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Stabilization of recombinant human growth hormone against emulsification-induced aggregation by Pluronic surfactants during microencapsulation

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Abstract

Protein aggregation upon exposing to the water/organic solvent interface is one of the most significant obstacles in developing poly(lactic-*co*-glycolic acid) (PLGA) microspheres with double emulsion process. The aim of present study is to devise a formulation strategy to prevent recombinant human growth hormone (rhGH) from aggregation during microencapsulation. The excipients used for stabilizing rhGH were selected from sugars, nonionic surfactants, polyol, and protein. Among the candidates, surfactants exhibited potentialities in protecting rhGH against emulsification-induced aggregation. It was also found that Pluronic F127 showed an outstanding as well as concentration-dependent stabilizing effect on rhGH, which was different to Pluronic F68 and Tween 20. After the rhGH solution comprising F127 and sucrose was emulsified with methylene chloride, the recovery of monomeric protein achieved 99.0%, principally attributed to the presence of F127. This solution was subsequently encapsulated as inner aqueous phase in the PLGA microspheres by a conventional double emulsion process, with the encapsulation efficiency higher than 98%. Improvement in the release of rhGH was observed for the microspheres co-encapsulating Pluronic F127 regardless in the presence or absence of sucrose, compared to the microspheres containing rhGH alone. The result further implied that co-encapsulation of Pluronic F127 in the microspheres played an important role in the stabilization of rhGH.

Keywords: Recombinant human growth hormone (rhGH); Protein stability; Emulsification; Microspheres; Pluronic

1. Introduction

Formulating effective delivery systems for proteins was one of the most highlighted, as well as the most challenging task over the past few decades, since numerous recombinant proteins have been developed for therapeutic applications. Although considerable interest has recently been focused on administrating proteins via oral, pulmonary, and transdermal routes, nevertheless due to their poor stability in gastrointestinal tract, low permeability to biological membrane barriers, and short in vivo half-life which always results in a therapeutic regimen of frequently dosing, parenteral depots consisting of biodegradable materials are the most suitable and feasible approach till today to maintain adequate plasma level and obtain long-lasting pharmacokinetic profiles (Sinha and Trehan, 2003).

Biodegradable injectable depots, especially poly(lactic-*co*-glycolic acid) (PLGA) microspheres, have been extensively investigated for delivering proteins and vaccines in a controlled mode (Cleland et al., 1997; Chen et al., 1997; Yang and Cleland, 1997; Schrier and DeLuca, 1999; Genta et al., 2001; Boehm et al., 2002). A lot of efforts have succeeded in incorporating proteins in PLGA microspheres with desired particle size, morphology, and encapsulation efficiency, however, the most significant obstacle in developing biodegradable microspheres for proteins has emerged as the instability of the protein during encapsulation and in vivo release (Van de Weert et al., 2000a; Schwendeman, 2002).

Double emulsification-solvent evaporation process is a conventional method for preparing protein-loaded PLGA microspheres, which possesses advantages including that high yields and encapsulation efficiencies are easily achieved, and

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scaling down is possible (Okada, 1997). Unfortunately, in order to be finely dispersed within the polymer matrix, the protein solution (inner aqueous phase) must be emulsified with PLGA-dissolved organic solvent, usually methylene chloride. This creates a very large water/organic solvent interface which is apt to be occupied by proteins, and where protein unfolding and other deleterious processes can occur. The presence of hydrophobic interfaces has been identified as a major cause of protein denaturation and aggregation (Cleland and Jones, 1996; Kim and Park, 1999; Sah, 1999a,b; Péan et al., 1999; Van de Weert et al., 2000b; Pérez and Griebenow, 2001), which will not only result in therapeutic inactivity and incomplete release, but also may cause unpredictable side effects, such as immunogenicity or toxicity (Cleland et al., 1993). Therefore, it is important to devise formulation strategies to preserve protein stability during microencapsulation.

Adding stabilizing agents to the inner aqueous phase for competing with proteins on the interface was a rational option to prevent proteins exposing to organic solvents, and consequently reduce emulsification-induced denaturation and aggregation. Stabilizing additives were diverse and principally included proteins (Johansen et al., 1998; Sah, 1999a,b), carbohydrates (Cleland and Jones, 1996; Sánchez et al., 1999), polyols (Péan et al., 1999; Pérez and Griebenow, 2001), and basic salts of bivalent metal ion (Johnson et al., 1997). Surfactants were also candidates for protein stabilizer, except that few efforts have proven to be successful. For instance, Tween could not prevent extensive protein aggregation during emulsification; on the contrary, partially hydrolyzed polyvinylalcohol (PVA) was promising in stabilizing proteins during the microencapsulation process (Van de Weert et al., 2000b).

Recombinant human growth hormone (rhGH) was liable to aggregate when contact with organic solvent, hence was a favorite model for investigating the stabilization of protein encapsulated in microspheres (Cleland and Jones, 1996; Kim and Park, 1999, 2001; Takada et al., 2003). Based on the denaturation mechanism of rhGH, it was hypothesized that additives which could shield it from hydrophobic interface might be effective stabilizers. Since surfactants have an intrinsic character of oriented locating on the biphasic interface, in the present study they were selected to protect rhGH during the process of microencapsulation. The capabilities of Pluronics preventing rhGH from emulsification-induced aggregation were evaluated and compared with other potential stabilizing agents, and the effects of Pluronics on the in vitro release of rhGH-loaded PLGA microspheres were subsequently assessed. The ultimate aim of this work was to develop a novel method of using Pluronics to stabilize rhGH encapsulated in biodegradable microspheres.

2. Materials and methods

2.1. Materials

rhGH (monomer content 95.7%) was supplied as a bulk solution by United Cell Biotechnology Co. (Shanghai, China). End-uncapped PLGA having a lactic/glycolic molar ratio of 75/25 and inherent viscosity of 0.38 dl/g (Mw 15 kDa) was purchased from DURECT (Pelham, USA). Pluronic F127 and F68 were donated by BASF (Mount Olive, USA). PVA (98–99% hydrolyzed, Mw 106–110 kDa), polyethylene glycol (PEG, 400 Da), trehalose, sucrose, mannitol, and methylene chloride were purchased from SinoPharm Chemical Reagent Co. (Shanghai, China). Tween 20 and sodium dodecyl sulfate (SDS) were obtained from Farco Chemical Supplies (Hongkong, China). Bovine serum albumin (BSA) was bought from Bo'ao Biological Technology Co. (Shanghai, China). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Desalinization and lyophilization of rhGH bulk solution

Using AKTA^{TM} explorer chromatography system (Pharmacia, Uppsala, Sweden), rhGH bulk solution was buffer-exchanged into 10 mM ammonium bicarbonate (pH 7) on a Sephadex G25 gel filtration column (16 mm × 190 mm). An aliquot of 10 ml bulk solution was loaded onto the column and eluted at 5.0 ml/min. The absorbance at 280 nm and conductance were simultaneously determined. Desalted rhGH solution was lyophilized (Christ Alpha 1-2, Germany) to yield an excipient- and buffer-free protein powder (Costantino et al., 1998). Lyophilization cycle initiated at −55 °C, including a primary drying at a shelf temperature of −35 °C and a chamber pressure of 15 μbar for 24 h followed by a secondary drying at 5 °C for 36 h.

2.2.2. Emulsification-induced rhGH aggregation

Lyophilized rhGH powder was reconstituted in 10 mM sodium phosphate buffer (pH 8.0) to form a solution containing 50 mg/ml rhGH. Various excipients were added to 0.2 ml above rhGH solution and allowed to dissolve, and then each aliquot of the obtained solution was mixed with 0.5 ml methylene chloride. In order to simulate the primary emulsification step in the process of microsphere preparation, the mixtures were either magnetically stirred (Sile Instrument Co., Shanghai, China) at about 2000 rpm or sonicated (ZhiSun Instruments Co., Shanghai, China) at 40 kHz for 1 min. After agitation or sonication, rhGH was extracted for three times from the organic solvent by 3 ml phosphate buffer (10 mM, pH 8.0), and the recovered solutions were merged and diluted to 10 ml. The diluent was centrifugated (Biofuge Primco R, Heraeus, Germany) at 3000 rpm and 4 °C for 30 min to remove undissolved protein, and the supernatant was determined by size exclusion chromatograph (SEC). The solution of same composition which has not been subject to emulsification was directly diluted and analyzed as control. Recovery was calculated by the ratio of detected protein concentration after emulsification to that of control.

2.2.3. SEC-HPLC analysis of rhGH

The amount of monomeric rhGH and soluble aggregates in each recovered sample was quantitated by SEC-HPLC. A set of TSK G2000 SW_{XL} and guard column (Tosoh, Japan)

was operated at 0.6 ml/min and 25 °C using a mobile phase of 0.063 M phosphate buffer (pH 7.0) containing 3% isopropanol. An aliquot of 20 μl sample was injected into the HPLC system (1100 Series, Agilent, Germany), and protein peak detection was performed at 214 nm.

2.2.4. Microsphere preparation

A double emulsion process was used to produce rhGH PLGA microspheres. At the primary emulsification step, 50 mg/ml Pluronic F127 with or without 45 mg/ml sucrose was used as protein stabilizing agents in 0.8 ml phosphate buffer (10 mM, pH 8.0) containing 75 mg rhGH. This inner aqueous phase was emulsified with the organic phase comprising 500 mg PLGA dissolved in 2 ml methylene chloride by magnetic stirrer at about 2000 rpm for 45 s. The resulting w/o primary emulsion was subsequently added to 50 ml PVA solution (6%, w/v) and agitated for 1 min using a mechanical stirrer (Eurostar, IKA, Germany) at 1800 rpm to create the double emulsion (w/o/w), which was then poured into 600 ml distilled water and stirred magnetically for 1 h at ambient temperature and pressure to allow solvent evaporation and microsphere formation. The microspheres were isolated by filtration, washed with 400 ml of distilled water and 200 ml of 0.2% F68 solution, and finally lyophilized (Virtis, USA) overnight to obtain a free flowing water dispersible powder. Unstabilized microspheres were prepared as the same process, except that the inner aqueous phase did not consist of F127 and sucrose. The size distribution and morphology of the microspheres were observed by an optical (UFX-II, Nikon, Japan) and a scanning electron microscopy (SEM, S-520, Hitachi, Japan), respectively.

2.2.5. rhGH loading and encapsulation efficiency

The amount of rhGH encapsulated in the PLGA microspheres was determined in triplicate by dissolving 10 mg of microspheres in 2.5 ml of 0.1 M NaOH solution containing 0.5% (w/v) SDS as reported by Kim and Park (1999). All samples were incubated at 37 °C for 2 days, and then centrifuged at 5000 rpm for 3 min. The contents of rhGH in the supernatants were measured at 292 nm by UV/Vis spectrophotometer (UV-2401PC, Shimadzu, Japan).

2.2.6. In vitro release of rhGH from microspheres

The release of rhGH from the PLGA microspheres was carried out using microcentrifuge filtration tubes (Durapore, 0.22 μm filter, Millipore, USA) as described by Cleland et al. (1997). A 20-mg amount of rhGH microspheres was suspended in 300 μl of release medium containing 10 mM HEPES (pH 7.4), 100 mM NaCl, 0.02% Pluronic F68, and 0.02% azide on the retentate side of the filter. The tube was incubated at 37 °C, and the sample was recovered at predetermined time intervals by centrifugation at 4330 rpm for 1 min. The filtrate was removed and the microspheres were resuspended with 300 μl of the release medium. The amount of released rhGH was quantitated with bichinchoninic acid (BCA) protein assay kits (Pierce, Rockford, USA) in a microplate format (PowerWave XS, Biotek, USA).

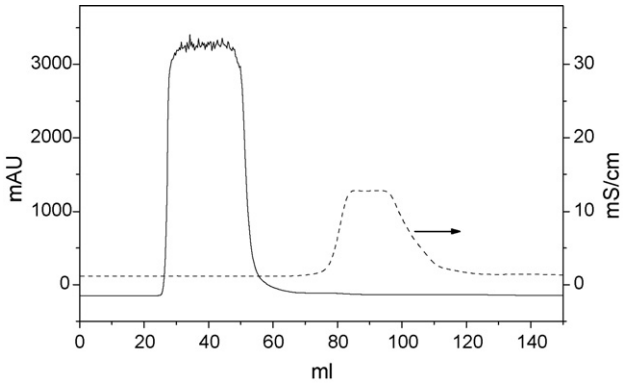

Fig. 1. Typical chromatogram of rhGH desalting. Eluting curve of rhGH (–) and electrolytes (- - -).

3. Results and discussion

3.1. Purification of rhGH

In order to eliminate the effects of undesired additives on the following stability study, rhGH bulk solution was desalted and purified using gel filtration chromatography system. Fig. 1 showed that rhGH and electrolytes in the bulk solution were completely separated based on the principle of size exclusion, and the solvent for rhGH was exchanged into ammonium bicarbonate. The collected protein solution was subsequently lyophilized, ammonium and bicarbonate were volatilized in this process, and hence an excipient- and buffer-free rhGH powder was obtained. The lyophilization did not adversely affect protein quality, particularly with regard to aggregation.

3.2. Effects of excipients on emulsification-induced rhGH aggregation

In order to simulate the primary emulsification process, the volume ratio of inner aqueous phase to organic solvent was set at 2:5, which was also used for preparing microspheres later. After the phosphate buffer solutions of rhGH at a concentration of 50 mg/ml were agitated or sonicated with methylene chloride for 1 min, approximately 30% of the protein was loss due to forming water insoluble precipitates. The recovered soluble protein contained more than 50% of protein aggregates, among which most were rhGH dimer, while the monomer contents decreased to less than 35% compared to its initial concentration (Fig. 2). To examine whether the observed rhGH aggregation was caused by contact with organic solvent or the continual creation of an extended air/water interface, rhGH solution containing 2 mg/ml Pluronic F68 was agitated for 3 min in the absence of methylene chloride. In this case, no considerable change in the content of rhGH monomer was observed (the recovery of monomeric protein was 98.3 ± 1.1% after agitation). Whereas when the same formulation was emulsified with methylene chloride for 1 min by agitation or sonication, the protein aggregation was similar to that of the excipient-free sample (data not shown). The results suggested that during microencapsulation, exposure to organic

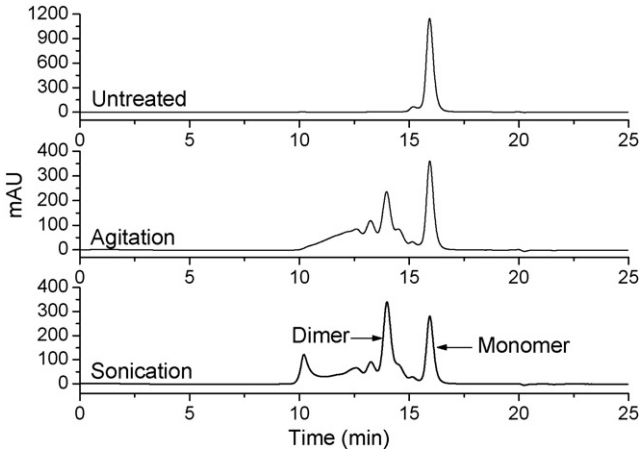

Fig. 2. Size exclusion chromatograms of rhGH in excipient-free phosphate buffer solution (pH 8.0) before and after emulsified with methylene chloride.

solvent was a critical factor leading to protein aggregation, and stabilizing excipients were indispensable to be incorporated in the microspheres formulation to prevent protein from aggregating.

The excipients used as candidates for stabilizing rhGH were selected from sugars, nonionic surfactants, polyol, and protein. Among the various kinds of excipients investigated in the present work, nonionic surfactants exhibited potentialities in protecting rhGH against emulsification-induced aggregation (Fig. 3). At a concentration of 20 mg/ml, Pluronic F68, Tween 20 and PVA increased the recovery of monomeric rhGH by 68.9, 71.7, and 52.5%, respectively, compared with the protein solution free of excipients. Pluronic F127 at the same concentration showed the most outstanding stabilizing effect on rhGH, resulting in a yield of rhGH monomer as high as $88.3 \pm 1.6\%$ after methylene chloride treatment. However, the recovery of both soluble and monomeric rhGH did not improved by adding 45 mg/ml sugars, including mannitol, trehalose, and sucrose, into the aqueous phase. Furthermore, no obvious improvement in the protein stability was observed when PEG 400 and BSA were present in the rhGH solution.

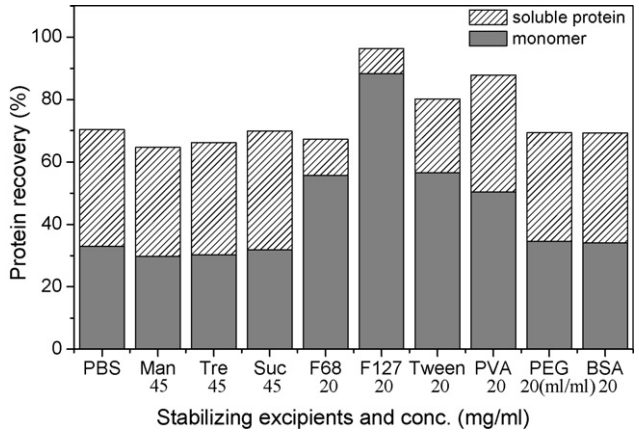

Fig. 3. Effects of various excipients on rhGH aggregation after emulsified with methylene chloride under agitation ($n = 3$). Man, Tre, and Suc represent mannitol, trehalose, and sucrose, respectively.

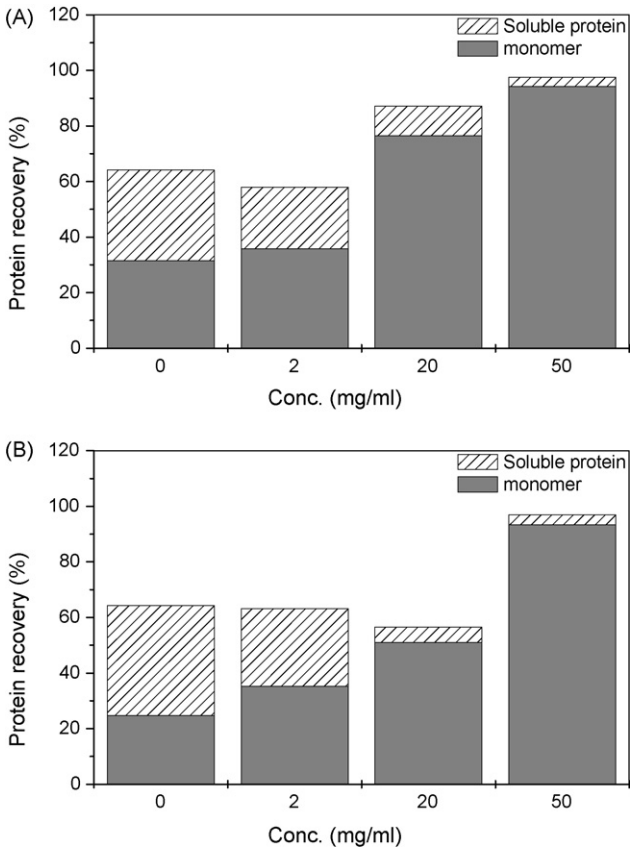

Fig. 4. The relationship between the concentration of Pluronic F127 and the aggregation of rhGH after emulsified with methylene chloride ($n = 3$). (A) Agitation and (B) sonication.

Based on the previous finding, the relationship between the concentration of Pluronic F127 and the aggregation of rhGH during emulsification with organic solvent was further explored. As illustrated in Fig. 4, the effects of Pluronic F127 upon protecting rhGH against aggregation presented an intensive dependence on its concentration. The recovery of monomeric rhGH increased with the concentration of F127, giving the correlative coefficients of 0.9496 and 0.9924 under agitation and sonication condition, respectively. When the aqueous phase contained 50 mg/ml of F127, the yields of rhGH monomer achieved 94.2 ± 1.7 and $93.4 \pm 0.5\%$ after treated with methylene chloride by agitation and sonication. The corresponding chromatograms were shown in Fig. 5, from which it could be seen that besides monomeric rhGH, just a little amount of dimer occurred in the recovered protein, indicating that Pluronic F127 at a high concentration stabilized rhGH efficiently against emulsification-induced aggregate.

Proteins tend to adsorb and unfold at water/organic solvent interfaces, because of their amphiphilic character and flexible conformation. This is detrimental to their stability, since exposure of hydrophobic moieties which are buried in the native state may lead to forming aggregates. Therefore, a rational stabilization strategy for proteins during emulsification should prevent it from contact with the hydrophobic surface.

Pluronics are ABA triblock copolymers consisting of hydrophilic polyoxyethylene (PEO) and hydrophobic poly-

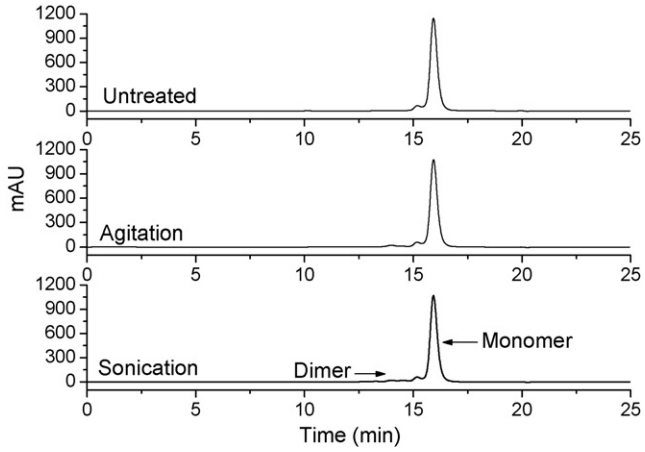

Fig. 5. The chromatograms of the rhGH solution containing 50 mg/ml of Pluronic F127 before and after emulsified with methylene chloride.

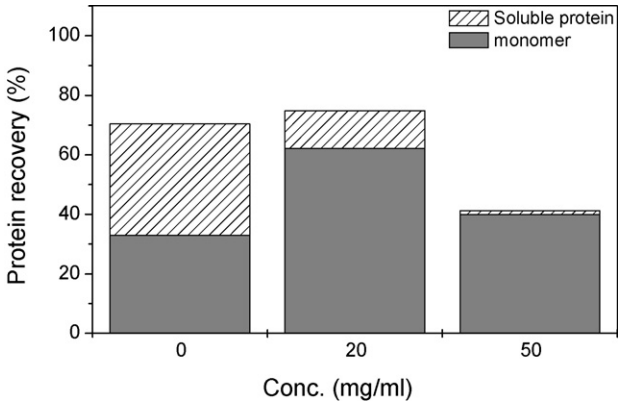

Fig. 6. Effects of Tween 20 on rhGH aggregation after emulsified with methylene chloride under agitation ($n = 3$).

oxypropylene (PPO). As a kind of nonionic surfactant, in a water-in-oil emulsion system Pluronics spontaneously insert their hydrophobic blocks into the oil phase while leaving hydrophilic blocks in the aqueous phase. The mechanism of surfactants stabilizing protein during emulsification may be due to their competing for adsorption and oriented locating on the biphasic interface, forming a protective barrier which prevented protein from exposing to organic solvent, hence protein unfolding and aggregating was restrained. This hypothesis could be confirmed by the result that various surfactants exhibited stabilizing effects upon rhGH to different extents during the emulsification process (Fig. 3). With the increase in surfactant concentration, there were more molecules competing with protein for the interfacial adsorption, and the probability that protein contact with organic solvent was consequently minimized, resulting in a reduced aggregation. However, a significant difference in the recovery of rhGH monomer was observed between Pluronic F127 and F68 at the same concentration and emulsifying condition. This could be explained from the view of the structure of Pluronic analogues. Pluronic F127 possesses longer PEO and PPO chains and higher PPO content compared to F68. Such a molecular structure endows F127 with a hydrophilic–hydrophobic balance value of 22, whereas that of F68 is 29 (Kabanov et al., 2002). Both of the characters made F127 more affinitive to the water/organic solvent interface, and the longer PEO chain remained in aqueous phase kept protein away from the interface more effectively, so F127 presented a much better stabilizing effect on rhGH than F68. This is consistent with the previous report by Katakam and Banga (1997), who found that F127 was the most effective stabilizer for rhGH against interfacial stress generated by vortex.

The stabilizing effects of nonionic surfactants on emulsification-induced protein aggregation were also proved by the increased rhGH recovery in the presence of PVA (Fig. 3). The surface active properties of PVA make it a popular stabilizer in the outer aqueous phase of w/o/w emulsions during the preparation of PLGA microspheres. Van de Weert et al., 2000b found that partially hydrolyzed PVA significantly reduced lysozyme aggregation during the encapsulation process, due to preferentially adsorb onto the water/organic solvent interface. In the present work, a similar phenomenon was observed on rhGH, although the PVA with different hydrolyzed degree and molecular weight was used.

It has been reported (Bam et al., 1998) that Tween could protect rhGH against agitation-induced denaturation via hydrophobic interactions, so the effects of Tween 20 on stabilizing rhGH were compared with Pluronics. After emulsified with methylene chloride under agitation, there was $62.1 \pm 3.1\%$ of rhGH monomer recovered from the formulation containing 20 mg/ml of Tween 20 (Fig. 6). The stabilizing effects shown by Tween 20 were slight better than Pluronic F68, but substantially worse than F127 at the same concentration. In contrast to Pluronic F127, when the concentration of Tween 20 increased to 50 mg/ml, the yield of monomeric rhGH decreased instead to $40.0 \pm 3.7\%$. The effect of surfactants on the physical stability of rhGH has been investigated by Katakam et al. (1995) using differential scanning calorimetry (DSC). At higher concentrations, a quite obvious tendency of decrease in the endothermic peak onset temperature was observed in Tween-contained rhGH formulation, indicating that rhGH was actually being destabilized. In addition, as the concentration of Tween increased, the endothermic peak energy also increased, suggesting that Tween might promote rhGH unfolding due to interaction with the protein. These results could explain the instable phenomenon of rhGH in the formulation comprising Tween of high concentration.

PEG and BSA were ineffective in stabilizing rhGH, probably attributed to the facts that PEG distributed anomalous in a water/methylene chloride system (Van de Weert et al., 2000b), and that the adsorptive equilibrium of BSA at the biphasic interface was slowly reached (Péan et al., 1999), during this process the emulsification of protein solution has already completed.

Sugars were usually used as protein protectants in lyophilized and liquid formulations, but in the present study failed in preventing rhGH against emulsification-induced aggregation under both agitation and sonication condition (Figs. 3 and 7). Cleland and Jones have reported that mannitol and trehalose could provide effective protect for rhGH during emulsification (1996). On the contrary, experiments with erythropoietin (Morlock et al., 1997), ovalbumin and lysozyme (Sah, 1999a) did not reach the same

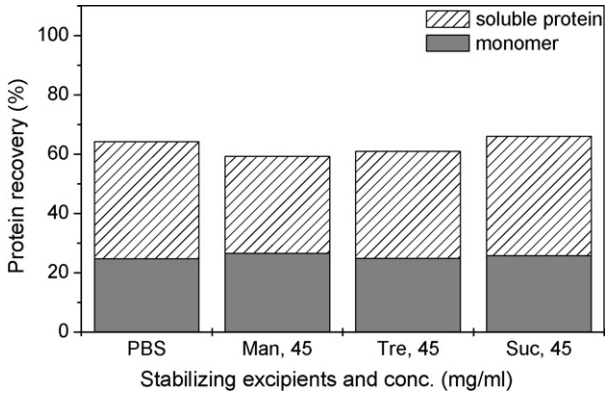

Fig. 7. Effects of sugars on rhGH aggregation after emulsified with methylene chloride under sonication ($n = 3$). Man, Tre, and Suc represent mannitol, trehalose, and sucrose, respectively.

conclusion, because the recoveries of these proteins after emulsified with organic solvent were not improved by using sugars as stabilizing agents. The discrepancy stemmed mainly from the protein concentration used in the aqueous phase. Since proteins may act as 'self-protectants' at high concentrations, acceptable stabilizing effects were obtained in the saturated rhGH formulations containing mannitol or trehalose, as described by Cleland and Jones (1996). Moreover, in the aqueous solution comprising sugars, proteins prefer to maintain their native conformation due to the preferential hydration effect. However, proteins are surface active, and prone to absorb to the huge water/organic solvent interface generated by emulsification. The adsorbed protein molecules tend to undergo various physical or chemical transformations. By contrast with proteins, sugars are not surface active and do not have any affinity to the biphasic interface. As a result, they seemed ineffective in restraining emulsification-induced protein aggregation (Sah, 1999a). However, considering

Table 1
Formulations and characteristics of rhGH PLGA microspheres (mean ± S.D., $n = 3$)

Formulation	Actual loading (%)	Encapsulation efficiency (%)
rhGH alone	12.4 ± 0.9	97.9 ± 2.2
rhGH and Pluronic F127	11.7 ± 0.3	91.6 ± 4.7
rhGH, Pluronic F127 and sucrose	11.3 ± 0.2	98.5 ± 0.6

that sucrose could reduce rhGH aggregation in aqueous solution (unpublished data), it was incorporated into the microspheres formulation to improve the protein stability during release. The inner aqueous phase, which was subsequently used to prepare the microspheres, contained 50 mg/ml Pluronic F127 and 45 mg/ml sucrose, and the recovery of rhGH monomer was 99.0 ± 1.4% after agitated for 1 min with methylene chloride.

3.3. Characterization of rhGH PLGA microspheres

The PLGA microspheres consisting of rhGH and stabilizing additives were prepared with a conventional double emulsion process. After optimization on the procedure parameters, the size distribution of the obtained microspheres was mainly in a range from 10 to 40 μm, with a mean size of 28.0 ± 15.4 μm and a span of dispersity of 1.15. The microspheres co-encapsulating rhGH with Pluronic F127 and sucrose were spherical observed by SEM, and had a smooth surface without pores, as shown in Fig. 8.

The encapsulation efficiency of rhGH in the PLGA microspheres was slightly affected by the additives (Table 1). When Pluronic F127 was incorporated in the microspheres, the encapsulation efficiency decreased from 97.9 ± 2.2 to 91.6 ± 4.7% with respect to the microspheres containing rhGH alone. The reduction of encapsulation efficiency in the presence of surfac-

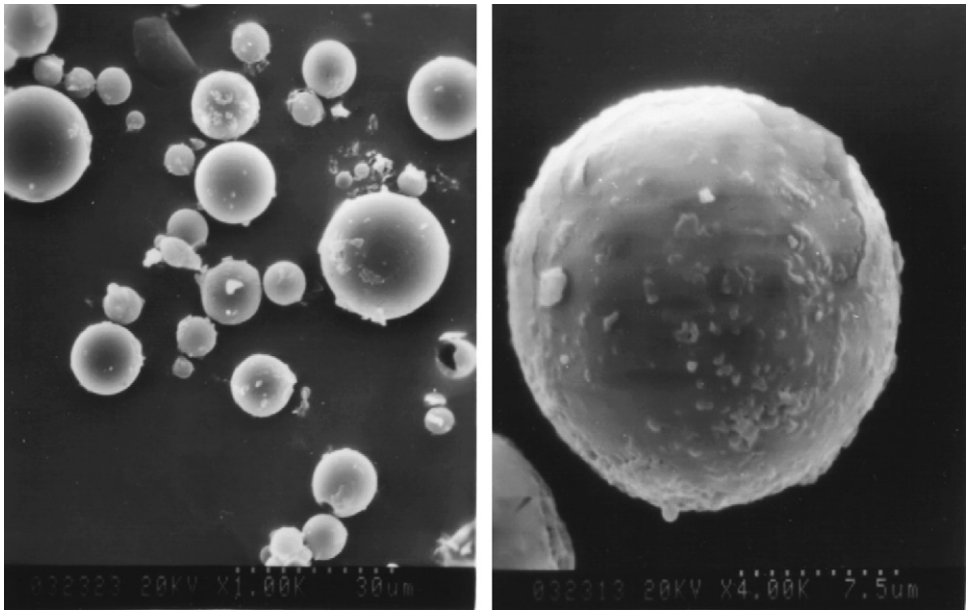

Fig. 8. Scanning electron micrographs of rhGH-loaded PLGA microspheres containing Pluronic F127 and sucrose as stabilizing additives (×1000 and ×4000, respectively).

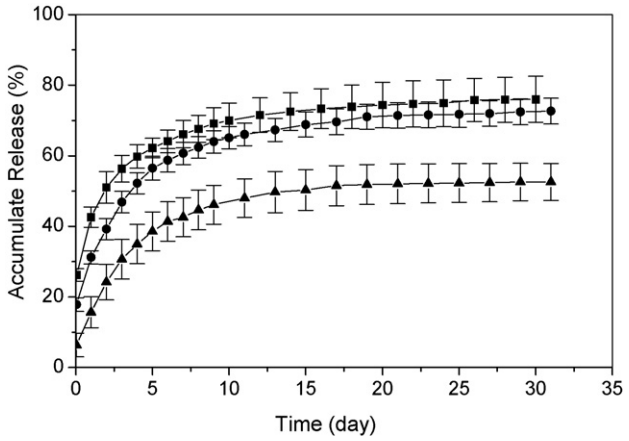

Fig. 9. Release profiles of rhGH from microspheres prepared with different components of inner aqueous phase ($n = 3$). (■) Containing F127 and sucrose; (●) containing F127; and (▲) without stabilizing additives.

tant could reasonably be attributed to a displacement of rhGH molecules from water/organic solvent interface, eventually decreasing the amount of rhGH located within the primary emulsion. This result is in agreement with previous reports (Blanco and Alonso, 1998; De Rosa et al., 2000), in which decreases in the encapsulation efficiency of proteins within PLGA microspheres were observed when F68 was added in the formulation. For the finally selected inner aqueous phase, the encapsulation efficiency was increased to $98.5 \pm 0.6\%$, due to preferential hydration of rhGH in the presence of sucrose (Johansen et al., 1998). The actual loadings of rhGH in the stabilized formulations with or without sucrose, and in the unstabilized formulation were 11.3 ± 0.2, 11.7 ± 0.3, and $12.4 \pm 0.9\%$, respectively.

3.4. In vitro release of rhGH PLGA microspheres

The release profiles of rhGH from the microspheres up to 1 month comprised a continuous release stage in the first 3 weeks followed by a plateau that release gradually leveled off, as shown in Fig. 9. Increments in both the burst releases and the total amounts of released rhGH were observed for the microspheres co-encapsulating Pluronic F127 regardless in the presence or absence of sucrose, compared to the microspheres containing rhGH alone. The recovered samples after 1 h of incubation were analyzed with SEC, and the contents of rhGH monomer were found similar and higher than 91% for both the stabilized and unstabilized formulations.

Incomplete release was frequently encountered for many proteins entrapped in PLGA microspheres, and was principally attributed to both protein aggregation and nonspecific protein adsorption occurring within the microspheres. The water soluble protein species which have not been subject to aggregation during microencapsulation were responsible for the continuous release upon incubation (Kim and Park, 1999). On the base of this theory, the improved stability of rhGH endowed by Pluronic F127 upon emulsification-induced aggregation could account for the increment in accumulative release of rhGH without significant structural changes. On the other hand, the PLGA used in the present study has uncapped terminal groups of carboxylic acid, which could establish ionic interactions with the positively charged amino acid residues on the protein surface. The increased release could also be interpreted that F127 reduced the fraction of rhGH anchored via ionic or hydrophobic interaction onto the polymer matrix by preventing the protein from contact with the PLGA-dissolved organic phase. Moreover, the enhanced hydrophilicity caused by the introduction of surfactant and sugar may contribute to the continuous release in the early stage (De Rosa et al., 2000). The results of in vitro release further implied that co-encapsulation of Pluronic F127 in the microspheres played an important role in the stabilization of rhGH.

To summarize, in the present study it was demonstrated that nonionic surfactants, particularly Pluronic F127, could provide protection for rhGH against emulsification-induced aggregation, attributed to their competing for adsorption and oriented locating on the water/organic solvent interface. The difference in the stabilizing effects on rhGH between Pluronic F127, F68, and Tween 20 implied that the structure of surfactants, such as the composition and length of the hydrophilic and hydrophobic chains, was determinant that should be considered when screen stabilizing agents for protein. Co-encapsulation of F127 in the microspheres resulted in an improved release of rhGH without significant aggregations, however, the conformation and bioactivity of the released protein should be confirmed in the further study. Anyway, from the viewpoint of preventing protein aggregation, co-encapsulation of Pluronic F127 was a promising approach to overcome the protein stability issue within biodegradable microspheres.

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References

Bam, N.B., Cleland, J.F., Yang, J., Manning, M.C., Carpenter, J.F., Kelley, R.F., Randolph, T.W., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Blanco, D., Alonso, M.J., 1998. Protein encapsulation and release from poly(lactide-*co*-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants. Eur. J. Pharm. Biopharm. 45, 285–294.

Boehm, G., Peyre, M., Sesardic, D., Huskisson, R.J., Mawas, F., Douglas, A., Xing, D., Merkle, H.P., Gander, B., Johansen, P., 2002. On technological and immunological benefits of multivalent single-injection microsphere vaccines. Pharm. Res. 19, 1330–1336.

Chen, L., Apte, R.N., Cohen, S., 1997. Characterization of PLGA microspheres for the controlled delivery of IL1α for tumor immunotherapy. J. Controlled Release 43, 261–272.

Cleland, J.L., Duenas, E., Daugherty, A., Marian, M., Yang, J., Wilson, M., Celniker, A.C., Shahzamani, A., Quarmby, V., Chu, H., Mukku, V., Mac, A., Roussakis, M., Gillette, N., Boyd, B., Yeung, D., Brooks, D., Maa, Y.-D., Hsu, C., Jones, A.J.S., 1997. Recombinant human growth hormone poly(lactic-*co*-glycolic acid) (PLGA) microspheres provide a long lasting effect. J. Controlled Release 49, 193–205.

Cleland, J.L., Jones, A.J.S., 1996. Stable formulations of recombinant human growth hormone and interferon-γ for microencapsulation in biodegradable microspheres. Pharm. Res. 13, 1464–1475.

Cleland, J.L., Powell, M.F., Shire, S.J., 1993. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit. Rev. Ther. Drug Carrier Syst. 10, 307–377.

Costantino, H.R., Carrasquillo, K.G., Cordero, R.A., Mumenthaler, M., Hsu, C.C., Griebenow, K., 1998. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. J. Pharm. Sci. 87, 1412–1420.

De Rosa, G., Iommelli, R., La Rotonda, M.I., Miro, A., Quaglia, F., 2000. Influence of the co-encapsulation of different non-ionic surfactants on the properties of PLGA insulin-loaded microspheres. J. Control. Release 69, 283–395.

Genta, I., Perugini, P., Pavanetto, F., Maculotti, K., Modena, T., Casado, B., Lupi, A., Iadarola, P., Conti, B., 2001. Enzyme loaded biodegradable microspheres in vitro ex vivo evaluation. J. Control. Release 77, 287–295.

Johansen, P., Men, Y., Audran, R., Corradin, G., Merkel, H.P., Gander, B., 1998. Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives. Pharm. Res. 15, 1103–1110.

Johnson, O.L., Jaworowicz, W., Cleland, J.L., Bailey, L., Charnis, M., Duenas, E., Wu, C., Shepard, D., Magil, S., Last, T., Jones, A.J.S., Putney, S.D., 1997. The stabilization and encapsulation of human growth hormone into biodegradable microspheres. Pharm. Res. 14, 730–735.

Kabanov, A.V., Batrakova, E., Alakhov, V.Y., 2002. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. J. Control. Release 82, 189–212.

Katakam, M., Banga, A.K., 1997. Use of poloxamer polymers to stabilize recombinant human growth hormone against various processing stresses. Pharm. Dev. Technol. 2, 143–149.

Katakam, M., Bell, L.N., Banga, A.K., 1995. Effect of surfactants on the physical stability of recombinant human growth hormone. J. Pharm. Sci. 84, 713–716.

Kim, H.K., Park, T.G., 1999. Microencapsulation of human growth hormone with biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. Biotechnol. Bioeng. 65, 659–667.

Kim, H.K., Park, T.G., 2001. Microencapsulation of dissociable human growth hormone aggregates within poly(D, L-lactic-*co*-glycolic acid) microparticles for sustained release. Int. J. Pharm. 229, 107–116.

Morlock, M., Knoll, H., Winter, G., Kissel, T., 1997. Microencapsulation of rh-erythropoietin, using biodegradable poly(D, L-lactide-*co*-glycolide): protein stability and the effects of stabilizing excipients. Eur. J. Pharm. Biopharm. 43, 29–36.

Okada, H., 1997. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv. Drug Deliv. Rev. 28, 43–70.

Péan, J.M., Boury, F., Venier-Julienne, M.C., Menei, P., Proust, J.E., Benoit, J.P., 1999. Why does PEG 400 co-encapsulation improve NGF stability and release from PLGA biodegradable microspheres? Pharm. Res. 16, 1294–1299.

Pérez, C., Griebenow, K., 2001. Improved activity and stability of lysozyme at the water/CH_2Cl_2 interface: enzyme unfolding and aggregation and its prevention by polyols. J. Pharm. Pharmacol. 53, 1217–1226.

Sah, H., 1999a. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. J. Control. Release 58, 143–151.

Sah, H., 1999b. Protein behavior at the water/methylene chloride interface. J. Pharm. Sci. 88, 1320–1325.

Sánchez, A., Villamayor, B., Guo, Y., McIver, J., Alonso, M.J., 1999. Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-*co*-glycolide) microspheres. Int. J. Pharm. 185, 255–266.

Schrier, J.A., DeLuca, P.P., 1999. Recombinant human bone morphogenetic protein-2 binding and incorporation in PLGA microsphere delivery systems. Pharm. Dev. Technol. 4, 611–621.

Schwendeman, S.P., 2002. Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems. Crit. Rev. Ther. Drug Carrier Syst. 19, 73–98.

Sinha, V.R., Trehan, A., 2003. Biodegradable microspheres for protein delivery. J. Control. Release 90, 261–280.

Takada, S., Yamagata, Y., Misaki, M., Taira, K., Kurokawa, T., 2003. Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique. J. Control. Release 88, 229–242.

Van de Weert, M., Hennink, W.E., Jiskoot, W., 2000a. Protein instability in poly(lactic-*co*-glycolic acid) microparticles. Pharm. Res. 17, 1159–1167.

Van de Weert, M., Hoechstetter, J., Hennink, W.E., Crommelin, D.J.A., 2000b. The effect of a water/organic solvent interface on the structural stability of lysozyme. J. Control. Release 68, 351–359.

Yang, J., Cleland, J.L., 1997. Factors affecting the in vitro release of recombinant human interferon-γ (rhIFN-γ) from PLGA microspheres. J. Pharm. Sci. 86, 908–914.