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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.
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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).

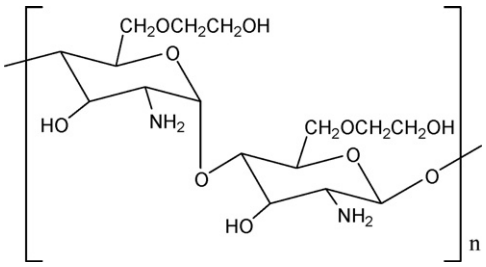

Fig. 1. The chemical structure of glycol chitosan (GC).

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

(40 mg) was dissolved in DCM (1 ml) and stirred for 30 min. This solution was centrifuged for 20 min at 5000 rpm and precipitates were collected for a next step. After adding 10 mM PBS (pH 5.1) to the precipitates, insoluble precipitate was separated by centrifugation and then dissolved in 1 ml of 6 M urea. It was known that insoluble fraction was 98% soluble in 6 M urea, indicating that the aggregates were non-covalent (Perez et al., 2002). The content of insoluble Lys aggregate in 6 M urea was measured by the TNBS method as described earlier.

2.6. In vitro Lys release studies

Protein release in PBS (pH 7.4, 0.01% sodium azide, 0.02% Tween 80) was monitored by BCA protein assay kit (Sandor et al., 2001). Thirty milligrams of microparticles were dispersed in 5 ml of PBS and incubated under mild stirring at 37 °C. Aliquots of supernatant were withdrawn at predetermined time intervals and an equal amount of fresh PBS added to maintain a constant volume of medium. The measurement of protein concentration by a microplate reader was performed at 562 nm.

The stability of Lys released from the PLGA microparticles for 4 h before a given time was evaluated by a bioactivity test. A 1.3 ml aliquot of Micrococcus lysodeikticus (ATCC 4698) cell suspension (0.2 mg/ml, 66 mM PBS 6.2) was incubated with either 20 μl of the Lys aqueous solutions obtained from the PLGA microparticles or a Lys standard solution (1–100 μl). The decrease in absorbance intensity at 450 nm over time was related to the bioactivity of the Lys against the Micrococcus lysodeikticus cells (van de Weert et al., 2000).

2.7. Evaluation of tissue reaction

Male Sprague–Dawley (SD) rats weighing 200–250 g were *s.c.* injected with the PLGA microparticles with GC at 100 mg microparticles per kg body weight. Each group of rats was sacrificed by carbon dioxide asphyxiation after 1 month of implantation. The tissues bearing microparticles were cut-off and fixed in a 10% neutral-buffered formaldehyde solution. The fixed tissues were then embedded in paraffin and sectioned with a 4 μm thick microtome. The tissue sections mounted on glass slides were stained with hematoxylin and eosin (H&E) to evaluate their cellular components' reactions on the implantation site (Anderson and Shive, 1997; Choi et al., 2002; Costantino et al., 2002).

3. Results

3.1. Lys loading of microparticles

Table 1 shows that the protein loading efficiencies of formed microparticles. They were 61–90 wt.% in all preparations and were improved significantly by increasing the amount of GC in them (Fig. 1). The actual protein content in the PLGA microparticles increased to 85.7 μg per 1 mg of microparticles as the amount of GC incorporation approached to 40 mg (nearly saturation concentration).

Table 1
Characterization of Lys encapsulated PLGA microparticles (mixture with GC) ($n = 3$)

Code	Amount of GC (mg)	Lys content (μg)[a]	Encapsulation efficiency (%)[b]	Average particle size (μm)
GC-0	0	62.3 ± 0.58	61.33 ± 3.05	17.62 ± 1.55
GC-1	10	60.3 ± 0.51	62.53 ± 2.05	20.53 ± 2.32
GC-2	20	76.8 ± 0.32	73.26 ± 1.99	26.32 ± 1.92
GC-3	40	85.7 ± 0.26	90.02 ± 2.03	26.65 ± 1.72

[a] Incorporated Lys content per 1 mg of microparticle.
[b] Weight fraction of incorporated Lys per weight fraction of added Lys.

The average particle size of the PLGA microparticles ranged from 17.62 to 26.65 μm as determined by a laser light scattering technique (Table 1), thus presenting unimodal and broad particle distribution (Fig. 2). Some PLGA microparticles were chosen to show their shapes and their shapes were relatively regular and spherical as visualized in a SEM photograph (Fig. 3), but they were affected by the GC concentration.

Fig. 4 shows the content of insoluble Lys aggregation in the PLGA microparticles. The insoluble Lys content of the GC-03 microparticles was significantly lower than that of any other microparticles. The GC additions considerably enhanced stability of Lys during the PLGA microparticle preparation.

3.2. In vitro Lys release and its bioactivity

Fig. 5 shows *in vitro* Lys release from microparticles. The PLGA microparticles prepared from the conventional composition (GC-0) had an initial burst release of approximately 52 wt.% on the first day, reaching a plateau of protein release after 2 days. As the GC amount increased, however, Lys' initial burst decreased remarkably to approximately 12 wt.%. On their long-term Lys release profile, GC-2 and GC-3 microparticles showed tri-phasic release behaviors, including continuous Lys release up to 67 wt.% and 78 wt.%, respectively, while GC-1 microparticles reached a plateau region (58 wt.% Lys release) after 22 days.

Fig. 6 shows the long-term stability of Lys released from the PLGA microparticles. The GC-03 microparticles maintained

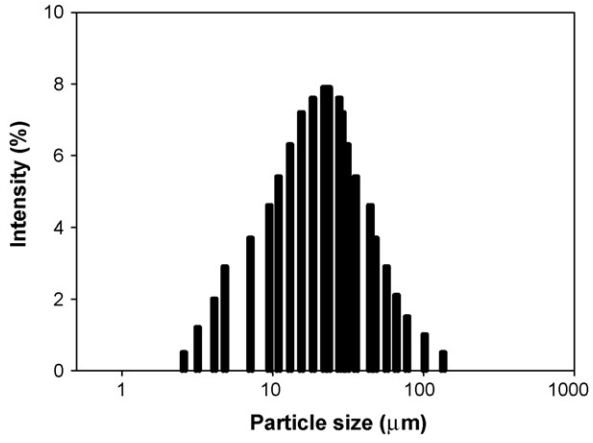

Fig. 2. The particle size distribution of GC-3 microparticles determined by laser light scattering technique.

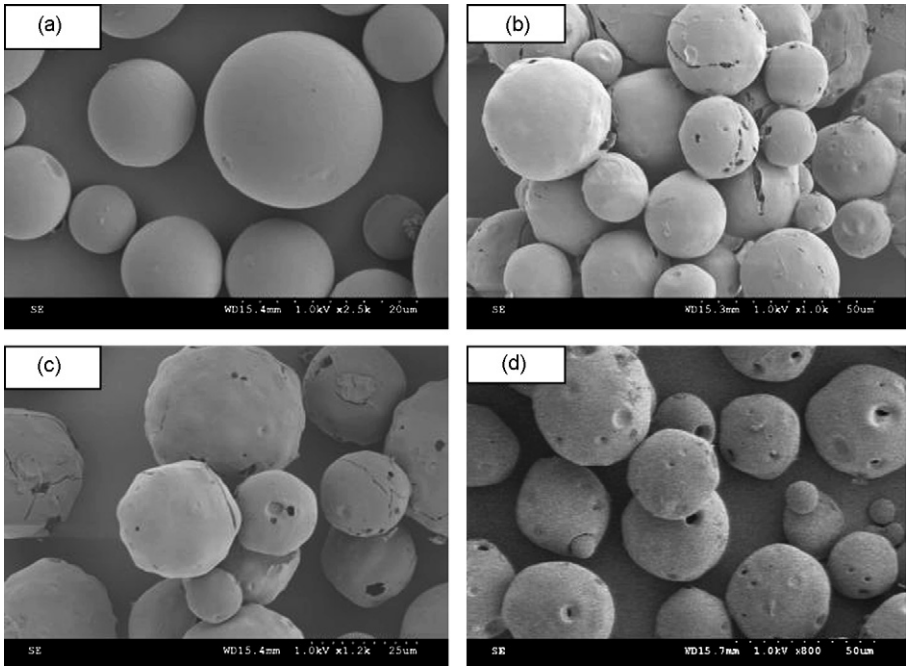

Fig. 3. The SEM image of the microparticles: (a) GC-0, (b) GC-1, (c) GC-2, and (d) GC-3.

their bioactive form with more than 95 wt.% of the Lys remaining for 28 days, but the control microparticles (GC-03) showed reduced protein bioactivity of approximately 92 wt.% after only 7 days. It was also observed that the bioactivity of Lys encapsulated into PLGA microparticles depends on the amount of GC. As the content of GC increases, so does Lys's bioactivity.

3.3. Tissue reactions

The PLGA microparticles (GC-0, GC-2, GC-3) were *s.c.* administered to Male Sprague–Dawley (SD) rat. The PLGA microparticles implanted on the rat's tissues were investigated to evaluate their biocompatibility (Fig. 7). In the case of the control (GC-0), a moderate small foreign body reaction was seen in the muscle layer, moderate fibrosis below the muscle layer, and many neutrophills. However, the PLGA microparti-

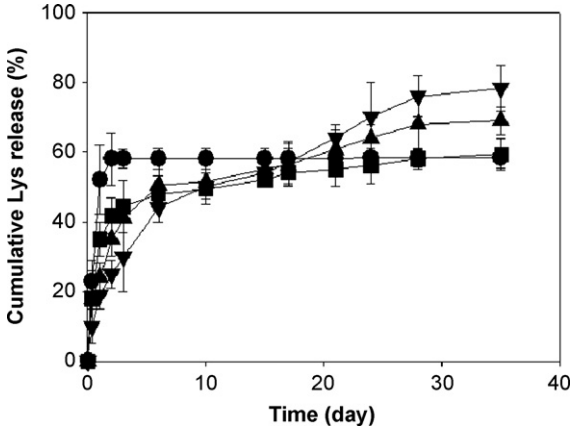

Fig. 5. The cumulative Lys release (wt.%) from GC-0 (●), GC-1 (■), GC-2 (▲), and GC-3 (▼) microparticles ($n = 3$).

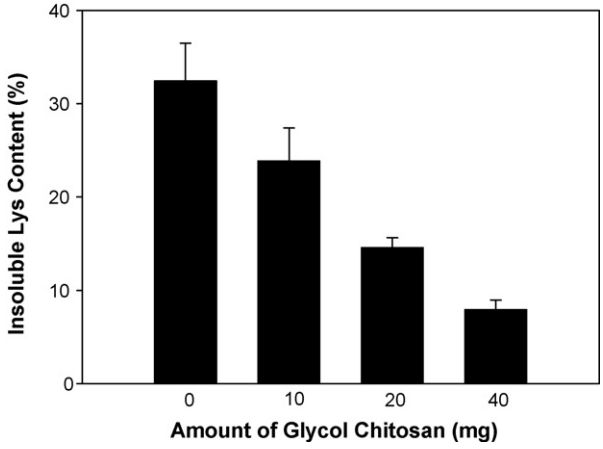

Fig. 4. The amount of insoluble Lys aggregates with GC concentration ($n = 3$).

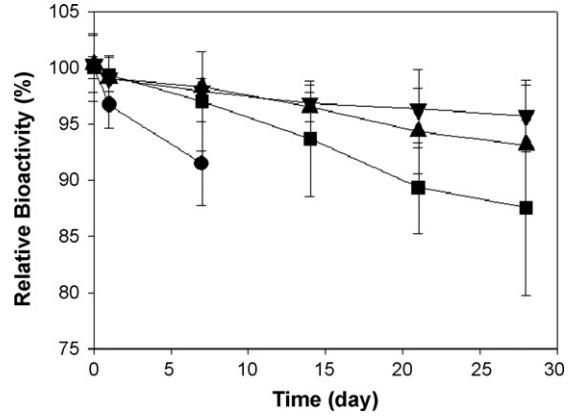

Fig. 6. The relative bioactivity of Lys against micrococcus lysodeikticus cells. Lys were released from GC-0 (●), GC-1 (■), C-2 (▲), and GC-3 (▼) microparticles with incubation time and were then analyzed ($n = 3$).

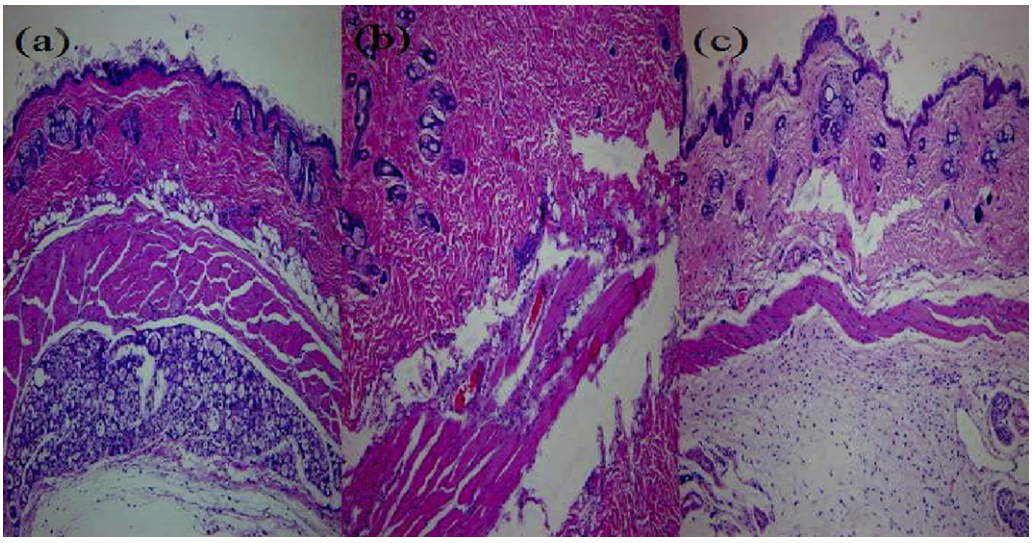

Fig. 7. H&E staining of tissues treated with (a) GC-0, (b) GC-2, and (c) GC-3 microparticles after 1 month.

cles with 20 mg GC showed a minimal foreign body reaction in the muscle layer, moderate fibrosis below the muscle layer, and negligible neutrophills. The PLGA microparticles with 40 mg GC decreased tissue responses compared to any other cases. No foreign body reaction was seen, but moderate fibrosis was present below the muscle layer, and negligible neutrophills were noted.

4. Discussion

The PLGA microparticles (17.62–26.65 μm size, Table 1) were prepared from multi-emulsion ($W_1/O/W_2$) method. The protein loading efficiency of these microparticles was dependent on the amount of GC incorporation. The protein loading efficiency increased as the amount of GC incorporation (Fig. 1) elevated. This event is probably due to that viscous GC in an aqueous phase entraps Lys, which is associated with the decline of Lys's leakage from the microparticles during the secondary emulsification. Of course, it was observed that increasing GC concentration rather made scars on the spherical surface of PLGA microparticles (Fig. 3). It may be reasonable that a viscous aqueous phase modified solvent evaporation rate and exerts surface morphology change of microparticles. Nevertheless, the GC additions considerably enhanced stability of Lys during the PLGA microparticle preparation (Fig. 4). These results are consistent with our previous investigations (Lee et al., 2006, 2007). The introduction of viscous materials such as starch, hyaluronate, and sucrose acetate isobutyrate (SAIB) enabled to safely encapsulate proteins into the PLGA microparticles (Lee et al., 2006, 2007), by decreasing interactions between proteins and organic solvent at the water/organic solvent interface.

Fig. 5 shows *in vitro* Lys release from microparticles. The GC-0 microparticles showed Lys release of 58 wt.% only for 2 days, whereas the GC-2 or GC-3 microparticles exhibits triphasic release behaviors for 1 month and completed the Lys release at 67 wt.% and 78 wt.% of given Lys content, respectively. Herein, GC-0 shows no further release of Lys after 2 days. This behavior may be due to that metabolites and acids liberated by degrading PLGA degrade proteins, which makes insoluble protein agglomeration and leads to no further protein release.

Considering the PLGA degradation products are related to the physically degradation like the formation of non-covalent protein aggregates (*i.e.*, consequently suppression of Lys release) and the chemically degradation of proteins during protein release (Perez et al., 2002; Kang et al., 2002; van de Weert et al., 2000), GC incorporated in the GC-2 or GC-3 microparticles seems to reduce these degradations of Lys, by deactivating the acidic PLGA degradation products through ionic complexation.

It is interesting to note that GC will be degraded by Lys under a hydrated condition, but the conjugation of ethylene glycol to GC (Hu et al., 2005) will suppress Lys's hydrolysis activity. In the step of microparticle preparation, GC's degradation by Lys may therefore be limited. Of course, GC in microparticles incubated over a long time would be gradually degraded by Lys and then may release small molecular hydrolyzed GCs (fragmented GCs) resulting from random scissoring. This hydrolyzed GC might have good mobility to interact with degrading acids (*e.g.*, lactic acid or glycolic acid). As a result, it is expected to enhance protein stability during long-term protein release. Furthermore, it was also observed that bioactivity of Lys encapsulated into PLGA microparticles depends on the amount of GC. As the content of GC increased, the bioactivity of Lys increased (Fig. 6). This suggests that GC helped to preserve Lys in a naive state.

To define the effect of small molecular hydrolyzed GCs on protein stabilization, bovine serum albumin (BSA)/GC loaded microparticles and BSA/GC/Lys loaded microparticles were prepared by the multi-emulsion method and analyzed for their BSA stabilizing effect after 20 days incubation. From this test, it is found that the former has relative lower BSA stability (from SDS-PAGE) than the latter over the incubation period (data not shown). Several significant bands below 66 kDa were detected in the case of BSA/GC loaded microparticles, but negligible bands below 66 kDa in the case of BSA/GC/Lys loaded microparticles. This indicates that hydrolysis activity of Lys on

GC facilitate further stabilization of proteins. Of course, monitoring of hydrolyzed GC and PLGA degradation products may be required for more proof of this hypothesis. This will be a next work.

In addition, Fig. 7 shows tissue responses of each microparticles (GC-0, GC-2, GC-3). In the case of the control (GC-0), there was moderate immune response. However, the PLGA microparticles with GC showed decreased immune response. In particular, the PLGA microparticles with 40 mg GC showed no foreign body reaction and negligible neutrophills. These results are continuous with Figs. 5 and 6. The GC incorporated microparticles are assumed to diminish leakage of the acidic PLGA degradation products, resulting in the reduction of immune response.

From these observations, GC is considered to the novel promising stabilizer for labile proteins upon the PLGA microparticles.

5. Conclusion

GC was incorporated to stabilize Lys in the PLGA microparticles. GC led to high encapsulation of proteins into microparticles and provided improved Lys stability during microparticle prepration and during its release. In particular, Lys, the model protein, was considered to facilitate GC's protein stabilization effect through ionic interaction between GC and PLGA degradation products, which is attributed to the improved mobility of GC fragmented by Lys' hydrolysis. On the biocompatibility test, the PLGA microparticles with GC exhibited negligible immune response. Therefore, co-administration of Lys and therapeutic proteins into microparticles or the introduction of a pro-stabilizer such as GC can be regarded as a promising strategy for leading long-acting protein delivery.

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