The freezing method used during lyophilization can substantially affect the structure of the ice formed, the water-vapor flow during primary drying, and the quality of the final dried product. Controlling how a solution freezes can shorten lyophilization cycles and produce more stable formulations.

Lyophilization (freeze-drying) is often used to prepare dry pharmaceutical formulations to achieve commercially viable shelf lives. The process comprises three steps: freezing, primary drying, and secondary drying. As water freezes in the first step, the dissolved components in the formulation remain in the residual liquid, a phase termed the freeze-concentrate. At the point of maximal ice formation, the freeze-concentrate solidifies between the ice crystals that make up the lattice. Under appropriate lyophilization conditions, the ice is removed by sublimation during primary drying, leaving the remaining freeze-concentrate in the same physical and chemical structure as when the ice was present. Residual water in the freeze-concentrate is removed in the secondary drying step.

Lyophilization cycle development typically focuses on optimizing the primary drying step. That is the most time consuming of the three steps, and the primary drying parameters are easily adjustable. They can affect both the time involved and the quality of the resulting cake. Extensive investigation of primary drying has demonstrated that two important parameters are chamber pressure and shelf temperature (1–9). They are usually adjusted to maximize the rate of heat transfer to each vial (speeding ice sublimation) without causing cake collapse.

Less attention has been paid to the freezing conditions and their potential effect on the primary and secondary drying processes and on the characteristics of the final product. Kochs et al. reported the effects of freezing conditions on primary drying in a specially designed aluminum and plastic sample cell (10). They observed variations in vapor diffusion coefficients (a measure of the ease of water-vapor flow) as a function of position and cooling rate. The variations appeared to be largely due to variations in sample morphology. Searles et al. reported some effects of freezing on the rate of primary drying in vials (11). They found that the primary drying rate was dependent on the ice nucleation temperature. Faster drying was also attributed to an increase in lamellar ice crystal content, formed when ice nucleants from Pseudomonas syringae were added in the vial contents. Searles and colleagues also showed that adding a properly specified annealing step in the freezing procedure increased the drying rate (12).

Our article describes the effects of several freezing methods on water-vapor flow during primary drying and on the final dried cake structure.

**NORMAL FREEZING AND SUBLIMATION**

When freeze-drying pharmaceutical products, manufacturers place vials containing aqueous formulations on shelves within a lyophilizer. Typically, the shelf temperature is then decreased (from 5 °C) over three hours and held at a low temperature (typically −40 °C to −50 °C) to ensure that all the vials freeze completely. As the temperature decreases, the liquid cools below the freezing point of the solution: Supercooled temperatures range from −10 °C to −20 °C with ice nucleation typically occurring at −15 °C. Freezing point depression accounts for a lowering of the freezing temperature by only 1.8 kelvin per molal. Most pharmaceutical manufacturing relies on supercooling because of the lack of nucleation centers in the formulations (such as particulates in solution or imperfections on the inside surface of the vials).

In a supercooled solution at −15 °C, ice can nucleate and propagate through the solution.
However, the entire solution cannot freeze immediately because the supercooled water can absorb only 15 cal/g of the 79 cal/g of heat given off by the ice formation. Therefore, the ice propagates from the nucleation site, and about 19% crystallizes in multibranching, tortuous paths while the vial contents warm to approximately −1 °C. After the initial ice network has formed, additional heat is removed from the solution by the shelf, and the remaining water freezes when the previously formed ice crystals grow.

**Primary drying.** At the completion of freezing, primary drying is initiated with the lowering of the chamber pressure and an increase in the shelf temperature. The ice sublimation front moves downward through the frozen plug, a process that makes the water vapor transit through the channels left by the sublimed ice above. Those narrow, often tortuous channels resist vapor flow, so sublimation is slowed. Consideration of the vapor channels illustrates how the method of freezing, by affecting ice crystal structure, can affect primary drying. Decreasing the resistance to mass transfer of water vapor increases the sublimation rate, which leads to shorter primary drying times while lowering the product temperature.

**MASS TRANSFER RESISTANCE**

An important parameter governing the relationship between independent variables (the shelf temperature and chamber pressure) and dependent variables (the ice sublimation rate and the product temperature) has been defined by Pikal as the **resistance to water-vapor flow** or the **mass transfer resistance** (MTR) (2).

**Determining MTR.** MTR describes the proportionality between the specific sublimation rate \( \frac{m}{A_p} \) and the pressure driving force \( P_o - P_v \):

\[
MTR = \frac{P_o - P_v}{m / A_p}
\]

where \( m \) is the sublimation rate, \( A_p \) is the cross-sectional area of the product in the vial, MTR is the normalized dried product resistance, \( P_o \) is the equilibrium vapor pressure of ice at the temperature of the subliming ice, and \( P_v \) is the pressure in the chamber. The relationship is analogous to that of electrical resistance: For a given pressure driving force (voltage), a decrease in MTR (electrical resistance) increases the rate of sublimation (electrical current). To determine MTR, measure the product temperature (from which we obtain \( P_o \) using the equation published by Jancso et al., 13) and the sublimation rate \( m \). These measurements can be obtained using a thermocouple and sequentially stoppering the vials during primary drying, then weighing the amount of the remaining solution. We previously published a complete description of this method for determining MTR (9).

**Effect of the freezing method on MTR.** Figure 1 shows MTRs during primary drying (10 °C shelf temperature, 100 mTorr chamber pressure) for vials frozen using the standard freezing process that we described earlier and for those frozen by alternative methods described below (plots used 25 mg/mL rhuMAb HER2, 20 mg/mL trehalose, 0.1 mg/mL polysorbate 20, and 5 mM histidine, with a 6.0 pH, filled at 5.0 mL in 10cc tubing vials). Following standard freezing, the resistance initially rose quickly, then more slowly as the sublimation front moved down the vial (increasing the thickness of the dried layer). That indicates that the upper layer of the dried cake contributed more to the resistance than did the lower portion, suggesting that the structure of the dried layer varied in the vertical direction.

Curves in Figure 1a represent the effect of the dried layer thickness on the cumulative MTR. The

<table>
<thead>
<tr>
<th>Freezing Method</th>
<th>Shelf Temp (°C)</th>
<th>Sublimation Rate (g/hr)</th>
<th>Product Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>10</td>
<td>0.36</td>
<td>−24.5</td>
</tr>
<tr>
<td>Vertical</td>
<td>10</td>
<td>0.46</td>
<td>−27.0</td>
</tr>
<tr>
<td>Vertical</td>
<td>20</td>
<td>0.48</td>
<td>−26.0</td>
</tr>
<tr>
<td>Vertical</td>
<td>30</td>
<td>0.55</td>
<td>−23.5</td>
</tr>
</tbody>
</table>

Table 1. Effect of freezing method and primary drying shelf temperature on sublimation rate and primary drying product temperature
Vacuum, the paraffin enters all the pores of the cake with no observable disturbance to the cake structure. The vial is then removed from the vacuum oven, cooled, and sectioned. The sectioned cakes are then observed under a fluorescence microscope. Interference from the fluorescence deeper in the cake is minimized by using a dark dye in the paraffin.

**Correlating MTR to cake structure.** The tortuous path that supercooled ice takes when propagating (Figure 3a) shows little directionality to the channels after sublimation. Those channels, roughly 25–100 μm in diameter, serve as conduits for water-vapor transport from the sublimation front to the vial headspace. Resistance to vapor flow out of the cake is a result of both the small, tortuous channels and thick skin-like material on the top of the cake (reaching a thickness greater than 1–2 mm). In the standard freezing scenario of Figures 1a and 1b, high MTR can be seen as the sublimation front moves down through the first 0.2 cm of the cake, corresponding to the thickness of the skin. Once the sublimation front passed the skin, the cumulative MTR increased more slowly (suggesting lower local MTR).

**Controlling ice nucleation.** One way to produce directional freezing is to freeze the vial contents rapidly by immersing the vial into a dry-ice/ethanol bath until it is completely frozen. The resulting pores left from the rapid freezing and ice propagation are very small (Figure 3b). They start at the walls of the vial, move toward the middle, then move up. Although directional, the pores are small and nonvertical. The effect of such fast freezing can be seen in the MTR: The cumulative MTR increases rapidly and to a higher level (Figure 1a) and shows very high local MTR at small cake depths (Figure 1b). For fast freezing, that high MTR is attributable to the small size of the pores and to the horizontal direction of the channels near the surface of the cake. The restricted vapor flow results in slower ice sublimation.

**Vertical freezing.** To obtain straight, vertical channels, solutions were cooled on wet ice, nucleated at the bottom with dry-ice, and placed on a −50 °C shelf. The ice crystals propagate vertically, and no thick skin forms on the cake surface (Figure 3c). As expected, a vertical channel orientation and the lack of thick skin on top lowers the MTR from that of samples from standard and fast-freezing protocols (Figure 1a). Figure 1b shows that the local MTR is also low and constant for all cake depths. Interestingly, Figure 1b also suggests that changing the freezing.

**CAKE STRUCTURE**

The structure of a lyophilized cake has been reported several times (14–20). In most cases, cakes came from a supercooled solution with little or no attempt at controlling ice nucleation or propagation direction. Samples were generally cut or pulled apart and viewed by scanning electron microscopy (SEM). Because the potentially fragile cake structure was unsupported, the area exposed may have been altered by the cutting. If the cake is pulled apart, the tear will likely follow a structural weakness in the cake, so the exposed area may not be representative of the rest of the cake.

In the paraffin investment technique we developed (Figure 2), paraffin acts as a support for the physical fine structure of the cake and also prevents water absorption during sectioning, observation, and storage (9). The method uses a fluorescent molecule (such as rhodamine B) that is added to the solution before lyophilization and distributes equally throughout the cake (with no visible phase separation or spatial partitioning). After lyophilization, the cake in the vial is placed under vacuum and warmed to 55 °C. Then paraffin is poured into the vial. While warm and under vacuum, the paraffin enters all the pores of the cake with no observable disturbance to the cake structure. The vial is then removed from the vacuum oven, cooled, and sectioned. The sectioned cakes are then observed under a fluorescence microscope. Interference from the fluorescence deeper in the cake is minimized by using a dark dye in the paraffin.

**Figure 2. Paraffin dye method**

Local MTR for various cake depths can be determined from the derivative of those curves (Figure 1b). The derivative curve (for standard freezing) also shows that the upper layer of the dried cake contributes more to the resistance than does the lower portion. That result is consistent with the results published by Kochs et al. (10). The relationship between resistance and dried cake structure can be investigated by studying cake structure after lyophilization.

**Figure 2. Paraffin dye method**

Filled vial → add fluorescent dye → lyophilize → warm, add black paraffin under vacuum → cool, release plug → image under a fluorescence microscope
method did not alter the cake structure at the vial bottom, because the local MTR values are equivalent for dried layer thickness greater than 0.5 cm.

Vertical freezing produces a higher sublimation rate and lower product temperature during primary drying (Table 1). The lower MTR allows faster sublimation, which results in lower product temperatures through increased heat removal from the latent heat of sublimation. The vertical freezing results are consistent with those found by Searles et al. that the presence of lamellar (instead of spheroidal) ice crystals increases drying rates (11).

SUPERCOOLING AND ANNEALING

Ice structure and MTR during primary drying can be altered by freezing the vial contents using the standard method (supercooling followed by spontaneous nucleation), then warming the frozen solution in the vial to a temperature just below the melting point (for example -2 °C) for 10 hours. This allows the ice crystals to rearrange and grow to a more stable state. The vials are then cooled again to -50 °C. Figure 3d shows results from that annealing process. The cakes produced after annealing tend to have larger pores than those from the standard freezing method, which would be expected to facilitate water-vapor transmission and lower the MTR during primary drying. Figure 1a shows that the annealed material has a much lower MTR than solutions frozen by the standard or fast processes and is comparable to solutions that were nucleated on the bottom with vertical ice propagation. The lower resistance observed for annealed material is consistent with a faster sublimation reported by Searles et al. (12). Figure 1b also shows that the annealed material has a local MTR that is low and constant throughout the cake. The effects of any skin appear to be minimal.

DECREASING MTR BY OTHER METHODS

Depending on solution composition, it may be possible to adjust the temperature (or the formulation) such that primary drying takes place at product temperatures near the glass transition temperature (Tg’) of the freeze-concentrate. That allows the freeze-concentrate to flow enough to create holes in the channel walls so water vapor can be transmitted in a less tortuous path, more directly up and out of the cake.

Figure 4a shows an SEM result in which holes have formed in the walls of the pores. These porous walls were not evident in formulations containing protein (Figure 4b) but were observed only in formulations using a protein-free placebo, which has a lower Tg’. The holes were less evident in the protein-free material when it was dried at lower temperatures. That implies that not all formulations are amenable to pore formation.

Figure 5 shows that the MTR for the placebo was significantly lower than that of the solution that contains protein (9). Obviously, the selection of processing conditions needs to consider final product quality. The possibility that such a small-scale collapse might alter the molecular structure or stability of the product would need to be evaluated by an appropriate analytical program.

Table 2. Effect of freezing method on the dried state stability of a therapeutic protein

<table>
<thead>
<tr>
<th>Freezing Method</th>
<th>Aggregation Rateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>2.3c</td>
</tr>
<tr>
<td>Annealed</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a2.5 mg/mL protein, 123 mg/mL total excipient
b at 50 °C
cpercent per month

OPTIMIZING THE CYCLE

The manner of freezing can have a significant impact on primary drying characteristics of a formulation. For a given primary drying shelf temperature and chamber pressure, a decrease in MTR will increase the rate of sublimation. In addition, an increase in the rate of sublimation will lead to a lower product temperature because of the enthalpy of ice sublimation (Table 1). Increasing the primary drying shelf temperature of vertically frozen vials increases the product tem-
perature for primary drying, which then matches that of a standard-frozen material dried at a colder shelf temperature. That higher shelf temperature adds driving force to the heat transfer, speeding primary drying. For equivalent product temperatures, the rate of sublimation was 40% faster for vertically oriented ice than for standard-frozen solutions (Table 1). That leads to a decrease in the time required for primary drying — which constitutes half of the time spent in the lyophilization process. Such a decrease should also be expected from annealed vials, so the duration of the lyophilization cycle should be reduced as well.

OTHER CONSEQUENCES OF FREEZING

The freezing process can have unexpected consequences on product quality. For example, some forms of sodium phosphate can crystallize upon slow freezing or annealing, resulting in a pH decrease in the freeze-concentrate (21–23). A decrease in pH has been shown to affect the stability of a drug when frozen and after lyophilization (24–26). Other excipients that crystallize during freezing (for example, mannitol) can lose their ability to stabilize proteins in the dried state (27–30). Some proteins undergo cold denaturation during slow freezing or annealing, which can have deleterious effects on product quality upon reconstitution.

Freezing methods can alter the void–solid interfacial area of a lyophilized cake and, inversely, the thickness of its channel walls. An increased surface area correlates with decreased dried product stability for some proteins (31). Freezing by immersing a vial into liquid nitrogen can result in increased protein aggregation (32) or decreased enzyme activity (33) when compared with freezer-cooled samples. Preliminary studies indicate that the freezing method has a significant effect on dried protein stability (Table 2). In addition, reconstitution time could be affected because increasing the surface area may allow faster reconstitution.

PRACTICAL ASPECTS

Freezing methods are seldom investigated because freezing is difficult to control. Normally, pharmaceutical products supercool to roughly −15 °C before nucleating, which is difficult to overcome by standard processes. Ice might be induced to nucleate at higher temperatures (closer to 0 °C) by conditioning the bottom surface of the glass vial. Nucleation would then occur on the bottom surface, and the ice would propagate in a vertical direction resulting in lower MTRs. Vials with that characteristic are not yet available. Annealing can be more practical but also has limitations. Whether annealing influences the time for lyophilization or the temperature variations of the product during freezing needs to be demonstrated.

Shortening the cycle. The freezing method used during lyophilization can have a significant effect on the structure of the ice formed, affecting both the water-vapor flow during primary drying and the final product. Freezing protein solutions in vials with vertically propagated crystals may prevent a thick skin from forming on the top of the lyophilized cake. The structure of vertically frozen material facilitates fast water-vapor flow during primary drying, resulting in lower product temperatures. By increasing the primary drying shelf temperature, the rate of sublimation was 40% faster in our studies for the vertically frozen material than for the standard-frozen material with an equivalent product temperature. We also observed increased sublimation in material annealed during the freezing segment. Decreased surface area correlates with increased product stability for some proteins. Understanding and controlling how a solution freezes can lead to shorter lyophilization cycles and, in some cases, more stable products.

ACKNOWLEDGMENTS

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