

available at [www.sciencedirect.com](http://www.sciencedirect.com)journal homepage: [www.elsevier.com/locate/ejps](http://www.elsevier.com/locate/ejps)

# Development of protein delivery microsphere system by a novel S/O/O/W multi-emulsion

Weien Yuan, Fei Wu, Meiyang Guo, Tuo Jin\*

Shanghai Jiaotong University, School of Pharmacy, 800 Dongchuan Road, Shanghai 200240, People's Republic of China

## ARTICLE INFO

### Article history:

Received 7 December 2007

Received in revised form

1 August 2008

Accepted 16 August 2008

Published on line 11 September 2008

### Keywords:

Protein stability

Sustained-release

PLGA microspheres

Freezing-induced phase separation

S/O/O/W multi-emulsion

## ABSTRACT

A novel method has been developed to protect protein drugs in poly (lactic-co-glycolic acid) (PLGA) microspheres using S/O/O/W multi-emulsion method. This method develops a novel protein drug sustained-release system, which is based on the combination of protein-loaded dextran glassy microparticles (protein-loaded AqueSpheres) and PLGA microspheres. The protein molecules are encapsulated in the dextran glassy particles and the drug-containing dextran glassy particles are further dispersed in the PLGA microspheres. The protein-loaded AqueSpheres based PLGA composite microspheres have spherical shape and a smooth surface. They possess a normal size distribution and a mean diameter of 67.08  $\mu\text{m}$ . The method may decrease protein aggregations and incomplete release due to avoiding protein contacting with oil/water interfaces and hydrophobic PLGA. The dextran glassy particles can stabilize proteins in the PLGA matrix, which is the major advantage of this novel protein sustained-release system over preparation for the PLGA microspheres using W/O/W double-emulsion method.

© 2008 Published by Elsevier B.V.

## 1. Introduction

Protein pharmaceuticals sustained-release formulation has been being developing since 1976 (Langer and Folkman, 1976). Because of their physical and chemical instabilities (Wang, 1999), protein drugs need to be administrated through injection for a long time and other routes are ineffective (for example oral delivery of low bioactivity (Wang, 1999), nanoparticles for lung protein delivery (Grenha et al., 2005), intranasal delivery of protein (Cheng et al., 2005) and other delivery (Jensen et al., 2002; Reis et al., 2007)). Presently, sustained-release microsphere is one of most popular dosages. Among all the methods for preparing microspheres, emulsion method is the most commonly used one, for example water-in-oil (W/O) (Jorgensen et al., 2006), water-in-oil-in-water (W/O/W) (Castellanos et al., 2002; Coombes et al., 1998; Freitas et al., 2005; Kim and Bae, 2004; Takada et al., 2003), water-in-oil-in-

oil (W/O/O) (Lu and Park, 1995) and O/W (Park et al., 1998). There are water/oil interfaces existing while preparing microspheres by these methods which lead to protein aggregation and inactivation (Van de Weert et al., 2000). Though solid-in-oil-in-water (S/O/W) (Morita et al., 2000) may decrease protein aggregates, some proteins still have aggregates due to protein in surfaces of microspheres contact with water/oil interfaces. Solid-in-oil-in-oil (S/O/O) can almost protect proteins from forming aggregates, but burst release is severe and residue of oil phase (Carrasquillo et al., 2001) may lead to immunity problem (Wang and Wu, 1998). Preparation of microspheres or microparticles using these methods exist still initial burst and the incomplete release of the encapsulated protein (Kim and Park, 1999; Kwon et al., 2001). We suggest a novel method of multi-emulsion preparing microsphere solid-in-oil phase 1-in-oil phase 2-in-water (S/O<sub>1</sub>/O<sub>2</sub>/W), in which solid phase is protein-loaded microparticles which their size is about 1–5  $\mu\text{m}$

\* Corresponding author. Tel.: +86 21 34205072; fax: ++86 21 34205072.

E-mail address: [tjin@sjtu.edu.cn](mailto:tjin@sjtu.edu.cn) (T. Jin).

0928-0987/\$ – see front matter © 2008 Published by Elsevier B.V.

doi:10.1016/j.ejps.2008.08.009

by low temperature induced phase separation (Yuan et al., 2007);  $O_1$  is oil phase of PLGA dichloromethane solution and  $O_2$  is a hydrophilic oil phase (hO) and can dissolve in water, so the oil phase is easily removed; W is aqueous phase containing 5% (W/W) sodium chloride aqueous solution.

## 2. Materials and methods

### 2.1. Materials

Poly (lactic-co-glycolic acid) (PLGA) 3A DL (lactide:glycolide = 50:50, MW 47,000) (PLGA 3A), 2A DL (lactide:glycolide = 50:50, MW 13,000) (PLGA 2A), 1A DL (lactide:glycolide = 50:50, MW 6500) (PLGA 1A), was obtained from Lakeshore Biopolymers Inc. (Cincinnati, OH). Polyethylene glycol (MW = 8000) (PEG), sodium dihydrogen phosphate, sodium chloride, and trehalose were purchased from Chinese medicine Group Chemical reagent corporation (AR). Polyvinyl alcohol (PVA) (MW 13,000–23,000), dextran (MW = 64,000–70,000), and horse myoglobin (MGB) were obtained from Sigma. Bovine serum albumin (BSA) was purchased from Siji Company.  $\alpha$ -Lactose was supplied by Shanghai hangxin chemical reagent company. Micro-BCA protein kit was purchased from Pierce Company.

### 2.2. Preparation of protein-loaded dextran microparticles and PLGA microspheres

Protein-loaded dextran microparticles (AqueSpheres) were prepared using freezing-induced phase separation (Yuan et al., 2007). In brief, a co-solution containing protein (0.2%, w/w), dextran (1%, w/w) and PEG (8%, w/w) was prepared, frozen at  $-20^\circ\text{C}$  overnight, and lyophilized to powder under  $5.25 \times 10^{-3}$  Pa for 24 h using a laboratory freeze-dryer (Christ ALPHA 1-2, Germany). During the freezing process, dextran separated out to form a temperature-stabilized dispersed phase in which the protein partitioned preferentially. The lyophilized powders were re-suspended in dichloromethane to dissolve the PEG continuous phase, followed by centrifugation at 12,000 rpm for 5 min on a micro-centrifuge (Eppendorf 5415D, Germany) to remove the dissolved PEG. This washing procedure was repeated three times, the pellets were evaporated under 1.33 Pa for 24 h using vacuum dryer (Fuma DZF-3, Shanghai Fuma Co. Ltd., China).

A sample of 10 mg protein-loaded dextran microparticles was suspended in 0.9 g of 10% PLGA solution dissolved in dichloromethane (forming oil phase 1). After vigorous stirring for 1 min, the suspension was emulsified into 5.5 ml hydrophilic oil phase 2 containing 72.7% (w/w) glycerol, 18.2% (w/w) 1,2-propylene glycol, 9.1% (w/w) water (containing 1% (w/w) PVA and 5% (w/w) sodium chloride) under stirring (using a Fluko FA25 homogenizer at 2000 rpm) for 30 s to form PLGA microspheres 40–100  $\mu\text{m}$  in diameter. This emulsion was immediately transferred into 1000 ml cold water ( $0-5^\circ\text{C}$ ) containing 5% (w/w) sodium chloride to harden the PLGA microspheres. The PLGA microspheres were ageing in the water at gentle stirring (100 rpm) using an electromotive stirrer (Xinhang JJ-1, Jintan Xinhang Co. Ltd., China) to extract the organic solvent for 3 h, then the system was warmed up to room temperature. The hardened microspheres were

rinsed with pure water for three times and then subjected to lyophilization again prior to storage.

As control, BSA and MGB were loaded into PLGA microspheres using conventional water-in-oil-in-water (W/O/W) process. The 0.1 ml protein solution containing different proteins including 5 mg BSA and 1 mg MGB was added into 0.9 ml of 10% (w/w) PLGA solution dissolved in dichloromethane under vigorous stirring (using a Fluko FA25 homogenizer at 2000 rpm) to form a water-in-oil (W/O) primary emulsion. Then, this W/O emulsion was added in 4 ml of an aqueous solution containing 1% (w/w) polyvinyl alcohol (PVA) and 5% (w/w) sodium chloride (NaCl) under the same stirring condition as above. At last, the double-emulsion sample was transferred in 1000 ml ( $0-5^\circ\text{C}$ ) 5% (w/w) sodium chloride solution and aged at gentle stirring (100 rpm) using an electromotive stirrer (Xinhang JJ-1, Jintan Xinhang Co. Ltd., China) to extract the organic solvent for 3 h to harden the microspheres and the harden microspheres were then rinsed using distilled water, followed by lyophilization prior to storage.

### 2.3. Scanning electron microscopy

The surface morphology of protein-loaded dextran microparticles and PLGA microspheres was determined using a scanning electron microscope (FEI SIRION 200 SEM, American). Samples were sputtered with gold and scanned at an accelerated voltage of 15 kV.

### 2.4. Protein content assay

To assay content of protein encapsulated within PLGA microspheres, the accurately weighed 20 mg of dry microsphere sample was re-dissolved in 5 ml of dichloromethane, followed at 12,000 rpm for 5 min to remove PLGA soluble in the solvent and to recover the solid parts. This dissolving-centrifugation process was repeated for three times, and the microparticles were collected after evaporation of solvent residues at a vacuum of 1.33 Pa for 24 h to remove the solvent residues. Protein-loaded dextran particles recovered as above were then dissolved in appropriate volume of phosphate buffer solution (PBS, 0.45 mol/l, pH 7.4) and Protein content was detected with a Micro-BCA kit method. The amount of protein recovered from each formulation was divided by the amount added in the formulation to give encapsulation efficiency. Loading was calculated using the following equation: loading (%) =  $P/M \times 100$ , where P is the actual total weight of protein encapsulated into PLGA microspheres and M is the actual total weight of harvested PLGA microspheres.

### 2.5. Microsphere size distribution

Average particle size and its distribution were detected using a Particle Size & Shape Analyzer (CIS-100, Ankersmid). The accurately weighed 10 mg of dry microsphere sample was suspended in the quartz full of isopropyl alcohol with stirring magnet bar.

**Table 1 – Effect of different MW and their mixture of PLGA 50/50 on encapsulation efficiency (BSA-loaded AqueSpheres based PLGA microspheres, n = 3).**

Formulation	Loading (%)	Encapsulation efficiency (%)
A	2.49 ± 0.14	54.78 ± 3.08
B	2.10 ± 0.15	46.26 ± 3.30
C	2.42 ± 0.13	53.24 ± 2.86
D	2.91 ± 0.15	64.02 ± 3.34
E	1.75 ± 0.13	38.50 ± 2.86

A: PLGA (3A); B: PLGA (3A): PLGA (2A): PLGA (1A) = 60:20:20 (w/w/w); C: PLGA (3A): PLGA (2A): PLGA (1A) = 60:30:10 (w/w/w); D: PLGA (3A): PLGA (2A) = 60:40 (w/w); E: PLGA (2A).

## 2.6. Size exclusion chromatography

Size exclusion chromatography (SEC) was performed on a TSK G2000 Sk-XL1 column a Younglin HPLC system (Younglin, Korea). Initial studies with an isotonic phosphate buffer (25 mM sodium phosphate, 100 mM NaCl, pH 7.4) used as the running buffer provided very little elution of the protein, suggesting nonspecific protein adsorption to the column. All samples were detected with 25 mM sodium phosphate 1.0 M NaCl, pH 7.25 as the running buffer to minimize protein adsorption. The sample was loaded onto the column. The protein was then eluted at a flow rate of 1.0 ml/min. Protein elution was monitored by absorbance at 214 nm.

## 2.7. In vitro release

To examine protein release kinetics, 20 mg of AqueSphere-loaded PLGA microspheres were suspended in 1 ml PBS buffer (20 mM sodium phosphate and 0.15 M sodium chloride, pH 7.4). The samples were placed in an incubator (Shanghai Fun Wa KYC 100C) and shook at 110 rpm and 37 °C. The release medium was replaced by same amount of fresh buffer each day and protein concentration was assayed using Micro-BCA or SEC-HPLC method.

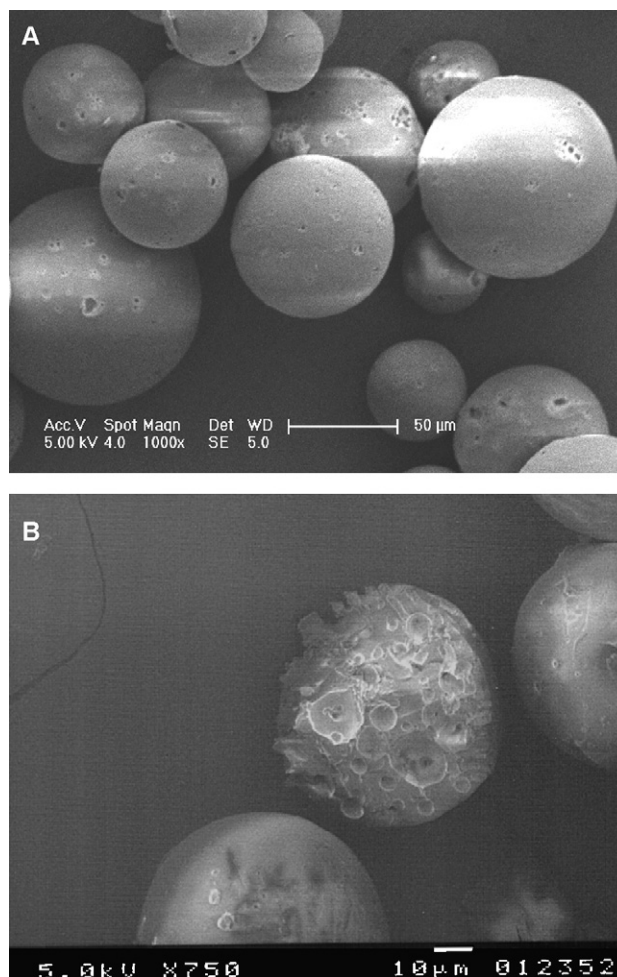
## 3. Results and discussions

### 3.1. Protein encapsulation efficiency

For both BSA and MGB, the protein encapsulation efficiency was measured by Micro-BCA kit or SEC-HPLC method. The efficiency for the first step, loading the proteins into dextran particles (AqueSpheres) was above 95% (w/w) (Yuan et al., 2009), and that for sequential two steps, then into protein PLGA microsphere was above 38–64% of the targeted encapsulation efficiency (Table 1).

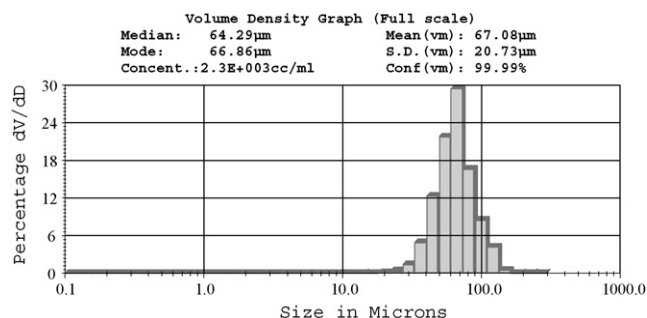
### 3.2. Morphology of AqueSphere-loaded PLGA microspheres

AqueSpheres dispersed in the PLGA matrix were visible during the ageing process of microsphere formation. As shown in Fig. 1, AqueSpheres (loaded BSA) are well distributed inside of the matrix of PLGA microspheres, 40–100 μm in diameter. The size distribution of AqueSpheres-loaded PLGA microspheres

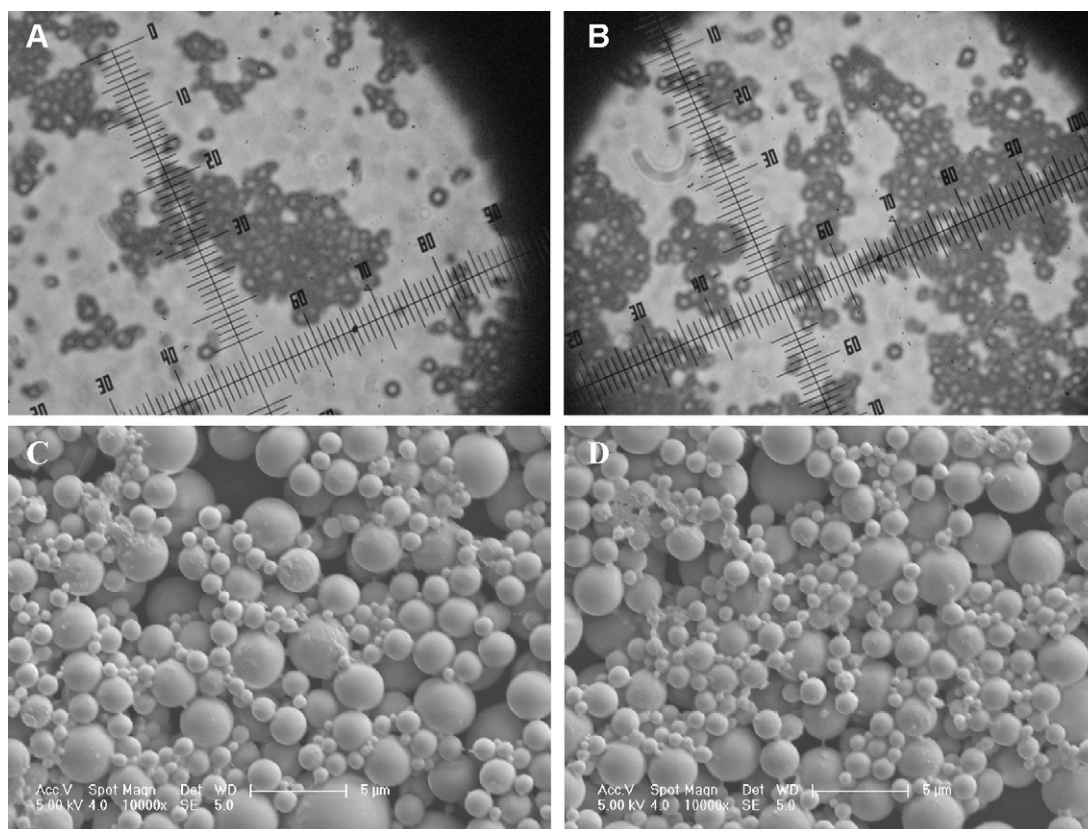


**Fig. 1 – SEM images of BSA-loaded AqueSpheres based PLGA 3A microspheres. (A) Surface morphology and (B) sector of MS.**

was examined using a Particle Size & Shape Analyzer (CIS-100, Ankers mid). Fig. 2 showed that the sizes of microspheres are 40–100 μm, mean size is 67.08 μm. This morphological image is consistent with that of scanning electron microscope (SEM) images. Fig. 1 showed the SEM image of whole AqueSphere-loaded PLGA microspheres (A) and that of fractured PLGA microspheres (B). The microsphere surfaces are smooth and the trace of AqueSpheres in the matrix of PLGA



**Fig. 2 – Distributions of protein-loaded AqueSpheres based PLGA microspheres. Sample: Fig. 1.**



**Fig. 3 – Optical microscopy and SEM images of BSA-loaded AqueSpheres. (A) Optical microscopy images of AqueSpheres before formulation; (B) optical microscopy images of AqueSpheres recovered from microspheres; (C) SEM images of AqueSpheres before formulation; (D) SEM images of AqueSpheres recovered from microspheres. Sample: Fig. 1.**

microspheres was comparable to the size of particles prior to loading into the microspheres (Fig. 1A and B). This result suggests that AqueSpheres were not substantially swollen during the microencapsulation process described above. In order to confirm whether the morphology of AqueSpheres emended in PLGA matrix changed, the AqueSpheres before embedded into and from the PLGA microspheres were taken photo by SEM and optical microscopy. The SEM and microscopy images (Fig. 3) showed that the morphology and sizes of AqueSpheres almost changed.

### 3.3. Aggregates study of formulations

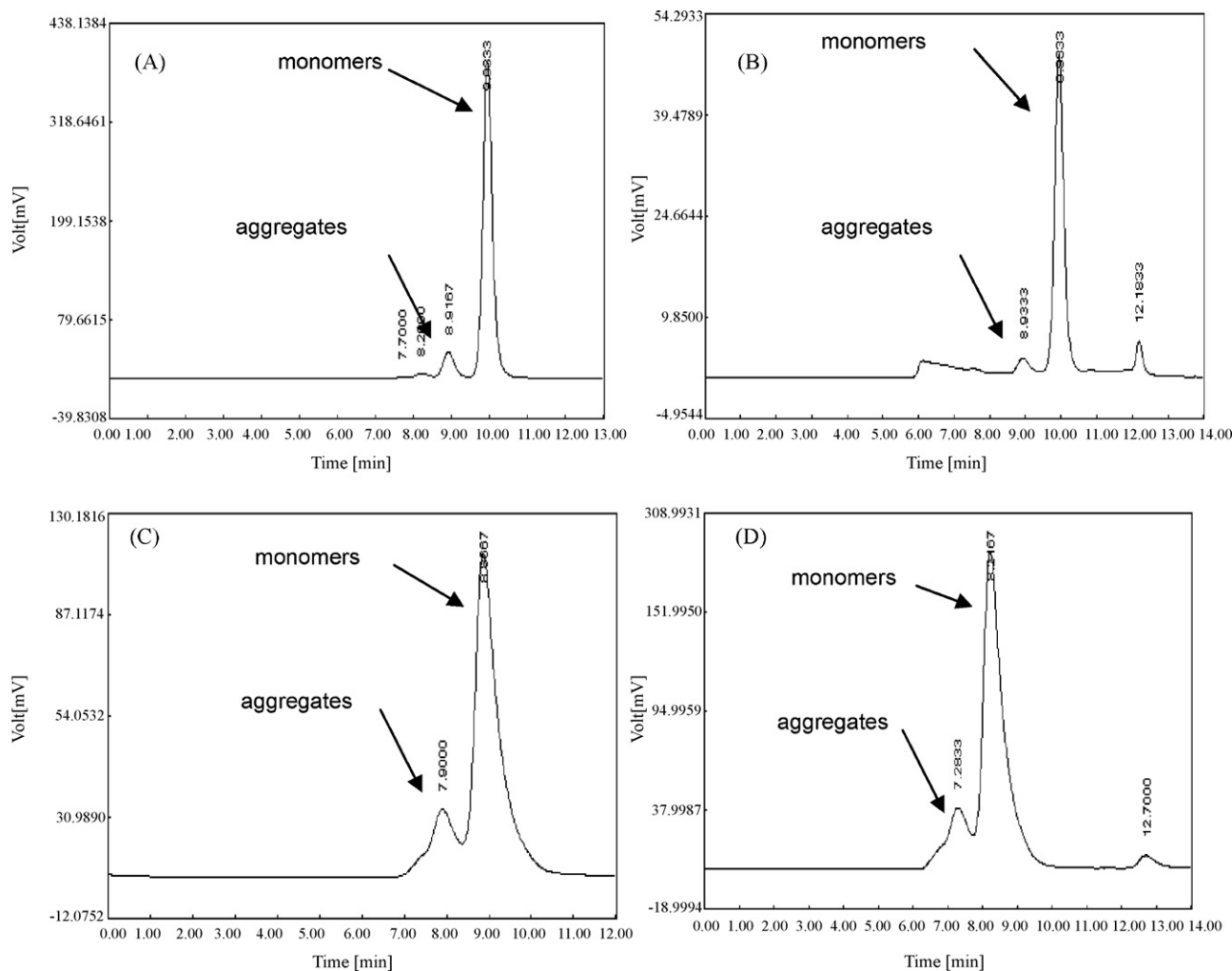
Aggregates formed by protein aggregation are well used indications for protein integrity during microencapsulation process (Cleland and Jones, 1996). Figs. 4 and 5 showed the SEC-HPLC results for aggregation of myoglobin after each microencapsulation step. Fig. 4A and B are the SEC-HPLC charts of original MGB solution, and MGB and BSA recovered from PLGA microspheres. The fractions of dimers and oligomers did not increase during the microencapsulation process as compared with the original sample. Similarly, there was no significant change in the contents of dimers for BSA recovered from PLGA microspheres as compared with original BSA sample (Fig. 4C and D). These results strongly suggest that the formulation process demonstrated in the present study offered a useful solution to microencapsulate proteins with-

out aggregation. As control, we detected aggregates of BSA and MGB from microspheres using multi-emulsion W/O/W. The result showed aggregates of proteins were much more than the present method in the paper. This is possible because oil-aqueous interfaces of multi-emulsion W/O/W exist more than S/O/O/W.

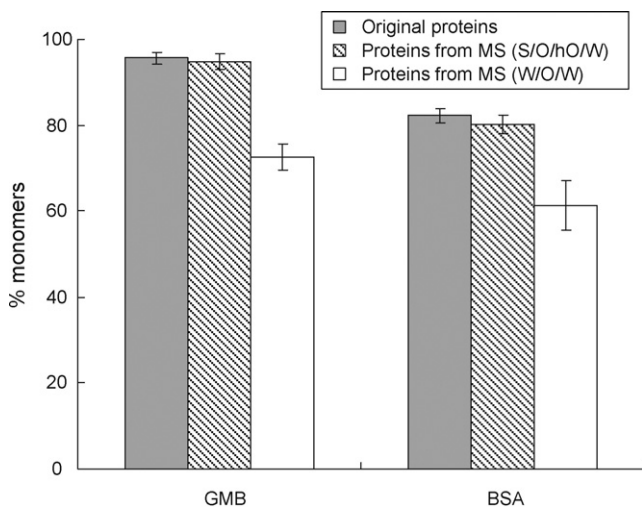
### 3.4. In vitro release profiles of proteins

Polysaccharides can be excellent protein stabilizers against temperature and moisture-induced protein denaturation. As their glassy state maintains, moisture residues even help to preserve original conformation of proteins (Breen et al., 2001; Jin et al., 2008). In the period of sustained release, the fine dextran glassy particles dispersed in the matrix of polymer devices may absorb water and become fully hydrated. However, unlike small molecular stabilizers, dissolved polysaccharides remain inside of the matrix of the polymer used for controlled release and slowly released in several weeks (Morlock et al., 1997). This hydrated polysaccharide phase may function as hydrophilic environment that isolates the protein molecules from each other and from the surrounding polymer matrix, the causes for protein aggregation and protein adsorption.

Fig. 6 showed the profiles of cumulative release of BSA from PLGA microspheres prepared using the present method. The microspheres were made of different molecular weight PLGA,



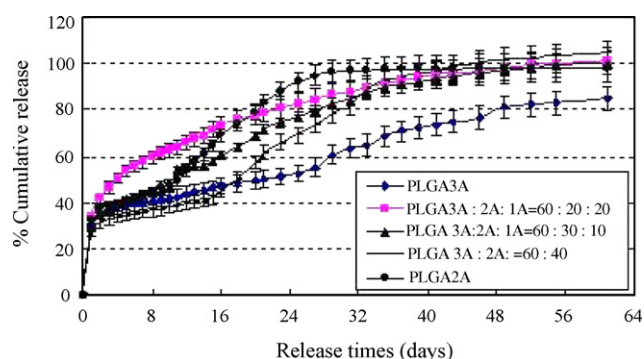
**Fig. 4 – Spectra of recovery proteins from PLGA microspheres by SEC-HPLC. (A) Original myoglobin; (B) myoglobin-loaded AqueSpheres based PLGA 3A microspheres; (C) Original BSA; and (D) BSA-loaded AqueSpheres based PLGA 3A microspheres.**



**Fig. 5 – % Monomers of recovery proteins from PLGA microspheres by SEC-HPLC (BSA or MGB-AqueSpheres based PLGA 3A microspheres, n = 3).**

and contained different amount of protein. Sustained-release profiles of proteins were achieved with a minimal burst of about 25% of the total BSA loadings released at day 1 and above 80% of loadings released up to 60 days (Fig. 6). As a comparison, proteins released from microspheres prepared using conventional W/O/W methods showed significantly severe burst and incomplete release (Aubert-Pouëssel et al., 2004). This result suggests that the polysaccharide dispersed phase play an important role in improving protein release kinetics.

A multi-emulsion W/O/W with oil/aqueous interfaces often results in protein aggregate and loses bioactivity (Morlock et al., 1997). Although a multi-emulsion S/O/W may decrease protein molecule and oil phase directly contact with, still some solidified protein particles may dissolve in aqueous phase and contact with oil/water interface led to a few protein aggregate during preparation producer (Fig. 5). As to a multi-emulsion S/O<sub>1</sub>/O<sub>2</sub>, the method may avoid of oil/water interfaces of the above and decrease protein aggregate, but oil phase-2 (O<sub>2</sub>) often is organic solvent (for example cottonseed oil, silicon oil, etc.) which need removing with a lot of other volatilization organic. These have some danger fac-



**Fig. 6 – Effect of different MW and their mixture of PLGA on *in vitro* release ( $n=3$ ). The ratio is w/w.**

tors including immunity *in vivo*, pollute environment, etc. We reported the method may overcome the disadvantage. As to a multi-emulsion  $S/O_1/O_2/W$ ,  $O_2$  is a hydrophilic oil phase (hO) which is easy to remove with water and cannot dissolve pre-loading proteins into polysaccharide particles. The hydrophilic oil phase is composed of 72.7% (w/w) glycerol, 18.2% (w/w) 1,2-propylene glycol, 9.1% (w/w) water (containing 1% (w/w) PVA and 5% (w/w) sodium chloride). The component may avoid dissolving the protein-loaded polysaccharide particles because the polysaccharide particles can be resistant to temperature and moisture (Breen et al., 2001). As long as its glassy state is maintained, moisture may stabilize proteins dispersed in its matrix (Chang et al., 2005). The hydrophilic oil phase may protect from protein during preparation of microspheres, too. We found that the size of microspheres decrease with increasing component of glycerol and the size of microspheres increase with increasing component of water. These are possible because polarity of water more than glycerol and result in forming more hydrophilic-hydrophobic interfaces with hydrophobic oil phase-1 ( $O_1$ ).

#### 4. Conclusion

We have demonstrated a novel method for preparing sustained-release protein microsphere formulation through multi-emulsion  $S/O/O/W$ . The method may avoid using multi-emulsion  $W/O/W$  with water/oil interface and  $S/O/O$  with hard to remove oil phase and initial burst. The present method may decrease protein aggregation and improve protein complete release. This finding suggests that the present method may be a novel one for preparing sustained-release protein microspheres system.

#### Acknowledgements

This work was partially supported by the program of National Science Foundation of China Committee (30472096), the joined program of National Science Foundation of China and the Hong Kong Research Grant Committee (30418006). The authors thank the Analytical Center of Shanghai Jiaotong University for Technical Support.

#### REFERENCES

- Aubert-Pouëssel, A., Venier-Julienne, M.-C., Clavreul, A., Sergent, M., Jollivet, C., Montero-Menei, C.N., Garcion, E., Bibby, D.C., Menei, P., Benoit, J.-P., 2004. *In vitro* study of GDNF release from biodegradable PLGA microspheres. *J. Control. Rel.* 95, 463–475.
- Breen, E.D., Curley, J.G., Overcashier, D.E., Hsu, C.C., Shire, S.J., 2001. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm. Res.* 18, 1345–1353.
- Carrasquillo, K.G., Staneley, A.M., Aponte Carro, J.C., De Jesús, P., Costantino, H.R., Bosques, C.J., Griebenow, K., 2001. Non-aqueous encapsulation of excipient-stabilized spray-dried BSA into poly(lactide-co-glycolide) microspheres results in release of native protein. *J. Control. Rel.* 76 (3), 199–208.
- Castellanos, I.J., Cruz, G., Crespo, R., Griebenow, K., 2002. Encapsulation-induced aggregation and loss in activity of  $\gamma$ -chymotrypsin and their prevention. *J. Control. Rel.* 81 (3), 307–319.
- Chang, L.L., Shepherd, D., Sun, J., Tang, X.C., Pikal, M.J., 2005. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: implications for the mechanism of protein stabilization in the solid state. *J. Pharm. Sci.* 94 (7), 1445–1455.
- Cheng, Y.-H., Dyer, A.M., Jabbal-Gill, I., Hinchcliffe, M., Nankervis, R., Smith, A., Watts, P., 2005. Intranasal delivery of recombinant human growth hormone (somatropin) in sheep using chitosan-based powder formulations. *Eur. J. Pharm. Sci.* 26 (1), 9–15.
- Cleland, J.L., Jones, A.J.S., 1996. Stable formulations of recombinant human growth hormone and interferon- $\gamma$  for microencapsulation in biodegradable microspheres. *Pharm. Res.* 13, 1464–1475.
- Coombs, A.G.A., Yeh, M.-K., Lavelle, E.C., Davis, S.S., 1998. The control of protein release from poly(DL-lactide co-glycolide) microparticles by variation of the external aqueous phase surfactant in the water-in oil-in water method. *J. Control. Rel.* 52 (3), 311–320.
- Freitas, S., Merkle, H.P., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. *J. Control. Rel.* 102 (2), 313–332.
- Grenha, A., Seijo, B., Remuñán-López, C., 2005. Microencapsulated chitosan nano-particles for lung protein delivery. *Eur. J. Pharm. Sci.* 25 (4–5), 427–437.
- Jorgensen, L., Moeller, E.H., van de Weert, M., Nielsen, H.M., Frokjaer, S., 2006. Preparing and evaluating delivery systems for proteins. *Eur. J. Pharm. Sci.* 29 (3–4), 174–182.
- Jensen, M., Hansen, P.B., Murdan, S., Frokjaer, S., Florence, A.T., 2002. Loading into and electro-stimulated release of peptides and proteins from chondroitin 4-sulphate hydrogels. *Eur. J. Pharm. Sci.* 15 (2), 139–148.
- Jin, T., Zhu, J., Wu, F., Yuan, W., Geng, L., Zhu, H., 2008. Preparing polymer-based sustained-release systems without exposing protein to water-oil or water-air interface. *J. Control. Rel.* 128, 50–59.
- Kim, H.K., Park, T.G., 1999. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. *Biotechnol. Bioeng.* 65, 659–667.
- Kim, J.-H., Bae, Y.H., 2004. Albumin loaded microsphere of amphiphilic poly(ethylene glycol)/poly( $\alpha$ -ester) multiblock copolymer. *Eur. J. Pharm. Sci.* 23 (3), 245–251.

- Kwon, Y.M., Baudys, M., Knutson, K., Kim, S.W., 2001. In situ study of insulin aggregation induced by water–organic solvent interface. *Pharm. Res.* 18, 1754–1759.
- Langer, R., Folkman, J., 1976. Polymers for sustained release of proteins and other macromolecules. *Nature* 263, 793–800.
- Lu, W., Park, T.G., 1995. Protein release from poly (lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J. Pharm. Sci. Tech.* 49 (1), 13–19.
- Morlock, M., Koll, H., Winter, G., Kissel, T., 1997. Microencapsulation of rherythropoietin, using biodegradable poly(D,L-lactide-co-glycolide): protein stability and the effects of stabilizing excipients. *Eur. J. Pharm. Biopharm.* 43, 29–36.
- Morita, T., Sakamura, Y., Horikiri, Y., Suzuki, T., Yoshino, H., 2000. Protein encapsulation into biodegradable microspheres by a novel S/O/W emulsion method using poly(ethylene glycol) as a protein micronization adjuvant. *J. Control. Rel.* 69 (3), 435–444.
- Park, T.G., Lee, H.Y., Nam, Y.S., 1998. A new preparation method for protein loaded poly(D,L-lactic-co-glycolic acid) microspheres and protein release mechanism study. *J. Control. Rel.* 55 (2–3), 181–191.
- Reis, C.P., Ribeiro, A.J., Houg, S., Veiga, F., Neufeld, R.J., 2007. Nanoparticulate delivery system for insulin: design, characterization and in vitro/in vivo bioactivity. *Eur. J. Pharm. Sci.* 30 (5), 392–397.
- 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. Rel.* 88 (2), 229–242.
- Van de Weert, M., Hoechstetter, J., Hennink, W.E., Crommelin, D.J.A., 2000. The effect of a water/organic solvent interface on the structural stability of lysozyme. *J. Control. Rel.* 68, 351–359.
- Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* 185, 129–188.
- Wang, N., Wu, X.S., 1998. A novel approach to stabilization of protein drugs in poly (lactic-co-glycolic acid) microspheres using agarose hydrogel. *Int. J. Pharm.* 166, 1–14.
- Yuan, W., Wu, F., Geng, Y., Xu, S.L., Jin, T., 2007. Preparation of dextran glassy particles through freezing-induced phase separation. *Int. J. Pharm.* 339, 76–83.
- Yuan, W., Geng, Y., Wu, F., Liu, Y., Guo, M., Zhao, H., Jin, T., 2009. Preparation of polysaccharide glassy microparticles with stabilization of proteins. *Int. J. Pharm.* 366, 154–159.