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Preparation of dextran glassy particles through freezing-induced phase separation

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Abstract

This report demonstrates a novel method to prepare fine polysaccharide glassy particles of uniform sizes under a condition without water/oil and water/air interfacial tension and cross-linking reagents. When a co-solution of dextran and polyethylene glycol (PEG) was frozen gradually, phase separation occurred during which dextran formed the dispersed phase and PEG remained in the continuous part. Fine dextran glassy particles were harvested after lyophilizing this frozen sample, followed by re-dissolving the continuous phase (PEG) in dichloromethane or acetonitrile. Desired mean particle diameter can be achieved within the range between 200 nm and 10 μm by selecting molecular weights of PEG and dextran, concentration of the co-solution, and PEG/dextran ratio. Increase in molecular weights, concentration or PEG/dextran ratio resulted in increase in particle sizes, and the vice versa. The dextran particles prepared as above showed smooth surface under an electron microscope, a phase transition temperature on thermogram, and sank in carbon tetrachloride (density = 1.592 g/ml), indicating that the particle matrix is dense and glassy. This particulate system and its forming process may have wide applications in formulating variety of pharmaceutical dosage forms and medical devices containing delicate biotech therapeutics.

Keywords: PEG; Dextran glassy particles; Uniform solution; Phase separation

1. Introduction

Formulating delicate biotech therapeutics, such as proteins into advanced pharmaceutical dosage forms (such as sustained-release depots) and therapeutic devices (such as cardiovascular stents) encounters series of difficulties not seen for small molecular chemical drugs and peptides due to the conformation instability of this type of macromolecules (Andya et al., 1999; Costantino et al., 2002; Frokjaer and Otzen, 2005; Fu et al., 2000). An approach to address this stability problem is to convert proteins to solid particles prior to subsequent formulation steps through freeze-drying with sugars or hydrophilic polymers, complexation with bivalent metal ions, and salting out with inorganic salts or PEG (Cleland and Jones, 1996; Johnson et al., 1996; Morita et al., 2000; Zale et al., 1998; Sanchez et al., 1999). Among these methods, loading proteins into polysaccharide fine particles is of especially interesting in that polysaccharide matrix protects proteins in a chemically compatible environment with resistance to moisture and elevated temperature (Al-Ruqaie et al., 1997; Breen et al., 2001; Yoshioka et al., 1997). Unlike small molecular protein stabilizers that are highly soluble and diffuse rapidly, polysaccharides remain inside of sustained-release depots for prolonged time thus may protect proteins during the entire process of in vivo action (Morlock et al., 1997).

However, to load proteins into fine polysaccharide particles is not an easy task. Freeze-drying a protein-polysaccharide solution results in formation of large proteins particles (diameter > 10 μm) of irregular shape (Cleland and Jones, 1996). Emulsifying such a solution into an oily phase or spraying the solution in air will expose proteins to a water/oil or a water/air interface, both are factors known to cause protein denaturation (Griebenow and Klibanov, 1996; Johnson et al., 1996; Sah, 1999; Wang et al., 1999; Yu et al., 2002). In addition, polysaccharide particles formed through spray drying are often loose and porous, and are less protective to proteins in subsequent formulation for which usage of organic solution is indispensable steps. To avoid hydrophilic/hydrophobic interface, Hennink et

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al. loaded proteins into a so-called aqueous two-phase system containing acrylate-conjugated dextran and PEG, followed by intra-particulate cross-linking (using a cross-linking reagent) to solidify the dispersed polysaccharide phase (Chung et al., 2005; Franssen et al., 1999; Hennink et al., 1997; Stenekes et al., 1998, 1999, 2000; Van Tomme et al., 2005). However, proteins of abundant functional groups may contact with reactive cross-linking reagents. A method to formulate proteins into fine solid polysaccharide particles without stability hazards is still an issue to be addressed.

It has been reported for a long time that polysaccharides and polyethylene glycol (EPG) may form an aqueous two-phase state when both are dissolved in water (Zaslavsky, 1995). Phase separation of the aqueous co-solution containing polysaccharide and PEG is a function of temperature, concentration, and molecular weight of these polymers. Heller et al. reported that phase separation occurred when a co-solution of dextran and PEG was frozen (Heller et al., 1997, 1999). These authors, however, regarded freezing-induced phase separation of dextran and PEG as a factor for protein denaturing based on their experiments on hemoglobin (a protein which needs high concentration of 2,3-diphosphoglyceric acid to complex its four subunits as inside of red cells (Shen et al., 1991)), thus they did not utilize this mechanism to prepare particles but tried to avoid it. We found that phase separation of dextran and PEG co-solution was not harmful for proteins other than hemoglobin (Jin et al., 2003); rather it may offer a convenient way to load proteins into fine solid polysaccharide particles without hydrophilic–hydrophobic interfacial tension.

In the present study, the effects of a number of formulation parameters for the freeze-induced particle-forming process, such as concentration and molecular weights of PEG and dextran, and PEG/dextran ratio on particle sizes and morphology were discussed based on a series of physical characterizations.

2. Materials and methods

2.1. Materials

Polyethylene glycol with various molecular weights (indicated as PEG 1000, PEG 2000, PEG 4000, PEG 6000 and PEG 8000 hereafter), sodium dihydrogen phosphate, potassium dibasic anhydrous, potassium chloride, and trehalose were purchased from Chinese Medicine Group Chemical Reagent Corporation. Dextran with molecular weights of 76,000–64,000 and 500,000 (indicated as dextran 70,000 and dextran 500,000 hereafter) were obtained from Sigma.

2.2. Preparation of dextran glassy particles

A co-solution (2 ml) of dextran and PEG of various concentration, molecular weight and PEG/dextran ratio were mixed by vortex for 5 min and then frozen in a freezer of −20 °C for over night. The frozen samples were lyophilized using a Christ ALPHA 1-2 laboratory freeze-dryer operating at a pressure of 0.05 mbar for 24 h. For a laboratory freeze-dryer, since the cooling sample is achieved by the sample's own sublimation heat, the sample vial must be placed without contact with the wall of the evaporation chamber to avoid sample melting by heat transfer. The lyophilized powders were suspended in 5 ml dichloromethylane or acetonitrile, followed by centrifugation at 12000 RPM on a Anker TGL-16C centrifuger to remove the PEG continuous phase which is soluble in the solvents. The above procedure was repeated for three times, and dextran glassy particles were obtained after the pellets were evaporated to remove the solvent residues.

2.3. Scanning electron and optical microscopic images

Scanning electron microscopic (SEM) images of dextran particles were taken using FEI SIRION 200 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. Optical microscopic images were taken using an Olympus CX41 microscope equipped with a digital camera (model μ710).

2.4. Particle size analysis

To determine mean size and size distribution, the dextran particles were suspended in dichloromethane and subjected to a Malvern Mastersizer 2000 particle sizer. All the measurements were carried out at a 90° scattering angle and 25 °C. The mean diameter of the particles was calculated based on the average of repeated three times measurements.

2.5. X-ray diffraction (XRD) patterns of dextran particles

X-ray diffraction patterns of the samples were recorded using a Rigaku D/MAX 2000 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, pure dextran and PEG were measured under the identical conditions.

2.6. Differential scanning calorimetry (DSC) of dextran particles

Differential scanning calorimetry (DSC) of the dextran particles was carried out using 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 250 °C at a rate of 10 °C/min. As controls, pure dextran and PEG were measured under the identical conditions.

2.7. Particle density

To estimate the density of the dextran particles, the samples were added into a series of organic solvents of different densities, such as dichloromethane (density = 1.325 g/ml), chloroform (density = 1.495 g/ml), and carbon tetrachloride

(density = 1.592 g/ml). The density of the particles was estimated by how they suspended in the solvents.

3. Results

3.1. Morphology of dextran particles prepared through freezing-induced phase separation

The morphological characteristics of the dextran particles prepared in the present study were examined using a scanning electron and optical microscopes. The results are shown in Figs. 1 and 2, respectively. For dextran 70,000 (Figs. 1A and 2A) and dextran 500,000 (Figs. 1B and 2B), spherical particles with smooth surfaces were obtained. Images of the same samples taken under optical microscope (for which larger sampling size can be achieved) consist with those under scanning electron microscope, showing that the particles of lower molecular weight dextran are slightly smaller in diameter (Fig. 2). The particles looked closely aggregated under the scanning electron microscope (Fig. 1), but not under the optical microscope (Fig. 2). When the particles were added in an organic solvent (dichloromethylane), a uniform suspension was formed without precipitation of large aggregates. Therefore, the particles aggregation under the electron microscopes was due to weak physical interaction.

3.2. Size distribution of dextran particles prepared through freezing-induced phase separation

To elucidate how formulation parameters affect particle size and distribution, we examined the effects of molecular weight of PEG, PEG/dextran ratio and concentration of small molecular sugars and salts added to the solution on dextran particle size distribution. The results are summarized in Figs. 4–7, respectively.

Size distribution of the dextran particles prepared in this study was determined based on laser scattering measurements. The volume-based distributions of the dextran particles formed of two different molecular weights are shown in Fig. 3. For the particles made of dextran 70,000, the particle diameters ranged from 2.0 to 3.5 μm (Fig. 3A), while for that of dextran 500,000, the range was 4.0 to 4.5 μm (Fig. 3B). These results consist with the microscopic images shown in Figs. 1 and 2.

The mean diameters of dextran particles are listed as a function of PEG molecular weight in Fig. 4. The diameters of the dextran particles (made of both dextran 70,000 and dextran 500,000) decreased as PEG molecular weight decreased from 8000 to 2000. For the samples co-dissolved with PEG 1000, large dextran pieces of irregular shape were harvested after lyophilization and washing with organic solvents. For the sample mixed with PEG 2000, there were also some large dextran particles of irregular shape formed during the freeze-drying process, but their fraction was much less as compared with that of PEG 1000. For samples mixed with PEG 4000 or PEG with larger molecular weight, no significant amount of large dextran pieces was observed.

The yield of spherical dextran particles (with the physical losses, such as sticking on the container taken into account) was between 95 and 105% (w/w) with experimental errors for the experiments involving PEG 8000 and PEG 6000. For the particles formed from solution with PEG 4000, the yield dropped to 80–90% (w/w) after the large and irregular dextran pieces were removed. Further reducing PEG molecular weight to 2000 resulted in a reduced yield of spherical dextran particles (about 60%). For the experiments involving PEG 1000, as mentioned above, only dextran pieces of irregular shape and diversified sizes were harvested.

According to the phase diagrams of dextran and PEG aqueous two-phase systems, the degree of phase separation of the two hydrophilic polymers is a function of their molecular weight (Zaslavsky, 1995). The higher their molecular weights are, the easier phase separation occurs, and the less of one ingredient partitioned in the phase of the counterpart. We presume, therefore, that for the sample mixed with PEG 1000, phase separation did not occur during the freezing process. The same hypothesis explains the dependence of dextran particle diameters on PEG molecular weight. For dextran mixed with PEG of higher molecular weight, phase separation occurs earlier during the freezing process so that the dispersed dextran droplets have more chances to associate to each other to form larger ones.

Fig. 5 compares the mean diameters dextran 70,000 particles formed from dextran-PEG co-solutions of different concentrations and PEG molecular weights. The dextran particle size increases with increasing the concentration of the system and PEG molecular weights.

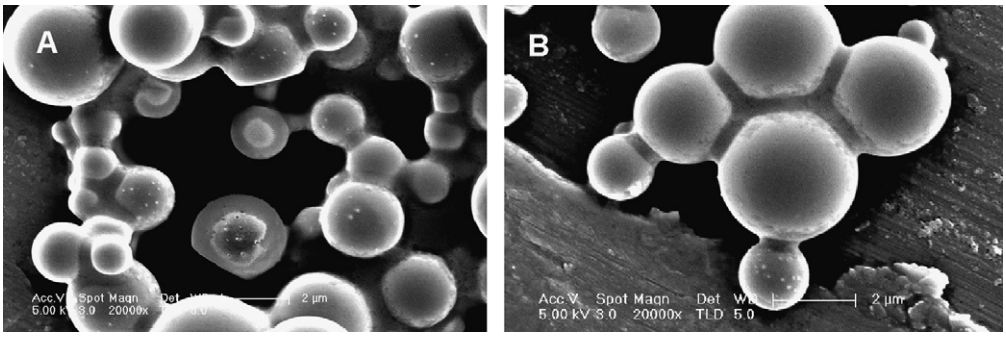

Fig. 1. SEM images of dextran glassy particles. The particles were formed through freezing-induced phase separation from a co-solution containing 0.45% dextran and 4.5% PEG 8000. (A) Particles of dextran 70,000; (B) particles of dextran 500,000.

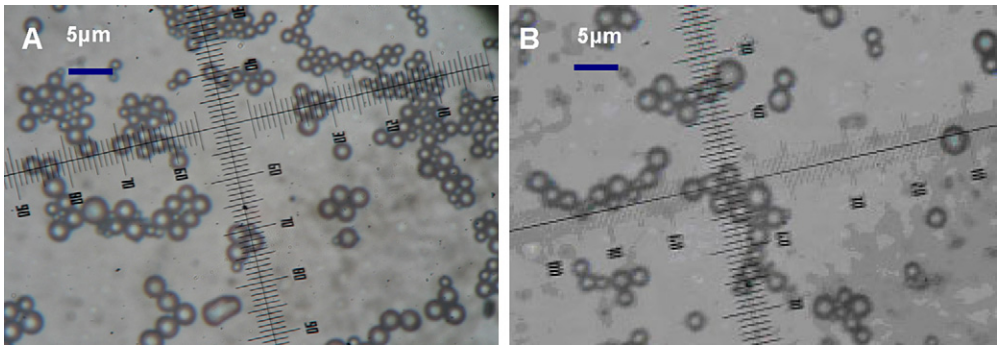

Fig. 2. Optical microscope images of dextran glassy particles. The particles were formed through freezing-induced phase separation from a co-solution containing 0.45% dextran and 4.5% PEG 8000. (A) Particles of dextran 70,000; (B) particles of dextran 500,000.

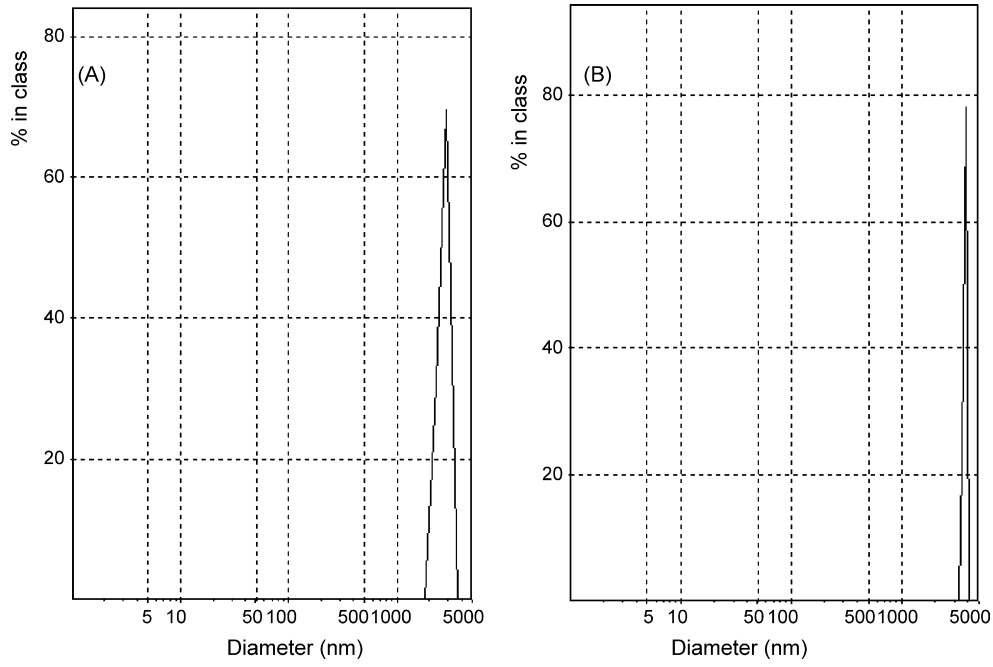

Fig. 3. Size distributions of dextran glassy particles. The particles were formed through freezing-induced phase separation from a co-solution containing 0.45% dextran and 4.5% PEG 8000. (A) Particles of dextran 70,000; (B) particles of dextran 500,000.

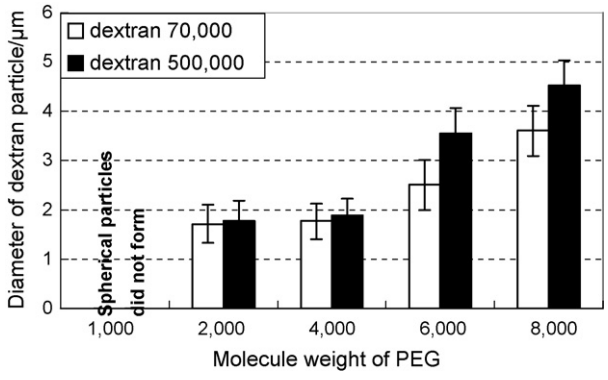

Fig. 4. Effect of PEG molecule weight on dextran particle sizes. The particles were formed through freezing-induced phase separation from co-solutions with concentrations of dextran and PEG of 0.91% and 9.1%, respectively. The error bar was calculated based on three repeated experiments.

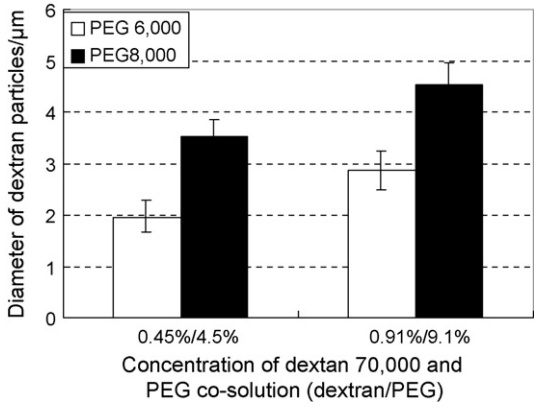

Fig. 5. Effect of PEG and dextran concentration on dextran particle sizes. The error bar was calculated based on three repeated experiments.

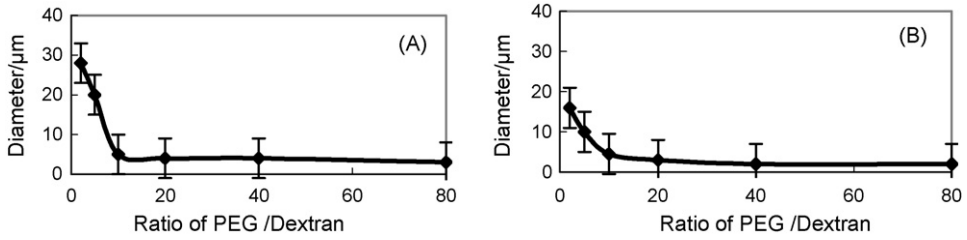

Fig. 6. Effect ratio of PEG/dextran on dextran particle size through freezing-induced phase separation from co-solutions. (A) 8% dextran 70,000 solution mixed 8% PEG 8000 at various ratio of PEG/dextran; (B) 8% dextran 70,000 solution mixed 8% PEG 6000 at various ratio of PEG/dextran. The error bar was calculated based on three repeated experiments.

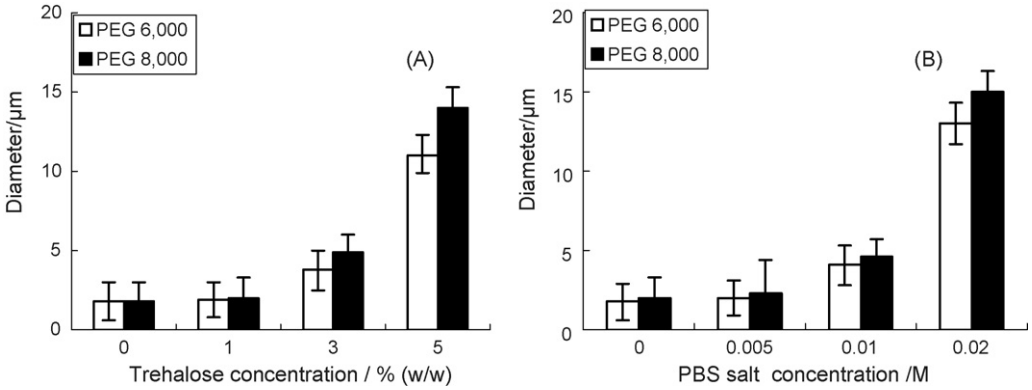

Fig. 7. Effect of trehalose and PBS salt on dextran 70,000 particle size. The particles were formed through freezing-induced phase separation from a co-solution containing 0.45% dextran and 4.5% PEG 8000. (A) Effect of trehalose; (B) effect of PBS. The error bar was calculated based on three repeated experiments.

Fig. 6 plots the mean diameters of dextran particles against ratio of PEG to dextran. For both samples formed from solutions of PEG 8000 and PEG 6000, the particles sizes decrease drastically from tens of microns to 3–5 microns as the PEG/dextran weight ratio increased from 1 to 10. Further increase in PEG/dextran ratio did not result in significant change in the dextran particle size. The similar phenomenon (the effect of PEG/dextran ratio on dextran particle size) has been reported by Stenekes et al. for acrylate-conjugated dextran (Stenekes et al., 1998, 1999).

Since small molecular sugars or salts are often used in pharmaceutical formulations as cryo-protectors, we examine the effects of trehalose and PBS (sodium dihydrogen phosphate, potassium dibasic anhydrous and potassium chloride) on dextran particle sizes. The results are shown in Fig. 7. For both trehalose and PBS salt, no effect was observed till their concentrations reached certain level (1% for trehalose and 5 mM for PBS salt). Above these concentrations, the mean diameters of dextran particles increased significantly, 15 μm for 5% trehalose and 20 mM PBS salt, respectively, as shown in (Fig. 7A and B). Probably these small molecular additives facilitated fusion of dextran dispersed phase. It has been reported that small molecular sugars additives improved protein stability especially protection in the lyophilization process by providing a hydroxyl rich environment (Cleland and Jones, 1996; Gnebenow and Klibanov, 1995). However to use small molecular sugars to protect proteins in this polysaccharide particles-forming process, the amount has to be controlled.

3.3. Crystalline structure and thermal properties of dextran particles

The dextran particles prepared by freezing-induced phase separation were subjected to X-ray diffraction (XRD) for its solid state. Fig. 8 summarizes the XRD patterns of the dextran particles together with PEG 8000 and dextran powder. PEG 8000 powder showed a XRD pattern with sharp diffraction peaks at 2θ

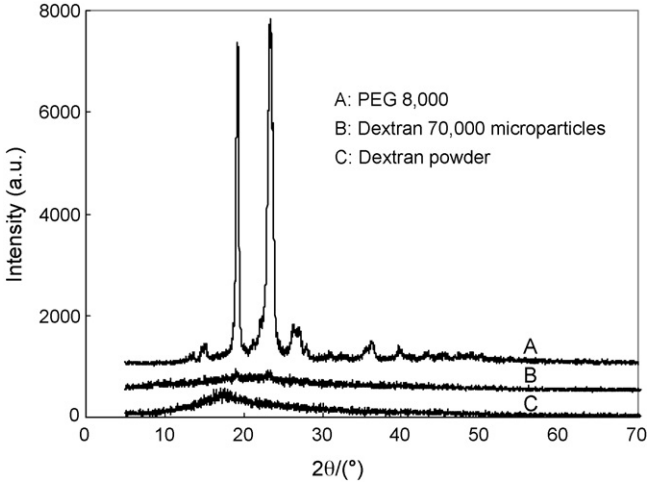

Fig. 8. X-ray diffraction (XRD) patterns of dextran particles formed through freezing-induced phase separation and of each component involved in the particle-forming process.

Table 1
Behaviors of dextran particles estimated as suspended in organic solvents

Type	Organic solvent		
	Dichloromethane	Chloroform	Carbon tetrachloride
Commercial dextran powder	↑	↑	↑
Dextran particles, $\Phi > 10\,\mu m$	↓	↓	↓
Dextran particles, $\Phi < 10\,\mu m$	↓	↓	—

Commercial dextran powder was obtained from Sigma. Dextran particles ($\Phi > 10\,\mu m$) were prepared through freezing-induced phase separation from a co-solution containing 5.0% dextran and 5.0% PEG 8000. Dextran particles ($\Phi < 10\,\mu m$) were prepared through freezing-induced phase separation from a co-solution containing 0.45% dextran and 4.5% PEG 8000. "Dextran particles" were those prepared through freezing-induced phase separation of a co-solution containing 0.45% dextran 70,000 and 4.5% PEG 8000. "↑": Floating at the surface of the solvents; "↓": sinking to the bottom of the solvents; "—": suspending within the matrix of the solvents.

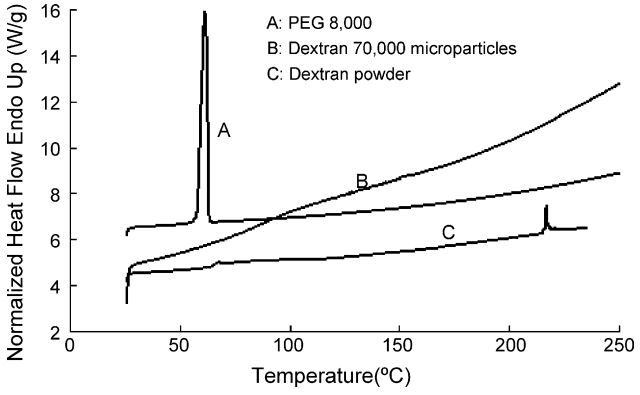

Fig. 9. DSC thermograms of dextran particles formed through freezing-induced phase separation and of each component involved in the particle-forming process.

of 19° and 23°. The dextran powder showed a broad peak centered at 2θ of 18°, suggesting some level of crystalline. The fine dextran particles showed no XRD patterns, suggesting a glassy state.

The XRD results consist with the results of differential scanning colarimetry (DSC). As shown in Fig. 9, PEG 8000 powder generated a sharp endothermic peak at 61.5 °C when being heated programmably, indicating its melting point. Heating the dextran powder resulted in a small endothermic peak at 217.0 °C, a temperature within the range of polysaccharide melting points (Kibbe, 2000). For the dextran particles, however, no endothermic peak was observed, but a bear bended region on its DSC curve at the temperature from 215.1 to 226.1 °C. We attribute this region to the glassy transition temperature of the dextran particles. While some peak assignments (for both XRD and DSC) may not be accurate, it is clear that the dextran particles formed via freezing-induced phase separation possess no crystalline structures.

3.4. Particle density

To estimate the true density of the dextran particles formed by phase separation and lyophilizaton, the samples were suspended in a series of organic solvent of different densities. The results are summarized in Table 1. All the particles readily sank to the bottom of the container when added into dichlormethane (density = 1.36 g/ml) and chloroform (density = 1.49 g/ml), but were suspending in carbon tetrachloride (density = 1.59 g/ml). We prepared some larger dextran particles using the same method and selected some with diameters over 10 μm and added into tetrachloride. These particles sank to the bottom of the solvent, indicating that the true density of the dextran particles formed by freezing and lyophilization is above 1.59. The size-effect on apparent buoyancy of the particles can be explained by interfacial tension between the hydrophilic particles and the hydrophobic solvent. Small particles have large specific surface area, thus the effect of interfacial tension (that drives the particles out of the solvent) on apparent particle buoyancy is more remarkable.

We further examined the density of commercially available dextran powders by adding them into the solutions, and found these particles, regardless of large or small (because the particles are not uniform) were floating on the top of all these three solvents, suggesting that their density is below 1.39. Table 1 summarizes the experimental results of the density estimates.

4. Discussion

Using freezing-induced phase separation, we demonstrated a convenient method to prepare fine polysaccharide particles under a condition free of hydrophilic–hydrophobic interfacial tensions and particle-solidification agents. The particles are uniform in size and desired sizes can easily be achieved by varying some formulation parameters, such as concentration of polysaccharide and PEG, ratio of PEG/polysaccharide and molecular weight of PEG.

The effect of the above formulation parameters on the particle size may be related to the temperature at which the dispersed phases were generated during the freezing-induced phase separation process. If the phase separation occurred at relatively higher temperature (like for more concentrated system), the polysaccharide dispersed phases will have more chance to fuse with each other and form larger droplets prior to reach a frozen state.

The reduced dextran particle sizes due to increase in ratio of PEG/polysaccharide (Fig. 6) may be explained by diluted dextran concentration and reduced popularity of the dextran droplets separated from the co-solution. On the other hand, since the aqueous two-phase separation is due to the incompatibility and immiscibility of two aqueous polymers of sufficient

molecular weight (Zaslavsky, 1995), phase separation might no longer occur when the molecular weight of PEG was below 2000 in the present study (Fig. 4).

The electron microscopic images (Fig. 1) and the density estimates (Table 1) are consist with each other to evidence the non-porous surfaces and solid glassy matrix of the dextran particles formed through freezing-induced phase separation. A glassy state of a polysaccharide particle is important for maintaining the nature conformation of proteins loaded by offering resistance against organic solvents, moisture and elevated temperature (Breen et al., 2001; Langer, 1991; Yoshioka et al., 1997).

One of the most important issues is whether the process of freezing-induced phase separation is harmful to protein stability. While it has been reported that PEG and dextran phase separation occurred during freezing caused protein to denature (Heller et al., 1997), the only example that we could find in literature is hemoglobin. Hemoglobin is a special protein which needs high concentration of 2,3-diphosphoglyceric acid to ionically conjugate its four subunits together. Therefore hemoglobin is only stable inside of red blood cells within which the equilibrium favors subunits conjugation due to high concentration of 2,3-diphosphoglyceric acid. We examine activity of β-galactosidase, a protein with four subunits and similar molecular weight as hemoglobin. After freezing in an aqueous emulsion system involving dextran-PEG interfaces, the enzymatic activity of the protein was not compromised (Jin et al., 2003).

Since well soluble proteins and glycoproteins (except PEGylated proteins) preferentially partition into the dextran phase of a dextran-PEG aqueous two-phase system (Zaslavsky, 1995), it is reasonably to assume that proteins partition into the dextran dispersed phase during freezing-induced phase separation. However, Morita et al. reported that proteins selves that were dissolved in a PEG solution formed particles by freeze-drying (Morita et al., 2000). In another word, freezing-induced phase separation may occur for protein-PEG co-solutions too. Therefore, proteins the possibility that form particles together with dextran by "co-precipitation" from a PEG solution during freezing, cannot be ruled out.

For whatever the particle-forming process, it has been well reported that once proteins are safely loaded into dense and solid particles of polysaccharides, they become highly resistant to the organic solvents used in microencapsulation (Breen et al., 2001; Hennink et al., 1997; Jin et al., 2003; Yoshioka et al., 1997). The unique polysaccharide particle-forming method demonstrated in this study should have wide applications in formulating structurally delicate biological therapeutics.

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