CHAPTER 1

GENERAL INTRODUCTION

1 INTRODUCTION

Starting in the early 1970s, the improved understanding of the biomolecular roots of human diseases (due to advances made in the fields of molecular biology and genetic engineering) paired with the development of new biotechnological techniques opened the door to tremendous possibilities of using proteins as pharmaceuticals to alleviate illnesses[1,2]. With the first production of rDNA (Paul Berg in 1972)[3] and the first recombinant plasmid transformation of E.coli (Herbert Boyer's team in 1973)[4] the era of recombinant protein pharmaceutics began.

Since the FDA-approval of the first human recombinant protein in 1982, (Humulin, Genentech) protein pharmaceuticals have become increasingly important in state of the art pharmacotherapy. Today more than 130 different proteins or peptides are approved for clinical use by the FDA and many more are under development[2]. The treatment of manifold diseases, such as diabetes mellitus, hemophilia or other enzyme deficiency diseases, various cancer afflictions, immune mediated diseases such as Multiple sclerosis or Hepatitis B/C as well as emergency medication such as clotlysis after acute myocardial infarctions has been tremendously improved by protein pharmaceuticals.

However, the same properties that contribute to the proteins' high potential as pharmaceutics, their complexity and compositional variety, also cause their high susceptibility to various stresses. Therefore, finding a formulation that stabilizes the protein during purification, production, storage and final application is one of the major challenges during biopharmaceutical development.

Because liquid formulations are more cost-conscious to handle during manufacturing, the desired drug product would be a stable liquid. What is more, liquids are more convenient for the patient[5]. Although efforts have been made to develop stable liquid formulations for biopharmaceuticals the method of choice for the stabilization of many protein pharmaceuticals is still lyophilization (e.g. Activase® (Alteplase), ReFacto® (Factor VIII), Enbrel® (Etanercept), Avonex® (Interferon beta-1a)[6]).

The freeze-dried state bears some advantages over the liquid state. As the protein ingredient is immobilized in the solid state the rate constant of most chemical and physical degradation reactions is decreased dramatically in the glassy state[7-9]. As the product thus becomes more stable, this might permit suitable long term storage at 5 °C or ambient temperatures (25 °C), leading to simplified product handling during storage and shipping, where exact temperature

control is not always feasible and considerably increases costs of goods. Another merit is that the risk of damage during abidance at sub-zero temperatures is eliminated. Furthermore the risk of interfacial-stress, e.g. during agitation is eliminated as the product is solid[5,7].

One major constraint of lyophilization is the time-consuming and costly production cycles. It is good pharmaceutical freeze-drying practice to keep the product at a low product temperature during the lyophilization process[10]. A rise in product temperature above the glass transition temperature of the maximally freeze concentrated protein-excipient solution (T_g') or even above the collapse temperature (T_c) is regarded as detrimental for product quality although data fully confirming this assumption is rare and not clear. The major purpose of this approach is to avoid the onset of structural cake collapse during the primary or secondary drying stage[11-14]. The onset of gross collapse in a protein pharmaceutical product causes the loss of the whole batch as reprocessing of collapsed material is not an option at the moment.

With the entry of biogenerics into the pharmaceutical market the development of stable and safe but cost-conscious products becomes even more important. “Conservative” freeze-drying cycles usually take several days up to a week to finish. Special attention has to be paid to optimization of lyophilization because any increase in product temperature dramatically decreases primary drying time[15]. Recently, there have been several patent applications, claiming the application of high primary drying temperatures to shorten drying times[16-18].

However, in order not to jeopardize product stability, exact monitoring of the freeze-drying process using state of the art process analytical technology (PAT) is important. Even more crucial is the thorough revision of the conventional approach of freeze-drying at low product temperatures. A detailed investigation of the implications of high product temperatures and collapse on protein stability during freeze-drying and the stability of such products during subsequent storage has to be performed in order to allow for a scientifically sound evaluation of the effect of collapse on protein stability and to provide a broad basis for the responsible handling and reprocessing of collapsed batches.

2 FREEZE-DRYING OF PROTEINS

2.1 THE FREEZE-DRYING PROCESS IN BRIEF

Lyophilization is a unit operation commonly employed as a drying technology in the food and pharmaceutical industry[19]. Freeze-drying was first mentioned by Ray in 1976[20]. Jennings gave the first definition to the term lyophilization emphasizing the intention to stabilize (heat-) sensitive materials by reducing the amount of solvent (most commonly water[21]) to a level that no longer allows biological growth or chemical reactions[22]. Lyophilization in its simplest form is defined as a process where the solution that shall be dried is first frozen and concomitantly the solvent is removed by first sublimation (primary drying) and second by desorption (secondary drying)[22,23].

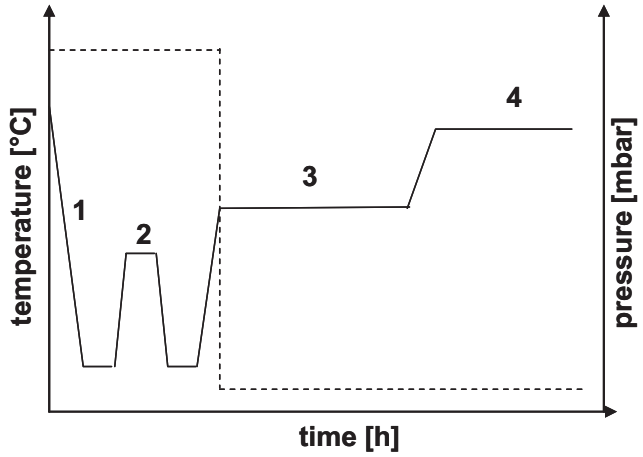

Figure 1.1: Scheme of a typical freeze-drying cycle.
Temperature (solid black line) and pressure (dashed black line) course during the different process stages: 1 = freezing, 2 = annealing (optional), 3 = primary drying, 4 = secondary drying.

The freeze-drying process can be divided into three major process stages, i.e. freezing, primary drying and secondary drying. An optional annealing step can be included between the freezing and the drying steps. Figure 1.1 depicts a scheme of a typical freeze-drying protocol.

In the following paragraph each step's key parameters are briefly described, focusing on characteristics important with regards to the collapse phenomenon.

FREEZING

During the freezing step, the temperature of the freeze-drying system is lowered below its solid-liquid phase transition temperature. Thereby the solvent is separated from the solute[23]. Figure 1.2 depicts the typical behavior of an amorphous system upon cooling:

Upon lowering the temperature either crystallization of both solvent and solute, forming an eutectic mixture below the eutectic temperature (T_{eut} in Figure 1.2), or crystallization of the solvent alone, resulting in phase separation and the formation of a crystalline ice-phase and an amorphous solute-phase, occur. In the case of phase separation, an increase in

concentration in the remaining solution (so-called freeze-concentration) is observed. Freeze-concentration continues until the viscosity exceeds a critical value, that defines the glass transition of the maximally freeze-concentrated solution (T_g' in Figure 1.2). Below the glass transition, the amorphous phase is solid and is referred to as a glass in the interstitials of the ice crystals[21].

The freezing step during lyophilization is considered at least as important for product quality as the drying steps due to its potential effect on protein stability[24]. The key parameter hereby is the cooling rate or rather the ice nucleation rate[25]. In general, a faster freezing rate results in smaller ice crystals[26,27]. Fast freezing causes a greater degree of super cooling, i.e. the retention of the liquid state below the equilibrium freezing point of the solution. Super cooling limits the ability to control the freezing rate by manipulation of shelf temperature, because the greater the degree of super cooling, the faster is the effective freezing rate once ice crystals nucleate[28-30]. Slow freezing in return results in larger ice crystals because ice crystal growth continues after ice crystal nucleation. However, a slow freezing rate also was observed to result in considerable super-cooling[31] and thus a fast effective freezing rate. Slow freezing also has the potential to increase the system's tendency for phase separation and prolongs the time where the protein exists in a highly concentrated fluid state[32]. In general, the ability to control the freezing rate is limited and an intermediate freezing rate is recommended[32].

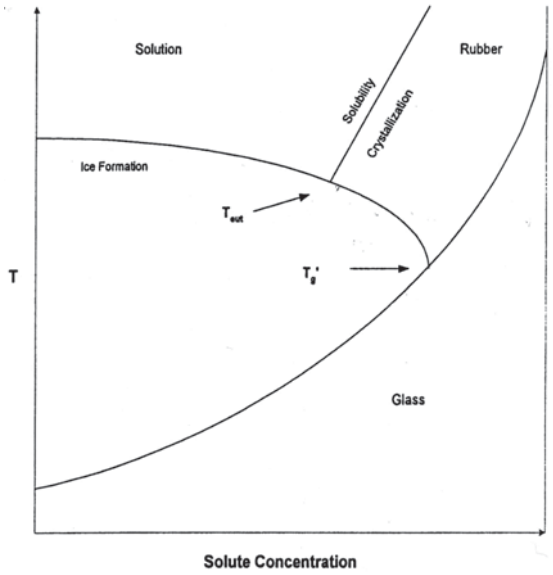

Figure 1.2: Theoretical phase diagram showing ice formation, solute crystallization, the eutectic melting point and the glass transition during freezing of an amorphous system. [25,28]

Small crystals generate small pores, as pores originate from sublimed ice crystals. During drying, water vapor has to diffuse through these pores out of the cake. Small pores oppose a higher resistance to water vapor flow than larger pores (a phenomenon referred to as dried layer resistance), thus decreasing the sublimation rate and turning primary drying less efficient. In contrast, larger pores greatly reduce the dry layer resistance to water vapor and

render primary drying more efficient. Annealing is sometimes applied to increase the size of ice crystals formed during the freezing step by Ostwald ripening and thus increase the efficiency of primary drying.

However, small pores also add up to a larger specific surface area as compared to large pores, resulting in a more efficient secondary drying[33].

PRIMARY-DRYING

During primary-drying the frozen solvent is removed from the product by sublimation. This is initiated by lowering the chamber pressure to a level lower than the vapor pressure of ice at the product temperature. The energy required for the phase transition is provided by adjusting the shelf temperature. Because the energy input is consumed by sublimation the resulting product temperature is much lower than the shelf temperature. Differences of up to 30 Kelvin (K) are not unusual. The product temperature approaching the shelf temperature due to the end of sublimation is a common way to detect the end of primary drying.

There are three mechanisms accounting for the heat transfer from the shelf to the vial: conduction, convection and radiation.

In order to prevent collapse the product temperature has to be maintained below the collapse temperature. When the collapse temperature is unknown, the glass transition or the eutectic melting temperature in amorphous or crystalline systems, respectively, is used as maximum allowable product temperature[33]. On the other hand, as each 1°K increase in product temperature decreases primary drying time by about 13%[15], the drying temperature should be maintained as high as possible.

The process is characterized by the sublimation rate v that can be expressed as:

$$v = \frac{A_p (P_p - P_0)}{R_p} \tag{1.1}$$

A_p is the cross sectional area of the vial, P_p is the product's vapor pressure at the sublimation front, P_0 is the partial vapor pressure in the vial and R_p is the dried layer resistance to water vapor flow[33,34].

The resistance of the already dried product, accounting for 90% of the total resistance to vapor flow, increases with progressing drying and can cause an increase of product temperature possibly leading to cake collapse[35]. However, the occurrence of small-scale collapse may decrease R_p and increase the sublimation rate[36].

SECONDARY DRYING

At the end of primary drying, only non-frozen water is present in the freeze-dried cake. This water cannot be removed by sublimation but has to be removed by desorption. Thus more energy is applied during secondary drying. To further promote the drying process some

authors advise to further decrease the chamber pressure in order to increase the concentration gradient of water vapor from the product to its surroundings.

Especially at the beginning of secondary drying it is important to bear in mind that the glass transition temperature is a function of residual moisture. As the residual moisture content at the end of primary drying can be as high as 30%, a too rapid increase in shelf temperature may cause the product temperature to rise above the collapse temperature resulting in cake collapse[37].

The residual moisture decreases rapidly during the first hours of secondary drying, approaching an equilibrium level dependent only on the shelf temperature and the specific surface area of the cake. It is independent of the chamber pressure or the height of the dried product layer[38].

2.2 FREEZE-DRYING OF PROTEIN PHARMACEUTICALS

Proteins are complex and labile molecules, sensitive towards various degradation pathways. As for economic viability a shelf life of 18-24 months is desirable[39], lyophilization is often the method of choice to develop stable market formulations for biopharmaceuticals. 46% of the FDA-approved biopharmaceuticals in 2003 were lyophilizates[40].

Although freeze-drying is used to stabilize labile products, the process itself generates both freezing and drying stresses and might often be harmful for the protein pharmaceutical[41]. Thus the careful development of a formulation that thoroughly stabilizes the active ingredient is as important as the lyophilization cycle development itself[40].

The formulation shall provide stability during manufacturing, freeze-drying, shipping, storage reconstitution and finally during administration of the final product in the patient. Degradation pathways of proteins are diverse and complex. Smallest amounts of degradation products can have severe consequences, for example as intermediates for further degradation, as e.g. oxidized species or heterogeneous nucleation sites as metals shed from vial filling pumps. Alternatively aggregated species may cause immune responses. Hence the formulation has to provide the highest possible level of stabilization[42].

In principle, a formulation for freeze-drying is composed of protein stabilizers (so-called cryo- and lyo-protectants), specific stabilizers as antioxidants or surfactants, bulking agents, tonicifiers and buffer agents[25,40]. Generally the total amount of solids in the formulation makes up between 2 and 10 % of the solution before freeze-drying, combining a sufficient mass to ensure a stable cake but allowing for good processability as well[5,43].

The choice of excipients is particularly governed by the level of stabilization required by the specific protein drug. If the concentration of active ingredient is low, a bulking agent like mannitol or glycine has to be used to ensure an elegant and mechanically stable cake with satisfying drying behavior. Furthermore, salts either serving as pH buffers or giving the required tonicity, should be added[9]. The addition of specific stabilizers such as surfactants,

antioxidants or preservatives can be necessary as well. To achieve a sufficiently short reconstitution time and adequate solubility, a solubilizer like arginine can be added.
The compatibility of the protein drug with excipients and container materials has to be kept in mind as well. Furthermore, the regulatory status of excipients, the route of administration or a desired modified release has to be taken into account.
The different components of freeze-drying formulations will be discussed in more detail below. Before, a short description of the stress situation arising during lyophilization is given.
During freezing, the decreasing temperature can cause cold denaturation, as the solubility of hydrophobic groups in water increases with decreasing temperature concurrently decreasing intramolecular hydrophobic interactions determining the protein's tertiary and quaternary structure[9,44-46]. Oligomeric proteins often show cold denaturation. One factor accounting for this phenomenon is, that association is amongst others determined by hydrophobic interactions[27,46].
Other instabilities arise due to the formation of ice crystals: Most obviously the formation of ice crystals creates new interfaces that are able to cause surface-induced denaturation[47].
Furthermore, due to the continuous freeze- concentration of the remaining amorphous phase the solution can change to a degree that might damage the protein. For example the manifold increase in ionic strength or destabilizing species such as oxygen might be deteriorating[27]. The relative concentration of formulation compounds can be changed as well, due to selective crystallization, causing e.g. pH changes or phase separation.
During drying the protein is subjected to dehydration stresses. As the level of residual moisture achieved after lyophilization is usually lower than the water content in the protein's hydration shell (0.3 - 0.35 g * g-1 protein[25,48]), this may disrupt the native state and cause changes in the protein's structure.
Various excipients are used to stabilize protein pharmaceuticals during freeze-drying and during subsequent storage. They can be classified into cryo- and lyo-protectants according to their ability to protect the protein during freezing and drying, respectively.
Although there are some proteins that do not need much stabilization and that can be dried without the addition of excipients, most biopharmaceuticals do need either cryo- or lyo-preservation or both[49].

CRYO-PROTECTANTS

The most widely accepted mechanism by which cryo-protection is mediated is preferential exclusion, meaning that the interaction between protein and excipient is thermodynamically unfavorable, leading to an accumulation of water molecules at the protein's surface (preferential hydration) as well as to a more compact structure of the molecule, burying the hydrophobic backbone[7,50-53].

Other mechanisms providing stabilization during freezing are modification of size of ice crystals, reduction of surface tension and restriction of diffusion of reacting molecules due to an increase in viscosity.

Commonly used cryo-protectants are polymers like polyvinylpyrrolidone (PVP), polyethylene glycol, e.g. PEG 3350, amino acids such as glycine or human serum albumin (HSA).

LYO-PROTECTANTS

Lyo-protection is mediated by two mechanisms: First, the lyo-protectant acts as a water substitute, forming hydrogen bonds with the protein's hydrophilic groups upon the removal of water[54,55]. This theory was first formulated by Crowe et al. and Allison et al. and is referred to as water replacement theory[56-59]. Because the interaction between carbohydrates and proteins necessarily requires both to be in the same (amorphous) phase, crystallization often has a destabilizing effect on solid protein formulations.

Excipients stabilizing by this mechanism are polyols and sugars, especially disaccharides, because with increasing molecular weight the steric hindrance renders effective hydrogen bonding more and more ineffective[5,7]. Sucrose and trehalose are most commonly used. Some amino acids, such as proline, arginine or sodium glutamate are used as well[55,60].

A second mechanism that stabilizes proteins in the dried state is vitrification: the active ingredients and all possible reacting species are immobilized in the glassy state where molecular mobility is strongly decreased. Common excipients stabilizing by this mechanism are high molecular weight carbohydrates like maltodextrins or polymers like polyvinylpyrrolidone (PVP).

The use of a disaccharide and a high molecular weight excipient together combines effective hydrogen bonding with a sufficiently high glass transition temperature (T_g) leading to maximum stabilization.

In contrast to this theory, Cicerone et al. reported that the addition of a plasticizer, e.g. glycerol to the amorphous phase leads to increased stability. The authors ascribe this observation to the filling of holes left by the glass former by the smaller plasticizer, thereby decreasing the fast glassy dynamics and increasing stability[61,62].