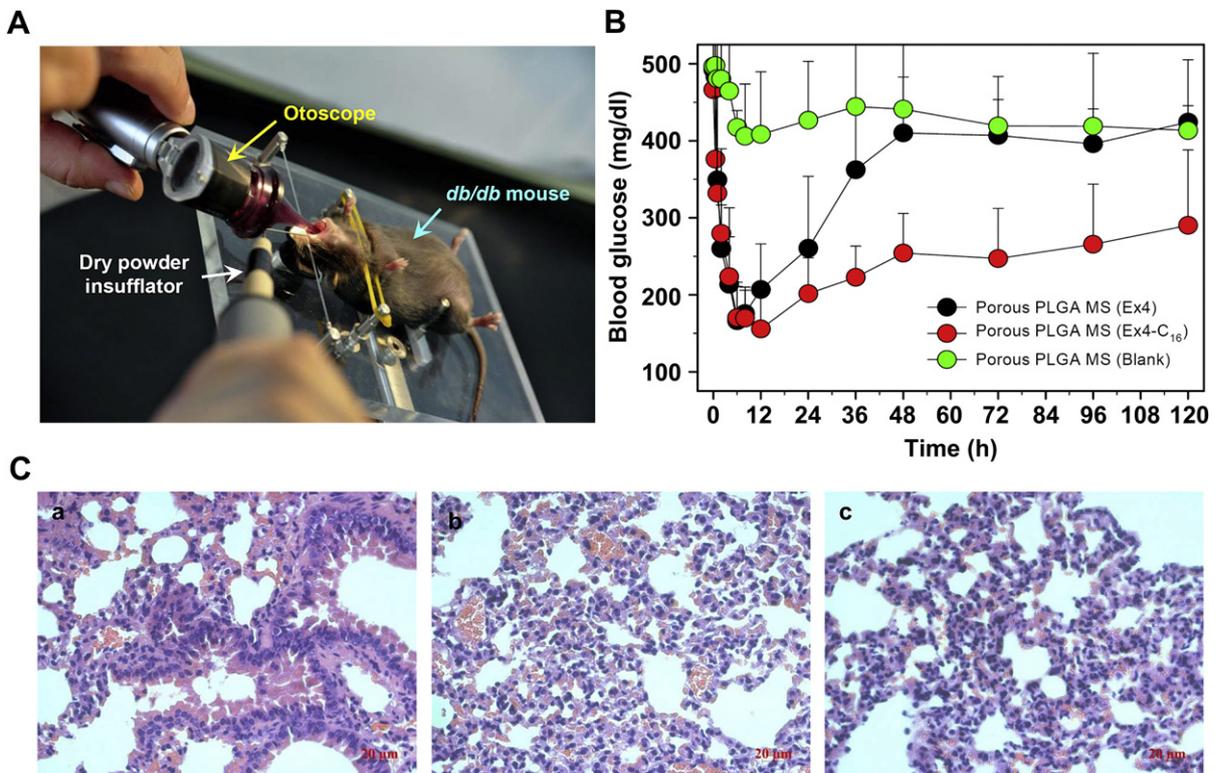


Fig. 6. (A) A picture of pulmonary administration of porous PLGA MS in db/db mice using an assembly of dry powder insufflator device and air pump. (B) Pulmonary hypoglycemic efficacies after administering porous PLGA MS adsorbed with Ex4 or Ex4-C₁₆ in non-fasted type 2 diabetic db/db mice. (C) Histology of lung tissues at 2 weeks after the administration of porous PLGA MS: a, control untreated; b, blank porous PLGA MS; c, porous PLGA MS adsorbed with Ex4-C₁₆.

Table 1
Finally chosen formulation and preparation conditions of porous PLGA MS adsorbed with Ex4-C₁₆ and their physicochemical characteristics.

Formulation & preparation conditions	PLGA type	RG504H
	Porogen type	Mixture (1:1) of Pluronic F68/F127
	DP/CP ratio (% v/v)	3.0
	Polymer : porogen	3:7
	Homogenization speed (rpm)	3000
	Hardening temperature (°C)	40
Microsphere characteristics	Particle size [MMAD ^a]	16.2 ± 2.3 [4.3 ± 0.5]
	Pore size range (μm)	0.5–2.0
	Actual loading (% w/w) [Ex4-C ₁₆ amount (mg)/PLGA MS (mg) × 100]	5.0 ± 0.7

Data are presented as means ± SD of at least 3 independent batches.

^a Mass median aerodynamic diameter.

weak ionic interaction at pH 4.0 was shown to be rapidly released out at pH 7.0 because its charge became negative and thus lost its ionic interaction.

The lung deposition results were a little disappointing because we expected that the porous PLGA MS particles would be directly located into the lower part of lungs due to their low aerodynamic density. However, if ever well-optimized, this possibility seems to seldom happen because experimental animals are unable to accomplish a complete inhalation because of anesthesia accompanied by weak breathing. Sometimes, perfect inhalation is known as a troublesome process to untrained adult humans. This fact can be considered why our porous PLGA MS did not immediately deposit the alveoli region after pulmonary dosing. Therefore, a single intubation into trachea does not result in the alveoli deposition without the cooperation of mice. Consequently, as reported previously [2], our intrapulmonary administered particles seemed to undergo three consecutive steps of (i) impaction by inertia, (ii) sedimentation by gravity, and finally (iii) Brownian diffusion, from trachea to alveoli. Our lung deposition images explain this fact.

The palmitoyl acylation of exendin-4 has another important advantage in terms of extended therapeutic duration. Fatty acid-conjugates of peptide or protein drugs are well known to extend half-lives by inducing albumin-binding [30–32]. HSA is known to possess five available binding sites for long fatty acids, and thus, fatty acylated peptide derivatives can bind tightly to circulating albumin [25]. Using this technology, a myristic acid-insulin (DesB30-Insulin levemir; Detemir[®], Novo Nordisk) was developed in 2005, and recently, a palmitic acid-glucagon-like peptide-1 analogue (Victoza[®]; previously Liraglutide[®], Novo Nordisk) was approved by FDA. Particularly, Victoza is administered once daily, whereas GLP-1, its parent, has a circulating half-life of only ~2 min. Furthermore, as we reported previously, a palmitic acylated exendin-4 was found to have greatly prolonged hypoglycemic efficacy in both fasted and non-fasted db/db mice [17–19]. In this study, the hypoglycemia induced by Ex4-C₁₆ was maintained for >~5 days after intrapulmonary dosing, which is much longer than that by native Ex4 (~36 h) (Fig. 6B). This protracted efficacy is primarily due to the sustained release from porous PLGA MS. Also, it seems evident that in terms of albumin-binding, Ex4-C₁₆ by itself offers

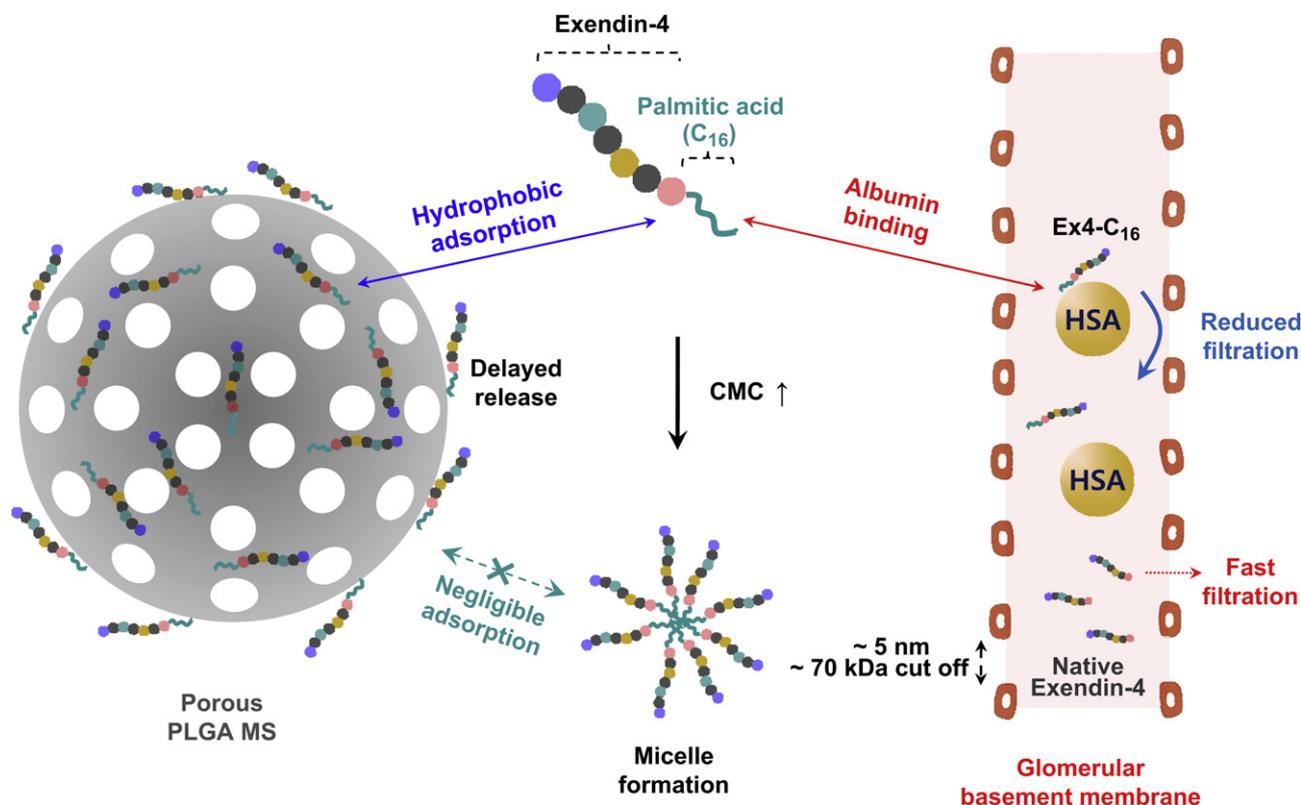


Fig. 7. An illustrated cartoon for the understanding of proposed dual-function concept underlying the design of Ex4-C₁₆-adsorbed porous PLGA MS.

the second benefit of extended therapeutic duration *in vivo*, after being deposited into the lungs and then entering into the systemic circulation (Fig. 7).

Inhaled particles are likely to invoke worrisome inflammatory/immune responses and sometimes toxicities, which is one of why Exubera[®] failed [33–35]. In addition, PLGA decomposes into lactic/glycolic acid unit, and degraded materials like these can markedly reduce micro-environmental pH values and induce necrosis [36]. In this study, the histology of lung tissues treated with porous PLGA MS did not show significant difference versus the control group. This is attributable to both relatively low amount of PLGA MS and fast decomposition of PLGA on account of porosity, when compared with long-term PLGA MS implants. Although the marginal duration for inhaled particle deposition is arguable, the much longer period of deposition of the inhaled particles could be evaded. Consequently, it can be a better choice to reduce a very long deposition duration of inhaled particles, e.g. from several weeks to months, and instead to introduce a longer systemic circulation of a loaded peptide drug as a compensation pharmaceutical tool.

5. Conclusion

We describe here a prototype of Ex4-C₁₆-adsorbed porous PLGA MS which has appropriate properties for inhalation in terms of particle size, porosity, drug loading, and sustained release. Especially, palmityl acylation of exendin-4 greatly induced its tight adsorption onto porous PLGA MS, delayed the release duration *in vitro*, and finally prolonged its hypoglycemic duration in the systemic circulation in diabetic rodents. We consider that this porous PLGA MS has considerable pharmaceutical potential as an inhalation delivery system for anti-diabetic peptides.

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Appendix

Figures with essential color discrimination. Figs. 3–7 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.10.045.

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