Component Crystallization and Physical Collapse during Freeze-Drying of L-Arginine–Citric Acid Mixtures

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Component crystallization and physical collapse during freeze-drying of aqueous solutions containing protein-stabilizing L-arginine and citric acid mixtures were studied. Freeze-drying microscopy (FDM) and thermal analysis of the solute-mixture frozen solutions showed collapse onset at temperatures (Tc) approximately 10°C higher than their Tg’s (glass transition temperatures of the maximally freeze-concentrated solute phase). Experimental freeze-drying of these solutions at a low chamber pressure showed the occurrence of physical collapse at shelf temperatures close to or slightly higher than the Tc. Slower ice sublimation at higher chamber pressures induced the physical collapse from lower shelf temperatures. The large effect of chamber pressures on the collapse-inducing shelf temperatures confirmed significance of the sublimation-related heat loss on the sublimation interface temperature during the primary drying. Drying of the single-solute L-arginine solution resulted in cake-structure solids composed of its anhydrous crystal. Thermal and powder X-ray diffraction (PXRD) analysis suggested slow crystal nucleation of L-arginine dihydrate in the frozen solutions. Characterization of the frozen solutions and freeze-dried solids should enable rational formulation design and process control of amino acid-containing lyophilized pharmaceuticals.

Key words freeze-drying; crystallization; glass; lyophilization; collapse; thermal analysis

Clinical applications of protein pharmaceuticals such as therapeutic antibodies are increasing. However, marginal storage stabilities of many proteins in the aqueous solutions emphasize the importance of developing freeze-dried formulations. Many lyophilized protein formulations contain disaccharides (e.g., sucrose and trehalose) that protect the proteins thermodynamically from structural changes during the freeze-drying process and kinetically from chemical degradation during subsequent storage. Some basic amino acid (e.g., L-arginine and L-histidine) and multivalent acid (e.g., H3PO4 and citric acid) mixtures are practical alternative stabilizers for protein formulations. The mixtures protect proteins from conformation-altering dehydration stress during the process. These mixtures form glass-state amorphous solids through networks of hetero-component molecular interactions between the amino and carboxyl groups, and thus improve chemical stability of the dried proteins during storage. L-Arginine also increases solubility of various proteins without apparent changes in their conformation.

Freeze-drying is a typical high-energy process, and varying its parameters significantly varies the formulation quality, including the cake appearance, component crystallinity, residual water, and stability of the active pharmaceutical ingredients (APIs). Failure of a biopharmaceutical lyophilization batch would lead to the loss of the therapeutic protein often more expensive than small molecular APIs. Freezing of aqueous solutions concentrates most solutes to a similar degree (70–80% w/w), regardless of their initial concentrations. Thus the physical properties of multi-solute frozen solutions (e.g., the solute crystallinity, crystal polymorph, and transition temperature of freeze-concentrated solutes) largely depend on their composition. Proteins and excipients are required to be concentrated into the non-crystalline solute-mixture phase to achieve the stabilizing effects.

Among the three segments comprising the lyophilization process, (freezing, primary drying, and secondary drying), optimization of the product temperature during the time-consuming ice-sUBLIming primary drying segment, by controlling the shelf temperature and chamber pressure is inevitable for achieving appropriate formulation quality and process efficiency. A higher product temperature during the primary drying allows faster ice sublimation, whereas decreased viscosity of the non-crystalline concentrated solute phase increases the risk of solid structure change (collapse) that starts from the sublimation interface. It is desirable to run the primary drying segment by maintaining the frozen solution, particularly the ice sublimation interface, slightly below the “highest allowable product temperature” of the solution to ensure process efficiency and product quality. Structurally disordered solids are usually not pharmacologically acceptable. This is because of their inelgant appearance and other changes that potentially affect functions of the formulation (e.g., higher residual water, pH change, slower dissolution, solute crystallization), although the collapse phenomena usually exerts only limited direct damage to the protein structure. The (glass transition temperature of maximally freeze-concentrated solute phase) obtained through thermal analysis has often been used as surrogate of the collapse temperature (Tc). Recent advances in freeze-drying microscopy (FDM) analysis have enabled direct observation of the collapse phenomena of frozen solutions in a small reduced-pressure cell that mimic the primary drying process. FDM analysis of frozen disaccharide solutions has shown onset of the collapse (Tc) at temperatures several degrees higher than Tg. The use of higher targeting product temperatures based on the FDM data should reasonably allow faster ice sublimation and thus reduce...
the segment time.

The objective of this study was to characterize the physical properties of frozen L-arginine and citric acid mixture solutions and their dried solids by thermal analysis ($T_d$), FDM observation ($T_r$), experimental freeze-drying, and powder X-ray diffraction (PXRD) analysis. It was expected that the high transition temperatures ($T_g$) of the L-arginine mixture frozen solutions would allow efficient primary drying at higher product temperatures. However, the different molecular interactions contributing to glass formation of the disaccharide and the amino acid–carboxylic acid mixture systems require the assessment of the relationship between the thermal transition and collapse phenomena for formulation and process development. Crystallization of L-arginine in frozen aqueous solutions was also studied.

Experimental

Materials L-Arginine and citric acid used in this study were of analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Thermal Analysis Thermal analysis of frozen solutions was conducted using a differential scanning calorimeter (DSC Q-10, TA Instruments, New Castle, DE, U.S.A.) with Universal Analysis 2000 software (TA Instruments). An aliquot ($10\mu L$) of aqueous solution in an aluminum cell was cooled to $-60°C$ at $5°C/min$ and then scanned at a heating rate of $1°C/min$. $T_g$ of the frozen solutions were determined from the maximum inflection point of the discontinuities in the heat flow curves. Larger volume solutions ($50\mu L$) were used to study the L-arginine crystallization process. Some frozen solutions were heat-treated by pausing the first heating scan at $-20°C$ and maintained at that temperature for 30 or 480 min. Thermograms were obtained during the second heating scans from $-60°C$. Thermal analysis of the freeze-dried solids was performed by heating the samples (1.03–1.53 mg) in open aluminum cells from $-10°C$ at $5°C/min$.

Freeze-Drying Microscopy An FDM system (Lyostat 2, Biopharma Technology Ltd., Winchester, U.K.) with an optical microscope (Model BX51, Olympus Co., Tokyo, Japan) was used to observe the behavior of the frozen aqueous solutions under vacuum. The sample temperature sensor was calibrated using the melting temperatures of ice, naphthalene crystal, and eutectic NaCl crystal as standards. Aqueous solutions (2 $\mu L$) sandwiched between cover slips (70 $\mu m$ apart) were frozen at $-40°C$ and maintained at that temperature for 5 min. Each sample was heated under vacuum (0.06 mbar) at $5°C/min$ to a temperature approximately $5°C$ below its $T_g$ and then scanned at $1°C/min$. The observation field was moved during the scan to follow the ice sublimation front. The $T_g$ of the frozen solution was determined by the appearance of translucent dots behind the ice sublimation interface ($n=3$).

Freeze-Drying A freeze-drier (Freezone-6, Labconco, Kansas City, MO, U.S.A.) equipped with temperature-controlling trays was used for lyophilization. The aqueous solutions (2.0 mL), in flat-bottomed borosilicate glass vials (60 vials, 21-mm diameter, SVF-10, Nichiden-rika Glass Co., Kobe, Japan), were placed on the shelves of the freeze-drier at room temperature. The shelves were cooled to $-36°C$ at $0.5°C/min$ and then held at that temperature for 2 h to freeze the aqueous solutions. Subsequently, the shelves were heated to the designated temperatures for primary drying at $0.2°C/min$, and then held at that temperature for $2h$ before applying vacuum for primary drying ($0.04\text{ mbar}$, $-20$ to $-12°C$, $20h$). The process segment was also performed at chamber pressures slightly lower than the vapor pressures of ice at the designated shelf temperatures to avoid a large drop in the product temperature due to rapid ice sublimation ($-24°C$: $0.52\text{ mbar}$, $-22°C$: $0.63\text{ mbar}$, $-20°C$: $0.85\text{ mbar}$, $-18°C$: $1.03\text{ mbar}$). Secondary drying was performed at $35°C$ for $4h$ ($0.04\text{ mbar}$) after gradual heating of the shelves ($0.2°C/min$). The vials were closed with rubber stoppers under vacuum. The structural integrity of the freeze-dried solids was visually inspected from their volume and surface texture (e.g., roughness, bubbles, etc.) to identify any physical collapse. Images of the some vials containing frozen L-arginine solutions (2.0 mL) were taken from outside of the lyophilizer window.

PXRD A powder X-ray diffractometer (Bruker D8 DISCOVER with GADDS, Bruker AXS) with CuKa radiation (40kV×40mA) was used for PXRD analysis. Diffraction patterns obtained in the range of 5–45° (2θ, 3 min total scan time) were recorded. The L-arginine dihydrate crystal was obtained by slow crystallization from the saturated aqueous solution. The aqueous L-arginine solution (800 m) in a glass vial was frozen ($-15°C$, overnight) and thawed to obtain the precipitated solid by paper filtration (freeze-thawed precipitate). The solid residue was immediately used for PXRD analysis.

Results

Figure 1 shows the thermal transition ($T_g$) and the collapse onset ($T_r$) temperatures of the frozen solutions containing L-arginine and citric acid at varying concentration ratios. The $T_g$ transition and the following gradual shift of the thermograms observed in the heating scans were typical for frozen solutions containing non-crystalline freeze-concentrated solutes surrounding ice crystals. Frozen mixture solutions showed the highest $T_g$ and $T_r$ at the 1:1 molar concentration ratio. All the frozen solutions showed the onset of physical collapse at $T_s$ approximately 8–10°C higher than the $T_g$ temperature. The temperature margins were larger than those reported for frozen disaccharide solutions. The effect of the total solute concentration on the $T_g$ and $T_r$ of L-arginine and citric acid mixture frozen solutions (1:1 molar ratio) is shown in Fig. 2. Increasing the total solute concentrations from 200 to 800 m slightly raised the $T_g$ without any apparent changes in the $T_r$.

The L-arginine and citric acid mixture solutions were freeze-dried at varying shelf temperatures and chamber pressures during the primary drying segment (Fig. 3). The structure of the resulting dried solids depended largely on solute concentration ratio, their total concentration, shelf temperature, and chamber pressure during the primary drying segment. Primary drying performed at a fixed lower pressure (0.04 mbar), popularly used in pharmaceutical lyophilization process, induced physical collapse at shelf temperatures close to or slightly higher than the $T_g$ of the respective frozen solutions obtained at the low pressure (Fig. 3A). Some of the cylindrical solids showed traces of bubbles on their surfaces, suggesting a large drop in the product temperature during the early part of the primary drying segment (data not shown). Primary drying of the frozen mixture solutions at chamber pressures slightly lower than the vapor pressure of ice at the particular shelf temperature induced physical collapse at shelf temperatures slightly higher than the $T_g$ (Fig. 3B). Some
frozen solutions were collapsed at the shelf temperatures below the $T_s$ obtained at the lower pressure FDM observation. The appearance of frozen solutions indicated slower ice sublimation during the primary drying segment at the higher chamber pressure. Freeze-drying of single-solute $L$-arginine solutions resulted in microporous cake solids across the entire range of primary drying shelf temperatures studied. Some translucent dots appeared and grew in these frozen solutions suggested eutectic crystallization of $L$-arginine (Fig. 4).

Varied total concentrations $L$-arginine and citric acid mixture solutions (1:1 solute molar ratio) were also lyophilized to clarify the effect of shelf temperature on the structural integrity of dried solids. The lower chamber pressure primary drying at shelf temperature below the $T_s$ resulted in the cake-structure solids. Lyophilization at higher shelf temperatures induced trace of bubbles on the surface of the cake solids (Fig. 5A). Primary drying at the relatively high chamber pressures induced physical collapse from lower shelf temperatures (Fig. 5B). The partial shrinking of solids obtained upon lyophilization of lower concentration $L$-arginine and citric acid mixture solutions (e.g., 50 mM each) at $-22^\circ$C suggested that they had a slightly higher propensity for physical collapse.

The physical properties of single-solute frozen $L$-arginine solutions and their freeze-dried solids were further studied. Figures 6 and 7 show the thermal and PXRD analysis data of $L$-arginine, $L$-arginine dihydrate, and freeze-dried $L$-arginine. The DSC scan of freeze-dried $L$-arginine showed 2 exotherms (approximately 214°C and 226°C) that suggested melting and accompanying decomposition at temperatures slightly lower than those of the anhydrous crystal powder.22) A small endotherm at approximately 47°C suggested loss of water from the solid. A broad (approx. 60°C) and a sharp (approx. 99°C) endotherms observed upon heating of the $L$-arginine dihydrate powder suggested removal of the crystallization water. The dominance of $L$-arginine anhydrate in the freeze-dried solids
was also confirmed in the PXRD patterns (Fig. 7). The structure of freeze-thawed L-arginine precipitate was not clear from the PXRD data.

The process of L-arginine crystallization was studied by thermal analysis of the frozen solutions. Figure 8 shows thermograms of the frozen solutions (50 µL) containing single-solute L-arginine or a mixture of L-arginine with citric acid obtained in the heating scans before and after a heat treatment at −20°C. The frozen L-arginine and citric acid mixture solution retained a $T_g$ transition and gradual endothermic shift of the thermogram upon exposure to the higher temperature ($−20°C$, 480 min), indicating stability of the non-crystalline concentrated solute phase. The first heating scan of the single-solute frozen L-arginine solution (800 mM) also indicated a non-crystalline concentrated solute phase. However, a broad exothermic peak at approximately $−10°C$ and a flat thermogram before the large ice melting endotherm induced after a shorter (30 min) and a longer (480 min) heat treatments, respectively suggested slow eutectic crystallization of L-arginine. Analysis of smaller volume heat-treated ($−20°C$, 30 min) frozen L-arginine solutions (800 mM, 10 µL) indicated apparently varied thermal properties. Some showed the solute crystallization exotherm, whereas others showed only gradual endothermic shift of the thermograms (data not shown).
Discussion  

Thermal Transition and Physical Collapse of Frozen Solutions  
The L-arginine and citric acid mixture frozen solutions showed varied thermal properties and propensities for structural collapse during the primary drying segment. Both the FDM observation (cell temperature) and the experimental freeze-drying (shelf temperature) showed occurrence of the physical collapse at temperatures higher than the $T_g$'s of the individual frozen solutions. The fact confirmed that $T_g$ is a reliable surrogate of $T_c$ for determining the highest allowable product temperature, particularly for smaller-scale lyophilization. Some studies define the thermal transition as softening temperature because of the large viscosity change of the non-crystalline solute phase.1,2) A large viscosity drop of the drying process at the lower chamber pressure, compared to the higher vapor pressure. Information on the sublimation interface temperature because of the large viscosity change of the non-crystalline solute phase.23) A large viscosity drop of the non-ice phase at the sublimation interface to the degree that is insufficient to maintain the physical integrity, was considered to induce the collapse phenomena at the product temperatures above the $T_g$.1)

It is plausible that different temperatures at the sublimation interfaces and the heat suppliers (e.g., lyophilizer shelf, FDM cell) explain the observed margin between the $T_g$'s and $T_c$'s or the collapse-inducing lyophilizer shelf temperatures. The product temperature at the sublimation interface is determined by the balance of heat supply (e.g., conduction, convection, radiation) and sublimation-related heat loss. The larger heat loss by faster ice sublimation anticipated during the primary drying process at the lower chamber pressure, compared to the limited heat supply mainly through the contact region of the vial bottom, should keep the sublimation interface much cooler than the shelf surface. Thus the collapse should occur only at the shelf temperatures higher than the $T_g$'s that supply sufficient heat. The traces of bubbles observed on the surface of some cake-structure solids indicated local collapse at the beginning of the primary drying segment. The ice sublimation may also induce the temperature gradients in the FDM cell and the inner frozen solution. In contrast, slower ice sublimation during the primary drying at the chamber pressures slightly lower than the vapor pressures of ice at the particular shelf temperatures should minimize the temperature difference between the lyophilizer shelf and sublimation interface. Thus physical collapse should occur at the shelf temperatures close to the $T_g$ of the corresponding frozen solution. Larger heat supply by convection through the gas phase may also contribute to raise the product temperature at higher chamber pressure. Information on the sublimation interface temperatures in the lyophilization vials and the FDM cell should improve relevance of the data obtained in the small-scale characterization.

Increasing the total L-arginine and citric acid mixture concentrations (1:1 molar ratio) resulted in a slightly higher $T_g$ without any apparent changes in the thermal transition temperature. A similar dependence of the $T_g$ on the solute concentration has been reported in frozen disaccharide systems.20,24) A spatially dense solid that forms upon drying of the higher solute concentration solutions would be a possible explanation for the increasing resistance to loss of the cake structure.

Crystallization of L-Arginine in Frozen Solutions  
Freeze-drying of single-solute L-arginine solutions resulted in cake-structure dried solids composed of anhydrous crystals at all the primary drying shelf temperatures studied. L-Arginine may crystallize either as an anhydrate or as dihydrate in the frozen solutions as observed in the freeze-thawed precipitate solid. The crystallization water may be removed readily during the secondary drying process. The thermal analysis data indicated slow crystallization of L-arginine in the frozen solution. The large translucent dots appeared on the surface of frozen L-arginine solutions, as well as the varied crystallization propensities of L-arginine in the heat-treated smaller volume solutions (10 μL), strongly suggested its inhomogeneous nucleation in the frozen solutions. Various formulation and process factors (e.g., solute concentration, co-solute composition, solution volume, freezing method, and thermal history) can affect the crystallinity of lyophilized L-arginine. Rapid cooling of the aqueous solutions by immersion in liquid nitrogen and immediate start of the vacuum drying resulted in amorphous L-arginine lyophilized solids.25,26)

Our results highlight the importance of characterizing frozen solutions for the formulation and process development of L-arginine-containing lyophilized formulations. Information on other formulation (e.g., volume and vial shape) and system (e.g., the number of vials and condenser temperature) factors should also be valuable for optimizing the process parameters by using mathematical methods.26) Application of several process analytical technology (PAT) tools, including manometric temperature measurement of the ice sublimation interface should ensure a robust and efficient lyophilization process based on the QbD principle, particularly for larger-scale repetitive manufacturing.27)

Acknowledgement  
This work was supported in part by the Japan Health Sciences Foundation (KHB1005).

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