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Preparation of polysaccharide glassy microparticles with stabilization of proteins

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ABSTRACT

This study investigates a method of preparing hazard-resistant protein-loaded polysaccharide glassy microparticles using freezing-induced phase separation method without exposure to water/oil, water/air interface and cross-linking reagents. Model protein (such as bovine serum albumin, myoglobin and β-galactosidase (β-Gal)) was dissolved in water together with dextran and polyethylene glycol (PEG), followed by a freezing process to form a temperature-stabilized aqueous–aqueous emulsion wherein dextran separated out as the dispersed phase with protein partitioned in preferentially. The frozen sample was freeze-dried and washed with dichloromethane (DCM) to remove the PEG continuous phase, after which protein-loaded polysaccharide particles, 1–4 μm in diameter, were harvested. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) patterns showed that the particles were in glassy state. These glassy polysaccharide microparticles can well protect the delicate structure of proteins and preserve their bioactivities under deleterious environment interacting with organic solvents, vortex and centrifugation processes that often involve during the formulation processes leading to polymer-based sustained-release systems. Therefore, this freezing-induced phase separation method is a mild and effective way to encapsulate protein into hazard-resistant polysaccharide glassy particles, which ensure its stability in subsequent formulating processes that leads to polymer-based sustained-release system.

1. Introduction

Most protein drugs need high frequency of administration due to their short *in vivo* half- life (Frokjaer and Otzen, 2005; Okada et al., 1994; Putney and Burke, 1998). Effective sustained-release systems of these drugs are required to meet the needs of decreasing adverse side effects, enhancing patient compliance, as well as reducing dosing frequency and avoiding unstable serum level. However, due to their sophisticated and delicate structure, protein drugs are highly susceptible of losing activity during polymer-based sustained-release formulation process and in long-term storage (Cleland et al., 1997; Frokjaer and Otzen, 2005; George and Abraham, 2006; Johnson et al., 1996; Manning et al., 1989; Putney and Burke, 1998). An approach to address this stability problem is to preload proteins into hazard-resistant protein-loaded particles before interacting with harsh conditions such as heat, organic solvent, oil/water interface or shear force that often involve during the formulation processes leading to polymer-based sustained-release systems.

Aqueous two-phase system is a compelling system to formulate protein-loaded polysaccharide particles for its mild conditions that are free of water-oil interface in the preparation process. However, this is not an easy task. Hennink et al. prepared protein-loaded dextran particles through aqueous two-phase systems using cross-linking agents which may react with chemical active groups of protein leading to its denaturation (Hennink and Franssen, 1998). Millqvist-Fureby et al. also prepared protein-loaded dextran particles through spray-drying aqueous two-phase systems, which caused protein's instability as well (Elversson and Millqvist-Fureby, 2005; Millqvist-Fureby et al., 2000). We reported that directly freeze-drying Zn^{2+}-protein suspension in PEG solution could obtain Zn^{2+}-protein microparticles under mild condition (Yuan et al., 2007a). However, this method is only applicable to limited proteins such as growth hormone, which forms a stable complex with zinc ions at nature state (Cunningham et al., 1991; Tracy, 1998).

To address this issue, we explored an aqueous two-phase system through freezing-induced phase separation method to prepare dextran glassy particles under a condition without water-oil, water-air interfacial tension and cross-linking reagents (Jin et al., 2008; Yuan et al., 2007b) and applied this system to model proteins bovine serum albumin, myoglobin and <beta>-galactosidase in order to evaluate the hazard-resistant ability of these polysaccharide particles. The protein-loaded polysaccharide glassy microparticles

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prepared using this method are spherical in shape with smooth surface and a narrow size distribution. Furthermore, they could endure the hazardous environment such as organic solvents, intense shear force and high temperature, and therefore protect the nature state of protein molecule and preserve its bioactivities. The particles may be directly used as inhaled dosage for their appropriate particle size, or further encapsulated into PLGA or other polymers to fabricate sustained-release system.

2. Materials and methods

2.1. Materials

Polyethylene glycol with various molecular weights of 2000, 4000, 6000 and 8000 Da (indicated as PEG 2000, PEG 4000, PEG 6000 and PEG 8000 hereafter) were obtained from Chinese Medicine Group Chemical Reagent Corporation (China). Dextran with molecular weight of 76,000–64,000 Da (indicated as dextran 70,000 or dextran hereafter), myoglobin (MGB), and β-galactosidase (β-Gal) were purchased from sigma (USA). Bovine serum albumin (BSA) was purchased from Siji Company (China).

2.2. Preparation of protein-loaded polysaccharide microparticles

A co-solution (3 ml) of dextran, protein and PEG with various molecular weight and PEG/dextran ratio was mixed by vortex for 0.5 min and then frozen in a refrigerator of −20 °C overnight. The frozen samples were lyophilized using a Christ ALPHA 1–2 laboratory freeze-dryer operating at a pressure of 5.25×10^{-3} Pa for 24 h. The lyophilized powders were suspended in 5 ml of dichloromethane (DCM), followed by centrifugation at 12,000 rpm for 5 min on an Anker TGL-16C centrifuge to remove the PEG continuous phase. The washing-centrifugation procedure was repeated three times, and the microparticles were evaporated under 1.33 Pa for 24 h using vacuum dryer (Fuma DZF-3, Shanghai Fuma Co. Ltd., China) to get rid of solvent residues. The obtained microparticles contain less than 0.5% (w/w) PEG after the washing process (Zaslavsky, 1995).

2.3. Scanning electron microscopy (SEM) images of protein-loaded polysaccharide particles

Scanning electron microscopy of protein-loaded polysaccharide microparticles was taken using FEI SIRION 2000 SEM system. All the samples were loaded on double-sided tape that was attached on a metal stub and sprayed with gold vapor for 10 min under argon atmosphere. The images were recorded at 5 kV sputtering energy under high vacuum.

2.4. X-ray diffraction (XRD) analysis of protein-loaded polysaccharide particles

X-ray diffraction patterns (XRD) of the samples were recorded using a Rigaku D/MAX2000 XRD system equipped with Cu-Kα radiation source (40 kV, 20 mA). The samples were loaded on the quartz samples-holder and scanned from 5° to 70° at a rate of 6°/min. As controls, dextran powders and PEG8000 powders were measured under the identical conditions.

2.5. Differential scanning calorimetry (DSC) analysis of protein-loaded polysaccharide particles

The physical state of solid samples was measured by differential scanning calorimetry (DSC) (a TA.CO-Q10 system equipped with a temperature-programmed heater capable of heating samples in a nitrogen stream at flow rate of 50 ml/min). The samples were loaded on the aluminum samples-holder and heated from 25 to 300 °C at a rate of 10 °C/min. As controls, dextran powders and PEG8000 powders were measured under the identical conditions.

2.6. Loading efficiency, encapsulation efficiency and recovery efficiency of protein-loaded polysaccharide particles

Protein-loaded polysaccharide microparticles (5 mg) were dissolved in 1 ml pH7.4 PBS. The concentration of protein was determined by MicroBCA kit (Pierce, USA). Loading efficiency was calculated using the following equation:

$$\text{Loading efficiency}\ (\%) = \frac{P}{M} \times 100 \quad (1)$$

where P is the actual total weight of protein encapsulated into polysaccharide microparticles and M is the actual total weight of harvested protein-loaded polysaccharide microparticles. In addition, the encapsulation efficiency was determined using the following equation:

$$\text{Encapsulation efficiency}\ (\%) = \frac{P}{P_t} \times 100 \quad (2)$$

where P_t is the theoretical amount of protein (obtained from feeding condition) encapsulated into polysaccharide microparticles.

$$\text{Recovery efficiency}\ (\%) = \frac{M}{M_t} \times 100 \quad (3)$$

where M_t is the theoretical weight of protein-loaded polysaccharide microparticles (obtained from feeding condition) including polysaccharide and protein.

2.7. Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) assay was carried out using a Younglin HPLC system equipped with a TSK G2000SW$_{XL}$ size-exclusion column (Japan). The mobile phase was composed of 0.15 M sodium chloride and 50 mM sodium phosphate (PBS, pH 7.25). The chromatographic charts were recorded at 214 nm. The dissolved protein sample was filtered with a 0.22 μm film and then injected into the HPLC system. The flow rate of mobile phase was 1.0 ml/min. The amount of monomer or aggregate protein was calculated using the Shimazu software, Chromato-Solution-Light.

2.8. Hazard-resistant ability of protein-loaded polysaccharide microparticles under deleterious environment

2.8.1. Organic solvents

Protein-loaded polysaccharide microparticles (5 mg) were suspended in 1 ml of dichloromethane, ethyl acetate, and acetonitrile for 4 h, respectively. The organic solvent (dichloromethane, ethyl acetate, or acetonitrile) was removed by evaporating under 1.33 Pa for over 24 h using vacuum dryer (Fuma DZF-3, Shanghai Fuma Co. Ltd., China). The obtained protein-loaded polysaccharide microparticles were dissolved in PBS (pH7.4) and the amount of monomer and aggregate proteins were quantified as described in Section 2.7.

2.8.2. Vortex and centrifugation processes

Protein-loaded polysaccharide microparticles (5 mg) suspended in 1 ml of dichloromethane, ethyl acetate, or acetonitrile were vortexed or centrifuged at 12,000 rpm for 5 min. And the process

was repeated five times. The organic solvents were removed as Section 2.8.1. Then the aggregates content of protein recovered from protein-loaded polysaccharide microparticles was determined according to Section 2.7.

2.8.3. Temperature

Temperature is often an effect factor of protein stability and protein store temperature, room temperature and *in vivo* normal temperature are often 4, 25 or 37 °C respectively, so we test protein stability from protein-loaded polysaccharide microparticles stored in these temperatures. Protein-loaded polysaccharide microparticles (5 mg) were placed in a seal vial (5 ml). The seal vials were placed in 4, 25 or 37 °C for 24 h, respectively. The aggregates content of reconstituted protein from microparticles was measured as described in Section 2.7.

2.8.4. Processes involved in freezing-induced phase separation method

The activity of β-Gal entrapped into lyophilized powders before removing PEG or recovered from β-Gal-loaded dextran particles (containing 1% trehalose) to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was determined as describe in reference (Volkin and Klibanov, 1989). Briefly, 0.1 ml of a solution of the protein, original or reconstituted, after each preparation step was mixed with the reaction system composed of 2.6 ml of PBS buffer (pH 7.3), 0.1 ml of 30 mM $MgCl_2$ solution, 0.1 ml of 3.36 M 2-mercaptoethanol solution and 0.1 ml of 68 mM ONPG. The mixed solution was incubated at 37 °C for 5 min, then immediately cooled to 0 °C. β-Gal activity was determined by detecting absorbance of the reaction product of ONPG at 420 nm.

3. Results

3.1. Morphology of protein-loaded polysaccharide microparticles

Scanning electron microscope (SEM) images of protein-loaded polysaccharide microparticles are shown in Fig. 1. BSA-loaded dextran particles are spherical shape with smooth surface (Fig. 1A). The diameter is less than 4 μm (Fig. 1A), similar to that of blank dextran microparticles (Fig. 1D). MGB-loaded dextran particles (Fig. 1B) and β-Gal-loaded dextran particles (Fig. 1C) also had smooth surface and uniform diameter (about 2 μm). These sizes meet the criterion for formulating composite sustained-release polymer microspheres without causing severe burst release in that the diameter of the inner particles should be less than 1/20 of that of the microspheres self (Cleland and Jones, 1996).

3.2. X-ray diffraction (XRD) and differential scanning colarimetry (DSC) patterns of protein-loaded polysaccharide microparticles

Fig. 2 summarized the X-ray diffraction patterns of the BSA-loaded dextran microparticles, BSA, dextran, PEG8000 and blank dextran microparticles. PEG 8000 powder showed an XRD pattern with sharp diffraction peaks at 2θ of 18° and 24°, suggesting its crystalline state. The dextran powder showed a broad peak centered at 2θ of 18°, suggesting some level of crystalline. BSA showed an XRD pattern with small diffraction peaks at 2θ of 8° and 21°, suggesting some yet little level of crystalline state. The blank dextran particles prepared using freezing-induced phase separation method showed no sharp diffraction peak. XRD pattern of the BSA-loaded dextran particles prepared using freezing-induced phase separation

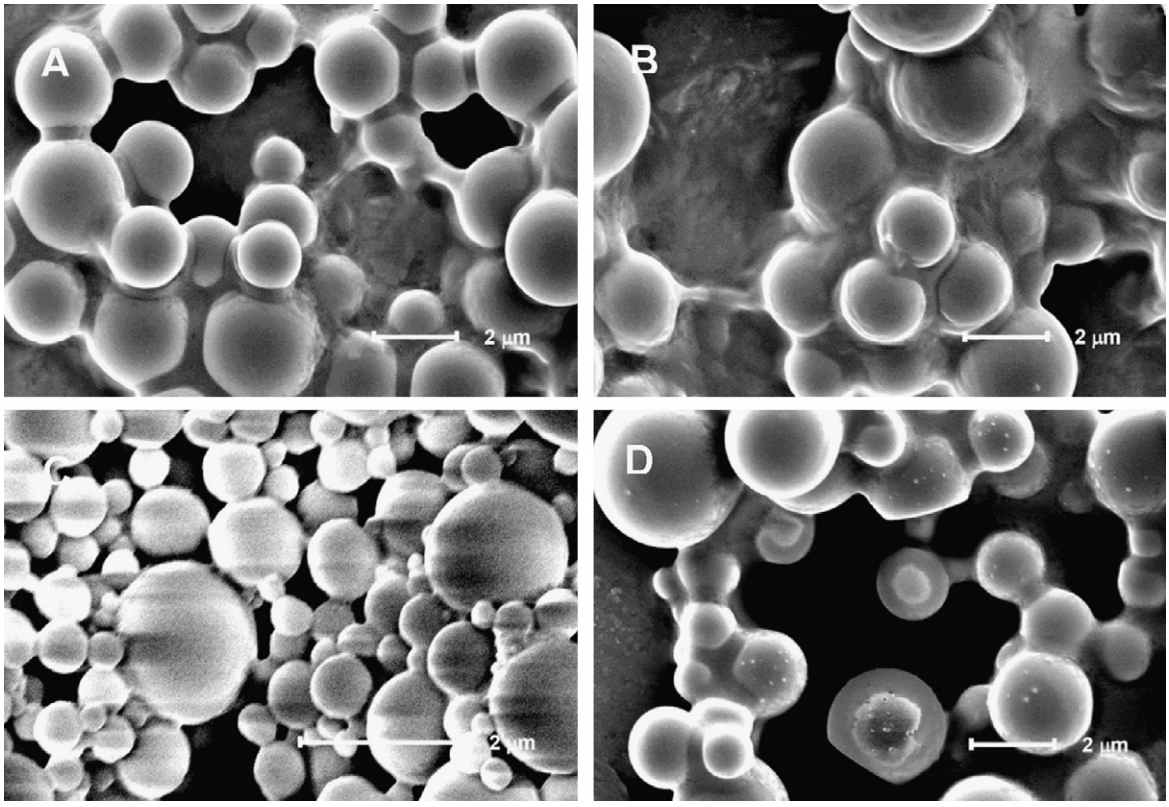

Fig. 1. Scanning electron microscope (SEM) images of protein-loaded dextran particles prepared using freezing-induced phase separation method. (A) BSA-loaded dextran particles, original BSA (5%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w); (B) MGB-loaded dextran particles, MGB (1%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w); (C) β-Gal-loaded dextran particles, β-Gal (0.1%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w); (D) blank dextran particles prepared using the same freezing-induced phase separation method, dextran70,000 (5%, w/w): PEG8000 (5%, w/w) = 1:10 (w/w). Magnification: (A) 20,000×; (B) 20,000×; (C) 40,000×; (D) 20,000×.

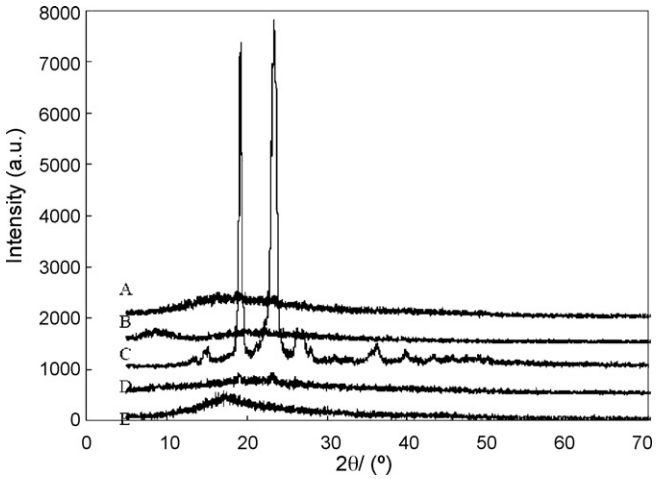

Fig. 2. XRD patterns of protein-loaded dextran particles prepared using freezing-induced phase separation method. (A) BSA-loaded dextran particles, original BSA (5%, w/w): dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w); (B) BSA powders; (C) commercial PEG8000 powders; (D) blank dextran particles prepared using the same freezing-induced phase separation method, dextran70,000 (5%, w/w): PEG8000 (5%, w/w) = 1:10 (w/w); (E) commercial dextran powders.

method consisted of a flat and broad peak across 10–30°, suggesting that the protein-loaded dextran particles are amorphous in physical state.

The differential scanning colarimetry patterns suggested the glassy state of BSA-loaded dextran microparticles. As shown in Fig. 3, PEG 8000 powder generated a sharp endothermic peak at 62.6° (melting point). For BSA, there was an endothermic peak at about 300°. For BSA-loaded polysaccharide microparticles, there was no endothermic peak but a bear bended region on the DSC curve at the temperature from 224.2° to 236.5°, which suggested the high glassy transition temperature of BSA-loaded dextran microparticles. A polysaccharide glassy matrix reduces the mobility of encapsulated proteins and is more resistant to temperature and moisture (Breen et al., 2001; Yoshioka et al., 1997).

3.3. Loading efficiency, encapsulation efficiency and recovery efficiency

Figs. 4 and 5 showed the effects of molecular weight of PEG (Fig. 4) and dextran/PEG ratio (Fig. 5) on loading efficiency (LE),

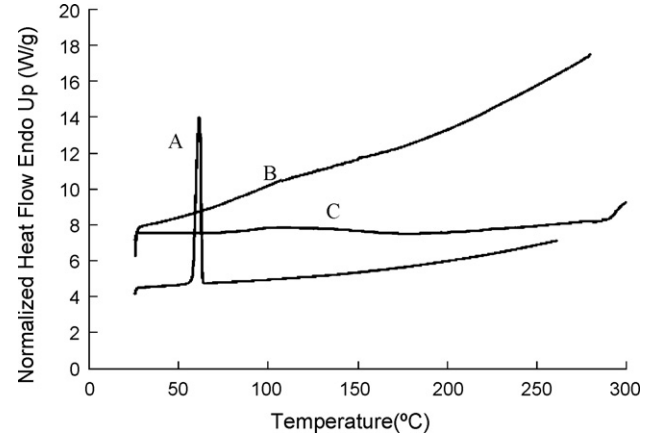

Fig. 3. DSC patterns of protein-loaded dextran particles prepared using freezing-induced phase separation method. (A) commercial PEG8000 powders; (B) BSA-loaded dextran particles, original BSA (5%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w); (C) BSA powders.

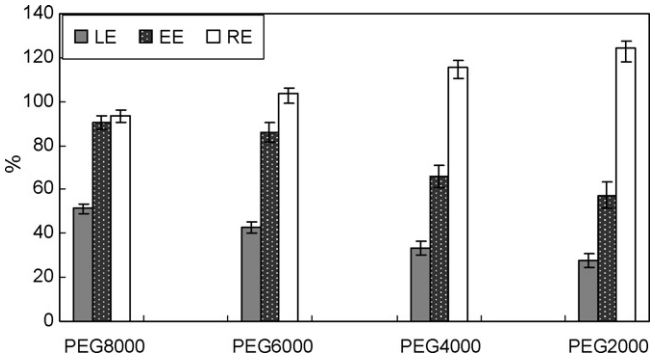

Fig. 4. Effect of different molecular weight of PEG on loading efficiency (LE), encapsulation efficiency (EE) and recovery effeciency (RE) of BSA-loaded dextran particles prepared using freezing-induced phase separation method ($n = 3$). BSA (5%, w/w):dextran (5%, w/w):PEG (5%, w/w) = 1:1:10 (w/w/w).

encapsulation efficiency (EE) and recovery efficiency (RE). Fig. 4 showed that loading and encapsulation efficiency increased as PEG molecular weight increased from 2000 to 8000 Da, but recovery efficiency decreased at the same time. Fig. 5 displayed that the loading efficiency, encapsulation efficiency and recovery efficiency of the BSA-loaded dextran particles increased as PEG/dextran ratio increased from 5 to 10, while they decreased as PEG/dextran ratio increased from 10 to 40. Therefore, PEG8000 is preferred in this system in terms of loading efficiency and encapsulation efficiency, and the appropriate PEG8000/dextran ratio of this protein-loaded system is 10 in terms of loading efficiency, encapsulation efficiency and recovery efficiency.

3.4. Hazard-resistant ability of protein-loaded polysaccharide particles against deleterious environment

Protein drugs easily aggregate and lose activity during polymer-based sustained-release formulating process, which involves deleterious environment such as organic solvents, intense shear force and high temperature. Table 1 showed the monomer content of proteins reconstituted from protein-loaded polysaccharide microparticles after suspension in different organic solvents including dichloromethane, ethyl acetate and acetonitrile. Monomer content of original myoglobin was 96.64%, and that of myoglobin reconstituted from myoglobin-loaded polysaccharide microparticles after suspended in various organic solvents decreased slightly (still over 96%, Table 1), and but non-encapsulated

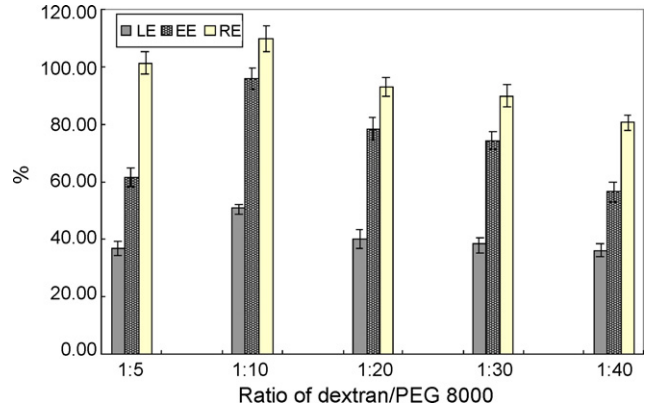

Fig. 5. Effect of dextran/PEG8000 ratio on loading efficiency (LE), encapsulation efficiency (EE) and recovery effeciency (RE) of BSA-loaded dextran particles prepared using freezing-induced phase separation method ($n = 3$). The ratio of BSA (5%, w/w) to dextran (5%, w/w) was 1:1 (w/w).

Table 1
Resistant ability of protein-loaded dextran glassy particles against deleterious environment ($n = 3$)

Deleterious environment[a]	%Monomers of MGB[b]	% Monomers of BSA[c]	% Monomers of MGB[d]	% Monomers of BSA[e]
Protein solution	96.64 ± 1.00	82.21 ± 1.03	96.74 ± 1.01	82.31 ± 1.23
Suspended in dichloromethane for 4 h	96.04 ± 1.45	81.91 ± 1.51	89.32 ± 1.65	76.53 ± 1.79
Suspended in ethyl acetate for 4 h	96.01 ± 1.42	81.94 ± 1.44	87.61 ± 1.77	74.56 ± 1.61
Suspended in acetonitrile for 4 h	96.05 ± 1.52	81.98 ± 1.45	86.55 ± 1.59	74.98 ± 1.85
Protein solution	96.60 ± 1.01	82.21 ± 1.07	96.65 ± 1.21	82.32 ± 1.07
Vortexed in dichloromethane for 5 min (repeated five times)	96.54 ± 1.3	82.01 ± 1.37	84.44 ± 1.38	73.01 ± 1.87
Vortexed in ethyl acetate for 5 min (repeated five times)	96.08 ± 1.30	81.94 ± 1.37	81.68 ± 1.50	72.97 ± 1.67
Vortexed in acetonitrile for 5 min (repeated five times)	96.07 ± 1.80	81.98 ± 1.65	81.27 ± 1.79	71.65 ± 1.91
Protein solution	96.64 ± 1.03	82.21 ± 1.04	96.59 ± 1.53	81.91 ± 1.54
Centrifuged in dichloromethane at 12,000 rpm for 5 min (repeated five times)	96.34 ± 1.07	81.01 ± 1.53	82.39 ± 1.17	71.61 ± 1.93
Centrifuged in ethyl acetate at 12,000 rpm for 5 min (repeated five times)	96.04 ± 1.01	80.34 ± 2.36	80.54 ± 1.81	70.39 ± 1.76
Centrifuged in acetonitrile at 12,000 rpm for 5 min (repeated five times)	96.05 ± 1.21	81.98 ± 1.08	79.25 ± 1.71	70.18 ± 1.78
Protein solution (4 °C)	96.64 ± 1.04	82.21 ± 1.06	96.64 ± 1.04	82.21 ± 1.06
Placed in seal vial at 4 °C for 24 h	96.64 ± 1.12	82.21 ± 1.10	96.64 ± 1.04	82.21 ± 1.06
Placed in seal vial at 25 °C for 24 h	96.54 ± 1.03	81.99 ± 1.22	96.50 ± 1.23	81.89 ± 1.22
Placed in seal vial at 37 °C for 24 h	96.24 ± 1.21	80.94 ± 1.26	95.04 ± 1.01	79.12 ± 1.21

[a] Protein-loaded dextran particles were treated under deleterious environment as described in Section 2.8.
[b] MGB recovered from MGB-loaded dextran particles prepared using freezing-induced phase separation method, MGB (1%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w).
[c] BSA recovered from BSA-loaded dextran particles prepared using freezing-induced phase separation method, original BSA (5%, w/w):dextran70,000 (5%, w/w):PEG8000 (5%, w/w) = 1:1:10 (w/w/w).
[d] Non-encapsulated MGB.
[e] Non-encapsulated BSA.

myoglobin decreased much (less than 90% Table 1), suggesting organic solvent-resistant property of protein-loaded polysaccharide microparticles. Monomer content of original BSA was 82.21%, while that of BSA reconstituted from BSA-loaded polysaccharide microparticles after suspended in various organic solvents also decreased slightly (over 81%, Table 1) and non-encapsulated myoglobin decreased much (less than 77%, Table 1).

Table 1 showed the vortex effect on protein-loaded dextran particles. Monomer content of original myoglobin and BSA was 96.60% and 82.21%, respectively, and the monomer content of myoglobin reconstituted from myoglobin-loaded and BSA recovered from BSA-loaded polysaccharide microparticles after vortex in various organic solvents for 5 min (repeated 5 times) decreased slightly (still over 96% and near 82% respectively, Table 1), and non-encapsulated proteins decreased much (less than 84% and 74%, Table 1), suggesting that the polysaccharide microparticles could endure intense shear force.

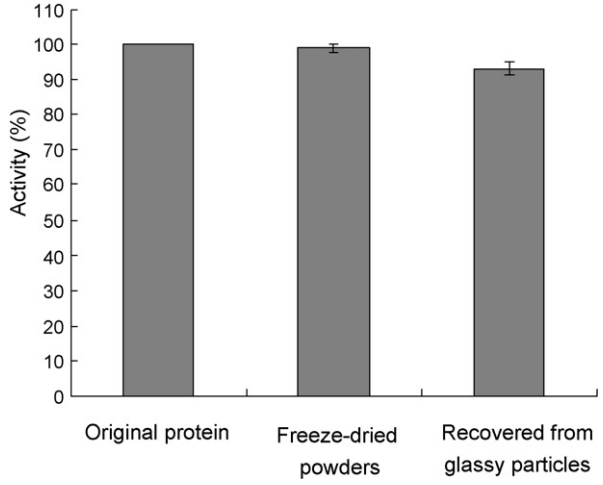

Fig. 6. Activity of β-Gal after freeze-dry process (before washing by DCM) and reconstituted from β-Gal-loaded polysaccharide microparticles prepared using freezing-induced phase separation method ($n = 3$). β-Gal (0.1%, w/w): dextran70,000 (5%, w/w): PEG8000 (5%, w/w) = 1:1:10 (w/w/w).

Table 1 showed the resistant ability of protein-loaded dextran particles against centrifugation process. Monomer content of original myoglobin and BSA was 96.64% and 82.21% respectively, and the monomer content of myoglobin reconstituted from myoglobin-loaded and BSA-loaded polysaccharide microparticles after centrifugation at 12,000 rpm for 5 min (repeated five times) decrease slightly (near 96% and over 80% respectively, Table 1), and non-encapsulated proteins decreased much (less than 83% and 72%, Table 1), suggesting that the polysaccharide microparticles could endure intense centrifugation force.

Myoglobin-loaded dextran particles and BSA-loaded dextran particles were placed in different temperature for 24 h. The monomer content of myoglobin or BSA reconstituted from particles above mentioned decreased slightly from 96.64% to 96.24% (myoglobin) or 82.21% to 80.94% (BSA) as the temperature increased from 4 to 37 °C (Table 1). The results indicated that protein-loaded polysaccharide microparticles could protect proteins from 4 to 37 °C.

3.5. Activity of β-Gal reconstituted from β-Gal-loaded polysaccharide microparticles

The β-Gal-loaded lyophilized powders before removing PEG prepared as Section 2.2 (sample 1) and β-Gal recovered from β-Gal-loaded polysaccharide microparticles (sample 2) were dissolved in PBS respectively and the activities of these samples to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside were detected. The activity of sample 1 is 98.56% if we supposed that the original β-Gal activity to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside was 100%, while activity of β-Gal recovered from β-Gal-loaded dextran particles (sample 2) was 93.34% (Fig. 6).

4. Discussion

The so-called aqueous two-phase system (polysaccharide and PEG) well used for protein purification has offered convenient mechanism to prepare polysaccharide particles in aqueous environment (Zaslavsky, 1995). Two water-soluble polymers dissolved in water may form two immiscible aqueous phases with increase

in their molecular weight, concentration or with decrease in temperature (Zaslavsky, 1995). Due to designed volume ratio and the less hydrodynamic nature volume (than PEG), the polysaccharide will separate out as the dispersed phase and forms particles upon successive lyophilization. The two separated aqueous phases can be two block phases or in an 'emulsion' form if the dispersed phase can be stabilized from fusion to each other. In our previous work, creating a Zeta potential around each dispersed phase is effectively ways to stabilize dispersed polysaccharide phase in PEG continuous phase (Jin et al., 2008). Since reduced temperature is also a factor to induce phase separation of two aqueous polymers (Zaslavsky, 1995), a particle-forming process can be designed in such way that polymer phase separation and dispersed phase stabilization occur at the same temperature (Yuan et al., 2007b). We applied this system to model protein and found that protein could be effectively encapsulated into polysaccharide particles under appropriate conditions. The encapsulation efficiency of BSA-loaded dextran particles (dextran: PEG8000 = 1:10) was higher than 95% (Fig. 5).

BSA-loaded dextran particles are spherical shape with smooth surface, and the diameter is less than 4 μm, similar to that of MGB-loaded dextran particles, β-Gal-loaded dextran particles and blank dextran particles (Fig. 1). The large specific surface area (meant high surface energy) of the fine polysaccharide droplets drove the dextran particles to be spherical. And different protein can be well encapsulated into these small and uniform particles which are not only suitable for inhaled dosage for their appropriate diameter, but also for microencapsulation into polymer-based sustained-release systems without causing severe initial burst. Diameter of the particles to be encapsulated into polymer-based microspheres without causing severe burst should be less than 1/20 of the diameter of the composite microsphere itself (Cleland and Jones, 1996). For the diameter of common microspheres was less than 100 μm, the size of particles to be encapsulated should be less than 5 μm.

XRD and DSC patterns of BSA-loaded dextran particles showed that these protein-loaded polysaccharide microparticles are not crystal but glassy state (Figs. 2 and 3). The glassy state of the protein-loaded polysaccharide particles is the key point to protect the nature state of protein molecule because a polysaccharide glassy matrix can effectively reduce the mobility of encapsulated proteins and is more resistant to organic solvents, moisture and elevated temperature (Breen et al., 2001; Robert Liu et al., 2004; Yoshioka et al., 1997). Size-exclusion high-performance liquid chromatography (SEC-HPLC) results showed that the monomer content of BSA or MGB encapsulated into polysaccharide particles under those harsh conditions such as interacting with organic solvents, vortex and centrifugation processes only slightly reduced (Table 1). And the activity of β-galactosidase entrapped into polysaccharide particles to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside also slightly reduced (Fig. 6). These glassy polysaccharide microparticles can well protect the delicate structure of proteins and preserve their bioactivities under deleterious environment. The polysaccharide glassy particles can effectively protect BSA (PI = 4.7), MGB (PI = 7.0) and β-galactosidase (having delicate quaternary structure consisted of four subunits) against deleterious environments, suggesting its wide application in developing hazard-resistant protein-loaded polysaccharide glassy microparticles.

Acknowledgements

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