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Glycol chitosan as a stabilizer for protein encapsulated into poly(lactide-*co*-glycolide) microparticle

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Abstract

Glycol chitosan (GC), a chitosan derivative conjugated with ethylene glycol, is soluble in water at a neutral/acidic pH and is viscous. This GC was incorporated into poly(lactide-*co*-glycolide) (PLGA) microparticles (prepared by the multi-emulsion $W_1/O/W_2$ (water-in-oil-in-water) method) to stabilize lysozyme (Lys) used as a model protein. Herein, GC's viscous property helped to improve Lys encapsulation efficacy and reduce Lys denaturaton at the water/organic solvent interface. When the GC concentration in the W_1 phase increased, the formation of non-covalent Lys aggregates decreased. This may be because the aqueous microdroplets surrounded by the firm viscous interface protect Lys from the degrading environment formed by the water/organic solvent interface. In an *in vitro* Lys release test, 40 mg incorporation of GC led to continuous Lys release of up to 78 wt.% for 1 month and presented bioactivity of more than 95% for Lys released from microparticles. In addition, there was negligible immune response in the tissue treated with the GC-incorporated PLGA microparticles, whereas there was a moderate foreign body reaction in the muscle layer and many configurations of neutrophils in the tissue treated with the PLGA microparticles without GC. It is expected that GC facilitates a decrease in immune responses exacerbated as a consequence of PLGA degradation.

Keywords: Glycol chitosan; Lysozyme; Protein stabilization; Poly(lactide-*co*-glycolide) degradation; Microparticle

1. Introduction

The biodegradable polymer, PLGA has been widely used in proteins delivery system in order to prolong their therapeutic effect (Sinha and Trehan, 2003; Sanchez et al., 2003; Tracy et al., 1999; Wang et al., 2005; Lagrace et al., 2006). However, proteins that encapsulated into PLGA microparticles have often showed reduced bioactivity and structural degradations (Sinha and Trehan, 2003; Tracy et al., 1999; Kang et al., 2002). It is known that PLGA degrades in a hydrolytic atmosphere and liberates acidic degradation products such as lactic acid and glycolic acid (Bilati et al., 2005; Freiberg and Zhu, 2004; Morlock et al., 1998; Bezemer et al., 2000; Francis et al., 1998). These acids facilitate a further degradation of PLGA and impair the physiological properties of the labile protein embedded in the PLGA (Sinha and Trehan, 2003; Bilati et al., 2005; Freiberg and Zhu, 2004; Morlock et al., 1998; Bezemer et al., 2000; Francis et al., 1998). The proteins susceptible to this acidic environment can easily be degraded by non-covalent aggregation and peptide bond hydrolysis (Sinha and Trehan, 2003).

To overcome this problem, stabilizers such as non-ionic surfactants, cyclodextrin derivatives, and poloxamers have been used to try to protect labile proteins from the degrading environment. However, their stabilizing effects on labile proteins were not consistent among various proteins (Castellanos et al., 2006; Costantino et al., 2002; Tobio et al., 1999; Kang et al., 2002). Therefore, Zhu et al. (2000) co-incorporated magnesium hydroxide (up to 3 wt.%) into the PLGA microparticles to neutralize the acidic environment formed by the degrading

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PLGA. However, it is difficult to preserve the effective concentration of magnesium hydroxide in microparticles for a long period because small molecules may be easily released from the PLGA microparticles. In a contrast, Kim et al. (2005) utilized positively charged macromolecule, poly(ethylene glycol)-*b*-poly(L-histidine) (PEG-PH) diblock copolymer in order to neutralize the acids. However, in practice it is difficult to use PEG-PH in PLGA microparticles because of the complicated PEG-PH synthesis (Kim et al., 2005; Lee et al., 2003). On the other hand, a few researchers have investigated the development of novel microparticle preparation methods such as solid-in-oil-in-water (S/O/W) (Castellanos et al., 2006), spray freeze drying (Costantino et al., 2002), and supercritical fluid (SCF) (Sarkari et al., 2003) method. There trials intend to improve protein bioactivity, but their effects are still unclear.

Chitosan has been intensively investigated during recent year because of its biocompatibility, biodegradability, nontoxicity, and remarkable affinity to proteins (Dai et al., 2005; Shahidi and Abuzaytoun, 2005; Martino et al., 2005). However, chitosan is normally insoluble above pH 6 due to its rigid crystalline structure and requires acids to be protonated. This solubility is not in agreement with fragile proteins sensitive to acidic conditions. This solubility is probably the major factor limiting its protein-related utilization.

Recently, various chemical modifications have been introduced to increase its water solubility (Gerasimenko et al., 2004; Chung et al., 2005; Cho et al., 2006). Among them, GC prepared from ethylene glycol is water-soluble at entire pH ranges (Hu et al., 2005). This property can help to formulate protein drugs and cells at neutral pH, without acids presence, and even hydrophobic drugs. Sakai et al. (2000) made GC/alginate polyion complex microcapsules to protect encapsulated islet from the host immune response. Kim et al. (2006) utilized hydrophobically modified GC as a nano-carrier for antitumor drugs. Park et al. (2006) conjugated GC to doxorubicin for antitumor activity. Another group prepared GC/poly(vinyl alcohol) interpenetrating polymer network type superporous hydrogels (Park and Kim, 2006).

In this study, we select GC as a protein stabilizer for preparing protein-loaded microparticles. Lys was used as a model protein. It is known that Lys is the primary enzyme responsible for chitosan degradation (Martino et al., 2005). In this regards, it is interesting to note that Lys will hydrolyze GC and then may produce reactive small molecules (fragmented GCs) during the incubation, and highly mobile small molecules of GCs will probably interact with the acidic PLGA degradation products (*e.g.*, lactic acid and glycolic acid), consequently stabilizing proteins from the degrading environment.

2. Materials and methods

2.1. Materials

Lys (from Chicken egg white, 50,000 Eu/mg), GC (M_w: 250,000 Da; degree of deacetylation: 82.7%) (Fig. 1), sodium azide, Tween 80, sodium chloride, urea, trinitrobenzene sulfonic acid (TNBS), polyvinyl alcohol (PVA) (M_w 13,000–23,000 Da) and micrococcus Lysodeikticus were purchased from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) was bought from J.T. Baker (Deventer, The Netherlands). PLGA, RG 502H (M_w 8000 Da) were provided from Boehringer–Ingelheim (Petersburg, USA). BCA protein assay Kit was purchased from Pierce (Milwaukee, USA).

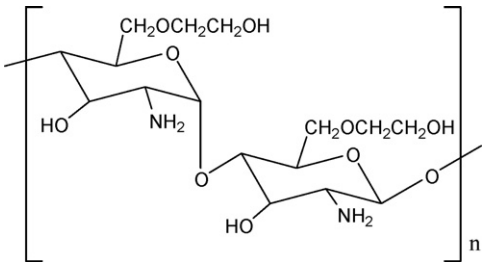

Fig. 1. The chemical structure of glycol chitosan (GC).

2.2. Preparation of PLGA microparticles

PLGA microparticles were fabricated by the conventional $W_1/O/W_2$ multi-emulsions (Lee et al., 2006, 2007). Lys (20 mg) was dissolved in 0.2 ml of de-ionized water containing GC (0–40 mg). RG 502H (100 mg) was added to 1 ml of DCM solution. Two solutions were then mixed together and emulsified by vigorous vortexing for 30 s and then injected into 1.0 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.

2.3. Encapsulation of Lys

The actual protein loading efficiency in the microparticles was measured by the TNBS method described in the literature (Bezemer et al., 2000). The absorbance of each sample was read on a microplate reader at a test wavelength of 450 nm. The Lys concentration is proportional to the absorbance at 450 nm (Lee et al., 2006, 2007).

2.4. Morphology and particle size distribution

The morphology of microparticles was confirmed with scanning electron microscopy (SEM, Hitachi S-3000 N). A laser light scattering technique (Mastersizer 2000, Malvern) was employed to confirm the particle size distribution of microparticles. The compressed air system was utilized to inhibit the aggregation of dried microparticles (Shinha and Trehan, 2005).

2.5. Insoluble Lys aggregation

The formation of insoluble Lys aggregate was determined after extracting Lys from the microparticles (Perez et al., 2002; Lee et al., 2006, 2007). Briefly, the solution of microparticles