Lyophilization or freeze-drying is often used to stabilize various pharmaceutical products, including virus vaccines, protein and peptide formulations, liposome, and small-chemical drug formulations (1–4).

Often a pharmaceutical product may be susceptible to physical and chemical degradation when stored as a ready-to-use solution. The goal of the formulations scientist is to identify the right formulation conditions, the right excipients in optimal quantities, and the right dosage form to maximize stability, biological activity, safety, and marketability of a particular product.

Desired characteristics of a lyophilized product

A lyophilized product should possess certain desirable characteristics, including

- long-term stability
- short reconstitution time
- elegant cake appearance
- maintenance of the characteristics of the original dosage form upon reconstitution, including so-

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lution properties; structure or conformation of proteins; and particle-size distribution of suspensions
- isotonicity upon reconstitution (in some cases, also for bulk solution).

The lyophilization process
The lyophilization process consists of three stages: freezing, primary drying, and secondary drying.

**Freezing.** During this stage the formulation is cooled. Pure crystalline ice forms from the liquid, thereby resulting in a freeze concentration of the remainder of the liquid to a more viscous state that inhibits further crystallization. Ultimately, this highly concentrated and viscous solution solidifies, yielding an amorphous, crystalline, or combined amorphous–crystalline phase.

**Primary drying.** The ice formed during freezing is removed by sublimation at subambient temperatures under vacuum. This step traditionally is carried out at chamber pressures of 40–400 Torr and shelf temperatures ranging from −30 °C to +10 °C. Through out this stage, the product is maintained in the solid state below the collapse temperature of the product in order to dry the product with retention of the structure established in the freezing step. The collapse temperature is the glass transition temperature ($T_g$) in the case of amorphous products or the eutectic temperature ($T_e$) for crystalline products.

**Secondary drying.** The relatively small amount of bound water remaining in the matrix is removed by desorption. During this stage, the temperature of the shelf and product are increased to promote adequate desorption rates and achieve the desired residual moisture.

Possible destabilizing effects of the lyophilization process
**Freezing.** Freezing damage can occur with labile products such as liposomes, proteins, and viruses (5,6). Initial ice-crystal size depends on the relative contributions of nucleation and crystal growth of ice. A rapid nucleation and growth rate resulting from a large degree of supercooling leads to a larger number of small ice crystals, which in turn presents a large ice–water interface (7). Exposure of proteins to this ice–water interface can lead to denaturation. Freezing stresses also can disrupt the liposome bilayer and emulsion structure.

The freezing step will determine the structure of the final dried cake as well as the drying rate. Small ice crystals produce pores with lower volume–surface area, thus resulting in lower diffusive flux and slower sublimation rates (7).

**Drying.** Removal of the hydration shell from proteins and products such as liposomes during drying in the absence of the appropriate stabilizers can cause destabilization of the protein structure and fusion of liposomes (8,9). Extremely low water content in the final product can result in destabilization, and optimal water content should be determined (10). The desired residual moisture must be correlated to stability during long-term storage as
part of development studies.

**Excipients in a lyophilized formulation**

The design of a lyophilized formulation is dependent on the requirements of the active pharmaceutical ingredient (API) and intended route of administration. A formulation may consist of one or more excipients that perform one or more functions. Excipients may be characterized as buffers and pH adjusters, bulking agents, stabilizers, and tonicity modifiers.

**Buffers.** Buffers are required in pharmaceutical formulations to stabilize pH. In the development of lyophilized formulations, the choice of buffer can be critical. Phosphate buffers, especially sodium phosphate, undergo drastic pH changes during freezing (6,11,12). A good approach is to use low concentrations of a buffer that undergoes minimal pH change during freezing such as Tris, citrate, and histidine buffers (13).

**Bulking agents.** The purpose of the bulking agent is to provide bulk to the formulation. This is important in cases in which very low concentrations of the active ingredient are used. Crystalline bulking agents produce an elegant cake structure with good mechanical properties. However, these materials often are ineffective in stabilizing products such as emulsions, proteins, and liposomes but may be suitable for small-chemical drugs and some peptides (14,15). If a crystalline phase is suitable, mannitol can be used. Sucrose or one of the other disaccharides can be used in a protein or liposome product.

**Stabilizers.** In addition to being bulking agents, disaccharides form an amorphous sugar glass and have proven to be most effective in stabilizing products such as liposomes and proteins during lyophilization (1,8,9,16). Sucrose and trehalose are inert and have been used in stabilizing liposome, protein, and virus formulations. Glucose, lactose, and maltose are reducing sugars and can reduce proteins by means of the maillard reaction (17–19).

Two hypothesis have been postulated to explain the stabilizing effects of the disaccharides.

- The water replacement hypothesis: Disaccharides have been found to interact with these products by hydrogen bonding similarly to the replaced water.
- The vitrification hypothesis: Disaccharides form sugar glasses of extremely high viscosity. The drug and water molecules are immobilized in the viscous glass, leading to extremely high activation energies required for any reactions to occur (8,9,16,20,21).

**Tonicity adjusters.** In several cases, an isotonic formulation might be required. The need for such a formulation may be dictated by either the stability requirements of the bulk solution or those for the route of administration. Excipients such as mannitol, sucrose, glycine, glycerol, and sodium chloride are good tonicity adjusters. Glycine can lower the glass-transition temperature if it is maintained in the amorphous phase. Tonicity modifiers also can
be included in the diluent rather than the formulation.

**Glass-transition temperature and its significance**

When heated, sugar glasses undergo a second-order transition from a rigid state to a viscoelastic rubbery state. The temperature at which the vitreous transformation occurs is the glass-transition temperature ($T_g$). When a product exceeds the $T_g$ value, the rigid glass softens to become a highly viscous rubbery material and collapses. The $T_g$ value of a formulation can be determined by differential scanning calorimetry (DSC), and the collapse temperature is measured by freeze-drying microscopy (22–24). Primary drying is always performed at the highest possible temperature while maintaining the product below the collapse temperature. A 5 °C increase in product temperature can lead to a decrease in drying time by a factor of two (13).

The dried amorphous product material also has a $T_g$ value. As water is removed during secondary drying, $T_g$ increases. Storage below $T_g$ is important for several products to maintain the rigid-glass structure and hence stability of the product (25,26).

**Formulation example 1: development of a lyophilized liposome formulation**

Preformulation studies were performed to select optimal pH, ionic strength, and excipients to optimize stability of the drug and lipids (F.K. Bedu-Addo, R. Coe, S. Bhamadi-
A diacyl phosphatidylcholine was used as the matrix lipid, cholesterol was included in the formulation to improve rigidity of the bilayer, and a negatively charged lipid was included to improve blood circulation time. Mannitol was selected as the bulk- ing agent, and maltose was a stabilizer. Dynamic light scattering and microscopy were used to monitor liposome fusion and disintegration. Lipid and drug stability were monitored by reverse-phase high-performance liquid chromatography (RP-HPLC). Water content was evaluated by Karl Fisher titration.

Lipid and drug concentrations were determined on the basis of the required dosage, therefore optimal sugar:lipid ratios were investigated by altering maltose concentrations. Maltose concentrations of 200, 125, and 100 mg/mL were investigated, thus resulting in sugar:lipid weight ratios of 3.4, 2.1, and 1.7, respectively. The effect of water content between 1 and 9% also was investigated.

Glass-transition enthalpy was evaluated using DSC to obtain a quantitative estimate of glass structure existing in the formulation. As shown in Figure 1, the effect of water content on glass content was dependent on the maltose:lipid ratio. Glassy structure content decreased with a decrease in water content. This effect diminished as the sugar:lipid ratio was increased. The $T_g$ value, as measured by DSC, increased linearly with water content. $T_g$, which is an indicator of the rigidity of the sugar glass, also increased with an increase in the sugar:lipid ratio. Liposome stability increased linearly with an increase in the amount of glassy structure existing in the formulation. Fusion leading to increase in particle size was observed at lower maltose:lipid ratios as water content was decreased. At a 1.7 ratio, liposome disintegration was observed below 2.7% water (see Table I).

Fusion leading to an increase in liposome size and potential drug leakage was observed at the lower maltose:lipid ratios and water con-

### Table I: Effect of water content and sugar–lipid ratio on liposome stability.

<table>
<thead>
<tr>
<th>Residual water content (%)</th>
<th>Sugar:lipid ratio 3.4</th>
<th>Sugar:lipid ratio 2.1</th>
<th>Sugar:lipid ratio 1.7</th>
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<td>7.2</td>
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</tr>
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<td>4.8</td>
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<td>132</td>
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tent. A maltose: lipid ratio of 3.4 provided good stability during lyophilization. The $T_g$ value and glass-transition enthalpy were good indicators of liposome stability.

**Formulation example 2: development of a lyophilized protein formulation**

A study was conducted to develop a lyophilized protein formulation (F.K. Bedu-Addo, R. Moreadith, and S. Advant, Diosynth-RTP/Thrombogenics Ltd, 2000). The first step in developing any formulation is determining the solubility and liquid stability at various pH, ionic strength, and excipient conditions conducted as part of the preformulation study. At this stage, it is necessary to have the right analytical tools to fully characterize the product. In the case of proteins, one must also understand the effects of various potential formulation conditions and excipients on conformation and conformational stability. This is typically achieved using biophysical techniques such as circular dichroism (CD), fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry, and fluorescence spectroscopy (27–32). FTIR can also be used to evaluate protein structure in the dried cake (27).

On the basis of the results of a preformulation study, five formulations were developed and evaluated using a conservative lyophilization cycle:

- **Formulation 1** contained a disaccharide and no crystalline bulking agent.
- **Formulation 2** contained a low ratio of disaccharide to bulking agent.
- **Formulation 3** contained a high ratio of disaccharide to bulking agent with 0.005% of a nonionic surfactant.
- **Formulation 4** contained a high ratio of disaccharide to bulking agent with 0.01% of a nonionic surfactant.
- **Formulation 5** contained a high ratio of disaccharide to bulking agent with no nonionic surfactant.

A nonionic surfactant was included in two of the formulations because in some cases, even in the presence of the appropriate stabilizers, a surfactant is required to inhibit aggregation upon reconstitution (33,34). Studies also have shown that the long-term stability of a lyophilized protein formulation and also the tendency to form aggregates upon reconstitution correlates with the extent of deviation from the protein’s native conformation.

Deamidation and oxidation were evaluated by RP-HPLC, formation of insoluble aggregates by UV400, soluble aggregate formation by size exclusion chromatography (SEC), protein concentration by UV280 and conformational change by CD.

Formulation 2 underwent significant conformational change during freezing and resulted in 2% aggregates by SEC. All formulations except Formulation 1 exhibited some conformational change during the freezing step. No further conformational changes were observed during drying, no chemical changes were observed in any formulation. No in-
soluble aggregates were observed during reconstitution.

Formulation 1 exhibited the longest drying and reconstitution times and also had the least elegant-looking cake structure. Formulations 3 and 4 containing surfactant did not provide any advantages over Formulation 5 and were eliminated. Formulations containing a crystalline bulking agent provided an elegant cake. Formulation 5 was selected. The very minor differences between the formulations were expected because preformulation studies had been performed to identify optimal conditions and the formulations optimized with respect to pH, buffer, stabilizer and bulking agent before lyophilization.

**Effects of the formulation on the lyophilization process**

One must understand that the process will be determined by the formulation. For example, the use of disaccharides will result in a low collapse temperature, which causes primary drying to be performed at low temperatures and implying a long process. A large volume fill or high solids content in the formulation will provide increased resistance to mass transfer, hence a longer process (35).

The process also can determine the properties of the formulation. The freezing process can influence crystallization of excipients such as mannitol and glycine (36–39). Incomplete crystallization will depress the collapse temperature.

Significant crystallization of the bulking agent will reduce drying time. However, large amounts of crystalline bulking agent can reduce stabilizing effects of the amorphous stabilizer especially with proteins.

**Summary**

Always optimize the formulation (pH, buffer, ionic strength, stabilizers) to provide maximum stability of the bulk solution before lyophilization as well as dried product upon long term storage.

Select the right excipients (amount and type) to provide product stability and efficient drying. For example, a dried small-chemical drug might be stable in only a crystalline bulking agent, whereas a dried protein product may require an amorphous stabilizer.

Select the lyophilization process to allow maximum drying efficiency while maintaining product integrity.

Understand the effect of each step of the process on the formulation to design the right process for the product.

**References**

25. S.P. Duddu and P.R. Dal Monte, “Effect of Glass-Transition Temperature on the Stability of Lyophilized Form-


