Colloids and Surfaces A: Physicochem. Eng. Aspects 340 (2009) 115–120

Contents lists available at ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

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

Biocompatible microspheres based on acetylated polysaccharide prepared from water-in-oil-in-water ($W_1/O/W_2$) double-emulsion method for delivery of type II diabetic drug (exenatide)

Hyun Jung Yang[a], In Suh Park[b], Kun Na[a,*]

[a] Department of Biotechnology, The Catholic University of Korea, 43-1 Yokkok 2-dong, Wonmi-gu, Bucheon, 420-743, Republic of Korea
[b] Department of Pathology, Inha University College of Medicine, Incheon, 402-751, Republic of Korea

ARTICLE INFO

Article history:
Received 10 November 2008
Received in revised form 27 February 2009
Accepted 5 March 2009
Available online 19 March 2009

Keywords:
Polysaccharide
Microspheres
Pullulan
Exenatide
Tissue reaction

ABSTRACT

Polysaccharide microspheres (PAMs) from acetylated pullulan were designed for the long-term delivery of peptide/protein drugs, as an alternative to a PLGA depot system. Three kinds of samples were obtained according to their different degrees of acetylation (0.8(PA1), 1.5(PA2), 2.3(PA3) acetyl groups in one glucose unit in pullulan), and then utilized to prepare a microsphere via a water-in-oil-in-water ($W_1/O/W_2$) emulsion method. The mean particle size of PAMs was shown to be in a range between 35 and 110 μm, as determined by a particle size analyzer. In order to evaluate their potential as a depot for protein/peptide delivery, exenatide, a drug used for the treatment of type II diabetes, was employed. The encapsulation efficiency of exenatide in PAMs was 69.1%, 80.4%, and 90.3% in PAM 1, PAM 2, and PAM 3, respectively. Although the release of exenatide from the PLGA microspheres evidenced a fast and high-burst behavior, PAMs evidenced a sustained release profile for 21 days. After 16 days, the released peptide was found to have a molecular weight almost identical to that of native exenatide, indicating that the stability of the peptide in the PAMs was maintained. The tissue reaction evidenced by the PAM was characterized by minimal foreign body reaction and minimal configurations of immune cells such as neutrophils and macrophages, but that of the PLGA microspheres was characterized by relatively elevated inflammation. On the basis of these results, we have concluded that the PAM may provide new insights into the development of new protein/peptide depots in long-term delivery.

1. Introduction

Poly(DL-lactide-co-glycolide) (PLGA) microspheres have been investigated for their utility in the long-term delivery of protein/peptide drugs, and certain such products have already been commercialized [1]. However, recent publications have underlined their disadvantages, which ultimately result in the loss of protein/peptide drug activity over long-term delivery. These disadvantages are induced principally by acidic environments in PLGA microspheres, and by acylation between protein/peptide and PLGA [2–7]. It has been established that an acidic condition induced by protons generated from the hydrolysis of ester linkages in PLGA facilitates the denaturation, aggregation, and deamidation of peptide/protein in PLGA microspheres. Additionally, acylation induced by the nucleophilic attack of amine groups in peptide/protein to a carbonyl group in PLGA can damage incorporated peptide/protein drugs [8,9], and effect an alteration of receptor specificity [10]. In a study investigating peptide denaturation in PLGA films, Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA) (Asn-hexapeptide) underwent three types of chemical degradation when encapsulated in PLGA: deamidation, amide bond cleavage and acylation [11]. Thus, the development of a new protein/peptide delivery depot to replace the PLGA system is clearly required.

Morimoto previously described a cationic microsphere based on aminated gelatin with ethylenediamine (CGMS). This system was characterized by a slower release and suppression of the burst release of acidic peptide/protein [12]. Additionally, chitosan-based microspheres have been evaluated for the nasal delivery of insulin. Mucoadhesive chitosan microspheres evidenced superior reduction of blood glucose levels, and controlled drug release [13].

Pullulan has been selected as a polymer for the preparation of microspheres, because it has a biocompatible and biodegradable polymer, which is degraded into non-toxic oligomers or monomers [14]. Thus, it has been investigated for use in biomedical and biomaterial applications [15,16].

This polymer was modified chemically via the addition of acetic anhydride (pullulan acetate; PA) to endow the appropriate properties for microsphere preparation via the double-emulsion method,

* Corresponding author. Tel.: +82 2 2164 4832; fax: +82 2 2164 4865.
E-mail address: kna6997@catholic.ac.kr (K. Na).

0927-7757/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.colsurfa.2009.03.015

because pullulan is soluble in water and insoluble in volatile organic solvents such as dichloromethane (MC). Acetylated pullulan (PA) evidenced an alteration in character, from solubility in water to insolubility. The acetylated pullulan is eliminated from human plasma by endogenous esterase and glycosidase [17,18]. The polymer was already utilized for lysozyme delivery by our group to identify its potential in peptide/protein delivery [1]. The release behaviors of lysozymes from PA microspheres (PAMs) were controlled by the degree of acetylation. High levels of acetylation effectively improved lysozyme release kinetics by reducing initial burst release and extending continuous release over a period of time.

Here, in an effort to determine whether or not the PA microsphere system is effective in a therapeutic peptide delivery system, exenatide (synthetic exendin-4) was employed as a model drug. Exendin-4 is a 39 amino acid peptide isolated from the salivary secretions of the Gila monster lizard (*Heloderma suspectum*) [19]. It shares a sequence homology of approximately 53% with the mammalian glucagon-like peptide-1 (GLP-1) [19], and is a highly potent GLP-1 receptor (GLP-1R) agonist, both *in vitro* and *in vivo*. Additionally, a glycine residue at position-2 confers resistance to cleavage by the DPP-IV enzyme, resulting an increase in the half-life of exenatide of approximately 6–8 h [20]. Therefore, exenatide is currently under investigation as a therapeutic agent for type II diabetes.

In this study, PAMs encapsulated with exenatide were prepared via the water-in-oil-in-water ($W_1/O/W_2$) double-emulsion method, and the particles were characterized by their morphology and particle size. Their peptide loading efficiency and release profile of exenatide *in vitro* were investigated, and its stability was also assessed via molecular weight (Mw) analysis. Moreover, its tissue compatibility was evaluated by the hematoxylin and eosin (H&E) staining of the implantation site. The peptide loading efficiency, release profile, and tissue compatibility results of PAMs were compared with those of the PLGA microsphere results.

2. Experiment

2.1. Materials

Pullulan, with a number-average molecular weight of 100,000, was purchased from Hayashibara Company, Japan. The others, including pyridine, acetic anhydride, formamide, and dimethyl sulfoxide (DMSO) were acquired from Sigma Company (St. Louis, MO, USA). Exenatide was purchased from the American Peptide Company. PLGA, RG 502H (Mw 8000 Da) were provided by Boehringer-Ingelheim (Petersburg, USA), All of the reagents were of extra reagent grade, without any need for further purification. The dialysis membranes, with a molecular weight cut-off (MWCO) of 10,000 g/mol, were purchased from Spectrum Laboratories, Inc.

2.2. Pullulan acetylation

To impart the hydrophobicity, pullulan was chemically modified via acetylation (Fig. 1). 500 mg of pullulan was suspended in 10 mL of formamide, then dissolved by 50 min of vigorous stirring at 50 °C until dissolved. Pyridine (2–4 mL) was added and dissolved via 1 h of vigorous stirring at room temperature. Then, acetic anhydride (1–7 mL) was added and dissolved via vigorous stirring. The mixture was stirred for 24 h at room temperature. The reaction mixture was poured into distilled water (DW) in order to remove impurities. The brown precipitates were then filtered and washed with 100 mL of DW several times, and dried for 3–4 h at 40 °C in a vacuum oven. For detail purification, the powder was re-dissolved in 15 mL of DMSO, then dialyzed with membrane (cut-off = 10,000) for 2 days against

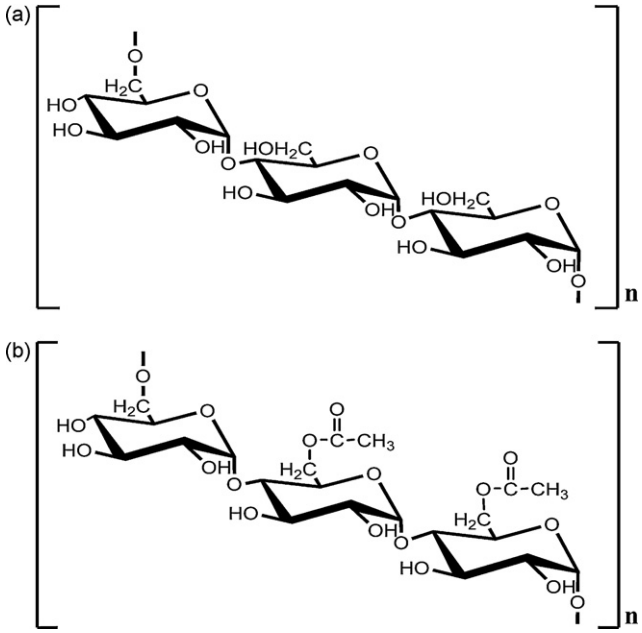

Fig. 1. Chemical structure of pullulan (a) and pullulan acetate (b).

DW. The final solution was freeze-dried and a white powder was obtained. The synthesized PA was identified via FT-IR and H-NMR. To determine the degree of acetylation for PA, the integration ratio of peaks at 1.94–2.09 ppm ($COCH_3$) and 3.96–5.23 (saccharide ring) was used [16].

IR (KBr): 3402 (s, OH), 2954 (m-2, CH_2), 1752 (vs, C=O) and 1375 (s, CH_3) (Fig. S-1)

H-NMR (DMSO-d_6): δ = 1.94–2.09 (d, $-COCH_3$), 3.96–5.23 (m, saccharide ring) (Fig. S-2)

2.3. Formulation of microspheres

Pullulan acetate microspheres (PAMs) were prepared via a double-emulsion-solvent evaporation technique (W/O/W, water-in-oil-in-water). 2 mg of exenatide was dissolved in 0.1 mL of distilled water and added to 1.0 mL of methylene chloride with 7.7 mg of PA, followed by 45 s of vortexing to form a W/O emulsion. The emulsion was then injected slowly (with 1 mL syringe and a needle with the 23 G) into 30 mL of 0.5% (w/v) aqueous PVA solution containing 0.9% NaCl. The mixture was stirred for 4 min at 1600 rpm with a Silverson L4R homogenizer (Silverson Machines Ltd, Waterside, UK). Methylene chloride was removed via 2 h of agitation at room temperature, and the PAMs were solidified. The hardened white PAMs were centrifuged, washed five times in DW, freeze-dried (OPR-FDU-7003) for 24 h, and stored at −20 °C until use. As a control, PLGA microspheres (RG 502H (Mw 8000 Da)) was also prepared by same method for PAM and compared to PAM.

2.4. Morphology and particle size distribution of microspheres

The surface morphology of PAMs was evaluated using an environmental scanning electron microscope (Model XL30 ESEM-FEG, PHILIPS). The freeze-dried microspheres were mounted on copper stubs with double-sided adhesive tape. After vacuum-coating, the specimens were examined by ESEM at 15 kV. To determine the size distribution, PAMs (1 mg/mL sample concentration) were first dispersed in distilled water via ultrasound dispersion, and then the particle size distribution was assessed using a Laser Particle Size Analyzer (Mastersizer Microplus).