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Research paper

Preparation and characterization of microparticles containing peptide produced by a novel process: spray freezing into liquid

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Abstract

The objective of this study is to evaluate excipient type on the physicochemical properties of insulin microparticles produced by spray freezing into liquid (SFL). A novel process was developed to produce microparticles containing bioactive peptides and proteins. The microparticles were formed by atomization of an aqueous feed solution containing insulin beneath the surface of a cryogenic liquid (e.g. liquid nitrogen). In this study, bovine insulin was dissolved in deionized water alone or with tyloxapol, lactose or trehalose. The aqueous solution was sprayed directly into liquid nitrogen through a polyetheretherketone capillary nozzle under high pressure to form frozen microparticles. Lyophilization was used to sublime the ice. The SFL insulin powder was characterized by different techniques, including X-ray diffraction, reverse-phase high pressure liquid chromatography, size exclusion chromatography, scanning electron microscopy (SEM), particle size distribution and surface area. The mean diameter of the insulin microparticles was 5–7 μm. SEM revealed that the microparticles were highly porous, and the morphology of the microparticles was influenced by the excipient type. The total surface area of the insulin microparticles ranged from 20 to 40 m^2/g, and the magnitude depended on the specific composition and total solids content of the aqueous feed solution. X-ray diffraction results indicated lack of crystallinity. No change in the level of the degradation product, A-21 desamido insulin, was found in the SFL insulin samples processed alone or with trehalose or tyloxapol. Similarly, no change in formation of high molecular weight transformation products (e.g. covalent insulin dimer) was detected in the samples processed with excipients. The results demonstrated that SFL is a feasible technique for forming porous microparticles containing insulin. The physicochemical properties of insulin were preserved by the SFL technique.

Keywords: Peptide; Spray freezing into liquid; Microparticle; Cryogenic liquid; Nozzle

1. Introduction

Recent advances in molecular biology have led to an explosive growth in the number of peptide and protein drugs derived from both recombinant technology and conventional peptide drug design [1]. However, development of peptide and protein therapeutics has proved to be very challenging because of the complexities of protein production and purification, low membrane permeability, inadequate physical and chemical stability, and relatively short half-lives of proteins and peptides [2].

To overcome the instability, proteins and peptides are often formulated into solid forms to achieve an acceptable shelf-life as pharmaceutical products. Most small particles to be used for pharmaceutical applications are prepared using conventional techniques such as lyophilization, milling, grinding and spray drying. Methods using supercritical fluids have been reported in recent years [3]. The most commonly used method for preparing solid protein powder is lyophilization [4]. Lyophilization produces particles in the desired particle size range under specific conditions, but with a broad size distribution. This process also generates a variety of freezing and drying stresses due to solute concentration, formation of ice crystals, pH changes, and freeze-induced phase separation. All of these stresses can denature proteins to various degrees. Thus, stabilizers are often required in a protein formulation to protect protein stability during freezing and drying processes.

In addition, particle size can be reduced by mechanical milling processes, including ball mills, colloid mills, hammer mills and jet mills. Milling produces particles having diameters greater than 1 μm, however the particle size distribution of the powder generated by milling is polydisperse. The heat generated during inter-particle collisions can cause changes in the solid state of the material. Furthermore,

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chemical decomposition of thermally labile molecules has been observed during micronization [5]. Jet mills can produce particles in the 1–10 μm range. However, the process involves the use of high velocity compressed air, which leads to electrostatically charged powders. In addition, particle size reduction tends to be more efficient for hard and brittle materials such as salts and minerals, but much less so for soft powders, such as proteins and peptides [5].

Spray drying is another method for preparing dry powders. The feed solution is atomized into droplets that dry rapidly because of their high surface area and intimate contact with the drying gas. The drying time for droplets is a function of the process conditions and has a wide range from less than 100 ms to s. Spray drying can reduce particle diameter to less than 5 μm, but biologically active proteins can become inactivated in the process due to denaturation at the aqueous-air interface and by exposure to heat during solvent evaporation [5]. Furthermore, the final product yield can be low, particularly in small-scale unit operations.

Supercritical fluids have been used in a variety of extraction and analytical methods. This process can also be used to prepare pharmaceutical powders. For example, Yeo et al. [6] have used this technique to prepare protein and peptide microparticles. However, this technique involves exposing proteins to organic solvents. This environment has been shown to induce significant protein conformational changes which may be accompanied by losses in biological activity [6–8].

Spray freezing into liquid is a novel particle engineering process for forming drug microparticles. Spraying the liquid formulation directly into the cryogenic liquid results in rapid freezing of the atomized droplets and formation of microparticles. The frozen particles are collected and lyophilized. The particle size can be controlled by the processing parameters. The present method results in extremely fast rates of freezing due to the small droplet size and very low temperatures. Studies have suggested that the rate of denaturation increases with the ice-water interfacial area, and this interfacial area increases with the freezing rate [9,10]. However, Heller et al. [11] suggested that ice formation could be avoided in spray freeze drying of polymeric solutions, resulting in a completely amorphous aqueous solution. Rapid cooling of aqueous solutions minimizes the formation of ice nuclei and reduces formation of crystalline water, and therefore minimizes solute concentration and pH change. Also, the rapid cooling rates may cause formation of an amorphous glass before any relaxation processes occur in the freeze concentrated solution, and this leads to reduced protein and peptide denaturation [11]. In addition, no organic solvents or elevated temperatures are utilized in the spray freezing into liquid (SFL) technology, when applied to water soluble peptides and proteins.

The objective of this study is to demonstrate that SFL is a suitable particle forming technology for peptides. Formulations were investigated to optimize processing conditions while maintaining integrity of the peptide. Furthermore, the stability of the peptide particles through the SFL process was confirmed.

2. Materials and methods

2.1. Materials

Bovine (Zinc) insulin was purchased from Spectrum Laboratory Products, Inc. (Gardena, CA). The potency was 23.5 IU/mg. Ammonium sulfate, triethylamine, tyloxapol, hydrochloric acid, sodium hydroxide and L-arginine were also purchased from Spectrum Laboratory Products, Inc. All chemicals were A.C.S. grade or higher. Acetic acid and acetonitrile from Spectrum Laboratory Products, Inc. were high-performance liquid chromatography (HPLC) grade.

2.2. Spray-freezing into liquid process

In a typical SFL process, the aqueous feed solution was prepared by dissolving 100 mg of insulin powder in a HCl solution (pH = 2), then the acidified insulin solution was titrated to pH 7.4 using 0.1 N NaOH. A schematic diagram of the SFL apparatus is shown in Fig. 1. The neutralized insulin solution was loaded into solution cell (B) and then sprayed beneath the surface of the cryogenic liquid, liquid nitrogen (D), through a 63.5 μm polyetheretherketone (PEEK) tubing nozzle (C) at a pressure of 4000 PSI which was provided by an ISCO syringe pump (A; Isco, Inc., Lincoln, NE) (Fig. 1A). The frozen particles were collected and lyophilized using a Virtis Advantage Lyophilizer (The Virtis Company Inc., Gardiner, NY).

2.3. Particle size analysis

The particle size distribution of micronized bulk insulin and the SFL powder formulations was measured with an Aerosizer LD (Amherst Process Instrument, Inc., Amherst, MA) according to the time-of-flight method in a fluidized state. The dual laser beam instrument measures the time taken by a fluidized particle to cover the distance between two laser beams and calculates the particle size distribution using cross correlation techniques. A density of 1.35 g/ml was used. The particle size distribution is reported as mass median diameter. The polydispersity of the particle size distribution is indicated by the Span Index (29). The particle size distribution is based on volume.

2.4. Differential scanning calorimetry (DSC)

DSC was performed with a DSC Model 2920 Modulated DSC and Thermal Analysis 2000 software (TA Instruments, New Castle, DE). Two to three milligrams of each test sample was hermetically sealed in aluminum pans. An empty sealed pan was used as the reference. The heating program was conducted using the modulated setting at 10°C/min over a range of 20–350°C.

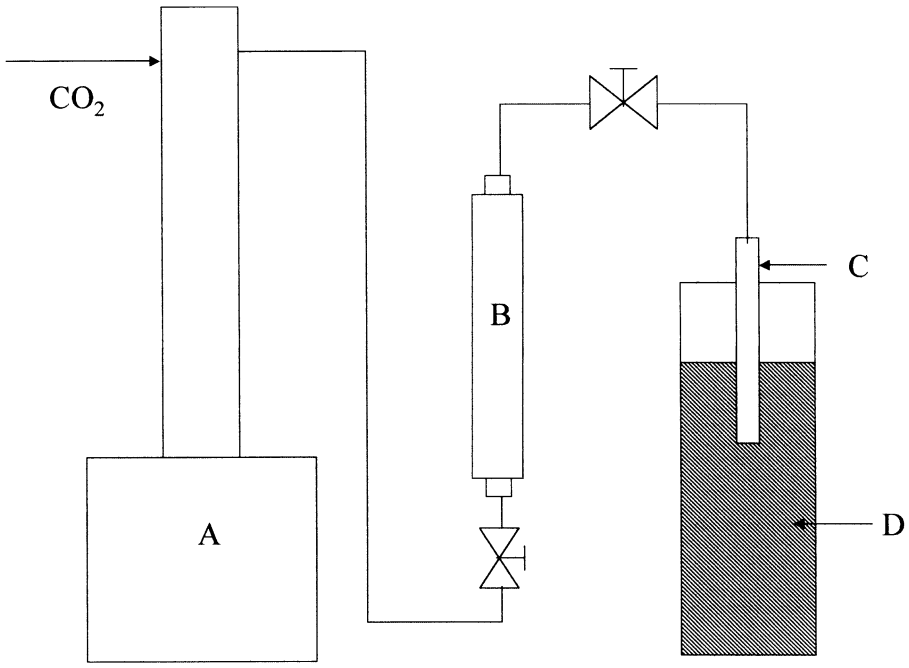

Fig. 1. Experimental apparatus for the spray freezing into liquid process. (A) Syringe pump. (B) Solution cell. (C) Atomization nozzle. (D) Cryogenic liquid.

2.5. Scanning electron microscopy (SEM)

A Hitachi Model S-4500 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) was used to obtain the SEM photographs of gold coated SFL powder. The dried powders were mounted on aluminum stages using double adhesive carbon conductive tape and coated with gold for 30 s using a Pelco Model 3 sputter-coater on argon atmosphere.

2.6. X-ray diffraction

Powder X-ray diffraction of samples was obtained by a Philips Model 1710 X-ray diffractometer with a copper target and nickel filter (Philips Electronic Instruments, Inc., Mahwah, NY) and Jade 5 XRD pattern processing software (Materials Data, Inc., Irvine, CA) was used to obtain the X-ray diffraction patterns.

2.7. Reverse-phase HPLC

The HPLC system consisted of a LC-10AT vp pump, SIL-10A autoinjector, and SPD-MI10A vp diode array detector (Shimadzu USA Manufacture Inc., Columbia, MD). The data were analyzed using Class-vp chromatography data acquisition system (Version 4.2). Reverse-phase chromatography was conducted with a 250 × 4.6 mm i.d., Macrosphere RP 300 C18, 5 μm particle size column (Alltech Associates, Inc. Deerfield, IL). A 200 mM ammonium sulfate buffer solution containing 0.5% (v/v) triethylamine was prepared for use in the mobile phase (pH 2.3). Mobile phase A consisted of 40% acetonitrile and 60% ammonium sulfate buffer. Mobile phase B consisted of 27% acetonitrile and 73% ammonium sulfate buffer solution. The flow rate was 1.0 ml/min. A linear gradient elution from 0% A and 100% B to 26% A and 74% B over 20 min was used. A second linear gradient elution from 26% A and 74% B to 100% A and 0% B between 21 and 25 min was then used. A third linear gradient elution from 100% A and 0% B to 0% A and 100% B between 25 to 45 min was used. The detection wavelength was 214 nm and the injection volume was 20 μl 0.25 mg/ml. System suitability requirements included accuracy (greater than 0.9998), precision (less than 2%), and peak asymmetry (less than 1.2) The limit of quantitation was 0.001 mg/ml.

2.8. Size exclusion chromatography

The HPLC system used for size exclusion chromatography (SEC) consisted of a LC-10AT vp pump, SIL-10A autoinjector, and SPD-MI10A vp Diode array detector (Shimadzu). Based on criteria of selectivity and resolution, a 30 cm × 7.8 mm Protein-Pak™ 125 column (Waters Corporation, Milford, MA) was chosen for the SEC studies. The mobile phase consisted of 15% (v/v) acetic acid, 20% (v/v) acetonitrile, 0.1% (w/v) L-arginine and 64.9% (v/v) deionized water. The flow rate was 0.5 ml/min and the injection volume was 20 μl of a 0.5 mg/ml solution. The eluent was detected using UV absorbance at 280 nm.

2.9. Statistical analysis

The insulin stability data were compared using a Student's *t*-test of the two samples assuming equal variances to evaluate the differences. The significance level ($\alpha = 0.05$) was based on the 95% probability value ($P < 0.05$).

3. Results and discussion

3.1. Preparation of microparticles

SFL is a novel particle engineering process developed for forming drug loaded microparticles by atomizing an aqueous feed solution beneath the surface of a cryogenic liquid. In this study, the cryogenic liquid was liquid N_2. The frozen microparticles are then sublimed at low temperature and low pressure. In order to preserve the higher order structure of the peptide by avoiding the formation of ice crystals, it is essential for the feed solution containing insulin to pass through the critical temperature zone very rapidly. The critical temperature zone is defined as the temperature range between crystallization and the glass transition temperature of the solution [12]. Because the insulating nozzle is inserted beneath the surface and directly into the cryogenic liquid, the droplets begin to freeze at or immediately after they are formed. The intense atomization of the droplets resulting from the high pressure drop through the small orifice nozzle produces extremely small droplets. The low temperature of liquid nitrogen (−196°C) and small droplet sizes produced by high pressure atomization through a 63.5 μm PEEK tubing nozzle (Fig. 1D) allowed for very rapid freezing rates to be achieved. It has been reported that the maximum cooling rates achievable with liquid nitrogen are on the order of 10^3 Ks^{-1} when the droplet size is less than 10 μm [13,14]. Under typical industrial freeze drying conditions, solutions are cooled relatively slowly, perhaps on the order of 1–10 Ks^{-1} [11]. Rapid freezing rates do not influence the activity of proteins and peptides because formation of dendritic ice crystals and the time for phase separation are minimized [11,15]. Furthermore, the rapid freezing rates utilized in the SFL process generate significant supersaturation and thus rapid nucleation rates of dissolved substances [11]. The rapid nucleation and restricted growth that occurs after the fine droplets are formed and frozen leads to extremely small primary particles following lyophilization.

3.2. Particle morphology and particle size

Large porous particles of insulin were prepared by SFL and optimized by varying the composition of the aqueous feed solution. The surface area of the SFL samples is given in Table 1. Compared to the bulk insulin particles (Fig. 2A), the particles (Figs. 2B–D) generated by the SFL process are highly porous. The surface of the particles is irregular and rough in appearance. Compared to the dense particles generated by other methods, such as jet milling [5], supercritical precipitation [16] and spray-drying [17,18], the particles produced by the SFL process were much less dense and more porous. Spray-drying also has been used to produce porous primary particles [19,20]. However because of the high processing temperatures used in the spray drying process, the use of that process to prepare powder of thermally labile molecules without significant degradation may be limited [5].

The particle size and size distribution are important parameters for incorporation of drug particles into drug delivery systems. In this study, the median particle size of the SFL insulin particles was in the range of 5–7 μm (Table 1). Span Index indicated that the particle size distribution was narrow (Table 1), and this is also observed in Fig. 3. The median particle size of the SFL insulin particles was smaller than that of the bulk insulin powder. It can be seen in Figs. 2B–D that the 5–7 μm diameter particles were aggregates of small discrete particles measuring less than 500 nm, depending on the composition of the aqueous feed solution containing the insulin. The bulk density of the SFL samples was between 0.002 g/ml to 0.015 mg/ml (Table 1). Production of large porous particles has been described by Edwards et al. [21]. These particles were produced by a double emulsion technique. Edwards et al. [22] also demonstrated the utility of particles having a bulk density of less than 0.4 g/cm^3 and a median diameter greater than 5 μm in drug delivery systems. The results presented in this study demonstrate that the SFL process was capable of generating large porous particles.

The composition of the SFL feed solution (Table 2) also influenced the particle size and particle morphology. From the SEM picture shown in Fig. 1, the SFL particles appeared highly porous and irregularly shaped. However, the surface area of these samples was different. The surface area of the SFL insulin/tyloxapol powder was the highest (44.44 m^2/g), whereas the surface area of the SFL insulin/tyloxapol/lactose powder was 19.84 m^2/g (Table 1).

3.3. Crystallinity of the particles

In this study, the insulin powder was generated by the

Table 1
The particle size distribution and surface area

Sample	Median diameter (μm)	Span Index[a]	Bulk density (g/cm^3)	BET surface area (m^2/g)
Bulk insulin	14.24	1.29	–	–
SFL insulin	5.06	0.92	0.002	30.16
Insulin/tyloxapol	6.87	1.16	0.003	44.44
Insulin/tyloxapol/lactose	5.72	0.86	0.012	19.84
Insulin/tyloxapol/trehalose	–	–	0.015	–

[a] Span Index = $(D_{90} - D_{10})/D_{50}$ [29].

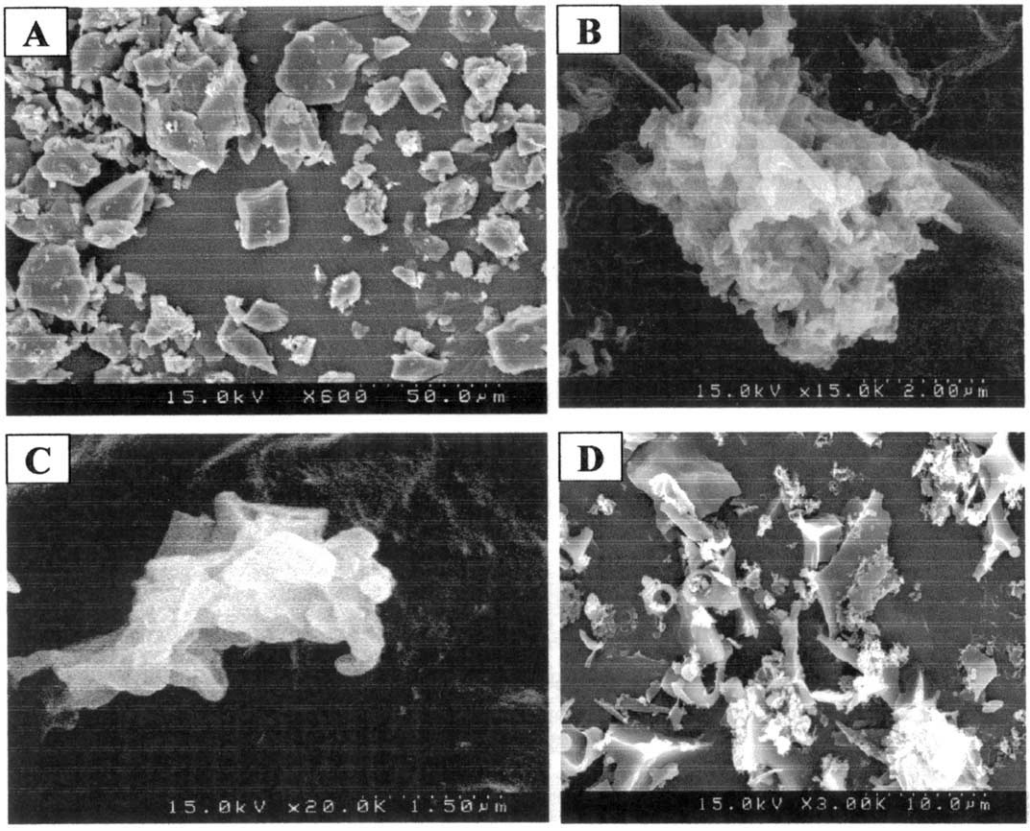

Fig. 2. SEMs of bulk insulin (A); SFL insulin (B); SFL insulin/tyloxapol (C); and SFL insulin/tyloxapol/lactose (D).

SFL process whereby an aqueous solution containing insulin was atomized directly into liquid nitrogen to form very small frozen particles. Because of the small droplet size of the atomized aqueous feed solution and the rapid freezing rate, the crystallinity of the particles was not evident. The X-ray diffraction patterns displayed in Figs. 4A,D,E show no crystallinity for SFL samples processed at pH 3.0. The X-ray diffraction curves in Figs. 4B,C from samples processed at pH 7.4 also indicate lack of crystallinity except for two small peaks of NaCl (formed from titration with NaOH). These two peaks were identified by comparing to a standard NaCl control sample (X-ray diffraction pattern not shown).

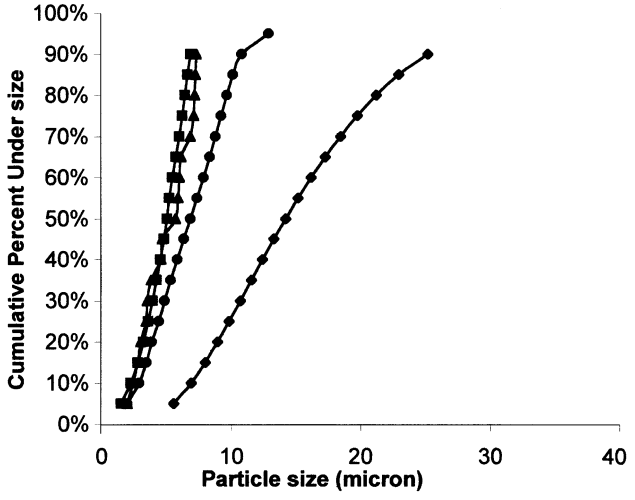

Fig. 3. Particle size distribution for the samples investigated. ◆ Bulk insulin; ■ SFL insulin; ● SFL insulin/tyloxapol; and ▲ SFL insulin/tyloxapol/lactose.

One of the major mechanisms of protein stabilization by cryoprotectants involves the formation of hydrogen bonds between a protein and an excipient at the end of the drying process to satisfy the hydrogen bonding requirement of the polar groups on the protein surface [23]. The amorphous state of proteins and stabilizers allows maximal hydrogen bonding between protein and stabilizer molecules to occur, therefore crystallization of any amorphous protein stabilizers causes destabilization due to inefficient hydrogen bonding. Therefore, amorphous sugar in the SFL samples is necessary in order to preserve the stability of insulin.

3.4. Stability of insulin

For peptides and proteins, the preservation of higher order structure, such as secondary, tertiary and quaternary conformation, is necessary in order to retain biological activity. Denaturation, degradation and aggregation are frequent manifestations of peptide and protein instability. Peptides and proteins undergo physical and chemical

Table 2
Composition of each formulation investigated in this study

Sample	Insulin (mg/ml)	Tyloxapol (mg/ml)	Lactose (mg/ml)	Trehalose (mg/ml)
SFL insulin	2.5	0	0	0
SFL insulin/tyloxapol	2.5	0.5	0	0
SFL insulin/tyloxapol/lactose	2.5	0.5	12.5	0
SFL insulin/tyloxapol/trehalose	2.5	0.5	0	12.5

degradation, and these instabilities present unique difficulties in the formulation, production and storage of protein pharmaceuticals. In this study, the degradation product, A-21 desamido insulin, was measured by reverse phase-HPLC method. The level of A-21 desamido insulin in the bulk insulin and SFL insulin samples is shown in Table 3. The level of A-21 desamido insulin in the bulk insulin sample prior to SFL processing was 3.03%. The percent of the A-21 desamido degradation product in the SFL insulin powder sample was 3.04%, indicating no differences in the level of A-21 desamido insulin between the SFL insulin powder sample and the bulk insulin sample ($P < 0.05$). Therefore, SFL processing did not cause chemical degradation of insulin.

Excipients can also influence the chemical stability of peptides and proteins. In this study, tyloxapol, lactose and trehalose were used as cryoprotectants. The mechanism of protein stabilization by cryoprotectants involves the preferential exclusion of water molecules from the surface of protein molecules [4]. The level of A-21 desamido insulin in the SFL insulin/tyloxapol sample was 3.06%, which was not different from the level in the bulk insulin (Table 3). The Tg of the SFL insulin/tyloxapol sample (95°C) was higher than the Tg of the bulk insulin sample (85°C) and the SFL insulin powder sample (80°C). This indicates that insulin molecules are stabilized by tyloxapol. The formation of ice-water interfaces during freezing may cause surface denaturation of proteins. Tyloxapol is a non-ionic surfactant which decreases the interfacial tension of protein solutions and reduces the driving force of protein adsorption and/or aggregation at these interfaces [24]. However, it has been reported that surfactants may denature or increase the degradation of proteins [25]. The results of this study indicate that when insulin/tyloxapol particles are prepared by SFL that tyloxapol does not cause degradation of insulin in the process.

In addition, sugars are often used to stabilize proteins or peptides. Lactose and trehalose were investigated in this study. The results shown in Table 3 reveal that the concentration of A-21 desamido insulin was 3.72% for the sample containing lactose, which was higher than the level of the degradation products in bulk insulin and the other SFL samples. Compared to the lactose sample, the concentration of A-21 desamido insulin in the trehalose sample was 3.05%, which was not different than the level in the bulk insulin sample prior to SFL processing ($P < 0.05$). The mechanism of protein stabilization by lyoprotectants involves the formation of an amorphous glass during lyophilization. It is the extreme viscosity in the glassy state that increases protein stability by decreasing the rate of interconversion of conformational substrates and conformational relaxation of the protein [26]. Since a higher Tg generally results in more stable protein formulations during lyophilization, sugars can increase the Tg of protein formulations [4]. Compared to lactose, trehalose seems to be a preferable cryoprotectant for biologically active macromolecules because trehalose does not have internal hydrogen bonds, which allow for

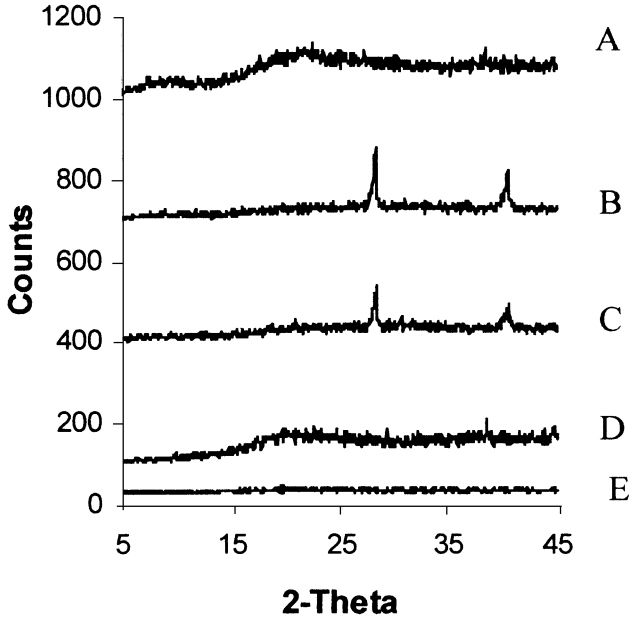

Fig. 4. The X-ray diffraction pattern of different samples investigated. Bulk insulin (A); SFL insulin (B); SFL insulin/tyloxapol (C); SFL insulin/tyloxapol/lactose (D); and SFL insulin/tyloxapol/trehalose (E).

Table 3
Influence of processing and excipient type on stability of insulin

Sample	Percent A-21 desamido	Percent dimer
Bulk insulin prior to SFL	3.03	0.25
SFL insulin	3.04	0.36
SFL insulin/tyloxapol	3.06	0.25
SFL insulin/tyloxapol/lactose	3.72	0.19
SFL insulin/tyloxapol/trehalose	3.05	0.18

more flexible formation of hydrogen bonds with proteins. Also trehalose has very low chemical reactivity [27]. Therefore, insulin was more stable when processed with trehalose.

Aggregation also occurs with proteins and peptides. Two or more protein or peptide molecules are held together by non-polar forces and hydrogen bonds. For insulin molecules, monomers can aggregate to form a covalent insulin dimer (CID). The two insulin molecules in the dimer are held together by non-polar forces and four hydrogen bonds arranged as a β sheet structure between the two antiparallel COOH terminal strands of the B chain. In the presence of zinc ions, three insulin dimers are assembled into a hexameric organization. The percentage of CID in both SFL processed samples and bulk insulin samples are shown in Table 3. Bulk insulin samples contained 0.25% CID. The CID concentration in the SFL insulin powder without excipients was 0.36% which is slightly higher than the CID level formed in the bulk insulin samples prior to SFL processing. The level of CID in the SFL insulin/tyloxapol sample was 0.25%, which is not different from the bulk insulin sample. However, the level of CID in the lactose and trehalose samples was not greater than the level of CID in the bulk insulin. Published studies have demonstrated that sugars can prevent aggregation of protein molecules. Hora et al. [28] reported that trehalose and lactose could inhibit dimer formation in tumor necorsis factor.

4. Conclusion

A novel particle engineering technology, spray freezing into liquid, used to generate porous microparticles containing a water soluble peptide was investigated and demonstrated. The particles are small, and have high surface area, low density and narrow particle size distribution. The results demonstrated that the SFL technology is feasible for the production of microparticles containing peptides and proteins.

Acknowledgements

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References

[1] J.J. Lin, J.D. Meyer, J.F. Carpenter, M.C. Manning, Stability of human serum albumin during bioprocessing: denaturation and aggregation during processing of albumin paste, Pharm. Res. 17 (2000) 391–396.
[2] M. Mackay, J. Phillips, J. Hastewell, Peptide drug delivery: colonic and rectal absorption, Adv. Drug Deliv. Rev. 28 (1997) 253–273.
[3] J. Kerc, S. Srcic, Z. Knez, P. Sencar-Bozic, Micronization of drug using supercritical carbon dioxide, Int. J. Pharm. 182 (1999) 33–39.
[4] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (2000) 1–60.
[5] K.A. Johnson, Preparation of peptide and protein powders for inhalation, Adv. Drug Deliv. Rev. 26 (1997) 3–15.
[6] S.D. Yeo, P.G. Debenedetti, S. Patro, T.M. Przybycien, Secondary structure characterization of microparticulate insulin powders, J. Pharm. Sci. 83 (12) (1994) 1651–1656.
[7] E. Reverchon, Supercritical antisolvent precipitation of micro- and nano-particles, J. Supercrit. Fluids 15 (1999) 1–21.
[8] M.A. Winters, B.L. Knutson, P.G. Debenedetti, H.G. Sparks, T.M. Przybycien, C.L. Stevenson, S.J. Prestrelski, Precipitation of proteins in supercritical carbon dioxide, J. Pharm. Sci. 85 (6) (1996) 586–594.
[9] B.S. Chang, B.S. Kendrick, J.F. Carpenter, Surface-induced denaturation of proteins during freezing and its inhibition by surfactants, J. Pharm. Sci. 85 (1996) 1325–1330.
[10] C.C. Hsu, H.M. Nguyen, D.A. Yeung, D.A. Brooks, G.S. Koe, T.A. Bewley, R. Pearlman, Surface denaturation at solid-void interface – a possible pathway by which opalescent particulates from during the storage of lyophilized tissue – type plasminogen activator at high temperatures, Pharm. Res. 12 (1995) 69–77.
[11] M.C. Heller, J.F. Carpenter, T.W. Randolph, Protein formulation and lyophilization cycle design: prevention of damage due to freeze-concentration induced phase separation, Biotechnol. Bioeng. 63 (1999) 166–174.
[12] Z.H. Chang, J.G. Baust, Ultra-rapid freezing by spraying/plunging: pre-cooling in the cold gaseous layer, J. Microsc. 162 (1991) 435–444.
[13] F. Franks, The properties of aqueous solutions at subzero temperatures, in: F. Franks (Ed.), Water. A Comprehensive Treatise. Water and Aqueous Solutions at Subzero Temperatures, 7, Plenum Press, New York, 1982, pp. 215–339.
[14] J.A.N. Zasadzinski, A new heat transfer model to predict cooling rates for rapid freezing fixation, J. Microsc. 150 (1988) 137–149.
[15] M.C. Heller, J.F. Carpenter, T.W. Randolph, Effects of phase separating systems on lyophilized hemoglobin, J. Pharm. Sci. 85 (1996) 1358–1362.
[16] R.T. Bustami, H.K. Chan, F. Dehghani, N.R. Foster, Generation of micro-particles of proteins for aerosol delivery using high pressure modified carbon dioxide, Pharm. Res. 17 (2000) 1360–1366.
[17] M. Adler, M. Unger, G. Lee, Surface composition of spray-dried particles of bovine serum albumin/trehalose/surfactant, Pharm. Res. 17 (2000) 863–870.
[18] L.A. Dellamary, T.E. Tarara, D.J. Smith, C.H. Woelk, A. Adractas, M.L. Costello, H. Gill, J.G. Weers, Hollow porous particles in metered dose inhalers, Pharm. Res. 17 (2000) 168–174.
[19] B. Cynthia, C. Lombry, B. Preat, R. Vanvever, Influence of formulation excipients and physical characteristics of inhalation dry powders on their aerosolization performance, J. Controlled Release 70 (2001) 329–339.
[20] R. Vanbever, A. Ben-Jebria, J.D. Mintzes, R. Langer, D.A. Edwards, Sustained-release of insulin from insoluble inhaled particles, Proc. Int. Symp. Controlled Release Bioact. Mater. 25 (1998) 261–262.
[21] D. Edwards, A. Ben-Jebria, R. Langer, Recent advances in pulmonary drug delivery using large, porous inhaled particles, J. Appl. Physiol. 84 (1998) 379–385.
[22] D.A. Edwards, J. Hanes, G. Caponetti, J. Hrkach, A. Ben-Jebria, M.L. Eskew, J. Mintzes, D. Deaver, N. Lotan, R. Langer, Large porous particles for pulmonary drug delivery, Science 276 (1997) 1868–1871.
[23] J.F. Carpenter, J.H. Crowe, T. Arakawa, Comparison of solute-induced protein stabilization in aqueous solution and in frozen and dried state, J. Dairy Sci. 73 (1990) 3627–3636.
[24] N.B. Bam, T.W. Randolph, J.L. Cleland, Stability of protein formulations: investigation of surfactant effects by novel EPR spectroscopic technique, Pharm. Res. 12 (1995) 2–11.
[25] M.D. Womack, D.A. Kendall, R.C. MacDonald, Detergent effects on enzyme activity and solubilization of lipid bilayer membranes, Biochim. Biophys. Acta 733 (2) (1983) 210–215.
[26] S.J. Hagen, J. Hofrichter, W.A. Eaton, Germinate rebinding and

conformational dynamics of myoglobin embedded in a glass at room temperature, J. Phys. Chem. 100 (1996) 12008–12021.
[27] B. Roser, Trehalose drying: a novel replacement for freeze-drying, Biopharmaceutics 4 (9) (1991) 47–53.
[28] M.S. Hora, R.K. Rana, F.W. Smith, Lyophilized formulations of recombinant tumor necrosis factor, Pharm. Res. 9 (1999) 33–36.
[29] A.H. Lefebvre (Ed.), Atomization and Sprays Hemisphere Publishing Corp, Washington, DC, 1989, pp. 89–103.