International Journal of Pharmaceutics 416 (2011) 69–76

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Stabilization and encapsulation of recombinant human erythropoietin into PLGA microspheres using human serum albumin as a stabilizer

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ARTICLE INFO

*Article history:*
Received 28 January 2011
Received in revised form 24 May 2011
Accepted 6 June 2011
Available online 14 June 2011

*Keywords:*
Recombinant human erythropoietin
Protein stabilization
Microspheres
Solid-in-oil-in-water (s/o/w) emulsion method
Human serum albumin

ABSTRACT

The aim of this study was to prepare recombinant human erythropoietin (rhEPO) loaded poly(lactic-co-glycolic acid) (PLGA) microspheres using human serum albumin (HSA) as a stabilizer. Prior to encapsulation, the rhEPO–HSA mixture microparticles were fabricated using a modified freezing-induced phase separation method. The microparticles were subsequently encapsulated into PLGA microspheres. Process optimization revealed that the polymer concentration in the organic phase and the sodium chloride (NaCl) concentration in the outer water phase of the s/o/w emulsion played critical roles in determining the properties of the resultant microspheres. An in vitro release test showed that rhEPO was released from PLGA microspheres in a sustained manner up to 30 days. A single injection of rhEPO-loaded PLGA microspheres in Sprague–Dawley rats resulted in elevated hemoglobin and red blood cell concentrations for about 33 days. The stability of the rhEPO within the PLGA microspheres was systematically investigated by size-exclusion high-performance liquid chromatography (SEC-HPLC), SDS–PAGE, western blot and in vivo biological activity assay. The stability of rhEPO released from rhEPO-loaded microspheres was also examined by western blot. The results suggested that the integrity of rhEPO was successfully protected during the encapsulation process and the release period from polymeric matrices.



## 1. Introduction

Erythropoietin (EPO) is a hormone primarily produced by kidney cells and is the main regulator of red blood cell production in mammals. Recombinant EPO is used to treat anemia resulting from renal failure, zidovudine treatment for HIV infection, bone marrow transplantation, and cancer chemotherapy (Eschbach et al., 1987; Markham et al., 1995; Spivak, 1994). Recombinant EPO is usually administered via two to three intravenous or subcutaneous injections per week for years. Except for these parenteral routes of administration, no other patient-friendly routes exist. To improve patients' compliance and therapeutic efficacy, a sustained-release delivery system that allows EPO administration once or twice a month is desirable.

Of the diverse drug delivery systems, biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microspheres are a promising depot formulation of protein and peptide drugs (Mundargi et al., 2008; Pisal et al., 2010). Over the last decade, many attempts have been made to develop biodegradable microsphere systems for recombinant human erythropoietin (rhEPO). Morlock et al. developed PLGA or PLGA–PEO–PLGA tri-block copolymer microspheres using a water-in-oil-in-water (w/o/w) double emulsion micro-encapsulation process (Morlock et al., 1997, 1998). With L-arginine, bovine serum albumin (BSA), or hydroxypropyl-β-cyclodextrin used as protein stabilizers, aggregated rhEPO was reduced to 3–5%. Pistel et al. prepared various PLGA and poly(ethylene oxide) copolymer microspheres using a modified w/o/w double-emulsion technique (Pistel et al., 1999). The amount of rhEPO aggregated inside the star-shaped block copolymers was found to be in the range of 3–10%. These results indicated that rhEPO was susceptible to micro-encapsulation processes and easily aggregated. The amount of aggregated rhEPO within the microspheres prepared using the w/o/w double-emulsion technique was higher than safety standards allow (≤2%) (for a solution from EPO) (Villalobos et al., 2005). To reduce rhEPO aggregation, a solid-in-oil-in-water (s/o/w) emulsion method has been investigated to develop rhEPO-loaded microspheres. Burke et al. encapsulated a rhEPO variant, Darbepoetin alfa, into a PLGA microsphere by spray-freeze drying and spray drying (Burke et al., 2004). While low levels of aggregation was achieved (≤2%), cumulative protein recovery over four weeks was very low (<30%). Moreover, covalent dimer (<6.5%) and high molecular weight aggregates (<2.3%) were recovered. Geng et al. reported a novel method to prepare erythropoietin-loaded PLGA microspheres (Geng et al., 2008). EPO was first formulated together with dextran to form EPO-dextran glassy particles. The mixture particles were subsequently encapsulated into PLGA microspheres

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doi:10.1016/j.ijpharm.2011.06.008

by a solid-in-oil-in-water (S/O/W) double emulsion method. The stability of EPO was preserved effectively during preparation process (aggregation of EPO <2%). In vitro release study showed that EPO could release from the composite PLGA microspheres in a sustained-release manner up to 60 days. However, in vivo efficacy of EPO maintained only about 30 days. In vitro release of EPO-loaded microsphere last far longer than the in vivo efficacy. Although the reason was not clearly illustrated, the protein aggregation and denaturation might be the important factors for the inefficacy of EPO during the late release period. Therefore, preserving rhEPO stability during the encapsulation process and release period from polymeric matrices remains a challenging issue that must be addressed in the development of rhEPO-loaded microspheres as drug delivery systems.

The aim of this study was to fabricate rhEPO-loaded PLGA microspheres using a modified s/o/w emulsion method with the purpose of preserving rhEPO stability during the encapsulation process and the sustained-release period. A large amount of HSA was used as a stabilizer to protect the rhEPO from denaturation and aggregation. Process optimization revealed that polymer concentration in the organic phase and NaCl concentration in the outer water phase of the s/o/w emulsion played critical roles in determining the properties of the resultant microspheres. The stability of the rhEPO within the PLGA microspheres was systematically examined by size-exclusion high-performance liquid chromatography (SEC-HPLC), SDS–PAGE, Western blot and in vivo biological activity assay. The in vitro release profile and in vivo pharmacodynamics of the rhEPO-loaded PLGA microspheres were also investigated.

## 2. Materials and methods

### 2.1. Materials

The rhEPO solution was obtained from NCPC GeneTech Biotechnology Development Co., Ltd. (Shijiazhuang, China). Polyethylene glycol, 4000, 6000, and 8000 Da in average molecular weight (called PEG or PEG 4000, 6000, 8000 hereafter), was purchased from Sigma (St. Louis, MO, USA). Polyvinyl alcohol (PVA) with a molecular weight range of 31,000–50,000 Da was obtained from Aldrich Chemical Company Inc. (USA). Poly (DL-lactic-co-glycolic acid) (PLGA, a copolymer with a ratio of 75:25 and with an average molecular weight of 23 kDa) was purchased from Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). Human serum albumin (HSA) was obtained from Shanghai RAAS Blood Products Co., Ltd (Shanghai, China). All other chemicals used were of analytical grade.

### 2.2. Preparation of the rhEPO–HSA mixture microparticles

The rhEPO–HSA mixture microparticles were prepared using a modified freezing-induced phase separation method (Morita et al., 2000a). In brief, a solution containing rhEPO (0.1%, w/w), HSA (1%, w/w), PEG (5–20%) and 0–0.2 M sodium phosphate was frozen at −80 °C overnight and subsequently lyophilized using a Christ ALPHA 1-2 plus freeze-dryer operating at a pressure of $5.0 \times 10^{-3}$ Pa for 20 h. The lyophilized powders were dispersed in 5 ml of dichloromethane at a high agitation speed, followed by centrifugation at 8000 rpm for 10 min to remove the PEG within the particles. This operation was repeated three times and the final precipitates were dried under vacuum overnight.

### 2.3. Preparation of the rhEPO-loaded PLGA microspheres

The rhEPO-loaded PLGA microspheres were prepared using a modified s/o/w method (Morita et al., 2000b). Briefly, 10 mg of the rhEPO–HSA mixture microparticles was suspended in 2.5 ml of dichloromethane solution containing 60–240 mg/ml PLGA using a magnetic stirrer at 20,000 rpm for 1.5 min. The resulting solid-in-oil (s/o) suspension was then added to 75 ml of 2% w/v aqueous PVA solution containing 0–5% NaCl and homogenized at 600 rpm for 1 min to form the s/o/w emulsion. For solvent extraction, the s/o/w emulsion was immediately diluted with 225 ml of 20 mM PB at pH 7.4 containing 0–5% NaCl and was stirred with a magnetic stirrer at 300 rpm for 6 h. The resulting rhEPO-loaded microspheres were collected by filtration and were washed three times with distilled water to remove the PVA and NaCl. The microspheres were then lyophilized using a Christ ALPHA 1-2 plus freeze-dryer operating at a pressure of $5.0 \times 10^{-3}$ Pa overnight and stored at −20 °C for use.

### 2.4. Particle size and morphology of the rhEPO–HSA mixture microparticles and the rhEPO-loaded PLGA microspheres

The rhEPO–HSA mixture microparticles and the rhEPO-loaded PLGA microspheres were examined for morphology and size using scanning electron microscopy (SEM, Hitachi S-520). The rhEPO–HSA mixture microparticles and the rhEPO-loaded PLGA microspheres were mounted onto metal stubs using double-sided adhesive tape and vacuum-coated with a thin layer of gold. Then the samples were examined by SEM. One hundred PLGA microspheres were randomly selected and measured for diameter and the results were given as an average value. To determine the mean particle diameter and size distribution, the rhEPO–HSA mixture microparticles were dispersed in dichloromethane and subjected to a Malvern Nano-S90 particle size analyzer. The mean diameter of the rhEPO–HSA mixture particles was calculated from the three time measurements.

### 2.5. Determination of encapsulation efficiency by size-exclusion high-performance liquid chromatography (SEC-HPLC)

The protein content in the microspheres was determined using an extraction method (Zhu et al., 2000). Thirty mg of dried microspheres were dissolved in methylene chloride. After centrifugation at 10,000 rpm for 15 min and the removal of the polymer solution, the remaining protein pellet was dissolved in 0.2 ml of 20 mM phosphate buffer (pH 7.4). After centrifugation at 10,000 rpm for 15 min, the concentrations of protein in the supernatant and precipitate were determined, respectively. The water-soluble protein was assayed by applying the samples to a Thermo Scientific BioBasic SEC-300 size-exclusion column (7.8 mm diameter × 150 mm height) using a Shimadzu LC-10Avp HPLC instrument (Shimadzu, Kyoto, Japan). 0.05 mol/L phosphate buffer with 100 mM sodium chloride, pH 7.4, was used as the mobile phase running at the flow rate of 0.5 ml/min. Absorbance was recorded at 280 nm. The rhEPO content in the supernatant was determined using a Quantikine in vitro diagnostic rhEPO enzyme-linked immunosorbent assay kit purchased from R&D Systems (Minneapolis, USA). The precipitate were incubated in phosphate buffered saline (PBS) containing 6 M urea at 37 °C for 30 min for denaturation and the water-insoluble protein percentage was then determined by Bradford protein assay kit (Bio-Rad Laboratories, USA). The amount of protein from both the water-soluble and insoluble parts was taken into account in the calculation of the actual protein loading (mg of encapsulated protein per 100 mg of microspheres). The encapsulation efficiency of protein in the microspheres was calculated as the ratio of actual and theoretical protein loadings.

### 2.6. Dyeing of the rhEPO-loaded PLGA microspheres

To confirm the existence of proteins on the surface of the rhEPO-loaded PLGA microspheres, Coomassie brilliant blue G-250 (CBB G-250) was used to dye the microspheres. CBB G-250 could selec-

tively stain the protein, and PLGA almost cannot be colored. PLGA microsphere with and without rhEPO were dipped in CBB G-250 aqueous solution for several minutes. All samples were subsequently washed using deionized water for several times, and then freeze-dried overnight.

### 2.7. Biological activity assay of rhEPO

In vivo biological assay of rhEPO was studied with BALB/C mouse (6–8 weeks of age) (Ramos et al., 2003). The animals were randomized into sample and standard group, with 8 mice each group. Standard and test samples were diluted appropriately in phosphate-buffered saline containing 0.1% bovine serum albumin.

A single dose of 10, 20 or 40 IU rhEPO/0.4 ml per mouse was injected subcutaneously on day 1. Then, 150–200 μl of blood sample was taken from the orbital venous sinus of each mouse on day 4 and immediately mixed with 0.2 mL of dilution solution containing EDTA-K2 anticoagulant. Then, the number of reticulocytes was detected by an automated reticulocyte analyzer R-3500 (Sysmex Corporation, Kobe, Japan) and reported as a percentage of total red cells. The activity of rhEPO in plasma sample was calculated by the correlation between reticulocyte percentage and the activity of rhEPO. The results were expressed as specific activity (IU/mg) which is biological activity per milligram of rhEPO.

### 2.8. Western blot analysis of rhEPO during encapsulation process and release period

Western blot assay of rhEPO recovered and released from PLGA microspheres was performed as described by Sambrook et al. (1989). Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out under non-reducing conditions using 12% polyacrylamide gels. Then, the proteins were transferred from the SDS–PAGE gels onto PVDF membranes at a constant voltage of 70 V for 4 h. After the PVDF membranes were blocked with 5% (w/v) dried skim milk powder overnight at 4 °C, the membranes were incubated with rabbit polyclonal antisera against rhEPO for 2 h at room temperature. The membranes were washed and incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate for 1 h. Blots were then visualized by chemiluminescence (West Pico SuperSignal substrate; Pierce).

### 2.9. In vitro release of proteins from PLGA microspheres

Thirty mg of dried microspheres was suspended in 1 ml of PBS (pH7.4) containing 0.05% (w/v) sodium azide and placed in a rocking incubator (SKY-211D, China) operating at 150 rpm and 37 °C. At each sampling time, the supernatant was withdrawn and replaced with the same volume of fresh PBS solution. The total protein content in the supernatant was assayed using a Bradford Protein Assay kit. The rhEPO content in the supernatant was determined using a Quantikine in vitro diagnostic rhEPO enzyme-linked immunosorbent assay kit purchased from R&D Systems (Minneapolis, USA). The amount of protein released within 24 h was defined as the initial burst.

### 2.10. Pharmacodynamic studies of rhEPO-loaded PLGA microspheres

The pharmacodynamics of the rhEPO-loaded PLGA microspheres were evaluated in male Sprague–Dawley (SD) rats (male, 180–210 g, Grade II, Certificate No. 06057) purchased from the Experimental Animal Center of Hebei Province in China. The rats were housed under conventional laboratory conditions in a room maintained at temperature of 24 ± 1 °C and were fed with commercial rat food and water ad libitum. Animals (five per group) were injected subcutaneously at the nape of the neck with 11 μg/kg rhEPO in microsphere form or the equivalent amount of blank microspheres (without rhEPO). Microspheres were dispersed in a sterile vehicle containing 2% sodium carboxylmethylcellulose, 0.9% sodium chloride, and 2% polysorbate 20 in 20 mM PB at pH 7.4. Then, 20 μl of blood samples were taken from the rats (at the tail vein) about twice per week after starting treatment, and immediately mixed with 0.5 mL of dilution buffer provided by the manufacturer. The hemoglobin (HGB) concentration and red blood cell number (RBC) in the blood samples was determined using an XE-2100 automated hematology analyzer (Sysmex, Japan).

## 3. Results and discussion

### 3.1. The formation of the rhEPO–HSA mixture microparticles

Although Morita et al. have prepared BSA microparticles using a freezing-induced phase separation method, the obtained microparticles were in the lyophilized mixture of protein and PEG (Morita et al., 2000a). Thus, the influence of formulation parameters for the freezing-induced phase separation process on the properties of protein microparticles was not indicated clearly. In the present work, the protein microparticles were firstly isolated from the lyophilized mixture of protein and PEG. Then the influence of PEG molecular weight, the ratio of PEG to proteins, and sodium phosphate concentration on the particle size and recovery of proteins were investigated in detail. Sodium phosphate is added because it is usually used as buffer to stabilize solution pH. The results are summarized in Table 1. The formation of microparticles was significantly affected by the sodium phosphate concentration. When no sodium phosphate was added, proteins aggregated together and no rhEPO–HSA microparticles were obtained (Fig. 1A). When the concentration of sodium phosphate in the mixture solution was 0.02 M, rhEPO–HSA microparticles formed no matter what kind of PEG was selected. The results suggested sodium phosphate facilitated the freezing-induced phase separation of the PEG/protein system. This phenomenon has been observed in other combinations of protein and nonionic polymers (Izutsu and Kojima, 2000). It has been conjectured that salts could alter the molecular interactions between polymers and proteins by covering the electrostatic effect and/or changing their hydration state (Tolstoguzov, 2000). Then, the miscibility between proteins and nonionic polymers in the frozen solutions were changed, which further enhanced the phase separation between proteins and polymers.

The obtained rhEPO–HSA mixture microparticles possessed a smooth surface and the microparticle diameters were smaller than 500 nm (Table 1 and Fig. 1B). The sizes of mixture particles were suitable for encapsulation within PLGA microspheres (Cleland and Jones, 1996). When the sodium phosphate concentration increased more than 0.1 M, rhEPO–HSA microparticles (~500 nm) and large, irregular particles (>10 μm) were simultaneously obtained (Fig. 1C). These large particles were crystal grains of sodium phosphate and were not suitable for encapsulation within PLGA microspheres because they would cause severe burst release (Cleland and Jones, 1996). When the ratio of PEG to proteins increased from 5:1 to 10:1, the recovery efficiency of proteins decreased remarkably from 93.7% to 77%. Moreover, the molecular weight of PEG and the ratio of PEG to proteins have some influence on the size and formation of rhEPO–HSA microparticles (Table 1). It has been reported that PEG could interact and formed complex with protein (Furness et al., 1998). When PEG was removed using dichloromethane, some protein might also be removed. Thus, with the increase of the ratio of PEG to proteins, the recovery efficiency of proteins decreased.

**Table 1**
Characterization of the rhEPO–HSA mixture microparticles.

| Code | Concentration of sodium phosphate | Molecular weight of PEG | Ratio of PEG to proteins | Mean particle size (μm) | Recovery of proteins (%)[a] |
|---|---|---|---|---|---|
| A | 0 | 6000 | 5:1 | NF[b] | – |
| B | 0.02 | 6000 | 5:1 | 0.43 ± 0.04 | 95.7 ± 3.1 |
| C | 0.02 | 6000 | 10:1 | 0.32 ± 0.04 | 77.0 ± 2.1 |
| D | 0.02 | 6000 | 20:1 | NF [b] | – |
| E | 0.02 | 4000 | 5:1 | 0.52 ± 0.06 | 98.8 ± 4.2 |
| F | 0.02 | 8000 | 5:1 | 0.43 ± 0.03 | 93.7 ± 3.3 |
| G | 0.1 | 6000 | 5:1 | >10 | 100.0 ± 2.6 |
| H | 0.2 | 6000 | 5:1 | >10 | 98.5 ± 2.1 |

[a] Mean ± S.D., $n = 3$.
[b] NF: no microparticle formed.

### 3.2. The influence of PLGA in the organic phase and NaCl in the outer water phase on the properties of microspheres

The influences of experimental factors on encapsulation efficiency, initial burst release, and particle size of PLGA microspheres were investigated using a single-factor experiment design. These factors included the PLGA concentration in dichloromethane, the volume of the external aqueous phase, the initial homogenizing time, the initial homogenizing speed, the second homogenizing speed, the second homogenizing time, and PVA and NaCl concentration in the external aqueous phase of the s/o/w emulsion. Of these factors, the PLGA concentration in dichloromethane and NaCl concentration in the external phase of the s/o/w emulsion affected the characteristics of the resultant microspheres remarkably.

The influences of NaCl on the properties of PLGA microspheres in the external water phase were investigated in detail and the results are summarized in Table 2. When NaCl concentration increased from 0 to 5%, encapsulation efficiency increased from 64.1% to 85.3%. How did NaCl concentration in the outer phase affect encapsulation efficiency? It has been reported that NaCl in the outer phase could balance the osmotic pressure between the inner phase and the external aqueous phase and then affect properties of microsphere (Han et al., 2001). Although the initial inner phase was in solid state, water could diffuse through the oil layer and rehydrate solid microparticles to form protein solution in the inner phase. The unbalanced osmotic pressure would enhance the material exchange between the inner phase and the outer water phase during the solidification of the microspheres (Zhou et al., 2010). This resulted in the decreased encapsulation efficiency and the formation of holes on the surface of the obtained microspheres (Table 2, Fig. 2A). When NaCl was added to the outer water phase, the osmotic pressure between the inner phase and the external aqueous phase was balanced. The material exchange between the inner phase and the outer phase was inhibited (Han et al., 2001; Zhou et al., 2010). It could be seen from Fig. 2 that no pores formed on the surface of the microspheres (Fig. 2B and C). The encapsulation efficiency increased significantly with the increase of NaCl concentration (Table 2). Moreover, with the influx of water from the outer phase into the inner phase being retarded, the resulting microspheres were denser and smaller. The sizes of PLGA microspheres decreased from 107 μm to 82 μm when NaCl concentrations in the external phase increased from 0% to 5% (Table 2). These results further confirmed our speculations.

NaCl in the outer water phase also plays a critical role for initial burst of rhEPO-loaded PLGA microsphere. When NaCl concentration increased from 0 to 5%, initial burst release decreased significantly from 73.3% to 19.9% (Table 2). It has been reported that initial burst was ordinarily attributed to rapid release of the protein adsorbed on the surface of the microspheres (Huang and Brazel, 2001). To investigate the protein distribution on the surface of rhEPO-loaded PLGA microspheres, microspheres were colored by CBB G-250. CBB G-250 could selectively stain the protein and PLGA almost cannot be colored (Fig. 3A). PLGA microsphere with the addition of 5% NaCl in the outer water phase (Fig. 3C) was almost not dyed, while PLGA microsphere prepared without addition of NaCl presented an obvious blue color (Fig. 3B). This indicated that the proteins were almost not present on the surface of PLGA microspheres when 5% NaCl was added in the outer water phase. Then the initial burst significantly reduced.

When PLGA concentrations in the organic phase were increased from 4% to 18%, the encapsulation efficiency increased remarkably from 12.3% to 79.3% (Fig. 4). When the PLGA concentration

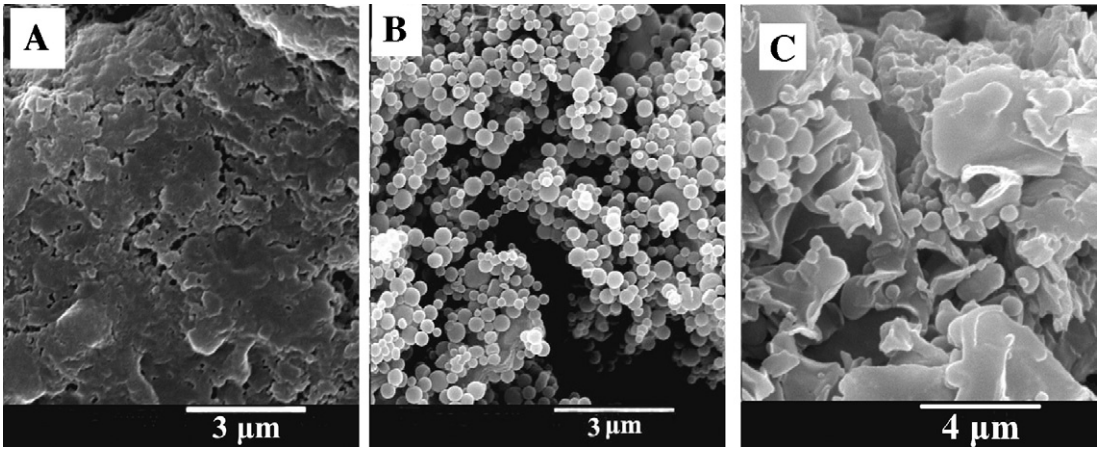


**Fig. 1.** Scanning electron microscopic images of freeze-dried rhEPO–HSA mixture microparticles corresponding to formulations A, B, and H in Table 1; these microparticles were prepared from the solutions in which the concentration of rhEPO was 1 mg/ml, HSA was 10 mg/ml, PEG6000 was 55 mg/ml, and sodium phosphate was 0 M (A), 0.02 M (B), or 0.2 M (C), respectively.

**Table 2**
Characterization of rhEPO-loaded PLGA microspheres.[a]

| Code | NaCl Concentr-ation (%) | Microsphere diameter (μm) | Yield efficiency (%) | Encapsulation Efficiency (%)[b] | Initial burst release (%)[b] |
|---|---|---|---|---|---|
| B-A | 0 | 104.0 | 83.0 | 64.1 ± 7.6 | 73.3 ± 4.6 |
| B-B | 1 | 87.2 | 83.7 | 70.5 ± 4.8 | 72.3 ± 3.2 |
| B-C | 2 | 83.3 | 85.0 | 75.0 ± 4.8 | 67.0 ± 3.3 |
| B-D | 3 | 81.3 | 91.7 | 78.6 ± 7.1 | 40.3 ± 10.3 |
| B-E | 4 | 69.7 | 89.0 | 83.2 ± 5.7 | 24.4 ± 2.52 |
| B-F | 5 | 63.0 | 89.5 | 85.3 ± 9.5 | 19.9 ± 1.5 |

[a] The rhEPO–HSA mixture microparticles corresponding to formulation B in Table 1 was used to fabricate the rhEPO-loaded PLGA microspheres.
[b] Mean ± S.D., $n = 3$.

further increased from 16% to 24%, encapsulation efficiency virtually did not change. It has been reported that the thickness of the PLGA layer would increase with increased PLGA concentration (Liu et al., 2007; Xie et al., 2008). Increasing the thickness of the PLGA layer would help prevent protein leakage from the s/o/w emulsion during solidification of the microspheres. As a result, increased PLGA concentration would result in improved encapsulation efficiency. Increased thickness of the PLGA layer would also hinder the leakage of drugs from PLGA microspheres. The increased PLGA concentration should also be helpful in reducing the rapid diffusion of drugs adjacent to the surface of the microspheres (Huang and Brazel, 2001). When PLGA concentration increased from 6% to 18%, the initial burst release of PLGA microspheres decreased significantly from 81% to 26% (Fig. 4). The experimental results support the above conjecture. The increased PLGA concentration also results in a more viscous (concentrated) polymer solution, which makes it difficult to form small s/o/w emulsion droplets and leads to increased particle size. Analysis of PLGA microspheres confirmed that the microspheres increased in size from 56 μm to 93 μm when the PLGA concentration increased from 6% to 18%.

### 3.3. The stability of rhEPO during formulation processes

The protein stability within rhEPO–HSA microparticles and rhEPO-loaded microspheres were firstly examined by SEC-HPLC and the results were shown in Fig. 5. The rhEPO, HSA and rhEPO–HSA microparticles only displayed one elution peak on SEC-HPLC (Fig. 5A–C). The elution peak of HSA was completely overlapped with that of rhEPO. Clearly, the process for fabricating rhEPO–HSA microparticles did not cause obvious protein aggregation. Proteins recovered from the rhEPO-loaded PLGA microspheres showed one main elution peak and two minor peaks eluted before the main component. Obviously, the minor peaks should be related to protein aggregates (Fig. 5D). To elucidate the protein aggregation during encapsulation process, SDS–PAGE and Western blot was further used to analyze proteins recovered from rhEPO–HSA microparticles and rhEPO-loaded microspheres. SDS–PAGE analysis indicated that a small amount of proteins recovered from rhEPO–HSA microparticles and rhEPO-loaded microspheres formed dimmers or polymers (Fig. 6A). However, No blot of aggregated rhEPO was developed with rabbit polyclonal antisera against rhEPO (Fig. 6B). These results suggested that the aggregated proteins were obviously not from rhEPO but HSA. In vivo biological activity assay was further used to assess integrity of rhEPO recovered from rhEPO–HSA microparticles and rhEPO-loaded microspheres. The results showed that the specific activity of rhEPO recovered from rhEPO–HSA mixture microparticles and rhEPO-loaded PLGA microspheres was comparable to that of fresh rhEPO solution (Fig. 7). These results suggested that the integrity of rhEPO was successfully protected during the encapsulation process.

To examine water-insoluble protein aggregates, the extracts recovered from rhEPO-loaded PLGA microspheres were washed in phosphate buffer to remove soluble part. The water-insoluble aggregates were then determined. The results showed that the content of water-insoluble aggregates was less than 0.3%. The water-insoluble aggregates were also analyzed by Western-blot (Section 2.8). The content of rhEPO in the samples was below the detection limit of Western-blot (about 20 pg). The results suggested that water-insoluble rhEPO aggregates formed during encapsulation process were not significant.

### 3.4. In vitro release of proteins from PLGA microspheres

In vitro release profiles of the total proteins and rhEPO from the PLGA microspheres were very similar (Fig. 8A). Both in vitro release profiles presented a biphasic pattern: (1) an initial burst release of about 20% of the total proteins or rhEPO occurred within one day; (2) after the initial burst, protein release profiles displayed a sustained release at a near zero-order release kinetic stage. The

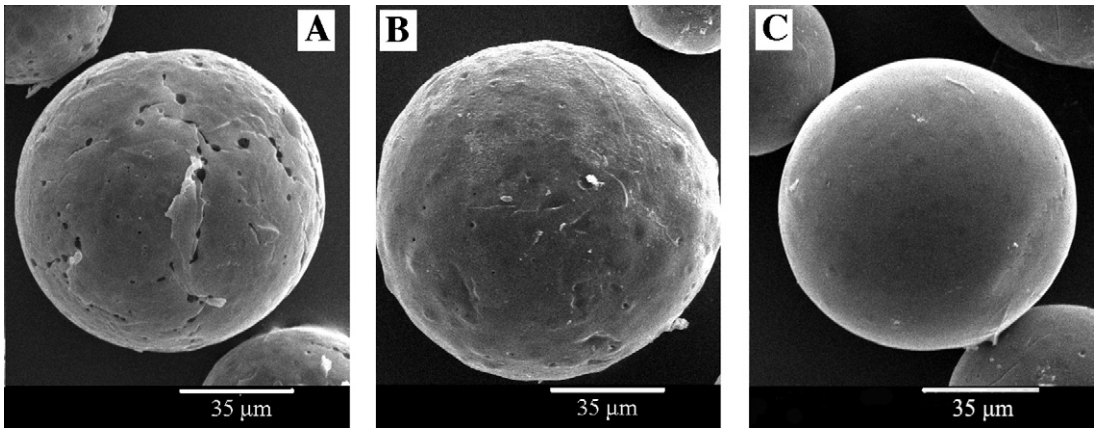


**Fig. 2.** Scanning electron microscopic images of microspheres corresponding to formulations B-A, B-C, and B-F in Table 2, which were prepared with 0% NaCl (A), 2.0% NaCl (B), and 5.0% NaCl (C) in the external water phase of the s/o/w emulsion.

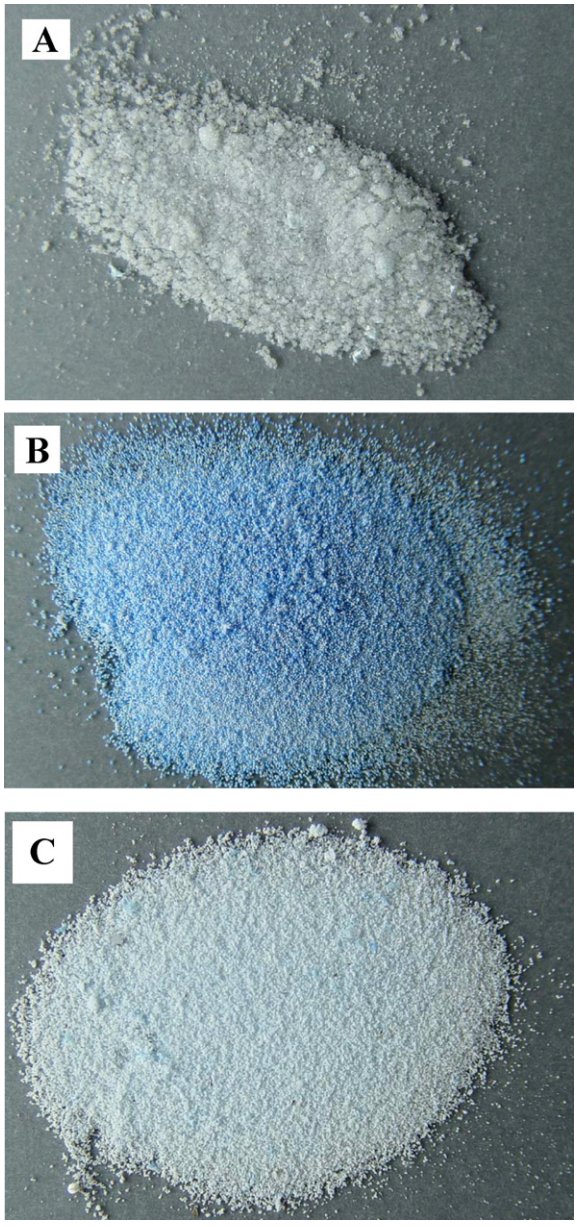


**Fig. 3.** The protein distribution on the surface of blank microspheres (without proteins) (A), rhEPO-loaded PLGA microspheres prepared with 0% NaCl (B), and 5.0% NaCl (C) in the external water phase of the s/o/w emulsion. Coomassie brilliant blue was used as the dye which could selectively color only proteins.

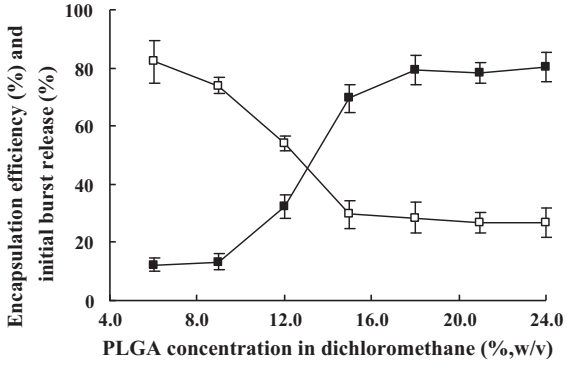


**Fig. 4.** The influence of the PLGA concentration in the dichloromethane on encapsulation efficiency (■) and initial release (□). The mean ± SD was derived from three separate experiments.

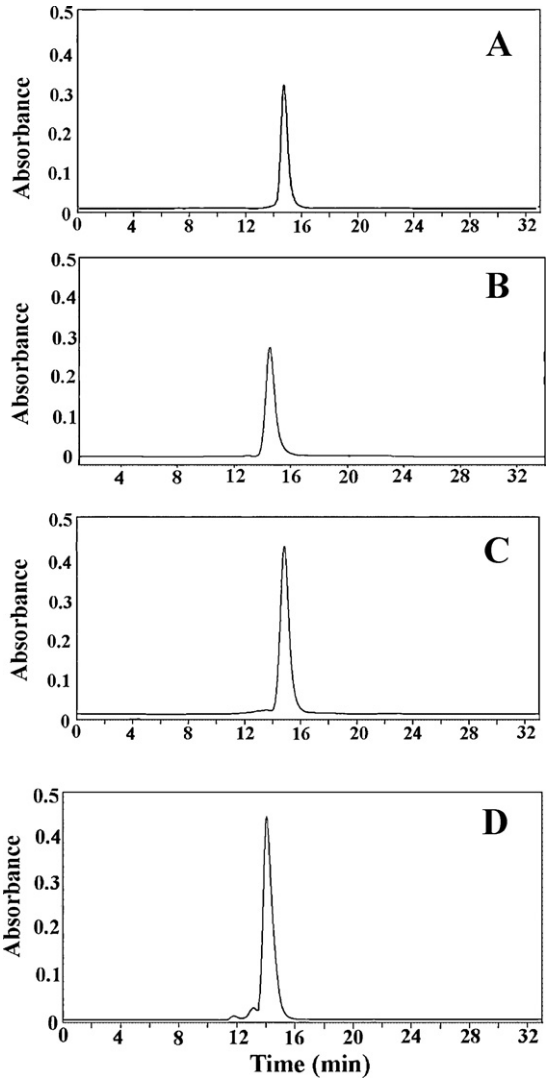


**Fig. 5.** SEC-HPLC plots of rhEPO recovered from various formulations. (A) HSA solution; (B) Fresh rhEPO solution; (C) rhEPO recovered from rhEPO–HSA mixture microparticles; (D) rhEPO recovered from rhEPO-loaded PLGA microspheres corresponding to formulation B-F in Table 2.

total amount of released proteins or rhEPO over a period of 30 days was more than 70%. The cumulative release of proteins was almost complete (more than 90%).

Western blot was subsequently used to detect aggregation of in vitro released rhEPO from PLGA microspheres. No blot of aggregated rhEPO was developed with rabbit polyclonal antisera against rhEPO (Fig. 8B). These results suggested that the released rhEPO should be in active state and not in polymeric state.

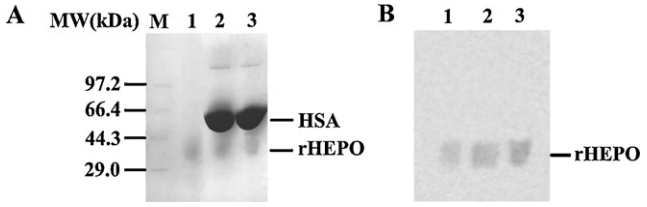


**Fig. 6.** SDS/PAGE (A) and Western-blot (B) analysis of rhEPO recovered from various formulations. M, molecular-mass marker proteins; lane 1, Fresh rhEPO solution; lane 2, rhEPO recovered from rhEPO–HSA mixture microparticles; lane 3, rhEPO recovered from rhEPO-loaded PLGA microspheres corresponding to formulation B-F in Table 2.

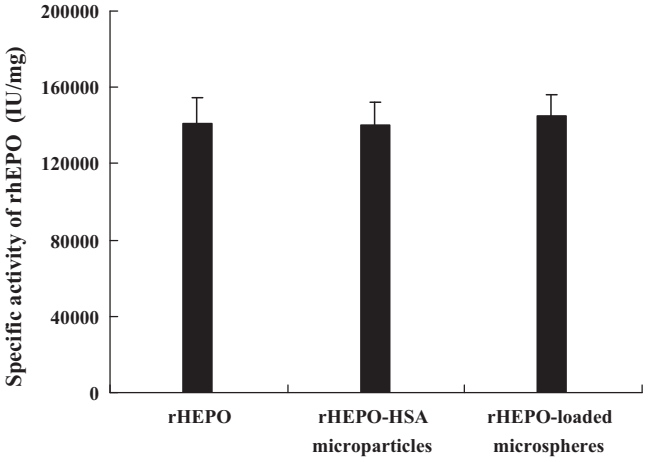


**Fig. 7.** In vivo biological activity assay of rhEPO recovered from various formulations.

### 3.5. *In vivo pharmacodynamics of rhEPO-loaded PLGA microspheres*

In vivo pharmacodynamics of rhEPO-loaded PLGA microspheres were examined based on elevated hemoglobin (HGB) concentration and red blood cells (RBCs) in male SD rats receiving a single injection of the rhEPO-loaded PLGA microsphere formulation or blank microspheres (without rhEPO). The HGB concentration and RBCs in the blood samples taken from the treated mice were determined using an automated hematology analyzer and the results are shown in Fig. 9A and B, respectively. A significant elevation of RBCs and HGB concentration was presented from the fourth day to the thirty seventh day following a single injection of rhEPO-loaded microspheres ($P < 0.05$). The elevation of RBC and HGB levels was sustained over 30 days, which is similar to that during in vitro release. The results suggested that the released rhEPO were in biologically active form during the sustained-release period.

## 4. Conclusion

The rhEPO-loaded PLGA microspheres were successfully prepared using a modified s/o/w emulsion technique with a large amount of HSA as a stabilizer. Prior to encapsulation, the rhEPO–HSA mixture of microparticles was fabricated using a modified freezing-induced phase separation method. The protein microparticles obtained, smaller than 500 nm in diameter, were suitable for encapsulation within PLGA microspheres. The influences of the main experimental factors on the properties of rhEPO-loaded microspheres were evaluated using a single-factor experiment design. The results revealed that polymer concentration and NaCl concentration in the outer water phase of the s/o/w emulsion played critical roles in determining the properties of the resultant microspheres. An in vitro release test showed that the release profile of rhEPO and total proteins from the PLGA microspheres presented two phases: an initial burst release of about 20% of loadings within one day, followed by sustained release of more than 70% of loadings up to 30 days. A single injection of the rhEPO-loaded PLGA microspheres administered to SD rats resulted in elevated HGB and RBC concentrations for about 33 days. The stability of the rhEPO was systematically investigated by SEC-HPLC, SDS–PAGE, western blot and in vivo biological activity assay. The results suggested that the integrity of rhEPO was successfully protected during the encapsulation process and the release period from polymeric matrices.

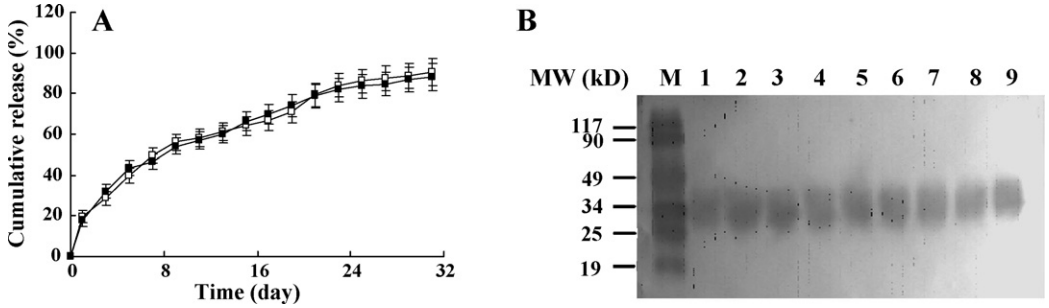


**Fig. 8.** (A) In vitro cumulative release profiles of total proteins (□) and rhEPO (■) from the PLGA microsphere corresponding to formulation B-F in Table 2. The mean ± SD was derived from three separate experiments. (B) Western blot analysis of rhEPO in the released samples. Lane M, molecular mass standards; Lanes 1–9, in vitro release samples on the first, third, fifth, ninth, thirteenth, seventeenth, twenty-first, twenty-fifth, twenty-ninth day, respectively.

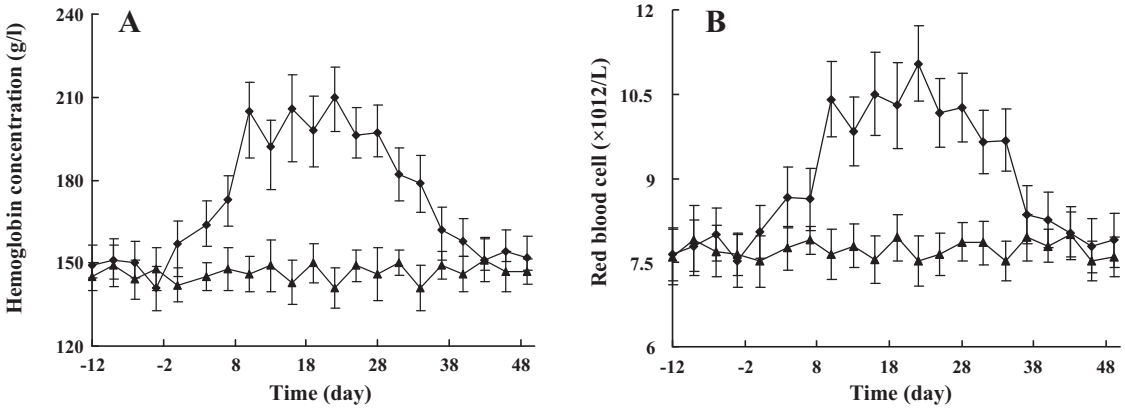


**Fig. 9.** Profiles of red blood cell (A) and hemoglobin (B) levels in SD rats after a single injection of rhEPO-loaded microspheres corresponding to formulation B-F in Table 2 (□) or blank microspheres (without rhEPO) (■). The mean ± SD was derived from five separate experiments.

## Acknowledgements

This work is partially supported by research grants from the Provincial Science & Technology Department of Hebei Province in China (09276418D-3) and the Science & Technology project of the Provincial Education Department of Hebei (200645) and Doctor Foundation of Hebei Normal University (L2005B23).

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