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Review

Lyophilization and development of solid protein pharmaceuticals

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Abstract

Developing recombinant protein pharmaceuticals has proved to be very challenging because of both the complexity of protein production and purification, and the limited physical and chemical stability of proteins. To overcome the instability barrier, proteins often have to be made into solid forms to achieve an acceptable shelf life as pharmaceutical products. The most commonly used method for preparing solid protein pharmaceuticals is lyophilization (freeze-drying). Unfortunately, the lyophilization process generates both freezing and drying stresses, which can denature proteins to various degrees. Even after successful lyophilization with a protein stabilizer(s), proteins in solid state may still have limited long-term storage stability. In the past two decades, numerous studies have been conducted in the area of protein lyophilization technology, and instability/stabilization during lyophilization and long-term storage. Many critical issues have been identified. To have an up-to-date perspective of the lyophilization process and more importantly, its application in formulating solid protein pharmaceuticals, this article reviews the recent investigations and achievements in these exciting areas, especially in the past 10 years. Four interrelated topics are discussed: lyophilization and its denaturation stresses, cryo- and lyo-protection of proteins by excipients, design of a robust lyophilization cycle, and with emphasis, instability, stabilization, and formulation of solid protein pharmaceuticals.

Keywords: Aggregation; Cryoprotection; Denaturation; Excipient; Formulation; Freeze-drying; Glass transition; Stability; Lyoprotection; Residual moisture

1. Introduction

Developing recombinant protein pharmaceuticals has proved to be very challenging because of both the complexity of protein production and purification, and the limited physical and chemical stability of proteins. In fact, protein instability is one of the two major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs (Wang, 1996). To over-

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come the instability barrier, proteins often have to be made into solid forms to achieve an acceptable shelf life.

The most commonly used method for preparing solid protein pharmaceuticals is lyophilization (freeze-drying). However, this process generates a variety of freezing and drying stresses, such as solute concentration, formation of ice crystals, pH changes, etc. All of these stresses can denature proteins to various degrees. Thus, stabilizers are often required in a protein formulation to protect protein stability both during freezing and drying processes.

Even after successful lyophilization, the long-term storage stability of proteins may still be very limited, especially at high storage temperatures. In several cases, protein stability in solid state has been shown to be equal to, or even worse than, that in liquid state, depending on the storage temperature and formulation composition. For example, a major degradation pathway of human insulin-like growth factor I (hIGF-I) is oxidation of Met[59] and the oxidation rate in a freeze-dried formulation in air-filled vials is roughly the same as that in a solution at either 25 or 30°C (Fransson et al., 1996). Similarly, the oxidation rate of lyophilized interleukin 2 (IL-2) is the same as that in a liquid formulation containing 1 mg ml^{-1} IL-2, 0.5% hydroxypropyl-β-cyclodextrin (HP-β-CD), and 2% sucrose during storage at 4°C (Hora et al., 1992b). At a high water content ($>50\%$), the degradation rate of insulin is higher in a lyophilized formulation than in a solution with similar pH-rate profiles in both states (Strickley and Anderson, 1996). The glucose-induced formation of des-Ser relaxin in a lyophilized formulation is faster than in a solution during storage at 40°C (Li et al., 1996). These examples indicate that stabilizers are still required in lyophilized formulations to increase long-term storage stability.

In the past two decades, numerous studies have been conducted in the areas of protein freezing and drying, and instability and stabilization of proteins during lyophilization and long-term storage. Many critical issues have been identified in this period. These studies and achievements have been reviewed elsewhere with emphasis on physical and chemical instabilities and stabilization of proteins in aqueous and solid states (Manning et al., 1989; Cleland et al., 1993); chemical instability mechanisms of proteins in solid state (Lai and Topp, 1999); various factors affecting protein stability during freeze-thawing, freeze-drying, and storage of solid protein pharmaceuticals (Arakawa et al., 1993); and application of lyophilization in protein drug development (Pikal, 1990a,b; Skrabanja et al., 1994; Carpenter et al., 1997; Jennings, 1999). Nevertheless, it appears that several critical issues in the development of solid protein pharmaceuticals have not been fully examined, including various instability factors, stabilization, and formulation of solid protein pharmaceuticals.

To have an up-to-date perspective of the lyophilization process and more importantly, its application in formulating solid protein pharmaceuticals, this article reviews the recent investigations and achievements in these exciting areas, especially in the past 10 years. Four interrelated topics are discussed sequentially, lyophilization and its denaturation stresses; cryo- and lyo-protection of proteins by excipients; design of a robust lyophilization cycle; and with emphasis, instability, stabilization, and formulation of solid protein pharmaceuticals.

2. Lyophilization and its denaturation stresses

2.1. Lyophilization process

Lyophilization (freeze-drying) is the most common process for making solid protein pharmaceuticals (Cleland et al., 1993; Fox, 1995). This process consists of two major steps: freezing of a protein solution, and drying of the frozen solid under vacuum. The drying step is further divided into two phases: primary and secondary drying. The primary drying removes the frozen water and the secondary drying removes the non-frozen 'bound' water (Arakawa et al., 1993). The amount of non-frozen water for globular proteins is about 0.3–0.35 g g^{-1} protein, slightly less than the proteins' hydration shell (Rupley and Careri, 1991; Kuhlman et al., 1997). More detailed analy-

sis of each lyophilization step is provided in Section 4.

Lyophilization generates a variety of stresses, which tend to destabilize or unfold/denature an unprotected protein. Different proteins tolerate freezing and/or drying stresses to various degrees. Freeze-thawing of ovalbumin at neutral pH did not cause denaturation (Koseki et al., 1990). Repeated (three times) freeze-thawing of tissue-type plasminogen activator (tPA) did not cause any decrease in protein activity (Hsu et al., 1995). Some proteins can keep their activity both during freezing and drying processes, such as α_1-antitrypsin in phosphate–citrate buffer (Vemuri et al., 1994), porcine pancreatic elastase without excipients (Chang et al., 1993), and bovine pancreatic ribonuclease A (RNase A, 13.7 kD) in the presence or absence of phosphate (Townsend and DeLuca, 1990).

However, many proteins cannot stand freezing and/or drying stresses. Freeze-thawing caused loss of activity of lactate dehydrogenase (LDH) (Nema and Avis, 1992; Izutsu et al., 1994b; Andersson and Hatti-Kaul, 1999), 60% loss of L-asparaginase (10 μg ml^{-1}) activity in 50 mM sodium phosphate buffer (pH 7.4) (Izutsu et al., 1994a), and aggregation of recombinant hemoglobin (Kerwin et al., 1998). Freeze-drying caused 10% loss of the antigen-binding capacity of a mouse monoclonal antibody (MN12) (Ressing et al., 1992), more than 40% loss of bilirubin oxidase (BO) activity in the presence of dextran or polyvinylalcohol (PVA) (Nakai et al., 1998), loss of most β-galactosidase activity at 2 or 20 μg ml^{-1} (Izutsu et al., 1993, 1994a), complete loss of phosphofructokinase (PFK) and LDH activity in the absence of stabilizers (Carpenter et al., 1986, 1990; Prestrelski et al., 1993a; Anchordoquy and Carpenter, 1996), and dissociation of *Erwinia* L-asparaginase tetramer (135 kD) into four inactive subunits (34 kD each) in the absence of any protectants (Adams and Ramsay, 1996).

2.2. Denaturation stresses during lyophilization

The lyophilization process generates a variety of stresses to denature proteins. These include (1) low temperature stress; (2) freezing stresses, including formation of dendritic ice crystals, increased ionic strength, changed pH, and phase separation; and (3) drying stress (removing of the protein hydration shell).

2.2.1. Low temperature stress

The first quantitative study on low-temperature denaturation of a model protein was conducted presumably by Shikama and Yamazaki (1961). They demonstrated a specific temperature range in which ox liver catalase was denatured during freeze-thawing. Cold denaturation of catalase at 8.4 μg ml^{-1} in 10 mM phosphate buffer (pH 7.0) started at −6°C. Loss of catalase activity reached 20% at −12°C, remained at this level between −12°C and near −75°C, then decreased gradually from −75 to −120°C. There was almost no activity loss between −129 and −192°C. Similar results were also obtained for ovalbumin by Koseki et al. (1990). Incubation of frozen ovalbumin solution caused structural change of ovalbumin, as monitored by UV difference spectra, which increased with decreasing temperature between −10 and −40°C. Further decrease in incubation temperature to −80°C caused less structural change, and no change at −192°C. Perlman and Nguyen (1992) reported that interferon-γ(IFN-γ) aggregation in a liquid mannitol formulation was more severe at −20°C than at −70, 5 and 15°C during storage. To prevent freezing-induced complication in studying cold protein denaturation, cold and heat denaturation of RNase A has been conducted under high pressure (3 kbar). Under this condition, RNase A denatured below −22°C and above 40°C (Zhang et al., 1995). All these examples are clear indication of low temperature denaturation rather than a freezing or thawing effect.

The nature of cold denaturation has not been satisfactorily delineated. Since solubility of non-polar groups in water increases with decreasing temperature due to increased hydration of the non-polar groups, solvophobic interaction in proteins weakens with decreasing temperature (Dill et al., 1989; Graziano et al., 1997). The decreasing solvophobic interaction in proteins can reach a point where protein stability reaches zero, causing cold denaturation (Jaenicke, 1990). While

normal or thermal denaturation is entropy-driven, cold denaturation is enthalpy-driven (Dill et al., 1989; Shortle, 1996). Oligomeric proteins typically show cold denaturation, i.e. dissociation of subunit oligomers, since association is considered to be a consequence of hydrophobic interaction (Jaenicke, 1990; Wisniewski, 1998). Theoretically, the calculated free energy of unfolding (ΔG_{unf}) for proteins has a parabolic relationship with temperature. This means that a temperature of maximum stability exists, and both high and low temperature can destabilize a protein (Jaenicke, 1990; Kristjánsson and Kinsella, 1991).

2.2.2. Concentration effect

Freezing a protein solution rapidly increases the concentration of all solutes due to ice formation. For example, freezing a 0.9% NaCl solution to its eutectic temperature of −21°C can cause a 24-fold increase in its concentration (Franks, 1990). The calculated concentration of small carbohydrates in the maximally freeze-concentrated matrices (MFCS) is as high as 80% (Roos, 1993). Thus, all physical properties related to concentration may change, such as ionic strength and relative composition of solutes due to selective crystallization. These changes may potentially destabilize a protein.

Generally, lowering the temperature reduces the rate of chemical reactions. However, chemical reactions may actually accelerate in a partially frozen aqueous solution due to increased solute concentration (Pikal, 1999). Due to solute concentration, the rate of oligomerization of β-glutamic acid at −20°C was much faster than at 0 or 25°C in the presence of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Liu and Orgel, 1997).

The increase in the rate of a chemical reaction in a partially frozen state could reach several orders of magnitude relative to that in solution (Franks, 1990, 1994).

The reported oxygen concentration in a partially frozen solution at −3°C is as high as 1150 times that in solution at 0°C (Wisniewski, 1998). The increased oxygen concentration can readily oxidize sulphydryl groups in proteins. If a protein solution contains any contaminant proteases, concentration upon freezing may drastically accelerate protease-catalyzed protein degradation.

2.2.3. Formation of ice-water interface

Freezing a protein solution generates an ice-water interface. Proteins can be adsorbed to the interface, loosening the native fold of proteins and resulting in surface-induced denaturation (Strambini and Gabellieri, 1996). Rapid (quench) cooling generates a large ice-water interface while a smaller interface is induced by slow cooling (also see Section 4.2). Chang et al. (1996b) demonstrated that a single freeze–thaw cycle with quench cooling denatured six model proteins, including ciliary neurotrophic factor (CNTF), glutamate dehydrogenase (GDH), interleukin-1 receptor antagonist (IL-1ra), LDH, PFK, and tumor necrosis factor binding protein (TNFbp). The denaturation effect of quench cooling was greater or equivalent to that after 11 cycles of slow cooling, suggesting surface-induced denaturation. This denaturation mechanism was supported by a good correlation ($r = 0.99$) found between the degree of freeze-induced denaturation and that of artificially surface-induced denaturation. The surface was introduced by shaking the protein solution containing hydrophobic Teflon beads. In a similar study, a correlation coefficient of 0.93 was found between the tendency of freeze denaturation and surface-induced denaturation for eight model proteins, including aldolase, basic fibroblast growth factor (bFGF), GDH, IL-1ra, LDH, maleate dehydrogenase (MDH), PFK, and TNFbp (Kendrick et al., 1995b). However, there was no significant correlation ($r = 0.78$) between freeze denaturation and thermal denaturation temperature (Chang et al., 1996b).

2.2.4. pH changes during freezing

Many proteins are stable only in a narrow pH range, such as low molecular weight urokinase (LMW-UK) at pH 6–7 (Vrkljan et al., 1994). At extreme pHs, increased electrostatic repulsion between like charges in proteins tends to cause protein unfolding or denaturation (Goto and Fink, 1989; Volkin and Klibanov, 1989; Dill, 1990). Thus, the rate of protein aggregation is strongly affected by pH, such as aggregation of

interleukin 1β (IL-1β) (Gu et al., 1991), human relaxin (Li et al., 1995a), and bovine pancreatic RNase A (Townsend and DeLuca, 1990; Tsai et al., 1998). Moreover, the solution pH can significantly affect the rate of many chemical degradations in proteins (Wang, 1999).

Freezing a buffered protein solution may selectively crystallize one buffering species, causing pH changes. Na_2HPO_4 crystallizes more readily than NaH_2PO_4 because the solubility of the disodium form is considerably lower than that of the monosodium form. Because of this, a sodium phosphate buffer at pH 7 has a molar $[NaH_2PO_4]/[Na_2HPO_4]$ ratio of 0.72, but this ratio increases to 57 at the ternary eutectic temperature during freezing (Franks, 1990, 1993). This can lead to a significant pH drop during freezing, which then denatures pH-sensitive proteins. For example, freezing of a LDH solution caused protein denaturation due to a pH drop from 7.5 to 4.5 upon selective crystallization of Na_2HPO_4 (Anchordoquy and Carpenter, 1996). LDH is a pH-sensitive protein and a small drop in pH during freezing can partially denature the protein even in the presence of stabilizers such as sucrose and trehalose (Nema and Avis, 1992). The pH drop during freezing may also explain why freezing bovine and human IgG species in a sodium phosphate buffer caused formation of more aggregates than in potassium phosphate buffer, because potassium phosphate buffer does not show significant pH changes during freezing (Sarciaux et al., 1998).

The pH drop during freezing can potentially affect storage stability of lyophilized proteins. Lyophilized IL-1ra in a formulation containing phosphate buffer at pH 6.5 aggregated more rapidly than that containing citrate buffer at the same pH during storage at 8, 30 and 50°C (Chang et al., 1996c). Similarly, the pH drop of a succinate-containing formulation from 5 to 3–4 during freezing appeared to be the cause of less storage stability for lyophilized IFN-γ than that containing glycocholate buffer at the same pH (Lam et al., 1996).

2.2.5. Phase separation during freezing

Freezing polymer solutions may cause phase separation due to polymers' altered solubilities at low temperatures. Freezing-induced phase separation can easily occur in a solution containing two incompatible polymers such as dextran and Ficoll (Izutsu et al., 1996). During freezing of recombinant hemoglobin in a phosphate buffer containing 4% (w/w) PEG 3350, 4% (w/w) dextran T500, and 150 mM NaCl, liquid–liquid phase separation occurred and created a large excess of interface, denaturing the protein (Heller et al., 1997). Addition of 5% sucrose or trehalose could not reverse the denaturation effect in the system (Heller et al., 1999a).

Several strategies have been proposed to mitigate or prevent phase separation-induced protein denaturation during freezing. These include use of alternative salts (Heller et al., 1999a), adjustment of the relative composition of polymers to avoid or to rapidly pass over a temperature region where the system may result in liquid–liquid phase separation (Heller et al., 1999c), and chemical modification of the protein such as pegylation (Heller et al., 1999b).

2.2.6. Dehydration stresses

Proteins in an aqueous solution are fully hydrated. A fully hydrated protein has a monolayer of water covering the protein surface, which is termed the hydration shell (Rupley and Careri, 1991). The amount of water in full hydration is 0.3–0.35 g g^{-1} protein (Rupley and Careri, 1991; Kuhlman et al., 1997). Generally, the water content of a lyophilized protein product is less than 10%. Therefore, lyophilization removes part of the hydration shell. Removal of the hydration shell may disrupt the native state of a protein and cause denaturation. A hydrated protein, when exposed to a water-poor environment during dehydration, tends to transfer protons to ionized carboxyl groups and thus abolishes as many charges as possible in the protein (Rupley and Careri, 1991). The decreased charge density may facilitate protein–protein hydrophobic interaction, causing protein aggregation.

Water molecules can also be an integral part of an active site(s) in proteins. Removal of these functional water molecules during dehydration easily inactivates proteins. For example, dehydra-

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