Biomaterials 32 (2011) 1685–1693

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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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ARTICLE INFO

Article history:
Received 7 October 2010
Accepted 22 October 2010
Available online 3 December 2010

Keywords:
Inhalation
Pulmonary delivery
Porous microspheres
Albumin-binding
Palmityl-acylated exendin-4
Hydrophobic interaction

ABSTRACT

A porous large poly(lactic-co-glycolic acid) (PLGA) microspheres (MS) adsorbed with palmityl-acylated exendin-4 (Ex4-C_{16}) 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-C_{16} was more efficiently adsorbed onto porous PLGA MSs than native exendin-4, and an approximately 5% loading of Ex4-C_{16} 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-C_{16} (approx. 100 μg/mouse) displayed much protracted hypoglycemic efficacy in non-fasted type 2 diabetic db/db mice. Porous PLGA MS with adsorbed Ex4-C_{16} 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 $\sim 1 \pm 0.5$ g/cm^3 and mean geometric diameters of 1–5 μm [6]. However, particles as heavy as ~ 1.0 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 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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0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.10.045

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-C_{16}) 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-C_{16} 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-C_{16} after intrapulmonary administration using dry powder insufflator were also evaluated.

2. Materials and methods

2.1. 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: 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 (Daejon, 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 μm 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-C_{16})

Palmityl-acylated exendin-4 (Ex4-C_{16}) 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 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). 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-C_{16} 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-C_{16} 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-C_{16}-adsorbed porous PLGA MS

The adsorption of Ex4-C_{16} onto porous PLGA MS was observed by CLSM after fluorescent derivatization. A portion (1 mg) of Ex4 or Ex4-C_{16} 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 μg/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-C_{16} onto porous PLGA MS

Aliquots (0.6 ml) of Ex4 or Ex4-C_{16} (each 30 μg/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-C_{16} release from porous PLGA MS

Aliquots (50 ml) of rhodamine-derivatized Ex4 or Ex4-C_{16} (30 μg/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-C_{16}-adsorbed porous MS (5 mg) was then suspended in 1 ml of PBS (pH 7.4) containing 0.02% (v/v) Tween 20, and gentle 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.

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