Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
International Journal of Pharmaceutics 309 (2006) 101–108
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Stabilization and encapsulation of a staphylokinase variant (K35R) into poly(lactic-*co*-glycolic acid) microspheres

Jin-Tian He, Hua-Bo Su, Guo-Ping Li, Xian-Mei Tao, Wei Mo, Hou-Yan Song *

Key Laboratory of Molecular Medicine, Ministry of Education, Fudan University, 130 Dong-an Road, Shanghai 200032, China

Received 27 October 2005; accepted 12 November 2005
Available online 4 January 2006

Abstract

The aim of this study is to prepare poly(lactic-*co*-glycolic acid) (PLGA) microspheres containing a staphylokinase variant K35R (DGR) with purpose of preserving the protein stability during both encapsulation and drug release. DGR-loaded microspheres are fabricated using a double-emulsion solvent extraction technique. Prior to encapsulation, the effect of ultrasonication emulsification of DGR solutions with methylene chloride on protein recovery was investigated. Moderate ultrasonic treatment of aqueous DGR/dichloromethane mixtures caused approximately 84% DGR aggregation. Polyvinyl alcohol (PVA) added into aqueous DGR solutions significantly improved DGR recovery to >90%. The effects of co-encapsulated PVA and NaCl in the external aqueous phase on the characteristics of the microspheres were investigated. When 2% PVA was co-encapsulated and 2.5% NaCl was added to the external water phase, DGR encapsulation efficiency was significantly increased from 7.1% to 78.1% and DGR was distributed uniformly throughout the microspheres. In vitro release test showed that DGR was released from PLGA microspheres in a sustained manner over 15 days. A large amount of released DGR was inactive in the absence of co-encapsulated PVA. On the contrary, when 2% PVA was co-encapsulated, the released DGR was almost completely intact within 9 days. In conclusion, PLGA microspheres can be an effective carrier for DGR and form a promising depot system.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Staphylokinase; Microspheres; Protein delivery; Polyvinyl alcohol; Protein stability

1. Introduction

Over the last 20 years, a large number of recombinant proteins have been investigated to find their therapeutic applications and many of them have been formulated as drugs, forming a new class of therapeutic agents. Ailments that can be treated effectively by this new class of therapeutic agents include cancers, autoimmune diseases, memory impairments, mental disorders, hypertension and certain cardiovascular and metabolic diseases (Banga and Chien, 1988; Sinha and Trehan, 2003). While proteins have many attractive properties and advantages compared to other kind of therapeutic agents such as small organic molecule drugs, they also have disadvantages, including short half-lives in vivo, physical and chemical instabilities, and low oral bioavailabilities. As a result, most of these proteins are therapeutically useful only by following a therapeutic regimen that requires infusion or frequent injections. This therapeutic regimen process is both tedious and expensive. On the other hand, the development of biodegradable polymeric microspheres as carriers for proteins is becoming a promising way to overcome the administrating problems of those proteins (Sinha and Trehan, 2003; Raymond et al., 1998). Some products have already been approved by the United States Food and Drug Administration (Sinha and Trehan, 2003). However, the preservation of full biological activity of incorporated proteins during processes of encapsulation and the drug release from polymeric matrices remains as a major challenge (Putney and Burke, 1998; Perez et al., 2002).

The water-in-oil-in-water ($W_1/O/W_2$) double-emulsion solvent extraction technique has been widely utilized for the encapsulation of therapeutic proteins, peptides and vaccines. During the microsphere preparation process, ultrasonication and the

* Corresponding author. Tel.: +86 21 64033738; fax: +86 21 64033738.
E-mail address: he_jintian@yahoo.com (H.-Y. Song).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.11.036

water–organic solvent interface have been suggested as the major cause for protein denaturation and aggregation (Perez et al., 2002; Morlock et al., 1997; Kang et al., 2002; Rosa et al., 2000). Therapeutic proteins after denaturation and/or aggregation are often inactive and potentially promote immune reactions, and thus, denaturation and aggregation processes should be avoided.

It is obvious that developing good formulation strategies to preserve protein stability is of great importance. Addition of stabilizing agents to therapeutic proteins is one of the approaches often used to stabilize the proteins. Stabilizing additives used in the formulation of protein drugs include proteins, sugars, polyols, amino acids, chelating agents and inorganic salts (Sinha and Trehan, 2003; Perez et al., 2002; Lu and Park, 1995; Cleland and Jones, 1996). Moreover, in the drug release processes, there are several potential factors that can cause the protein drug inactivation. These factors include the hydration of protein, the reduction of environmental pH around the drug produced by polymer degradation, and the presence of hydrophobic surfaces. The use of stabilizers has been proved to be a useful method for eliminating these unfavorable factors (Sinha and Trehan, 2003; Perez et al., 2002; Zhu et al., 2000; Putney and Burke, 1998). Since each excipient can preserve only a few protein drugs during encapsulation, a particular excipient must be found for a specific protein drug.

Staphylokinase (Sak), a 136-amino acid profibrinolytic bacteria protein, has been shown to be a promising thrombolytic agent (Collen, 1998). Pilot-randomized trial displayed that the recombinant Sak was equipotent to recombinant tissue-type plasminogen activator (rt-PA or alteplase) for coronary artery recanalization and significantly more fibrin-selective (Vanderschueren et al., 1996). Sak variant K35R (i.e., Lys[K] in position 35 substituted with Arg[R]) (code DGR) is a bifunctional protein that possess fibrinolytic and antiplatelet aggregation activities (Su et al., 2004). Moreover, DGR possesses greatly reduced immunogenecity in guinea pigs compared to wild type staphylokinase (Su et al., 2004). Thus, DGR is a promising drug to prevent and treat thromboembolic diseases.

From a common perspective, frequent injections of protein drugs appear inconvenient; therefore, long-term formulation for the DGR would be desirable. The aim of this study is to prepare DGR-loaded microspheres, which are able to preserve the protein stability during both encapsulation processes and drug release. A strategy is developed, in the present work, to ameliorate the aggregation of DGR at the water/methylene chloride interface by employing various excipients. These excipients were also demonstrated to be effective on the reduction of other protein aggregations at the water/methylene chloride interface (Perez et al., 2002). DGR recovery was significantly improved by the addition of polyvinyl alcohol (PVA) whereas no stabilizing effects were observed with other excipients during ultrasonication emulsification. We have also examined the effects of co-encapsulated PVA and NaCl in external water phase on PLGA microsphere properties such as morphology, encapsulation efficiency, actual loading, and DRG release. The reason for denaturation of DGR in microspheres during the late stage of drug release is also analyzed.

2. Materials and methods

2.1. Materials

Preparation and purification of DGR were carried out by a method described previously (Su et al., 2004). Poly(DL-lactic-*co*-glycolic acid) (PLGA, a copolymer with a ratio of 75:25 and with an MW of about 13 kDa) was purchased from Chengdu Hangli Fine Chemicals Company, Ltd. (Sichuan, China). $Mg(OH)_2$ nanoparticles and PVA with an MW range of 30 000–70 000 were obtained from Aldrich. All other chemicals used were of analytical grade.

2.2. Microsphere preparation

DGR-loaded microspheres were prepared by a $W_1/O/W_2$ technique. Briefly, a desired amount of DGR was dissolved in 0.25 ml of 20 mM phosphate buffer (pH 7.4) and poured into 2.5 ml methylene chloride containing 200 mg PLGA. DGR stabilizer, PVA and $Mg(OH)_2$ nanoparticles, were added to the internal aqueous phase when needed. The water-in-oil (W_1/O) emulsion was emulsified by ultrasonication (80 W, 30 s). Afterwards, the primary emulsion was added into 75 ml of 2% (w/v) aqueous PVA and homogenized at 600 rpm for 1 min to form the multiple emulsion ($W_1/O/W_2$). For solvent extraction, $W_1/O/W_2$ emulsion was subsequently diluted with 225 ml of 20 mM phosphate buffer (pH 7.4) and stirred with a magnetic stirrer at 300 rpm for 6 h. 2.5% NaCl solution was used as the external aqueous phase when needed. The resulting DGR-containing microspheres were collected by filtration and were washed three times with water. The microspheres were then vacuum-dried over night and stored at −20 °C for use.

2.3. Stability of DGR during ultrasonication

The effect of ultrasonication on DGR stability was studied in aqueous DGR solution and W_1/O-systems consisting of 0.20 ml aqueous DGR solution and 2.0 ml methylene chloride. The following excipients were added to the aqueous phase: Tween 80, PEG 400, sucrose, mannitol, and PVA. Ultrasonication was carried out for 30 s at 80 W. After emulsification, DGR was extracted into the aqueous phase by adding 2.5 ml of 0.02 M PB (pH 7.4) and then centrifuged at $3000 \times g$ for 20 min to accelerate phase separation. Residual methylene chloride partitioned in the aqueous solution was removed by vacuum for 10 min. The aqueous phase was subjected to the protein quantification and fibrinolytic activity assay (described in the latter section).

2.4. Particle size distribution and morphology of microspheres

The morphology of microspheres was observed by scanning electron microscopy (SEM, Hitachi S-520). Freeze-dried microspheres were re-dispersed in distilled water and the size of microspheres was measured by a particle size analyzer (Model 780 AccuSizer, Particle Sizing Systems, Inc., CA, USA). The results are reported as a volume size distribution.

2.5. DGR loading and encapsulation efficiency in microspheres

DGR content in the microspheres was determined by an extraction method (Zhu et al., 2000). A certain amount of dried microspheres was dissolved in methylene chloride. After centrifugation and removal of the polymer solution, the remaining DGR pellet was dissolved in 0.2 ml of 20 mM phosphate buffer (pH 7.4). The concentration of DGR in supernatant was measured; and the aggregates were incubated in phosphate buffered saline (PBS) containing 6 M urea at 37 °C for 30 min for denaturation from which the insoluble DGR percentage was then determined. The DGR concentration was determined by Bradford protein assay (Bradford, 1976). The amount of protein from both water-soluble and insoluble parts was taken into account in the calculation of the actual protein loading (mg of encapsulated DGR per 100 mg of microspheres). The encapsulation efficiency of DGR in the microspheres was calculated as the ratio of actual and theoretical DGR loadings.

2.6. Protein distribution in microsphere

A confocal image system (CIS, Zeiss LSM510) equipped with a 720 nm filter for 2-photon excitation was employed to observe DGR distribution in microspheres. Dry microspheres were dispersed on a glass slide; fluorescence images of cross-sections were taken by an optical sectioning. All the images were obtained using a single resolution.

2.7. In vitro release studies

A certain amount of dried microspheres was suspended in 1 ml of PBS (pH 7.4) containing 0.05% (w/v) sodium azide and was then incubated at 37 °C. At each sampling time, the supernatant was withdrawn and replaced with the same volume of fresh PBS solution. The DGR content and activity in the supernatant was determined by Bradford protein assay (Bradford, 1976) and by fibrinolytic activity assay (described in the next section), respectively. The amount of DGR released within 24 h was defined as the initial DGR burst.

2.8. Fibrinolytic activity assay

The fibrinolytic activity was measured by a radial caseinolytic assay (Hauptmann et al., 1995). In brief, casein plates were prepared in dishes containing 1.0% agarose, 2.7% skim milk powder and 15 μg/ml plasminogen. Equal-diameter wells were cut for the test samples. After 10 μl of standard Sak or DGR solution was added into each well, the plates were kept in a moister chamber at 37 °C for 10–12 h. The diameter of the halo around the well was measured and used to calculate the fibrinolytic activity.

2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A suitable amount of dried microspheres was dissolved in methylene chloride. After centrifugation and removal of the polymer solution, the remaining DGR pellet was dispersed in 0.1 ml of 20 mM phosphate buffer (pH 7.4). Then SDS-PAGE was performed using 15% gels and the gel was then stained with a solution of Coomassie brilliant blue.

3. Results and discussion

3.1. Stability of DGR during ultrasonication emulsification in the absence of PLGA

During the primary emulsion process, a large interface between the aqueous and organic phase is often formed and is regarded as the major cause for protein denaturation (Perez et al., 2002; Morlock et al., 1997; Kang et al., 2002). However, denaturation of protein can also occur during sonication even in the absence of methylene chloride (Krishnamurthy et al., 2000). Thus, the effect of ultrasonication on DGR stability in the presence and in the absence of methylene chloride is examined in the present work. In the absence of methylene chloride, the activity of DGR remains a level of above 90% even if ultrasonicated for 40 s at 80 W (Fig. 1). On the other hand, in the presence of methylene chloride, the loss of activity of DGR is very serious even if emulsification is processed for only 20 s at the same energy input. Moreover, a lot of white precipitates were observed at the interface between the aqueous and organic phase. SDS-PAGE analysis indicates that the precipitates are of the denatured DGR (Fig. 2, Lane 3). Interestingly, the residual DGR retains its full specific activity after emulsification (Table 1). These observations reveal that the large water–methylene chloride interface is the major destabilizing factor for DGR in the process of water–methylene chloride emulsion.

The protective effect of various excipients on DGR activity is examined during the emulsification process in the absence of PLGA. As shown in Table 1, the additions of sucrose, mannitol, PEG 400 and Tween 80 do not improve DGR recovery in the emulsions. In fact, the protein recovery is slightly decreased. In contrast, with PVA as a stabilizer, no protein precipitates are observed at the interface between water and methylene chloride. Moreover, the DGR recovery is significantly increased and its

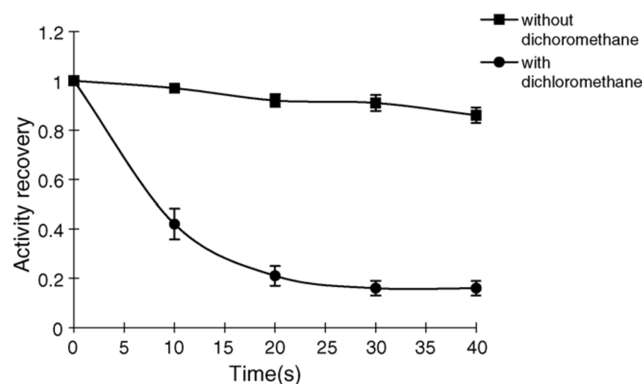

Fig. 1. Activity recovery of DGR after emusification of protein solution with or without methylene chloride.

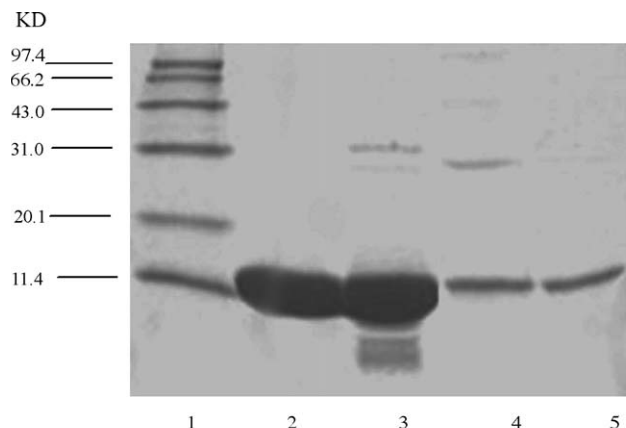

Fig. 2. The stabilization effect of $Mg(OH)_2$ for DGR within microspheres during in vitro release. Lane 1, molecular weight marker proteins: 97.4, 66.2, 43.0, 31.0, 20.1, 14.4 kDa; Lane 2, purified DGR; Lane 3, denatured DGR during primary emusification; Lane 4, DGR within microspheres after released over 20 days; Lane 5, DGR within microspheres with 3% $Mg(OH)_2$ as excipient after released over 20 days.

specific activity is completely maintained. It was suggested that the proteins adsorbed to the water–methylene chloride interface displace the water molecules surrounding the interface and the hydrophobic patches of the proteins (Sah, 1999). The adsorbed protein molecules are denatured and inactive at the water–methylene chloride interface. Sucrose and mannitol are not a surfactant and ineffective in blocking protein adsorption. PEG behaves anomalously in a water/methylene chloride system, preferentially distributing into the organic phase. Tween is composed of hydrophilic poly(ethylene glycol) (PEG) chains and a hydrophobic fatty acid chain grafted onto a sorbitan. As a result, both PEG and Tween have only an affinity for organic phase and cannot block denaturations of DGR at the interface (van de Weert et al., 2000a). PVA is a nonionic surfactant and has an affinity to the interface. Thus, PVA is an effective agent for preventing the protein from aggregations at the interface (van de Weert et al., 2000a).

3.2. Microsphere size, morphology and drug distribution within microspheres

As reported in Table 2 and Fig. 3, PVA in the internal water phase or/and NaCl in the external water phase affect the microsphere size and particle distribution. Under the conditions that the external water phase contains no NaCl, co-encapsulated PVA resulted in formation of slightly bigger microspheres, with an average size of 104.3 μm in diameter, compared to that of 95.1 μm in the control experiments. It has been suggested that PVA in the internal water phase could increase the viscosity of the primary emulsion. Since it is difficult to break up the solution into smaller droplets at the same mixing power, the formation of slightly bigger microspheres is not surprising (Yang et al., 2001). Whether the external aqueous phase contains 2% PVA or not, the

Table 1
Protein recovery and enzymatic activity recovery after emulsification of protein solutions with excipients in methylene chloride

DGR (mg/ml)	Excipients	Excipients concentration (%)	DGR recovery[a] (%)	Specific activity[a] (%)
10	–	–	17.7 ± 1.2	109 ± 5.1
10	Tween 80	0.1	8.9 ± 0.7	83.9 ± 0.9
10	Tween 80	1.0	9.1 ± 0.6	94.4 ± 5.0
10	PEG 400	2.5	8.3 ± 0.8	82.0 ± 4.6
10	PEG 400	25	7.7 ± 0.6	78.6 ± 3.4
10	Sucrose	0.5	8.6 ± 0.4	89.4 ± 5.3
10	Sucrose	4	7.9 ± 0.7	92.2 ± 9.1
10	Manitol	0.5	9.0 ± 0.3	95.9 ± 5.0
10	Manitol	4	7.6 ± 0.2	100 ± 9.5
10	PVA	2	98.2 ± 2.8	92.8 ± 4.9
10	PVA	10	93.2 ± 2.3	106 ± 2.7
45	–	–	18.7 ± 1.2	98 ± 3.4
45	PVA	5	96.8 ± 4.0	113 ± 5.9
45	PVA	10	94.3 ± 7.8	108 ± 10.3

[a] Mean ± S.D., $n = 3$.

Table 2
Effect of excipients on the properties of DGR microspheres

Batch	Initial DGR concentration (mg/ml)	PVA concentration in W_1 (%, w/v)	NaCl concentration in W_2 (%)	Microsphere diameter (μm)	Actual loading[a] (%)	Encapsulation efficiency[a] (%)
A	10	–	–	95.1	0.068	7.1 ± 0.4
B	10	–	2.5	93.7	0.46 ± 0.02	45.4 ± 1.6
C	10	2	–	104.3	0.12 ± 0.01	12.5 ± 0.8
D	10	2	2.5	75.4	0.74 ± 0.02	78.1 ± 2.2
E	45	–	2.5	98.7	3.8 ± 0.2	83.9 ± 4.3
F	45	5	2.5	86.1	3.4 ± 0.3	85.5 ± 3.4

[a] Mean ± S.D., $n = 3$.

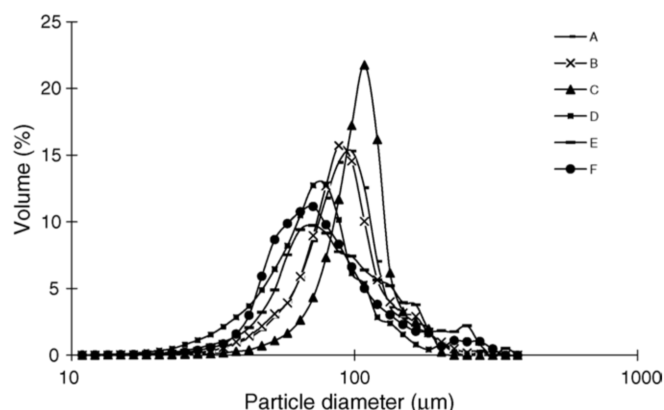

Fig. 3. Size distribution of DGR-loaded PLGA microspheres. The conditions for A–F corresponded to Table 2.

addition of 2.5% NaCl to the external aqueous phase produces smaller microspheres (Table 2, Fig. 3). It has been suggested that salt in the external water phase affect the size and particle distribution by changing the osmotic pressure between the internal and external water phase (Han et al., 2001). In the preparation of DGR-loaded microspheres, phosphate concentration in the internal water phase is higher than the salt concentration of the external water phase, leading to the influx of water from external water phase and resulting in porous and larger microspheres. But when NaCl is added to the external water phase, the osmotic pressure between inner water phase and outer water phase is balanced, giving rise to denser and smaller microspheres (Fig. 3).

NaCl in the outer water phase also plays a critical role in determining microsphere morphology. Microspheres prepared without balancing osmotic pressure have rough surfaces; many large pores exist on their surface, as shown in Fig. 4, panels A and C. These large pores may form channels through which outer water influxes into the microspheres. When osmotic pressure is balanced, the large pores disappear and the surfaces of microspheres are relatively smooth and compact (Fig. 4, panels B and D). The addition of PVA to the internal water phase could enhance the primary emulsion stability and then yields microspheres with smoother surface (Fig. 4D).

The distribution of DGR in the microsphreres was shown by confocal light microscopy (Fig. 5). With co-encapsulated PVA and NaCl in the outer water phase, DGR was distributed uniformly throughout the polymer matrix within microspheres (Fig. 5). The internal structure of most microspheres is a honeycomb style (Rosca et al., 2004). Thus, the DGR molecules are uniformly distributed within the particles (Fig. 5D). The similar effect of PVA on the BSA distribution within PCL microspheres was also observed by Yang and coworkers (Yang et al., 2001).

3.3. DGR loading and encapsulation efficiency

Our results show that the encapsulation efficiency is strongly affected by the osmotic pressure between the inner and outer water phase (Table 2). When 2.5% NaCl is added to the external water phase, DGR encapsulation efficiency is significantly increased, independent of if PVA in the internal water phase. NaCl in the outer water phase influences the osmotic pressure, thus, affecting the drug loading and encapsulation efficiency (Han et al., 2001). PVA in the internal water phase can also enhance the encapsulation efficiency (Table 2).

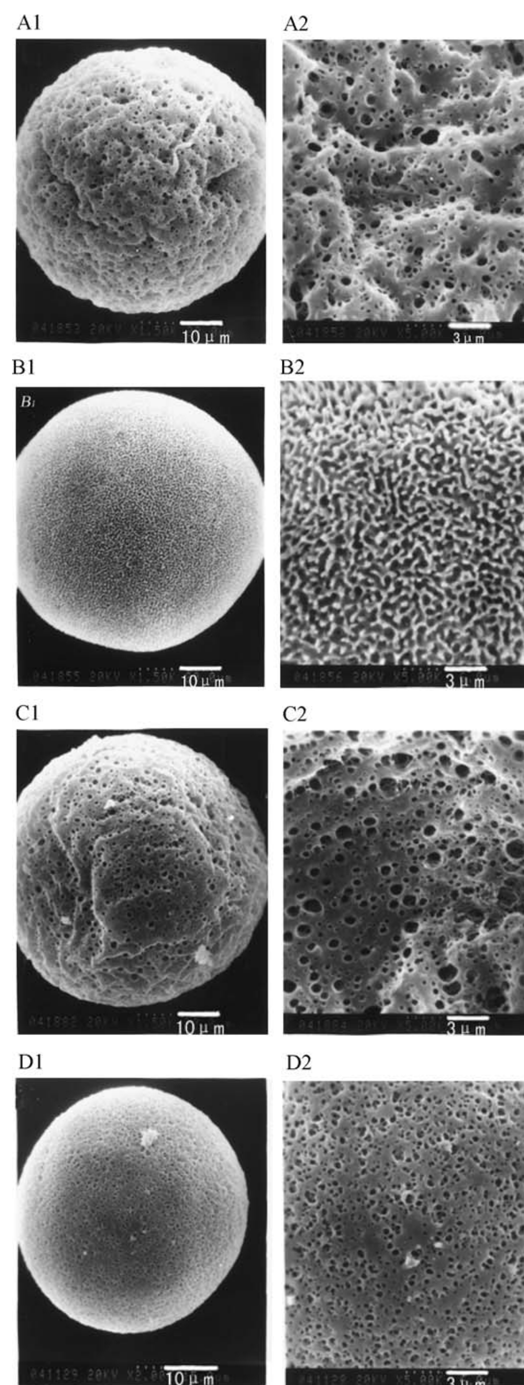

Fig. 4. SEM pictures of microspheres surface fabricated at the different condition: (A) without PVA and without NaCl in W_2; (B) without PVA and 2.5% NaCl in W_2; (C) 2% PVA and without NaCl in W_2; (D) 2.0% PVA and 2.5% NaCl in W_2. A1, B1, C1, D1 was taken with 1500× or 2000× magnification and A2, B2, C2, D2 with 5000× magnification.

Table 3
Stability of DGR within PLGA microspheres

Batch	Soluble[a] (%)	Specific activity[a] (%)	Insoluble[a] (%)
B	75.0 ± 5.4	99.0 ± 5.6	25.0 ± 7.4
D	91.4 ± 5.4	108 ± 2.6	8.6 ± 3.1
E	67.6 ± 1.7	97.0 ± 4.7	32.4 ± 4.0
F	96.0 ± 6.3	102 ± 9.8	4.0 ± 0.3

The conditions for all batches corresponded to Table 2.
[a] Mean ± S.D., $n = 3$.

3.4. Stability of DGR within PLGA microspheres

The study of emulsification with methylene chloride in the absence of PLGA can provide a valuable insight into the protein stability at the water/methylene chloride interface. However, the presence of PLGA cannot be neglected when protein stability is studied during microsphere preparation (Kang et al., 2002; Sah, 1999). When PLGA was dissolved in the organic phase, any interaction between the protein and PLGA, as well as the increased viscosity of the organic phase, may cause a different protein behavior during emulsification. Thus, the stability of DGR within lyophilized microspheres is examined in the present work and the results are shown in Table 3. Without PVA, the content of soluble DGR amounted to 75% and 67.6%. The recovery of active DGR was dramatically increased compared to that in the primary emulsification process without PLGA. When PVA is co-encapsulated, the recovery of DGR from the lyophilized microspheres is similar to that in the primary emulsification process without PLGA.

3.5. In vitro release

In vitro release of DGR from PLGA microspheres is illustrated in Fig. 6. Both the total amount and active amount of DGR released from the various fabrications are determined in the present work. When PVA is not co-encapsulated, a large amount of released DGR is inactive and the release profiles of the total DGR amount and the active DGR amount are significantly different (Fig. 6A and B). When PVA is co-encapsulated, the activity of DGR is almost completely preserved and the DGR release profiles of the total amount and the active amount are similar (Fig. 6C and D). The release profile exhibits a biphasic pattern: (1) an initial burst release: a large amount of DGR is release within 1 day; (2) after the initial burst, DGR release profile displays a sustained release. The total amount of released DGR over a period of 15 days is more than 60%.

The addition of PVA to the internal water phase does not significantly affect the initial burst and the release rate of DGR, but

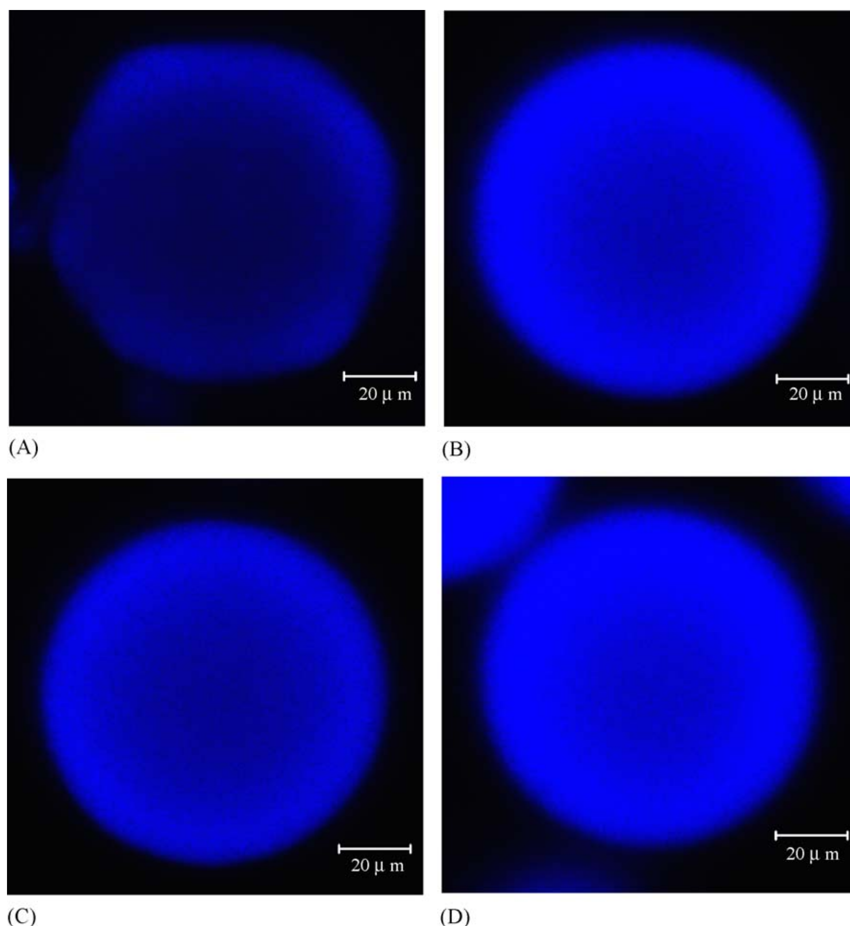

Fig. 5. CIS images of microspheres fabricated at different conditions: (A) without PVA and without NaCl in W_2; (B) without PVA and 2.5 NaCl in W_2; (C) 2.0% PVA and without NaCl in W_2; (D) 2.0% PVA and 2.5% NaCl in W_2.

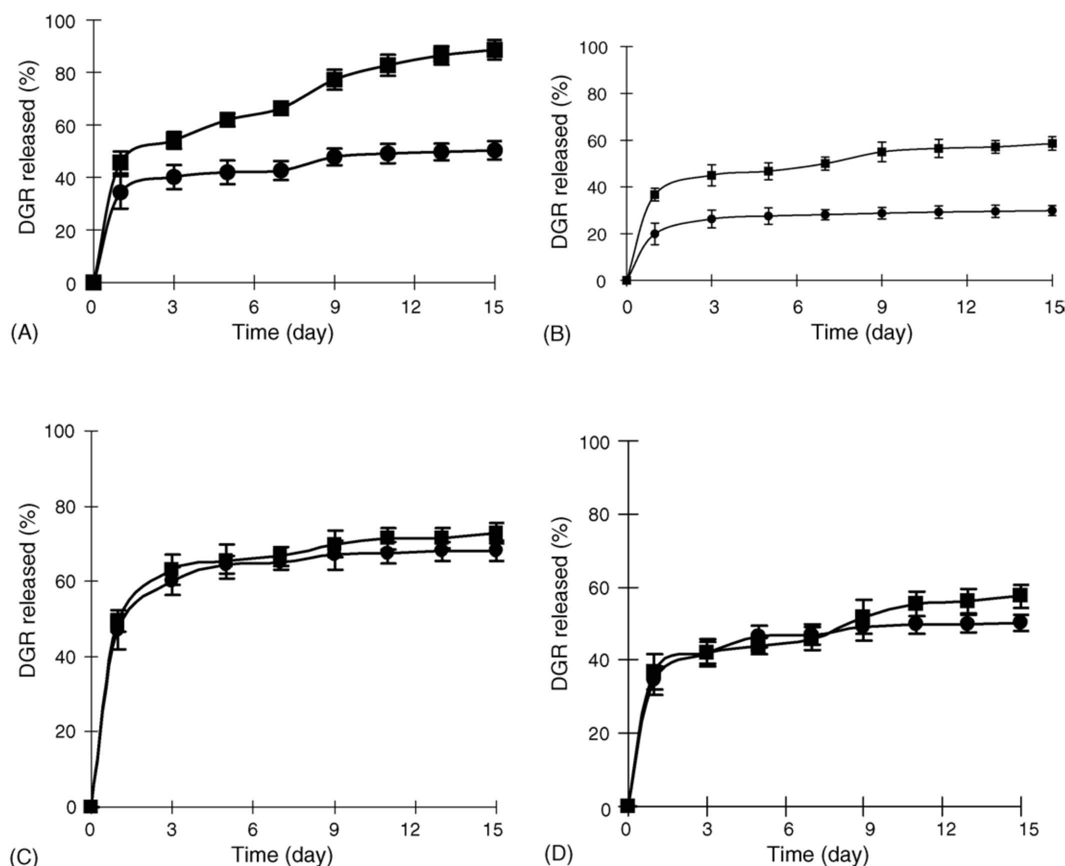

Fig. 6. In vitro release from different microspheres formulations. Headline describes used preparation condition for DGR microsphere: (A) without PVA and without NaCl in W_2; (B) without PVA and 2.5% NaCl in W_2; (C) 2.0% PVA and without NaCl in W_2; (D) 2.0% PVA and 2.5% NaCl in W_2. (■) DGR amount; (●) DGR activity.

the addition of NaCl to the outer water phase slightly decreases the initial burst and the release rate (Fig. 6). The connecting channels between pores on the surfaces and those inside microspheres were observed previously (Wang et al., 2002). The permeability of microspheres with porous surfaces was high and fluorescent probe molecules (MW: 467 and 3000 Da) could easily diffuse into the microspheres (Wang et al., 2002). Proteins within microspheres could be easily leached out from the large pores (∼1000 nm) (Huang and Brazel, 2001; van de Weert et al., 2000b). When NaCl is added to the outer water phase, the osmotic pressure is balanced and the microspheres had only small pores on their surfaces. Obviously, the rate of drug release from these microspheres is slower than that from porous microspheres. Yang and coworkers reported that PVA in the internal aqueous phase decreased the BSA release rate (Yang et al., 2001). Their results are not consistent with ours. This discrepancy is probably due to the different conditions for sample preparations, and to different microsphere sizes, copolymer compositions and properties of the incorporated drugs (Huang and Brazel, 2001).

DGR release from the microspheres is not complete (about 60–90%) and the denatured DGR increases gradually during release (Fig. 6). The residual DGR within microspheres is analyzed by SDS-PAGE after releasing for 20 days. A large amount of DGR within microspheres is not soluble and forms dimers and polymers (Fig. 2). The formation of dimers and polymers seems not reversible. The dimers and the polymers of the DGR have no biological activity. Dimeric and polymeric DGR cannot be derived from the encapsulation process because the denatured DGR at the water–methylene chloride interface contained only a little amount of dimeric DGR. They might be resulted from deleterious microclimate within microspheres. There might have several potential factors, which can result in irreversible denaturation of proteins: elevated moisture, acidic pH, and the polymer surfaces (Sinha and Trehan, 2003; Perez et al., 2002). Preliminary results show that acidic pH and the polymer surface might be the major causes for denaturation and aggregation of DGR. It is observed that DGR is sensitive to an acidic circumstance and is not stable at pH 4.0. About 60% DGR is denatured in 2 days in a pH 4.0 buffer. The degradation products of PLGA are acidic in nature, which can lead to a decrease in pH within the microspheres. It was reported that $Mg(OH)_2$ could maintain the microclimate at neutral pHs within microspheres (Zhu et al., 2000). When 3% $Mg(OH)_2$ nanoparticles is co-encapsulated, the polymeric DGR is not detected by SDS-PAGE (Fig. 2). Another factor leading to potential deterioration of DGR upon release from microspheres is non-specific adsorptions of proteins on the polymer by hydrophobic and ionic interactions. To test DGR adsorptions, DGR solutions in phosphate buffered saline (pH 7.4) containing different NaCl concentrations is incubated with

blank PLGA microspheres for 12 days at 37 °C. The results show that about 30% DGR activity is lost when NaCl concentration is lower than 0.5 M. When NaCl concentration is higher than 0.5 M, only about 10% activity is lost. The results suggest that the ionic interactions between DGR and free carboxyl end groups of PLGA chains are another important cause for denaturation of DGR during release.

4. Conclusion

This study has shown that the co-encapsulation of PVA in PLGA microspheres processing by the emulsification-solvent evaporation method is a useful method for preventing DGR from aggregation at the water/methylene chloride interface. A large amount of DGR aggregates at the water/methylene chloride interface upon ultrasonication emulsification. These aggregates are primarily caused by the presence of this interface, not by ultrasonication. DGR was fully protected during the primary emulsion process when PVA was added to the aqueous DGR solution. The co-encapsulated PVA and NaCl in the outer aqueous phase greatly affect the encapsulation efficiency, microsphere surface morphology, microparticle diameter, protein distribution, and the drug release profiles. The in vitro release study shows that DGR is released from PLGA microspheres in a sustained manner and the total amount of released DGR is more than 60% over a period of 15 days. When PVA is co-encapsulated, the stability of DGR is effectively protected during the encapsulation process and the release profile of the total amount is consistent with that of the active amount within 9 days. Moreover, it is found that acidic microclimate and interactions between the protein and the PLGA surface are the main causes for the denaturation of DGR during release.

Acknowledgements

We would like to express our gratitude to Dr. Tiesheng Shi for his invaluable assistance. The study was supported by national 863 projects (No. 863-2001AA215291).

References

Banga, A.K., Chien, Y.W., 1988. Systemic delivery of therapeutic peptides and proteins. Int. J. Pharm. 48, 15–50.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

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

Collen, D., 1998. Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. Nat. Med. 4, 279–284.

Han, K., Lee, K.D., Gao, Zh.G., Park, J.S., 2001. Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing. J. Control. Release 75, 259–269.

Hauptmann, J., Guhrs, K.H., Hartmann, M., Schlott, B., 1995. The fibrinolytic activity of staphylokinase mutants in the fibrin plate assay. Haemostasis 25, 272–276.

Huang, X., Brazel, C.S., 2001. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J. Control. Release 73, 121–136.

Kang, F., Jiang, G., Hinderliter, A., DeLuca, P.P., Singh, J., 2002. Lysozyme stability in primary emulsion for PLGA microsphere preparation: effect of recovery methods and stabilizing excipients. Pharm. Res. 19, 629–633.

Krishnamurthy, R., Lumpkin, J.A., Sridhar, R., 2000. Inactivation of lysozyme by sonication under conditions relevant to microencapsulation. Int. J. Pharm. 205, 23–34.

Lu, W., Park, T.G., 1995. Protein release from poly(lactic-*co*-glycolic acid) microspheres: protein stability problems. PDA J. Pharm. Sci. Technol. 49, 13–19.

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

Perez, C., Castellanos, I.J., Costantino, H.R., Al-Azzam, W., Griebenow, K., 2002. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J. Pharm. Pharmacol. 54, 301–313.

Putney, S.D., Burke, P.A., 1998. Improving protein therapeutics with sustained-release formulations. Nat. Biotechnol. 16, 153–157.

Raymond, T.B., Mark, A.T., Dwaine, F.E., Stephen, E.Z., 1998. Sustained delivery of proteins for novel therapeutic products. Science 281, 1161–1162.

Rosa, G.D., 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, 283–295.

Rosca, I.D., Watari, F., Uo, M., 2004. Microparticle formation and its mechanism in single and double emulsion solvent evaporation. J. Control. Release 99, 271–280.

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

Sinha, V.R., Trehan, A., 2003. Biodegradable microspheres for protein delivery. J. Control. Release 31, 261–280.

Su, H.B., Zhang, Y.G., He, J.T., Mo, W., Zhang, Y.L., Tao, X.M., Song, H.Y., 2004. Construction and characterization of novel staphylokinase variants with antiplatelet aggregation activity and reduced immunogenecity. Acta Biochim. Biophys. Sin. 36, 336–342.

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

van de Weert, M., van't Hof, R., van der Weerd, J., Heeren, R.M.A., Posthuma, G., Hennink, W.E., Crommelin, D.J.A., 2000b. Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques. J. Control. Release 68, 31–40.

Vanderschueren, S., Collen, D., Werf, F., 1996. A pilot study on bolus administration of recombinant staphylokinase for coronary artery thrombolysis. Thromb. Haemost. 76, 541–544.

Wang, J., Wang, B.M., Schwendeman, S.P., 2002. Characterization of the initial burst release of a model peptide from poly(D,L-lactide-*co*-glycolide) microspheres. J. Control. Release 82, 289–307.

Yang, Y.Y., Chung, T.S., Ping Ng, N., 2001. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials 22, 231–241.

Zhu, G., Mallery, S.R., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in injectable poly(lactide-*co*-glycolide). Nat. Biotechnol. 18, 52–57.