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Research paper

Water-free microencapsulation of proteins within PLGA microparticles by spray drying using PEG-assisted protein solubilization technique in organic solvent

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Abstract

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) microparticles encapsulating therapeutic proteins were prepared under a water-free formulation condition. Bovine serum albumin (BSA) and recombinant human growth hormone (rhGH) were homogeneously solubilized as nano-scale complexes in methylene chloride phase by using polyethylene glycol (PEG) as a complex-forming agent. The organic phase containing dissolved PLGA and PEG/protein complexes was directly spray dried to obtain PLGA microparticles encapsulating proteins. They exhibited sustained release profiles of BSA and rhGH up to 30 days with reduced initial bursts. The released protein molecules from the microparticles maintained structural integrity without aggregation, suggesting that the current single-step protein microencapsulation method without using water could be potentially applied for sustained delivery of a wide range of therapeutic protein drugs that are not soluble in organic solvents.

Keywords: Poly(D,L-lactic-*co*-glycolic acid) (PLGA); Microparticles; Protein; Spray drying; Sustained release

1. Introduction

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) copolymers have been popularly used for a prolonged delivery of various therapeutic proteins due to their biodegradability and biocompatibility [1–3]. However, it is extremely difficult to encapsulate hydrophilic proteins within hydrophobic microparticles without damaging their conformational structure, because proteins are very susceptible to denaturation/degradation during a series of harsh formulation steps. It is well known that various protein instability events including chemical degradation, covalent/non-covalent aggregation, and non-specific adsorption on the PLGA surface occur to varying extents during the formulation process as well as the release period [4–6]. Such undesirable stability problems not only reduce biological activities of microencapsulated proteins, but also directly influence on their release kinetic profiles from PLGA microparticles. Generally, proteins were encapsulated within PLGA microparticles by conventional water-in-oil-in-water (w/o/w) double emulsion/solvent evaporation methods [4]. During the formulation, proteins were mainly denatured and aggregated by an exposure to an interface created between water and oil phases [7–10].

Alternatively, micronized protein solid particles were directly dispersed in an organic phase containing PLGA. The organic phase was emulsified and solvent evaporated in a continuous water phase (S/O/W single emulsion/solvent evaporation method) or cryogenically sprayed into a liquid nitrogen medium for solvent extraction [11–14]. We also reported that the encapsulation of reversible protein aggregates could minimize the formation of irrevers-

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ible protein aggregates within PLGA microparticles [15]. Micronized protein particles and aggregates were prepared by spray drying, ultrasonic atomization, PEG-induced aqueous phase separation, supercritical fluid antisolvent technique (SAS), and organic solvent-induced controlled protein precipitation [16–22]. However, most dry protein powders prepared by the above methods had an average size around 1–5 μm, not suitable for homogeneous encapsulation within PLGA microparticles having an average size of 10–50 μm for injection with a syringe needle.

In our previous studies, we have shown that various biomacromolecules such as plasmid DNA, proteins, and carbohydrate polymers could be solubilized in selected organic solvents in the presence of polyethylene glycol (PEG) [23,24]. It was found that proteins and PEG could form stable nano-sized complexes in polar organic solvents by non-covalent interactions. In this study, based on the finding of PEG-assisted protein solubilization in organic solvents, a novel water-free protein microencapsulation method was presented. Bovine serum albumin (BSA) and recombinant human growth hormone (rhGH) were complexed with PEG in a methylene chloride phase containing PLGA. The organic phase was directly spray dried to obtain PLGA microparticles encapsulating proteins. It was hypothesized that PEG/protein complex particles dispersed in a volatile organic solvent were homogeneously microencapsulated within PLGA microparticles with a high loading amount, resulting in improved release profiles with enhanced protein stabilities. Various PLGA microparticles were prepared by different formulation conditions such as PLGA copolymer composition and molecular weight, and PEG/PLGA weight ratios. Protein release profiles and structural integrities after release were characterized.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) and polyethylene glycol (Mw 3.35 kDa) were from Sigma (St. Louis, MO). Recombinant human growth hormone (rhGH) was kindly donated from Dong-A Pharmaceutical Co. (Korea). Poly(D,L-lactic-*co*-glycolic acid) (PLGA) copolymers having 50/50 and 75/25 molar composition of lactic/glycolic acid were purchased from Boehringer Ingelheim (Ingelheim, Germany) (RG502, RG503, and RG756). Methylene chloride was purchased from Junsei Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Characterization of nano-sized PEG/protein complexes in methylene chloride

To eliminate salts, BSA (66 kDa) or rhGH (22 kDa) was dialyzed in deionized water for more than one day. Desalted BSA (1 mg) or rhGH (0.5 mg) dissolved in 500 μl of deionized water was mixed with PEG (Mw 3.35 kDa) dissolved in 500 μl of deionized water at various weight ratios of PEG/protein from 1 to 45, and then frozen in liquid nitrogen. The frozen samples were lyophilized at −50 °C under a pressure of 9 mm Torr without further annealing step. The lyophilized PEG/protein mixture was added in 1 ml of methylene chloride and vortexed briefly. To evaluate the solubility, transmittance value of methylene chloride containing PEG/BSA complexes was measured at 400 nm using a UV spectrophotometer (UV-1601, Shimadzu, Japan). The size of PEG/protein complexes in methylene chloride was measured by a dynamic light scattering instrument (Zeta plus, Brookhaven, New York) equipped with a He–Ne laser at a wavelength of 632.2 nm at various PEG/BSA weight ratios.

2.2.2. Preparation of PLGA microparticles by spray drying

Four different PLGA microparticles were prepared according to the formulations listed in Table 1. A mixture of co-lyophilized BSA (8.6 mg)/PEG (43 mg) (PEG/BSA weight ratio = 5) was dissolved in 20 ml of methylene chloride. RG502, RG503, or RG756 PLGA polymer was added to the methylene chloride solution. The molecular weights of RG502, RG503, or RG756 PLGA polymer were 14.5 kDa, 36 kDa, and 98 kDa, respectively [25–27]. Various amounts of RG756 PLGA polymer were also used for the formulation. As shown in Table 1, the weight ratio of PLGA to BSA was adjusted at 5, 14, and 52. rhGH microencapsulated PLGA microparticles were prepared by using RG503 polymer according to the similar process. Spray drying was carried out using a Buchi 190-Mini Spray Dryer (Buchi, Flawil, Switzerland). The operating conditions were as follows: nozzle diameter, 0.5 mm; air flow-rate, 800 L/h; sample pumping speed set, 5%; aspiration, 100%; inlet temperature, 45 °C; outlet temperature, 39 °C.

2.2.3. Characterization of PLGA microparticles

The size and surface morphology of PLGA microparticles prepared by varying formulation conditions were visualized by scanning electron microscopy (SEM, Philips 535M). The microparticles were mounted on a brass stub using a double-sided adhesive tape and vacuum coated

Table 1
Various BSA encapsulated PLGA microparticles prepared by changing weight ratio of BSA/PEG/PLGA and polymer composition

Sample	PLGA	BSA:PEG:PLGA weight ratio	Size (μm)	Leading amount (%)	Initial burst (%)
1	RG756	1:5:5	27.0 ± 16.8	8.4 ± 0.4	73.5 ± 2.8
2	RG756	1:5:14	23.8 ± 4.6	4.7 ± 0.4	56.3 ± 3.3
3	RG756	1:5:52	1.3 ± 0.5	2.3 ± 0.8	18.3 ± 2.8
4	RG502	1:5:52	3.8 ± 0.8	1.8 ± 0.2	19.2 ± 1.4

with a thin layer of gold particles. To show the morphological change of PLGA microparticles during incubation time in phosphate buffered saline (PBS, pH 7.4) solution, PLGA microparticles were incubated in a shaking incubator at 37 °C for 20 days and freeze-dried. To eliminate salts in the release medium, PLGA microparticles were washed with distilled water three times before freeze-drying. The average size of the microparticles was determined by measuring the diameter of at least 50 microparticles. The loading amount of proteins within PLGA microparticles was determined in triplicate after dissolving microparticles at a concentration of 10 mg/ml in PBS solution containing 1 N NaOH as previously reported [4]. The protein content in the clear solution obtained after overnight incubation at 37 °C was analyzed by micro-BCA protein assay kit (Pierce, Rockford, IL).

2.2.4. Release profile of proteins from PLGA microparticles

Dried PLGA microparticles (10 mg) were resuspended in 1 ml of PBS solution and placed in a shaking incubator at 37 °C. At pre-determined time intervals, the supernatant was collected after centrifugation at 5000 rpm for 1 min to determine the amount of released protein by micro-BCA protein assay kit. The same volume of fresh PBS buffer solution was replenished at each sampling time to maintain constant pH in the incubation medium.

2.2.5. Size-exclusion chromatography (SEC) and polyacrylamide gel analysis

Structural integrities of the released rhGH fraction from spray-dried microparticles were analyzed using size-exclusion chromatography and polyacrylamide gel electrophoresis [28]. An HPLC (1100 series, Agilent Technologies, Palo Alto, USA) with a UV detector was operated using Bio-Silect 250-5 (300 × 7.6 mm, Bio-Rad) as a size-exclusion column; PBS solution as an isocratic mobile phase; 1.0 ml/min as a flow-rate. The eluate was monitored by UV absorption measurement at 280 nm. Naked rhGH and released rhGH from spray-dried microparticles were also loaded in polyacrylamide gel (4–20% gradient gel, Invitrogen, Carlsbad, CA). Electrophoresis was carried out in a constant voltage mode at 120 V for 1 h. After staining with 0.1% coomassie blue solution, the gel was destained with an aqueous solution of 10% methanol and 10% acetic acid.

3. Results and discussion

A schematic flow diagram for preparation of spray-dried microparticles encapsulating PEG/protein complexes is shown in Fig. 1. To eliminate salts, BSA and rhGH proteins were extensively dialyzed before use. Protein and PEG at different weight ratios were dissolved together in deionized water and lyophilized. The aqueous PEG/protein mixture was co-lyophilized by rapid liquid nitrogen quenching/freezing method without further annealing step. After the lyophilized mixture was dissolved in a PLGA containing

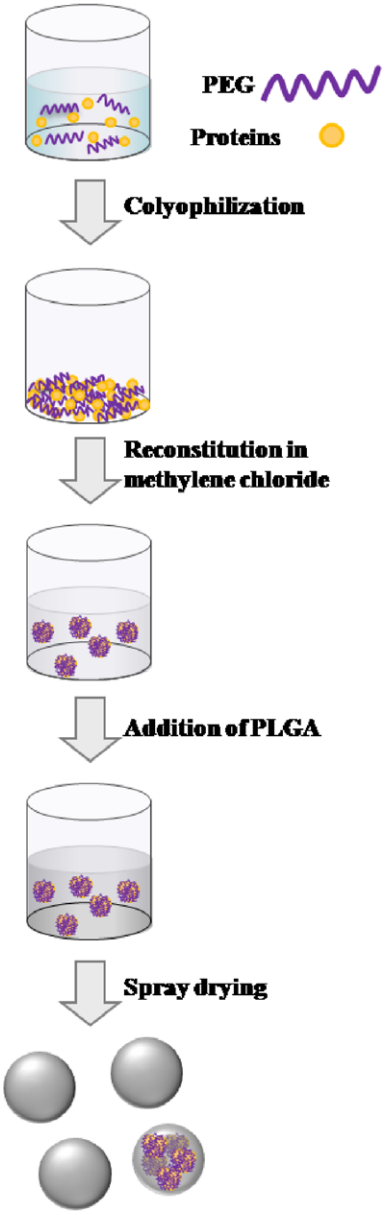

Fig. 1. Schematic illustration for the preparation of PLGA microparticles by spray drying.

methylene chloride phase by forming protein/PEG complex particles, the organic phase was directly spray dried to produce protein loaded PLGA microparticles. Quite recently, we reported that diverse biomacromolecules, such as protein, nucleic acids, and carbohydrate polymers, could be solubilized in organic solvents by using PEG as a nano-scale complex-forming agent [24]. The organic solvent phase dissolving a co-lyophilized protein/PEG mixture was transparent without showing any aggregates and precipitates. Previously it was reported that PLGA microparticles containing 2–3 μm protein powder were prepared by a single solid-in-oil-in-water (S/O/W) emulsion method [14]. It was claimed that micron-sized protein particles could be produced by controlled freezing-induced condensation followed by PEG-induced phase separation during

the lyophilization process. The diameter of phase separated protein particles decreased with increasing the weight ratio of PEG/protein. In our recent study, however, we proposed that co-lyophilized protein and PEG were actually complexed by non-covalent interactions in methylene chloride phase, rather than phase separated. We could attain nano-scale protein/PEG complexes by using much lower PEG amount than the reported PEG amount required for the phase separation between PEG and protein during the controlled freezing-lyophilization process. It was confirmed that BSA and PEG, labeled with two different fluorescent dyes, were co-localized in methylene chloride, suggesting that they were indeed complexed [24]. Nano-sized protein/PEG complexes were preferentially formed when the PEG/protein mixing ratio in an aqueous solution was higher than 5.

To show the solubility of PEG/BSA complexes in methylene chloride, transmittance values of the organic solution containing PEG/BSA complexes prepared with different PEG/BSA weight ratios were measured by UV spectrophotometer. According to our previous report, appropriate molecular weight of PEG was 3.35–10 kDa for the solubilization of proteins in organic solvent [24]. Thus, PEG with molecular weight of 3.35 kDa was chosen for this experiment. As shown in Fig. 2A, the transmittance value increases as raising the weight ratio of PEG/BSA and reaches at $83.4 \pm 0.3\%$ at the weight ratio of 45. The effective diameter of PEG/BSA complexes in methylene chloride decreases from 371.3 ± 26.4 to 107.4 ± 2.4 nm with increasing the weight ratio of PEG/BSA from 5 to 45, as determined by DLS. No detectable formation of complexes could be observed below PEG/BSA weight ratio of 2. It was postulated that protein and BSA were complexed by hydrogen bonding in methylene chloride. It is known that nonionic hydrophilic polymers such as poly(vinyl alcohol) (PVA) and poly(*N*-vinylpyrrolidone) (PVP) also form complexes with proteins via hydrogen bonding in aqueous solution [29,30]. In our previous study, PEG/BSA complexes in

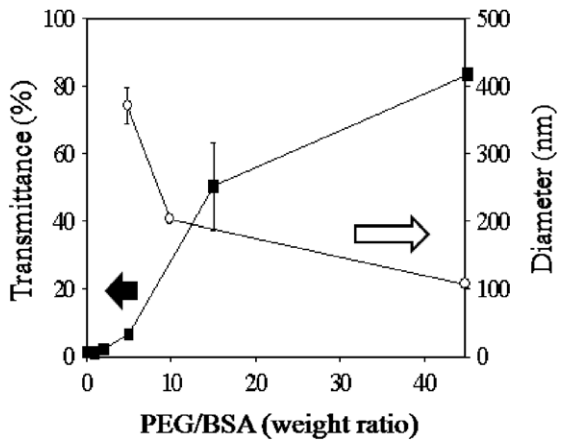

Fig. 2. Transmittance of 100% methylene chloride solution containing PEG/BSA complexes and effective diameter of PEG/BSA complexes at various weight ratios of PEG/BSA.

a diameter of 139.9 ± 51.1 nm in methylene chloride at the PEG/BSA weight ratio of 45 were visualized using AFM, similar to the size value as determined by DLS [24].

PLGA polymers with varying amounts and different compositions were also dissolved in the methylene chloride phase containing PEG/BSA complexes formulated at a fixed PEG/BSA weight ratio of 5, and directly spray dried. Four different PLGA microparticles were prepared according to the formulations listed in Table 1. The weight ratio of PLGA to the BSA was varied at 5, 14, and 52, while a total weight of PEG, BSA, and PLGA in methylene chloride was kept constant at 500 mg. Fig. 3A–C shows SEM pictures for three different RG756 PLGA microparticles prepared at different weight ratios of PLGA to BSA (sample 1–3). PLGA microparticles prepared at the PLGA/BSA weight ratio of 5 and 14 were highly aggregated with an average size of (A) 27.0 ± 16.8 μm and (B) 23.8 ± 4.6 μm. In contrast, PLGA microparticles prepared at the weight ratio of 52 were (C) 1.3 ± 0.5 μm. The size and morphological characters observed for aggregated PLGA microparticles shown in Fig. 3A and B could be attributed to the agglomeration of individually spray dried, fine PLGA microparticles having relatively higher PEG contents than those in Fig. 3C. It was likely that the partial melting of blended PEG during the hot-air spray-drying process could lead to the physical fusion of PLGA microparticles in the cyclone area [31]. The loading amount (%, w/w) of BSA within PLGA microparticles (μg of protein/μg of total microparticle × 100) prepared at PLGA/BSA weight ratios of 5, 14, and 52 were $8.4 \pm 0.4\%$, $4.7 \pm 0.4\%$, and $2.3 \pm 0.8\%$, respectively (Table 1). Two other PLGA polymers, RG503 (data not shown) and RG502 (Fig. 3D), were also used for the formulation at the same PLGA/BSA weight ratio of 52. There were no significant differences in the size, morphology, and BSA loading amount between the three spray-dried PLGA microparticles depending on the polymer composition and molecular weight. This suggests that the PLGA/PEG/BSA weight ratio of the formulation was a critical factor in determining the overall shape and size of the resultant PLGA microparticles. It can be also noticed that the larger PLGA microparticles prepared from the relatively higher PEG amount in the formulation, although the BSA loading amounts were concurrently increased, gave rise to higher initial burst releases due to the rapid dissolution of blended PEG fraction from the PLGA microparticles upon incubation in the buffer medium.

Fig. 4 shows *in vitro* release profiles of BSA from the two RG756 PLGA microparticles and one RG502 PLGA microparticles formulated at PLGA/BSA weight ratio of 14 and 52 (sample 2–4). The PLGA microparticles containing PLGA/BSA weight ratio of 14 (weight percent of PEG to the total components = 25.0%) exhibited an initial burst of $56.3 \pm 3.3\%$ at 1 day and a subsequent slow release up to 30 days, while those containing PLGA/BSA weight ratio of 52 (weight percent of PEG to the total components = 8.6%) showed continuous release profiles after

18.3 ± 2.8% (RG756) and 19.2 ± 1.4% (RG502) of initial bursts. The PLGA microparticles formulated with the weight ratio of 14 had the greater loading amount of BSA, but the higher PEG content within microparticles caused the significant extent of initial burst, compared to those with the weight ratio of 52. This was probably because entrapped BSA was easily released out through the pores generated by the rapid dissolution of incorporated BSA/PEG complexes. It was likely that BSA/PEG complexes physically entrapped within PLGA microparticles might be preferentially hydrated and swollen in aqueous media, and then quickly released out. On the other hand, PLGA microparticles with the weight ratio of 52 exhibited much reduced initial bursts with sustained release behaviors, although they were much smaller than the other one. Two PLGA microparticles prepared from different compositions (RG756 and RG502) showed a little influence on cumulative BSA release profiles, as long as the relative ratio of BSA/PEG/PLGA in the formulation was the same. This was because encapsulated BSA molecules were released out predominantly by diffusion, not by the erosion/degradation of PLGA polymers [32]. The BSA/PEG complexes entrapped with more densely fabricated PLGA microparticles were more slowly swollen upon hydration, dissociated, and free BSA molecules were released out in a controlled fashion.

PLGA microparticles containing a therapeutic protein, recombinant human growth hormone (rhGH), were also prepared. rhGH has been used for treating pediatric hypopituitary dwarfism, requiring subcutaneous administration for several years daily or three times a week [4,19]. A sustained release formulation of rhGH has great potential to provide patients with comfort as well as therapeutic efficacy. Prior to the formulation process, rhGH was mixed with PEG and co-lyophilized to form nano-sized complexes in methylene chloride. As shown in Fig. 5A, the methylene chloride solution containing PEG/rhGH complexes was transparent as increasing weight ratio of PEG/rhGH. Although the increasing tendency of transmittance value as increasing PEG/rhGH weight ratio was similar to that in the PEG/BSA complexes, the transmittance value of

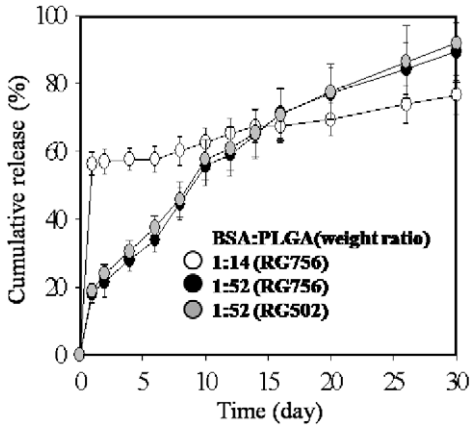

Fig. 4. Cumulative release profiles from PLGA microparticles prepared by RG756 and RG502 at PLGA weight ratio to BSA of 14 and 52.

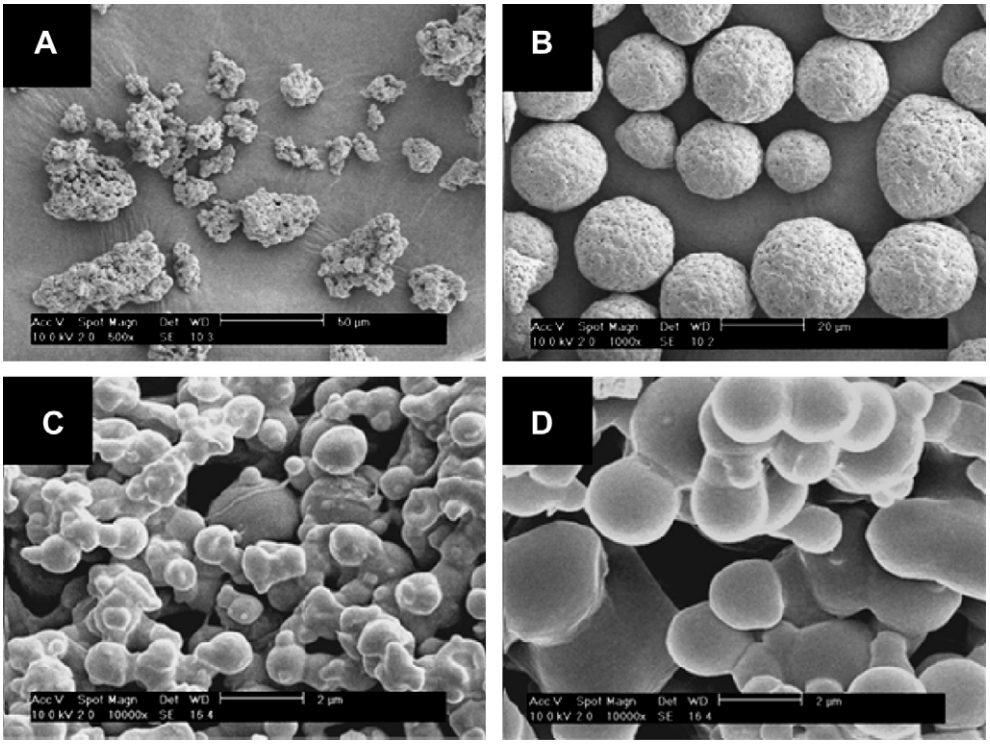

Fig. 3. Scanning electron microscopy (SEM) image of spray-dried microparticles at various PLGA weight ratios to the BSA of (A) 5, (B) 14, and (C and D) 52 prepared by various PLGA polymers, RG756 (A–C) and RG502 (D).

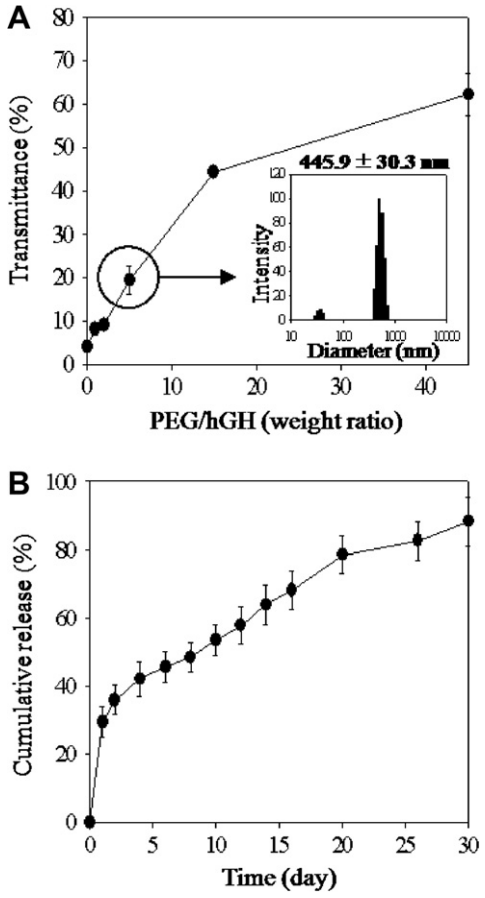

Fig. 5. (A) Transmittance of 100% methylene chloride solution containing rhGH at final concentration 0.5 mg/ml and effective diameter of PEG/rhGH complexes in methylene chloride. (B) Cumulative release profiles from PLGA microparticles prepared by RG503 at PLGA weight ratio to BSA of 52.

methylene chloride containing PEG/rhGH at weight ratio of 45 was lower than that containing PEG/BSA, which might be attributed to different PEG binding characters between BSA and rhGH. The effective diameter of PEG/rhGH complexes in methylene chloride was 445.9 ± 30.3 nm, which was a little bigger than that of PEG/BSA complexes.

rhGH was encapsulated within PLGA (RG503) microparticles at PEG/rhGH weight ratio of 5 by direct spray drying. The average diameter of PLGA microparticles containing PEG/rhGH complexes and the loading amount of rhGH within microparticles were 2.2 ± 0.6 μm and 2.9 ± 0.3 (w/w)%, respectively. rhGH was released out continuously from PLGA microparticles during 30 days after 29.4 ± 4.5% of initial burst at day 1 (Fig. 5B). To show the morphological change of PLGA microparticles during the release time, SEM image of PLGA microparticles incubated for 20 days in PBS solution was compared to that of as-prepared PLGA microparticles (Fig. 6). After incubation for 20 days, several PLGA microparticles were fused together with generating highly porous internal morphology (Fig. 6B and C). The fusion of PLGA microparticles containing rhGH/PEG complexes was caused by lowered glass transition temperature (Tg) of PLGA polymer chains by hydrolysis of ester bonds [32]. The porous internal morphology of degraded PLGA microparticles suggests that rhGH/PEG complexes were physically embedded in the PLGA matrices, but they were swollen and slowly diffused out, leaving behind a porous PLGA skeletal structure. Thus it appears that the direct encapsulation of rhGH/PEG complexes plays a crucial role in the sustained release profile of rhGH [33–35]. In our previous study, biodegradable microparticles with inter-connected nano-porous

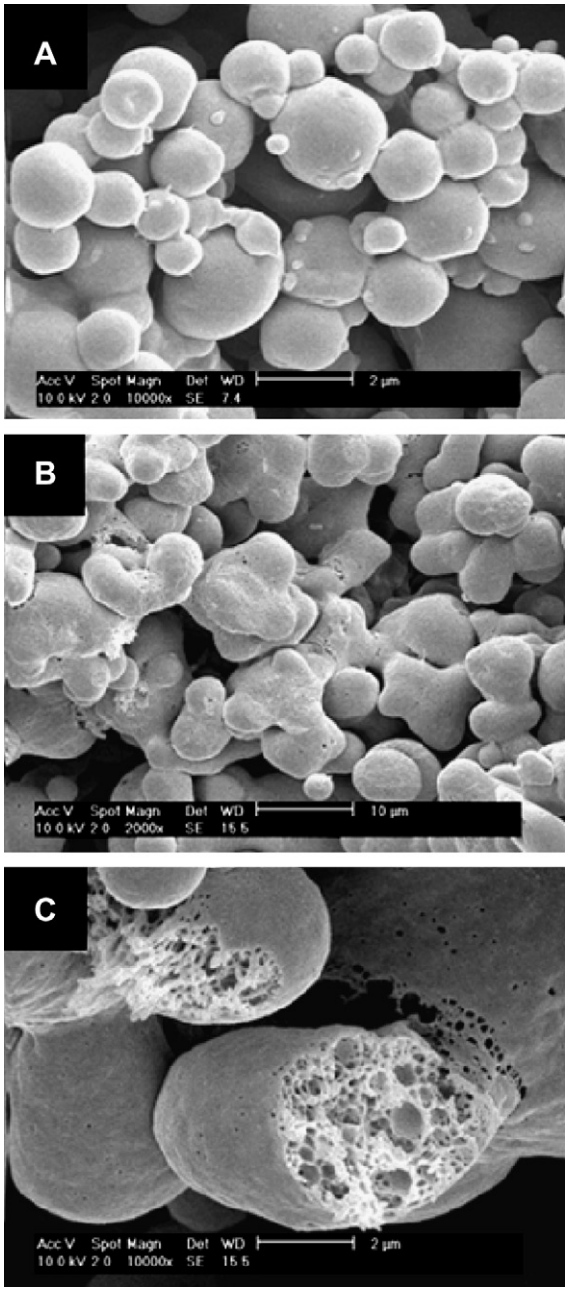

Fig. 6. Scanning electron microscopy of (A) as-prepared PLGA microparticles containing rhGH, and (B and C) incubated microparticles for 20 days in PBS solution.

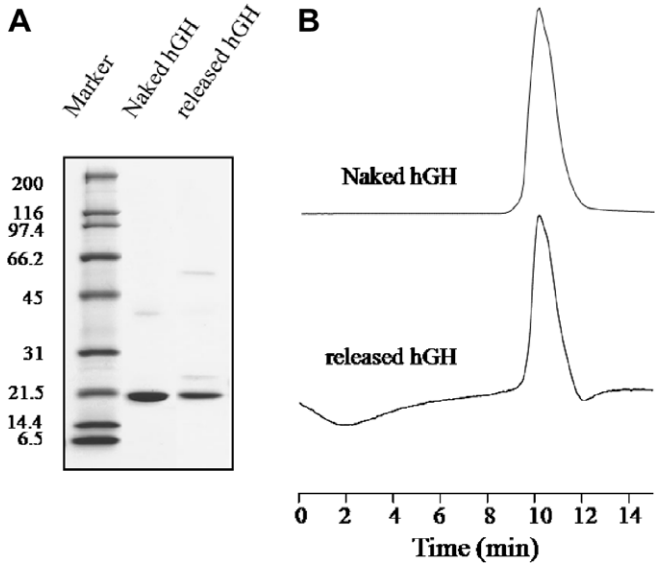

Fig. 7. (A) PAGE gel result and (B) size-exclusion chromatography (SEC) of naked rhGH and released rhGH (day1) from PLGA microparticles.

structure showed far better controlled protein release profiles [34]. The highly porous inter-connected channels within spray-dried microparticles, accompanying with influxing of water, were presumably produced via initial hydration of rhGH/PEG complexes.

The structural integrity of rhGH after release was analyzed by size-exclusion chromatography (SEC) and gel electrophoresis as shown in Fig. 7. It was previously reported that the aggregation of rhGH severely occurred during the conventional W/O/W formulation process by an exposure to an air/liquid interface [36]. The results of PAGE gel and SEC suggest that a negligible amount of rhGH was aggregated in the fraction of released rhGH from PLGA microparticles after 1 day. This was due to the fact that the compact structure of rhGH/PEG complexes in a single organic phase, enabled rhGH molecules to avoid from harsh processing conditions encountered during the spray-drying process, for example, shear-stress induced during an atomization [18]. While the spray-drying process is one of the preferred formulation methods for protein encapsulated microparticles due to easy operation, high encapsulation efficiency, and good maintenance of sample purity, it was reported that when an aqueous solution containing protein molecules was spray dried, approximately 25% of the feed protein was easily degraded by the surface-induced denaturation at an air–liquid interface of the droplets generated during the atomization as well as thermal degradation due to high inlet temperature during the formulation process [37,38]. In this study, protein molecules were complexed and directly spray dried at low inlet temperature in a water free condition to minimize such adverse effects on protein conformational change. The water-free microencapsulation of proteins within PLGA microparticles via direct spray drying would have high potential for achieving sustained protein formulations without the loss of bioactivities.

In conclusion, it has been demonstrated that two model proteins, BSA and rhGH, could be directly encapsulated within PLGA microparticles by forming complexes with PEG in pure methylene chloride without using water. Spray-dried PLGA microparticles containing proteins exhibited relatively low initial bursts and sustained release behaviors for 30 days *in vitro* due to the formation of self-generating interconnected porous channels within degrading PLGA bulk phase. The current non-aqueous solution spray drying technique protected proteins from denaturation after spray-drying process, and enabled complete release of proteins.

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