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Short communication

Protein release behavior from porous microparticle with lysozyme/hyaluronate ionic complex

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Abstract

Porous microparticles (PMs) with a low density (<0.4 g/cm^3) for pulmonary protein delivery were prepared by the water-in-oil-in-water ($W_1/O/W_2$) multi-emulsion method using a cyclodextrin derivative as a porogen. The complexation of positively charged lysozyme (Lys) and negative-charged hyaluronate (HA) was investigated for long-term protein release from PMs. The interaction of Lys and HA not only increased protein encapsulation efficiency but also stabilized Lys against a denaturing organic solvent (dichloromethane). Furthermore, PMs with Lys/HA complexes increased the Lys release period up to 7 days, as opposed to a 4 h Lys release time from PMs without Lys/HA complexes. In particular, PMs containing 10 mg of HA and 50 mg of Lys showed almost zero-order Lys release kinetic for 7 days and preserved the bioactivity of Lys more than 98% during its entire release period. This result suggests that PMs with Lys/HA complexes may be applied in long-term pulmonary administration of protein or peptide drugs, including those that require particles to arrive at a deep lung epithelium with the help of low density (high porosity) of PMs.

Keywords: Porous microparticle; Hyaluronate; Ionic complex; Cyclodextrin; Long-term pulmonary protein delivery

1. Introduction

Lungs exhibit favorable features for protein drug delivery such as ample blood supply, avoidance of hepatic first-pass metabolism, and low enzymatic metabolism for labile protein drugs. Protein particles that can be inhaled have therefore attracted much attention due to this protein delivery route being non-invasive and patient-convenient unlike intravenous injections [1–4]. However, this delivery system has been challenged by a limited deposition of aerosols in deep lung epithelial tissues [5,6], recognition by the alveolar macrophages [5,7], and the short-term therapeutic effect of hydrophilic protein drugs [8,9].

Several groups have reported that protein particles measuring less than approximately 1 μm are exhaled during normal tidal breathings. Particles measuring more than approximately 6 μm are deposited in the upper airways and are subject to a vigorous mucocilliary clearance [5,7]. Only protein particles of aerodynamic diameter between 1 and 5 μm can be well deposited throughout the lung [5,6], bypassing deposition in the mouth and throat. However, those particles could still be removed by alveolar macrophages that recognize and uptake small foreign particles [5,10]. These complicated events in the lung are what make pulmonary protein delivery so difficult.

Recently, porous particles of greater than 5 μm were investigated as an alternative method [11]. The low mass density (<0.4 g/cm^3, aerodynamic diameter of 1–5 μm) and large size (>5 μm) of the porous particles allowed a high accumulation of protein particles in the periphery of the lung, thus preventing phagocytic clearance [11–16]. In one study, an increased accumulation of porous particles led to a 24-fold higher therapeutic effect than that seen using non-porous particles [11].

However, the protein release behavior of these porous particles was unsatisfactory. The release of recombinant human growth hormone (rhGH) from the porous poly(lactide-*co*-glycolide) (PLGA) microparticles released up to 100 wt.% in only 1 day [17], presenting high initial bursts and short-term

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protein release [9]. Immunoglobulin release from the lipid-based hollow-porous microparticles was completed in approximately 18 h [18]. The porous poly(acrylic acid)–cysteine microparticles completed the drug release after 1 h [8]. Ungaro et al. [19] made long-term insulin releasing PMs, but the aerodynamic diameter of the particles was bigger than 5 μm.

This study investigated PMs with protein–polyelectrolyte complexes for long-term delivery. It is known that proteins interact with polyelectrolytes and form complexes dependent upon the ionic strength of the solution [20,21]. However, few papers have been published on the incorporation of polyelectrolytes into PMs. Lin et al. [22] reported porous chitosan/polyelectrolyte complex hydrogel microspheres with particle size of 700–900 μm. This is only formulated for oral administration. In this study, HA, a natural biodegradable polyelectrolyte, was selected for its strong interactions with positively charged proteins and was investigated about its effect on protein release behavior from PMs. Protein/HA complexes are expected to stabilize proteins in the presence of denaturing organic solvent (dichloromethane) and to allow long-term protein release from PMs.

2. Materials and methods

2.1. Materials

Lys (from Chicken egg white, 50,000 EU/mg), bovine serum albumin (BSA), sodium hyaluronate (HA) (Mw 1.5–1.8 × 10^6), sodium azide, Tween 80, sodium chloride trinitrobenzene sulfonic acid (TNBS), dichloromethane and polyvinyl alcohol (PVA) (Mw 12,000–23,000) were obtained from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) was bought from J.T. Baker (Deventer, Netherlands). Sulfobutyl ether β-cyclodextrin sodium salt (SBE-CD) was kindly provided by CyDex Corp (Calabasas, USA). PLGA, RG 502H (lactide:glycolide = 50:50, Mw 9300) and RG 504H (lactide:glycolide = 50:50, Mw 50,100) were purchased from Boehringer-Ingelheim (Petersburg, USA). BCA protein assay Kit was purchased from Pierce (Milwaukee, USA).

2.2. Preparation of protein loaded PMs

Microparticles were fabricated by the conventional $W_1/O/W_2$ multi-emulsions [23]. Proteins (BSA or Lys, 50 mg) were dissolved in 0.5 ml of a deionized water containing HA (2.5–20 mg) and SBE-CD (0–300 mg) for the preparation of aqueous phase. PLGAs, RG 502H (300 mg) and RG 504H (100 mg) were added to 3 ml of DCM solution for the preparation of organic phase (Table 1). Each solution was then mixed together and emulsified by vigorous vortexing for 30 s and then injected into 0.5 wt.% PVA and 0.9 wt.% NaCl aqueous solution. The emulsification was carried out for 5 min by a homogenizer (manufactured by Tokushu Kika Kogyo Corp.) at 4000 rpm. The resultant mixtures were hardened by gentle stirring for 40 min and then collected by centrifugation at 3000 rpm for 2 min. The particles obtained were washed three times with 0.9 wt.% NaCl aqueous solution and freeze-dried for 3 days. The residual DCM content in the obtained product was detected at below 500 ppm (by gas chromatography), fulfilling the USP XXIII requirement.

The content of intact protein was measured using a reverse phase-HPLC method. For the extraction of proteins from the microparticles, the solution of microparticles (40 mg) were dissolved in DCM (1 ml) and stirred for 1 min. This solution was centrifuged for 10 min at 5000 rpm and precipitates were collected for the next step. The protein particles were obtained after vacuum drying. HPLC analysis was done under following conditions: C_{18} μ Bondapak column (3.9 mm × 300 mm, Waters) using variable wavelength detector at 220 nm.

2.3. Protein loading

The actual protein loading efficiency in the microparticles was measured by the TNBS method described in the literature [24]. The actual protein (Lys or BSA) content in the microparticle was calculated using following equation:

$$\%\ \text{Protein} = \frac{P_t}{M_t} \times 100,$$

where P_t is the total amount of protein embedded in microparticles and M_t is the total amount of microparticle harvested.

Table 1
Compositions used for constituting PM and characterization of PM

Sample	Composition			% Protein ($n = 3$)	% Loading efficiency ($n = 3$)	Hydrolyzed protein (%)[a] ($n = 3$)	Mean particle size (μm)[b] ($n = 3$)
	Protein (mg)	SBE-CD (mg)	Hyaluronate (mg)				
PM_0	Lys (50)	0	0	8.5 ± 2.5	86.5 ± 3.6	13.5 ± 1.0	25.5 ± 2.3
PM_1	Lys (50)	100	0	4.4 ± 2.4	50.6 ± 2.5	7.2 ± 0.5	23.4 ± 1.6
PM_2	Lys (50)	150	0	4.2 ± 2.8	45.9 ± 1.8	6.5 ± 0.7	30.9 ± 1.8
PM_3	Lys (50)	200	0	3.3 ± 1.5	44.1 ± 3.3	6.1 ± 0.4	25.6 ± 1.5
PM_4	Lys (50)	250	0	1.3 ± 0.9	19.7 ± 1.5	6.0 ± 0.5	25.1 ± 1.9
PM_5	Lys (50)	300	0	1.2 ± 0.5	20.2 ± 4.5	5.8 ± 0.6	–
PM_6	Lys (50)	200	2.5	8.9 ± 1.1	88.0 ± 2.9	2.3 ± 0.5	25.5 ± 1.8
PM_7	Lys (50)	200	5	9.1 ± 0.8	90.3 ± 1.3	0.9 ± 0.3	25.1 ± 2.5
PM_8	Lys (50)	200	10	9.4 ± 0.3	92.4 ± 0.6	0.8 ± 0.5	27.3 ± 2.4
PM_9	Lys (50)	200	20	9.8 ± 0.6	93.1 ± 0.5	0.8 ± 0.4	26.6 ± 2.7
PM_{10}	BSA (50)	200	20	2.6 ± 0.9	29.1 ± 5.6	6.9 ± 1.0	32.9 ± 2.5

[a] Measured by a reverse phase-HPLC (Section 2.2).
[b] By a laser light scattering technique (Section 2.4).