Pharmaceutical Research, Vol. 25, No. 12, December 2008 (© 2008)
DOI: 10.1007/s11095-008-9647-7

Research Paper

Formation of Zinc–Peptide Spherical Microparticles During Lyophilization from *tert*-Butyl Alcohol/Water Co-solvent System

Feng Qian,[1,4] Nina Ni,[1] Jia-Wen Chen,[2] Sridhar Desikan,[3] Vijay Naringrekar,[1] Munir A. Hussain,[1] Nancy P. Barbour,[1] and Ronald L. Smith[1]

Received April 18, 2008; accepted May 30, 2008; published online June 14, 2008

Purpose. To understand the mechanism of spherical microparticle formation during lyophilizing a *tert*-Butyl alcohol (TBA)/water solution of a zinc peptide adduct.
Method. A small peptide, PC-1, as well as zinc PC-1 at (3:2) and (3:1) ratios, were dissolved in 44% (wt.%) of TBA/water, gradually frozen to −50°C over 2 h ("typical freezing step"), annealed at −20°C for 6 h ("annealing step"), and subsequently lyophilized with primary and secondary drying. Zinc peptide (3:1) lyophile was also prepared with quench cooling instead of the typical freezing step, or without the annealing step. Other TBA concentrations, i.e., 25%, 35%, 54% and 65%, were used to make the zinc peptide (3:1) adduct lyophile with the typical freezing and annealing steps. The obtained lyophile was analyzed by Scanning Electron Microscopy (SEM). The zinc peptide solutions in TBA/water were analyzed by Differential Scanning Calorimeter (DSC). The surface tension of the TBA/water co-solvent system was measured by a pendant drop shape method.
Results. With typical freezing and annealing steps, the free peptide lyophile showed porous network-like structure that is commonly seen in lyophilized products. However, with increasing the zinc to peptide ratio, uniform particles were gradually evolved. Zinc peptide (3:1) adduct lyophiles obtained from 25%, 35% and 44% TBA exhibit a distinctive morphology of uniform and spherical microparticles with diameters of ~3–4 μm, and the spherical zinc peptide particles are more predominant when the TBA level approaches 20%. Adopting quench cooling in the lyophilization cycle leads to irregular shape fine powders, and eliminating the annealing step causes rough particles surface. When TBA concentration increases above 54%, the lyophiles demonstrate primarily irregular shape particles.
Conclusions. A proposed mechanism of spherical particle formation of the 3:1 zinc peptide encompasses the freezing of a TBA/water solution (20–70% TBA) causing the formation of a TBA hydrate phase ("dispersed TBA hydrate"). Decreasing the temperature further causes the formation of a eutectic mixture between TBA hydrate ("eutectic TBA hydrate") and water. Due to its low aqueous solubility, the zinc peptide adduct accumulates in both of the dispersed and eutectic TBA hydrate phases to form a hydrophobic "oil" phase. Since the eutectic TBA hydrate phase is surrounded by ice, a "solid emulsion" forms to lower the interfacial energy, and gives rise to spherical zinc peptide particles upon solvent sublimation. Possibility of liquid–liquid phase separation during freeze-drying was also investigated, and no evidence was found to support this alternative mechanism.

KEY WORDS: eutectic system; lyophilization; phase diagram; spherical particles; *tert*-butyl alcohol (TBA).

INTRODUCTION

Lyophilization or freeze-drying of pharmaceutical solutions is a widely used process for the preparation of parenteral products (1,2). Although most lyophiles are made from aqueous solution, lyophilization with pure organic solvent (3,4) or organic/water co-solvents (5–13) were reported in some cases where such solvents provided process or formulation advantages, such as improved drug stability (3,4), short drying time (6,7), and increased solubility of hydrophobic drugs (10,13).

Most lyophilization products are amorphous, and exhibit porous and network-like physical appearance under microscopy. Non-aqueous solvents, such as isopropanol (IPA) and water co-solvent, or *tert*-Butyl alcohol (TBA) and water co-solvent, were also reported to be able to promote the crystallization of Cephalothin sodium (8) and Cefazolin sodium (14), respectively, so as to yield physically and chemically more stable products than the amorphous lyophiles (15).

tert-Butyl alcohol (TBA) and water combination is the most extensively evaluated non-aqueous co-solvent system,

[1] Biopharmaceutics R&D, Bristol-Myers Squibb Company, New Brunswick, New Jersey 08903, USA.
[2] Department of Industrial & Physical Pharmacy, Purdue University, West Lafayette, Indiana 47906, USA.
[3] Biopharmaceutics and Analytical R&D, Bristol-Myers Squibb India Private Limited, Biocon Park, Jigani Link Road, Bommasandra IV, Bangalore 560099, India.
[4] To whom correspondence should be addressed. (e-mail: feng.qian1@bms.com)

and it has been used in the manufacture of marketed injectable pharmaceutical products, such as CAVERJECT® Sterile Powder (5). TBA freezes completely in most commercial freeze-dryers, readily sublimes during primary drying due to its high vapor pressure, and possesses low toxicity. All of the above advantages contribute to the attractiveness of the TBA/water as a solvent system for lyophilization (5).

PC-1, a proprietary compound (PC) of Bristol-Myer Squibb Company is an amorphous polypeptide whereby its apparent aqueous solubility decreases dramatically in the presence of zinc, due to the formation of an amorphous adduct (termed "zinc PC-1"). Water is not a feasible solvent to make zinc PC-1 lyophile due to poor aqueous solubility of the adduct (<1 μg/mL for 3:1 zinc/PC-1 ratio). However, TBA/water co-solvent with 20–70% of TBA (weight percent in this report) offers satisfactory zinc PC-1 solubility (>2 mg/mL PC-1 concentration). A lyophilization process was investigated, wherein PC-1 and zinc PC-1 at (3:2) and (3:1) ratios were dissolved in 44% TBA/H_2O co-solvent to form a clear solution, gradually frozen to −50°C over 2 h ("typical freezing step"), annealed at −20°C for 6 h ("annealing step"), and subsequently lyophilized with primary and secondary drying steps.

A unique morphology of uniform and spherical particles evolved with the increase of zinc–peptide ratio. The zinc PC-1 (3:1) lyophile exhibits a very distinctive morphology of smooth surface particles that are spherical in shape, and have narrow particle size distribution of ~3–4 μm. The purpose of the current study aimed to investigate the mechanism of this unique particle formation phenomenon.

MATERIALS AND METHODS

Materials

PC-1 was synthesized by Bristol-Myers Squibb Company. Zinc acetate was purchased from Spectrum (Gardena, CA, USA). TBA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water for injection was obtained from B/Braun (Irvine, CA, USA).

Lyophilization of PC-1 and Zinc PC-1 Solution in TBA/Water

Solutions of PC-1 or zinc PC-1 (zinc peptide molar ratio 3:2 or 3:1) were prepared by weighing the predetermined amount of zinc acetate and PC-1 into TBA/water co-solvent (20–70% of TBA), followed by vortexing and stirring. Clear solutions were obtained and the concentration of the final solutions was 5–10 mg/mL (with respect to PC-1). The solution was filled into 5 mL glass tubing vials and lyophilized by a Virtis Genesis 25EL lyophilizer (Gardiner, NY, USA). Most samples were prepared with the following cycle:

1. Freeze the solution to −50°C in 2 h (typical freezing).
2. Ramp the temperature to −20°C and hold for 6 h (annealing step).
3. Ramp the temperature to −50°C in 1 h and hold for 4 hour.
4. Turn on the vacuum, and control it at 200 microns through the whole cycle. Ramp the temperature to −30°C in 1 h and hold for 12 h. Then ramp the temperature to −20°C in 1 h and hold for 12 h (primary drying).
5. Ramp the temperature to 5°C in 1 h and hold for 6 h. Then ramp to 35°C in 2 h and hold for 12 h (secondary drying).

Some samples were prepared by modified lyophilization cycles, where the freezing step was replaced by a quench cooling with liquid N_2, or the annealing step was eliminated. The residual TBA concentration in all of the final lyophilizes is below 2% (*w*/*w*).

Differential Scanning Calorimetry (DSC)

Zinc PC-1 (3:1) solutions (10 mg/mL) in various ratios of TBA/water were prepared. Approximately 10 mg of each solution was transferred into an aluminum pan that was sealed by an aluminum cap. A similarly prepared empty sample pan was used as the reference. A TA DSC Q1000 Differential Scanning Calorimeter equipped with a Refrigerated Cooling System (New Castle, DE, USA) was used for characterization of samples. The temperature scanning rate was 2.5°C/min for all samples. To mimic the "typical freezing step", some samples were frozen at −50°C first, then annealed at −20°C for 6 h before being finally heated to 40°C. Some other samples were used to analyze the effect of annealing, where frozen solutions were heated to 40°C at 2.5°C/min directly without the annealing step.

Scanning Electron Microscopy (SEM)

A scanning electron microscope (Philips XL 30ESEM, FEI Philips, Hillsboro, OR, USA) was used to study the morphology of zinc PC-1 lyophiles. Before observation with SEM, the lyophiles were mounted on the aluminum stubs by double-sided tape and sputter coated with Pd (Pelco SC-7 Auto Sputter Coater). The SEM analysis was carried out at an accelerating voltage of 20 kV.

Surface Tension Measurement of TBA/Water Co-solvent

Surface tension of the TBA/water co-solvent was measured at room temperature by a KRÜSS DSA 10 MK2 Drop

Table I. Solubility of PC-1 in Various Ratio of TBA/Water Mixture

	Ratio of TBA/water mixture									
TBA percentage in water (wt.%)	8	16	25	35	44	54	65	76	88	100
Solubility of PC-1 (mg/mL)	<7	<7	<13	~24	~55	~64	~45	<40	<40	<7

PC-1 is less soluble in either pure water or pure TBA. The highest solubility was obtained in ~50 wt.% of TBA.

Shape Analysis System (Matthews, NC, USA). A pendant drop shape method was used and the measurement was duplicated for each TBA/water ratio.

RESULTS AND DISCUSSION

Morphology of the PC-1 and Zinc PC-1 Lyophiles

As listed in Table I, the solubility of PC-1 reaches the maximal of ~60 mg/mL in 40–50% TBA, and the value is lower in either pure water or TBA. Zinc binds reversibly with PC-1 to form zinc PC-1 adduct, and the apparent aqueous solubility of zinc PC-1 decreases continuously with the increase of zinc peptide ratio. At 3:1 ratio, the aqueous solubility of zinc PC-1 is about two orders of magnitude lower than the free PC-1. However, the zinc PC-1 dissociates in TBA/water co-solvents where PC-1 solubility is high (such as 44% TBA/water), which enables the favorable lyophilization process starting with a clear, homogenous solution.

After lyophilization from 44% TBA/water solution with the typical freezing, annealing and drying steps (i.e., Step 1–5 in "MATERIALS AND METHODS"), the PC-1, 3:2 zinc PC-1 and 3:1 zinc PC-1 lyophiles were examined visually and by SEM. The free PC-1 lyophile has the appearance of a porous and uniform lyophilized cake, which maintains its shape and volume after gentle shaking. Under SEM (Fig. 1A and B), the PC-1 lyophile shows typical network-like amorphous structure of a lyophilized material. The zinc PC-1 lyophiles (both 3:2 and 3:1) also appear to be uniform, but their cake volume and structure are less well preserved after drying. The lyophilized cakes collapse into powder with gentle shaking, which is more evident in the 3:1 zinc PC-1 product. Under SEM, uniform particles started to form at 3:2 zinc–peptide ratio (Fig. 1C and D), although they are less spherical and smooth than those from the 3:1 ratio (Fig. 1E and F). Although some irregular shape fine particles (mostly <1 μm) disperse throughout the powder bed, spherical particles with a narrow size distribution around 3–4 μm were

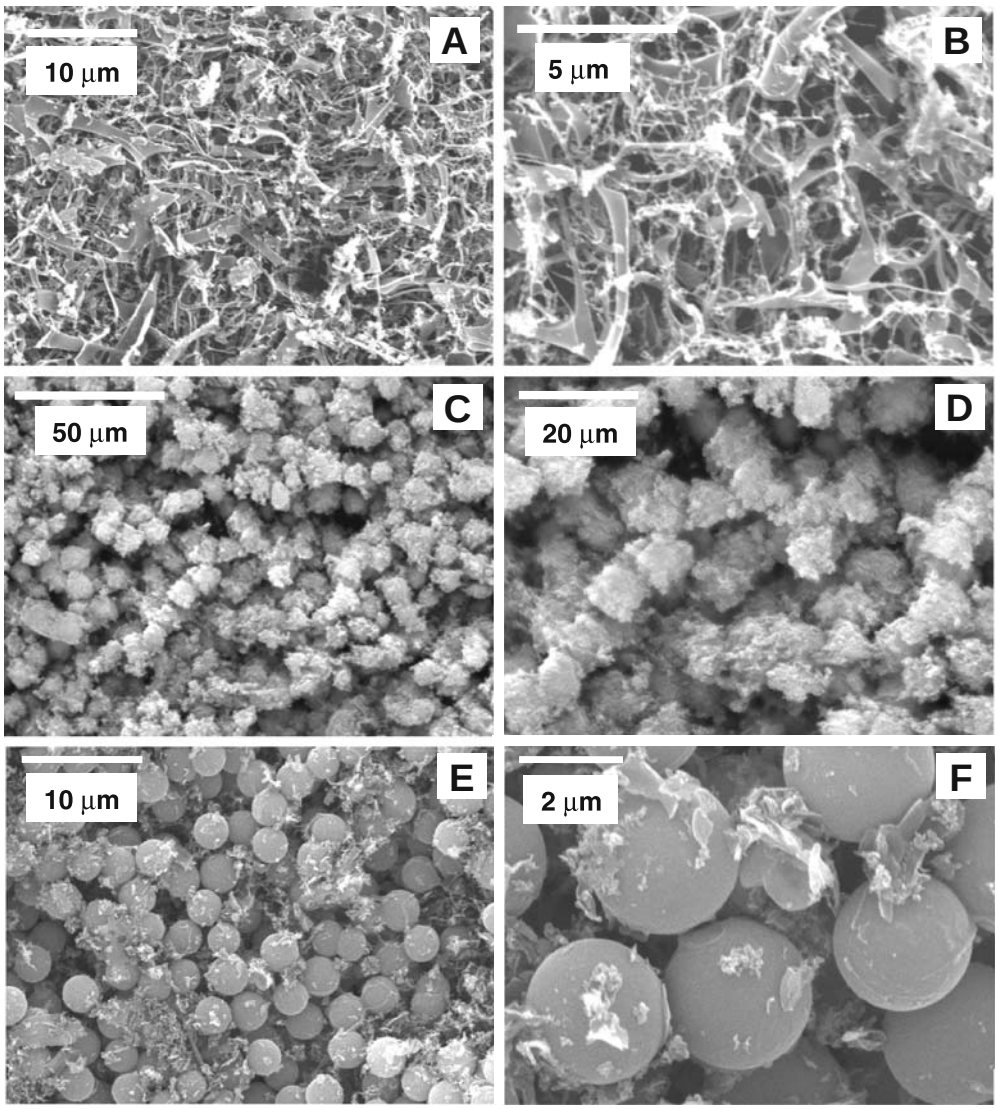

Fig. 1. Lyophilized PC-1 (**A**, **B**), 3:2 zinc PC-1 (**C**, **D**), and 3:1 zinc PC-1 (**E**, **F**). The materials were dissolved in 44% TBA/water co-solvent first to form clear solution (10 mg/mL PC-1 concentration). The solution was then lyophilized with the "typical freezing"; "annealing", primary and secondary drying steps (see "MATERIALS AND METHODS" section).

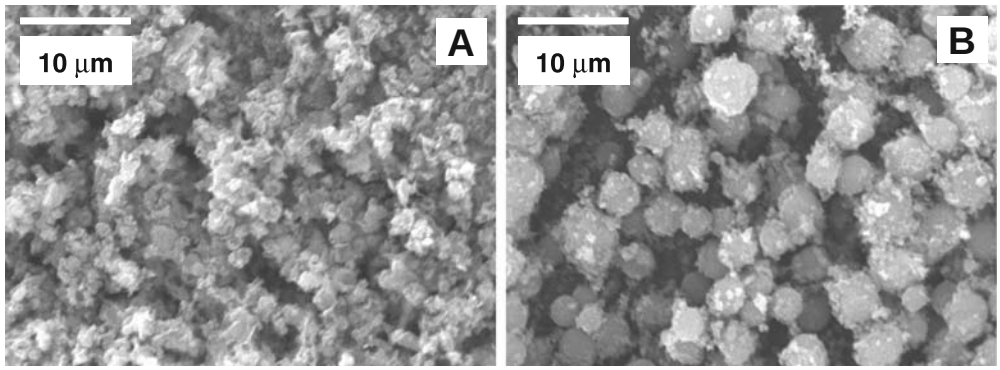

Fig. 2. Zinc PC-1 (3:1) lyophile obtained by: **A** Quench cooling with liquid N_2, followed by same primary and secondary drying steps. **B** Typical 2-h freezing, eliminated the annealing step, followed by same primary and secondary drying steps.

dominant in the 3:1 zinc PC-1 lyophile. Overall, the evolution of spherical particle is accompanied by the increase of zinc to PC-1 ratio, or the decrease of the apparent aqueous solubility of zinc PC-1.

Effects of Freezing History on the Morphology of Zinc PC-1 Lyophiles

In order to study the dependence of spherical microparticle formation on cooling and annealing, modified lyophilization cycles were also used to generate the 3:1 zinc PC-1 lyophiles. In one modified lyophilization cycle, the zinc PC-1 solution was first frozen with liquid N_2 instead of the 2-h, "typical freezing step", and then subjected to primary drying immediately without annealing. As shown in Fig. 2A, the lyophile did not retain the spherical microparticles as obtained with the more gradual, "typical freezing step" (Fig. 1E and F); instead, much finer and irregular shape particles were generated. In another modified lyophilization cycle, the "annealing step" was eliminated while other steps were preserved. The particles obtained thereafter are somewhat spherical but their surface is much rougher, with many fines existing in the lyophile (Fig. 2B).

Apparently, the formation of zinc PC-1 spherical microparticles is also dependant on the thermal history of the frozen solution prior to the primary drying. A slow, 2-h freezing step combined with a 6-h annealing step renders spherical zinc PC-1 (3:1) particles as shown in Fig. 1E and F, whereas, omitting any of the two steps eliminates the spherical particle formation (Fig. 2A) or results in fine, irregular powders on the particle surface (Fig. 2B). It appears that an equilibrated frozen state of zinc PC-1 in TBA/water is a prerequisite for spherical microparticle formation. Since the solution used for lyophilization only contains ≤10 mg/mL (1 wt.%) zinc PC-1, the freezing behavior of TBA/water binary system may explain the observed behavior, which led to further study of the TBA/water phase diagram.

The Frozen Structure of TBA/Water Binary System

Kasraian and DeLuca reported a TBA/water binary phase diagram (16), shown in Fig. 3A, whereby TBA forms a hydrate that contains 70% TBA, and the TBA hydrate forms eutectic mixture with either pure water or TBA. Shown in the phase diagram, Eutectic A (20% TBA) is the eutectic mixture between water and TBA hydrate, and Eutectic B (90% TBA) is between TBA hydrate and pure TBA.

In this present study, only TBA/water solutions with 20–70% of TBA (between Eutectic A and TBA hydrate) were used for lyophilization, due to the otherwise low solubility of zinc PC-1 in the TBA/water system. When freezing a TBA/water co-solvent within this composition range, e.g., 44%, the following events occur sequentially:

1. A TBA hydrate phase forms first in TBA/water solution once the temperature drops below the phase boundary between liquid and liquid/TBA hydrate (~3°C, point "1" in Fig. 3A). Since the TBA hydrate phase crystallizes from solution without extra size

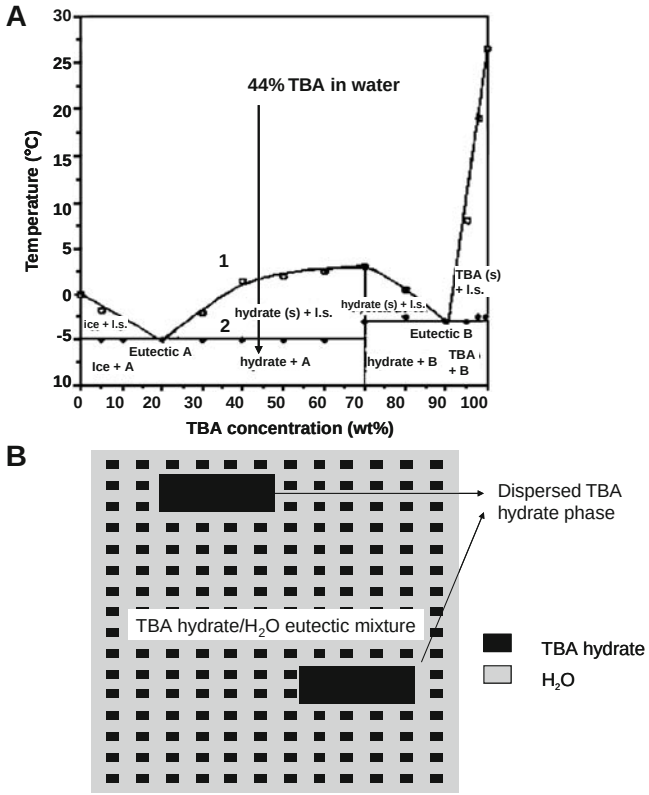

Fig. 3. A Phase diagram of the TBA/water system (16). **B** An illustration of the frozen structure of 44% TBA/water based on the TBA/water phase diagram.

limitation, relatively large crystals form, which is defined as "dispersed TBA hydrate". The "dispersed TBA hydrate" was observed by cold-stage microscopy and was reported to be needle shape large crystals (16).

2. When the temperature further decreases towards the eutectic melting temperature (−5°C, point "2" in Fig. 3A), more dispersed TBA hydrate crystallizes while the composition of the remaining TBA/water solution approaches Eutectic A.
3. Eutectic A, a eutectic mixture between ice and TBA hydrate (eutectic TBA hydrate), forms after the temperature drops below the eutectic melting temperature. Eutectic A was reported to be very fine crystalline mixture that can not be resolved under optical microscopy (16). This is expected since eutectic mixtures usually consist of very fine (~micrometer range) crystalline phases of each individual component, due to size reduction caused by the simultaneous crystallization process (17).

Finally, a phase separated frozen structure is obtained as illustrated in Fig. 3B, where the large blocks represent the dispersed TBA hydrate in Eutectic A (TBA hydrate/water eutectic mixture). Based on the phase diagram, one can conclude that within Eutectic A, the eutectic TBA hydrate is the dispersed phase (~30%) while water is the continuous phase (~70%). Therefore, the eutectic TBA hydrate phase in Eutectic A is represented by the isolated, small blocks that are surrounded by water. It's worth noting that there are only two chemically different phases in the frozen TBA/water: TBA hydrates (both dispersed and eutectic TBA hydrates) and water, and they were represented by the dark (TBA hydrate) and light (water) regions in Fig. 2B.

Validation of TBA/Water Phase Diagram in the Current Multi-phase System

The zinc PC-1 solution in TBA/water is a multi-component system that contains zinc acetate and PC-1. In order to validate the applicability of the TBA/water binary phase diagram in this system, zinc PC-1 (3:1) solutions (10 mg/mL) in 20% (Eutectic A composition), 44% and 70% (TBA hydrate composition) TBA were analyzed by DSC. In one set of experiments (Fig. 4A), all three solutions were frozen at −50°C, annealed at −20°C for 6 h, then heated to 40°C. The 20% TBA solution of zinc PC-1 showed a single melting endothermic peak at −7.9°C; the 44% solution showed two melting endothermic peaks at −6.8°C and −0.6°C, respectively; and the 70% solution showed a single melting peak at −0.7°C. These DSC results are in close agreement with the TBA/water binary phase diagram (Fig. 3A) even with the existence of zinc PC-1, which indicates that a small amount of zinc PC-1 (i.e., ≤1 wt.%) does not change the freezing behavior of TBA/water significantly.

Figure 4B showed DSC results of 10 mg/mL zinc PC-1 solution in 44% TBA/water. One sample was frozen to −50°C first, and then annealed for 6 h at −20°C before being heated to 30°C. The other sample was heated to 30°C immediately after freezing without the annealing step. Consistent with the literature (16), a sample without annealing showed two metastable phases that melt before the eutectic melting. This observation again supports that the small amount of zinc PC-1 does not alter the frozen habit of TBA/water significantly.

Origin of the Zinc PC-1 Spherical Microparticles

As mentioned earlier, the aqueous solubility of zinc PC-1 decreases dramatically with the increase of zinc ratio, and the apparent aqueous solubility of 3:1 zinc PC-1 is two orders of magnitude lower than the free PC-1. However, due to the much higher solubility of PC-1 in some TBA/water co-solvents, zinc PC-1 dissociates readily and is effectively much more soluble in these co-solvents. We hypothesize that during the freezing and phase separation of TBA/water, zinc PC-1 has a preferred accumulation in the TBA containing phases rather than in the water phase, and this tendency is more evident with the increase of the zinc ratio, or the decrease of zinc PC-1 aqueous solubility.

In frozen and equilibrated TBA/water (i.e., after annealing), zinc PC-1 should be rich in the TBA hydrate phases. Since both the dispersed TBA hydrate and the eutectic TBA hydrate have the same chemical composition (70% TBA), zinc PC-1 should have the same concentration in these two TBA hydrate phases. The remaining question would be which TBA hydrate phase is the origin of the spherical zinc PC-1 particles.

To address this question, TBA/water with different ratios, 25%, 35%, 54% and 65% were used to make zinc PC-1 (3:1) lyophilized cakes and their morphology is shown in

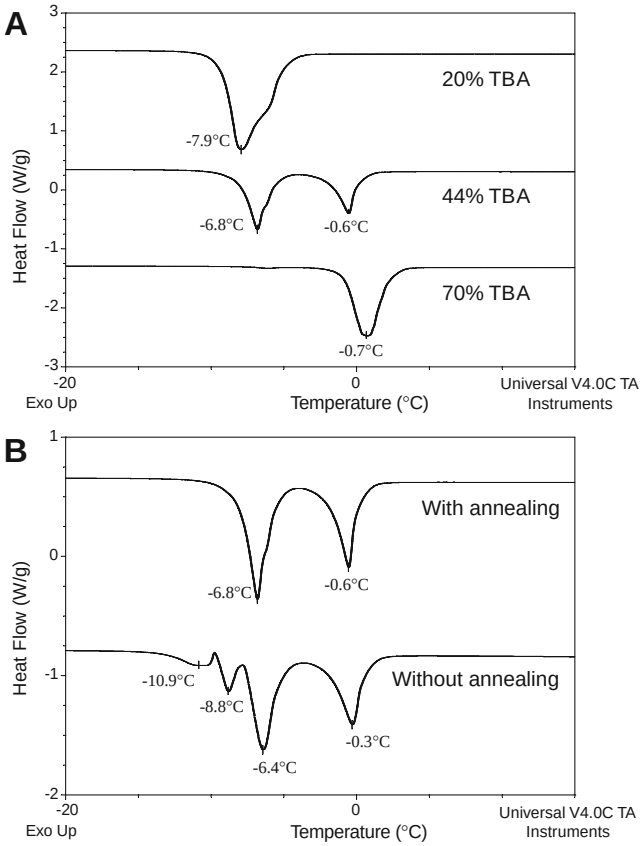

Fig. 4. A DSC of zinc PC-1 (3:1) in 20%, 44%, 70% TBA/H_2O solution (*w*/*w*). The samples were annealed at −20°C for 6 h (**B**), DSC of zinc PC-1 in 44% (*w*/*w*) TBA/H_2O solution, with or without annealing step.

Figure 5. Along with the 44% TBA/water that was also used (Fig. 1E and F), the investigated solvent ratio covers the entire range between Eutectic A composition (20% TBA) and the TBA hydrate composition (70% TBA) (Fig. 3A). Increasing TBA in the solution obviously increases the amount of dispersed TBA hydrate and decreases the amount of the eutectic TBA hydrate.

As the most striking observation (Figs. 1E, F and 5), increasing TBA apparently led to the diminishing of the 3–4 μm spherical particles: when TBA was increased to more than 54%, the lyophile became mostly irregular shape fine powder. The other observation is that the irregular fines appear to increase with the increase of TBA (see Figs. 5A, B and 1E where TBA was 25%, 35% and 44%, respectively). When 25% of TBA was used (Fig. 5A), almost the entire lyophile was 3–4 μm spherical microparticles with only a small amount of fines, whereas, the amount of fines increased proportionally with TBA, as shown in Figs. 5B and 1E where 35% and 44% of TBA were used, respectively.

These collective observations lead us to conclude that the zinc PC-1 (3:1) spherical microparticles stem from the eutectic TBA hydrate. Also, the size of the eutectic TBA hydrate, rather than the dispersed TBA hydrate, is more consistent with the size of the zinc PC-1 spherical particles. The exact domain size of the eutectic TBA hydrate crystals was not reported, but it could be in the micron range due to the physics of eutectic crystallization (17), while the dispersed TBA hydrate is much bigger (16).

The irregular shape fine powder in the lyophile could originate from the dissolved zinc PC-1 in the dispersed TBA hydrate phase, due to the observation that the amount of the fines increase with the TBA percentage. While in a non-equilibrium frozen TBA/water (i.e., without annealing), metastable phases exist that could also dissolve the zinc PC-1, and further give rise to fine powders upon solvent removal by vacuum. This could explain the presence of more fines and the rough particle surface in the lyophiles made without annealing (Fig. 2B).

Driving Force for the Zinc PC-1 Spherical Microparticles Formation

The formation of a curved interface (i.e., spherical particles) is caused by the interfacial tension between the two neighboring regions to lower the interfacial energy (18). In a frozen zinc PC-1 solution in TBA/water, zinc PC-1 accumulates in the dispersed and the eutectic TBA hydrate phases, the two interfacial tensions between each TBA hydrate phase and its environment are of interest for further investigation. For the eutectic TBA hydrate, the interface is the TBA hydrate *vs*. water; while for the dispersed TBA hydrate, the interface is the TBA hydrate *vs*. Eutectic A. The interfacial tension between two solid phases is difficult to determine, only the interfacial tension between two immiscible liquids can be estimated by the surface tension measurement.

Figure 6 shows the surface tension of TBA/water at room temperature. With the increase of TBA, the surface tension of TBA/water decreases from 74 mN/m (pure water)

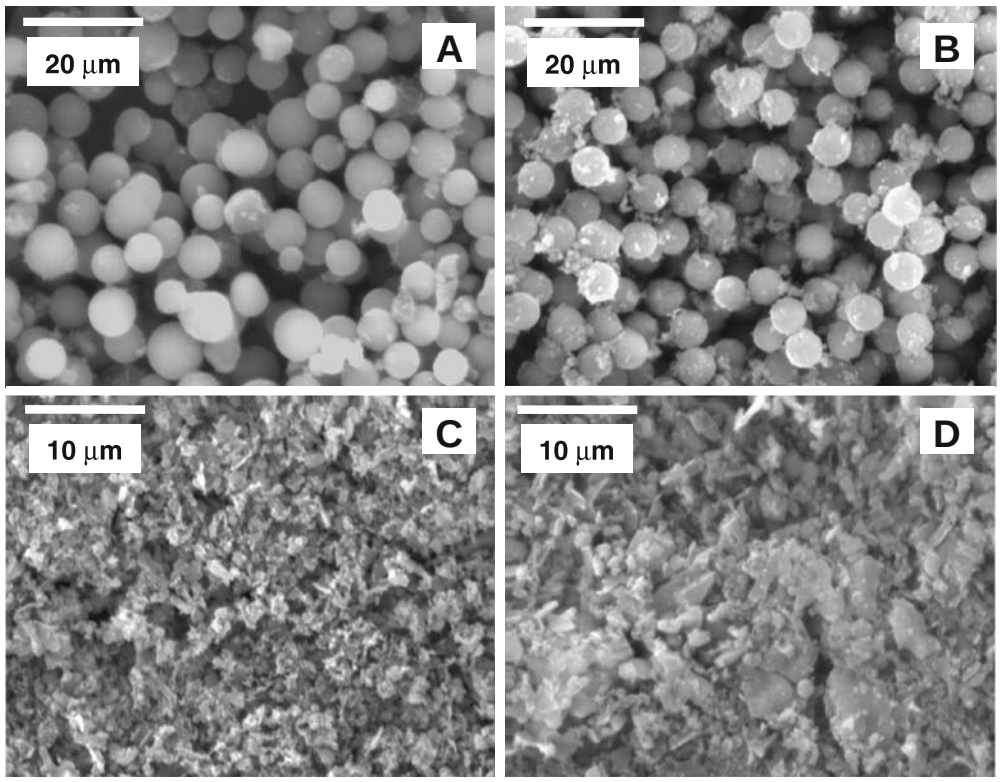

Fig. 5.. Lyophilized zinc PC-1 (3:1) with 25% (**A**), 35% (**B**), 54% (**C**), 65% (**D**) of TBA in water (*w*/*w*). The lyophilization process includes the "typical freezing"; "annealing", primary and secondary drying steps (see "MATERIALS AND METHODS" section). The *scale bars* represent 20 μm in **A** and **B**, and 10 μm in **C** and **D**.

to a plateau of 24 mN/m after TBA is at more than 30%. The following surface tension values are of particular interest: 74 mN/m (water), 29 mN/m (20% TBA, Eutectic A composition), and 24 mN/m (70% TBA, TBA hydrate composition). Thus, one conclusion is that the surface tension difference between 70% TBA (TBA hydrate composition) and water is much larger than that between 70% TBA and 20% TBA (Eutectic A composition). Assuming a similar relationship exists in frozen state, one could hypothesize that the interfacial tension between the eutectic TBA hydrate and water could be much larger than that between the dispersed TBA hydrate and eutectic A. Therefore, the eutectic TBA hydrate could form a "solid emulsion" in water, and the strong interfacial tension causes the formation of the spherical particles. It should be noted that TBA/water is miscible at all ratios and there is no interfacial tension between two TBA/water solutions. The above discussion is a theoretical hypothesis to explain the mechanism of spherical zinc PC-1 particle formation under the conditions of this study. A more definitive understanding may be realized through additional experiments.

Other Potential Mechanism: Liquid–Liquid Phase Separation

In a multi-component solution, when enthalpical expulsion between the incompatible components overcomes the entropy gained by complete mixing, liquid–liquid phase separation occurs. A single phase solution is thermodynamically favorable when the concentration of the incompatible components is low, while liquid–liquid phase separation occurs once a critical concentration of these components is reached (12,19–22). It was reported that during the freezing of a solution, the solutes can reach concentrations as high as 20–50 times of their initial concentration (23–25). Therefore, during the freezing of a relatively dilute and single phase solution with liquid–liquid phase separation potential, critical concentration for phase separation could be reached sometimes.

Heller, Carpenter and Randolph demonstrated that a solution containing poly (ethylene glycol) (PEG) and dextran, which is initially below critical concentration for phase separation, did experience a liquid–liquid phase separation induced by freeze concentration (19–22). In one of their studies, a single phase phosphate buffer solution containing 4% PEG, 4% dextran and 10 mg/mL hemoglobin was lyophilized and the lyophile morphology showed somewhat spherical features that was attributed to the liquid–liquid phase separation during freezing (20).

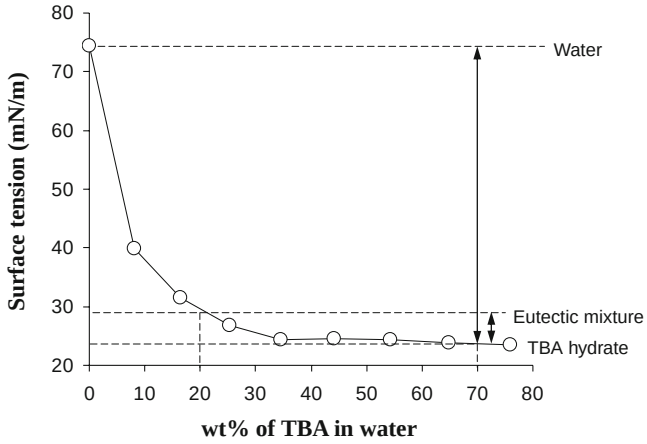

Fig. 6. Surface tension of TBA/water cosolvent system. The *longer double-arrow* represents the surface tension difference between water and 70% TBA/water (TBA hydrate composition), the *shorter double-arrow* represents the surface tension difference between 20% TBA/water (eutectic mixture composition) and 70% TBA/water (TBA hydrate composition).

Wittaya-Areekul and coworkers also reported liquid–liquid phase separation during the freeze drying of a tobramycin sulfate solution in TBA/water (12). By visual examination, the authors determined that TBA concentration above 20% and tobramycin sulfate concentration above 12% showed liquid–liquid phase separation at room temperature, where the solution separated into tobramycin sulfate-rich and TBA-rich phases. In frozen solutions, concentration of TBA below 8% and concentration of tobramycin sulfate below 8% result in a single-phase system, and higher concentration gives rise to two-layer frozen system, which subsequently leads to two-layered lyophilized cake after drying.

Considering the above, we have investigated if liquid–liquid phase separation occurred in our system; and if so; could this be a potential mechanism for the spherical zinc PC-1 microparticle formation.

First, acknowledging the freeze concentration potential, the difference between systems and processes, it is still worthwhile to note that the initial concentration of our solution is much lower than those where liquid–liquid phase separation occurred. In the TBA/water solution for lyophilization, the PC-1 concentration is 5–10 mg/mL (0.5–1 wt.%), and the zinc acetate concentration is <0.3% wt.%. While in the tobramycin sulfate TBA/water solution, at least 8% initial tobramycin sulfate concentration was needed to cause liquid–liquid phase separation (12). It was also reported that more than 5% of both PEG 3350 and dextran T500 are needed for phase separation to occur at 0°C (20).

Second, with visual examination of the frozen solution and the lyophilized cake, we did not see any macroscopic phase separation, such as two-layered system. All frozen solutions and lyophilized cakes appeared to be uniform, single phase system. Under SEM, the morphology of the spherical zinc PC-1 microparticles is dramatically different from what was reported earlier by others. Although it was reported that liquid–liquid phase separation during freeze drying did cause some degree of spherical feature in some lyophiles (20), such uniform and smooth microparticles of almost perfect spherical shape (Figs. 1E, F and 5A,B) in our system has not been reported before, and we consider the similarity between them and the prior observation as low.

Third, we investigated the possibility of liquid–liquid phase separation experimentally with similar procedure as Wittaya-Areekul *et al.* used (12). As discussed earlier, critical concentration of incompatible components have to be reached to obtain liquid–liquid phase separation. Therefore, PC-1 and zinc acetate were added into TBA/water of different ratio at room temperature both individually and in combination with each other until saturation, and the resultant solution was examined visually for liquid–liquid phase separation. We observed no sign of phase separation with either PC-1, or zinc acetate, or zinc PC-1 in combination.

Based on the above consideration and observation, we found no evidence of liquid–liquid phase separation in our

system. The collective facts, including the spherical particle formation only with 3:1 zinc PC-1, which has extremely low aqueous solubility (Fig. 1); and the correlation between the amount of spherical microparticles in the final lyophilized cake and the TBA/water ratio (Figs. 1 and 5), support our hypothesis that the spherical microparticle formation is due to the eutectic formation in the TBA/water system, and the preferred accumulation of the zinc PC-1.

CONCLUSION

In this work, a unique phenomenon was observed where uniform spherical microparticles of a zinc peptide adduct were obtained after lyophilization of a TBA/water solution. The mechanism of spherical particle formation was investigated. A hypothesis is proposed where phase separation occurs during the freezing of TBA/water solution; zinc peptide, whose aqueous solubility decreases with the increase of zinc peptide ratio, accumulates in the dispersed and the eutectic TBA hydrates to form a hydrophobic, oil phase. The eutectic TBA hydrate is surrounded by water and a "solid emulsion", similar to an oil-in-water emulsion, is formed. Spherical interface formed to lower the interfacial energy and spherical zinc peptide particles were obtained after solvent removal. No evidence was observed in this system to support a liquid–liquid phase separation, which could be a potential alternative mechanism. Understanding the mechanism of spherical particle formation during lyophilization of zinc PC-1 may offer new insights into future formulation applications.

REFERENCES

1. S. L. Nail, S. Jiang, S. Chongprasert, and S. A. Knopp. Fundamentals of freeze-drying. *Pharm. Biotechnol.* **14**:281–360 (2002).
2. N. A. Williams and G. P. Polli. The lyophilization of pharmaceuticals: a literature review. *J. Parenter. Sci. Technol.* **38**(2):48–59 (1984).
3. N. Ni, M. Tesconi, S. E. Tabibi, S. Gupta, and S. H. Yalkowsky. Use of pure *t*-butanol as a solvent for freeze-drying: a case study. *Int. J. Pharm.* **226**(1–2):39–46 (2001) Medline. DOI 10.1016/S0378-5173(01)00757-8.
4. D. W. Flamberg, D. L. Francis, S. L. Morgan, and G. F. Wickes. Low temperature vacuum drying of sterile parenterals from ethanol. *Bull. Parenter. Drug. Assoc.* **24**(5):209–217 (1970).
5. D. L. Teagarden and D. S. Baker. Practical aspects of lyophilization using non-aqueous co-solvent systems. *Eur. J. Pharm. Sci.* **15**(2):115–133 (2002) Medline, DOI 10.1016/S0928-0987(01)00221-4.
6. K. Kasraian and P. P. DeLuca. The effect of tertiary butyl alcohol on the resistance of the dry product layer during primary drying. *Pharm. Res.* **12**(4):491–495 (1995) Medline, DOI 10.1023/A:1016285425670.
7. J. Oesterle, F. Franks, and T. Auffret. The influence of tertiary butyl alcohol and volatile salts on the sublimation of ice from frozen sucrose solutions: implications for freeze-drying. *Pharm. Dev. Technol.* **3**(2):175–183 (1998) Medline, DOI 10.3109/10837459809028493.
8. C. Telang and R. Suryanarayanan. Crystallization of cephalothin sodium during lyophilization from *tert*-butyl alcohol–water cosolvent system. *Pharm Res.* **22**(1):153–160 (2005) Medline, DOI 10.1007/s11095-004-9021-3.
9. D. J. Van Drooge, W. L. Hinrichs, and H. W. Frijlink. Incorporation of lipophilic drugs in sugar glasses by lyophilization using a mixture of water and tertiary butyl alcohol as solvent. *J. Pharm. Sci.* **93**(3):713–725 (2004) Medline, DOI 10.1002/jps.10590.
10. Z. Wang, Y. Deng, and X. Zhang. The novel application of tertiary butyl alcohol in the preparation of hydrophobic drug–HPbetaCD complex. *J. Pharm. Pharmacol.* **58**(3):409–414 (2006) Medline, DOI 10.1211/jpp.58.3.0017.
11. J. Cui, C. Li, Y. Deng, Y. Wang, and W. Wang. Freeze-drying of liposomes using tertiary butyl alcohol/water cosolvent systems. *Int. J. Pharm.* **312**(1–2):131–136 (2006) Medline, DOI 10.1016/j.ijpharm.2006.01.004.
12. S. Wittaya-Areekul, G. F. Needham, N. Milton, M. L. Roy, and S. L. Nail. Freeze-drying of *tert*-butanol/water cosolvent systems: a case report on formation of a friable freeze-dried powder of tobramycin sulfate. *J. Pharm. Sci.* **91**(4):1147–1155 (2002) Medline, DOI 10.1002/jps.10113.
13. Y. Koyama, M. Kamat, R. J. De Angelis, R. Srinivasan, and P. P. DeLuca. Effect of solvent addition and thermal treatment on freeze drying of cefazolin sodium. *J. Parenter. Sci. Technol.* **42**(2):47–52 (1988).
14. M. J. Pikal, A. L. Lukes, J. E. Lang, and K. Gaines. Quantitative crystallinity determinations for beta-lactam antibiotics by solution calorimetry: correlations with stability. *J. Pharm. Sci.* **67**(6):767–773 (1978) Medline, DOI 10.1002/jps.2600670609.
15. M. J. Pikal, A. L. Lukes, and J. E. Lang. Thermal decomposition of amorphous beta-lactam antibacterials. *J. Pharm. Sci.* **66**(9):1312–1316 (1977) Medline, DOI 10.1002/jps.2600660927.
16. K. Kasraian and P. P. DeLuca. Thermal analysis of the tertiary butyl alcohol–water system and its implications on freeze-drying. *Pharm. Res.* **12**(4):484–490 (1995) Medline, DOI 10.1023/A:1016233408831.
17. K. A. Jackson and J. D. Hunt. Lamellar and rod eutectic growth. *Trans. Metall. Soc. AIME.* **236**:1129–1142 (1966).
18. A. Martin. *Physical Pharmacy.* Lippincott Williams & Wilkins, Baltimore, MD, 1993, pp. 362–368.
19. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Effects of phase separating systems on lyophilized hemoglobin. *J. Pharm. Sci.* **85**:1358–1362 (1996) Medline, DOI 10.1021/js960019t.
20. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Manipulation of lyophilization-induced phase separation: implications for pharmaceutical proteins. *Biotechnol. Prog.* **13**:590–596 (1997) Medline, DOI 10.1021/bp970081b.
21. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Protein formulation and lyophilization cycle design: prevention of damage due to freeze-concentration induced phase separation. *Biotechnol. Bioeng.* **63**:166–174 (1999) Medline, DOI 10.1002/(SICI)1097-0290(19990420)63:2<166::AID-BIT5>3.0.CO;2-H.
22. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Conformational stability of lyophilized PEGylated proteins in a phase-separating system. *J. Pharm. Sci.* **88**:58–64 (1999) Medline, DOI 10.1021/js980257j.
23. F. Franks. Freeze-drying: from empiricism to predictability. The significance of glass transitions. *Dev. Biol. Stand.* **74**:9–18 (1992).
24. F. Franks. Freeze-drying: a combination of physics, chemistry, engineering and economics. *Jpn. J. Freezing Drying.* **38**:5–16 (1992).
25. R. H. Hatley and A. Mant. Determination of the unfrozen water content of maximally freeze-concentrated carbohydrate solutions. *Int. J. Biol. Macromol.* **15**:227–232 (1993) Medline, DOI 10.1016/0141-8130(93)90042-K.