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

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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.

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

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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 × *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.