Characterization of Murine Monoclonal Antibody to Tumor Necrosis Factor (TNF-MAb) Formulation for Freeze-Drying Cycle Development

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Purpose. This study was designed to characterize the formulation of protein pharmaceuticals for freeze-drying cycle development. Thermal properties of a protein formulation in a freezing temperature range are important in the development of freezing and primary drying phases. Moisture sorption properties and the relationship between moisture and stability are the bases for the design of the secondary drying phase.

Methods. We have characterized the formulation of TNF-MAb for the purpose of freeze-drying cycle development. The methods include: DTA with ER probes, freeze-drying microscopy, isothermal water adsorption, and moisture optimization.

Results. The DTA/ER work demonstrated the tendency to "noneutectic" freezing for the TNF-MAb formulation at cooling rates of –1 to –3°C/min. The probability of glycine crystallization during freezing was quite low. A special treatment, either a high subzero temperature holding or annealing could promote the maximum crystallization of glycine, which could dramatically increase the T_g' of the remaining solution. The freeze-drying microscopy further indicated that, after the product was annealed, the cake structure was fully maintained at a T_p below –25°C during primary drying. The moisture optimization study demonstrated that a drier TNF-MAb product had better stability.

Conclusions. An annealing treatment should be implemented in the freezing phase in order for TNF-MAb to be dried at a higher product temperature during primary drying. A secondary drying phase at an elevated temperature was necessary in order to achieve optimum moisture content in the final product.

KEY WORDS: TNF-Mab; protein pharmaceuticals; freeze-drying; formulation characterization.

INTRODUCTION

Freeze-drying (lyophilization) technology has provided desirable stability for many pharmaceutical proteins during shipping and long-term storage. In general, the freeze-drying process consists of three essential phases, namely: 1) Freezing phase: freeze an aqueous product; 2) Primary drying: reduce pressure and increase shelf temperature to sublime ice from the frozen product; 3) Secondary drying: further increase shelf and, consequently, product temperature *in vacuo* and remove additional water from the product. A well-designed freeze-drying cycle not only increases the efficiency of the manufacturing process, but also ensures product stability and aesthetic acceptability (1–4). Greater understanding of protein drug formulation characteristics enables rational design and development of a freeze-drying cycle. During cycle development, the process parameters, such as shelf temperature and chamber pressure at each freeze-drying phase, can be specified as a function of time according to the characteristics of a particular protein formulation. The impact of these parameters on the quality of a final product needs to be taken into consideration. In this respect, the information on the thermal properties of a protein formulation in a frozen temperature range is important in the development of freezing and primary drying phases. The thermal properties usually include eutectic crystallization temperature (T_x), eutectic melting temperature (T_e), glass transition temperature of the maximally frozen concentrated amorphous matrix (T_g'), and ice melting temperature (T_{im}). On the other hand, moisture sorption properties, relationship between moisture and stability, and glass transition temperature (T_g) of a lyophilized product are the bases for the design of secondary drying. Therefore, formulation characterization provides crucial information for designing and developing a freeze-drying cycle.

Many approaches have been explored to determine the thermal properties of formulations for lyophilization, including differential thermal analysis (DTA) (5,7,8), differential scanning calorimetry (DSC) (10), electrical resistance analysis (ERA) (5,7–9,11,13), freeze-drying microscopy (6–8,12,14,15), and cryoenviromental scanning electron microscopy (CESEM) (16). Moreover, moisture sorption challenged by saturated salt solutions and dynamic vapor sorption (DVS) techniques have been used to characterize moisture sorption properties and to determine optimum moisture conditions for product stability (17).

In this study, we have characterized the formulation of Murine Monoclonal Antibody to Tumor Necrosis Factor (TNF-MAb) for the purpose of freeze-drying cycle development. TNF-MAB is a protein with a molecular weight of approximately 150,000 Daltons. The formulation characteristics were investigated by DTA with electrical resistance (ER) probes, freeze-drying microscopy, isothermal water sorption, and moisture optimization studies. The work reported here on the formulation characterization may provide useful information and practical guidelines for the development of freeze-drying cycles with other biological products.

MATERIALS AND METHODS

Materials

TNF-MAb formulation produced by Bayer was used as the test solution. The formulation was mainly composed of TNF-MAb (20 mg/ml), maltose (10 mg/ml) and glycine (20 mg/ml).

DTA/ER Analysis

DTA (differential thermal analysis) was equipped with ER (electrical resistance) probes (11,13). Freezing-thawing behavior of TNF-MAb was monitored by the simultaneous measurement of thermal properties by DTA and electrical

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properties by ER (the combination apparatus was designated DTA/ER). DTA/ER was conducted on 250 microliter sample volumes in accordance with a method described by MacKenzie (11). The procedure contrasts favorably with other current and earlier thermal analytical methods, e.g., that of Rasmussen and MacKenzie (18), that employed 10 microliter sample volumes. Aqueous TNF-MAb samples were frozen to –75°C at rates of 1.5°C/min and, then rapidly cooled to –150°C. The frozen sample was warmed at a warming rate of 1.5°C/min, which was shown not to introduce measurable thermal gradients in the sample. ER determinations were conducted on 0.25-ml sample volumes in the same apparatus. The VirTis EPC-1 control unit was employed in a low frequency AC mode in the determinations. A timed reversing switch was inserted between the sample cell and the control unit to permit the periodic depolarization of the sample electrodes. Cooling was achieved by the controlled aspiration of liquid nitrogen. Warming was obtained with the aid of electrical heating. The temperature differences between sample and reference, as well as the sample's electrical resistance were recorded as a function of temperature. In the DTA thermogram, the upward peak represents an exothermic transition and downward peak indicates an endothermic transition. In the ER plot, electrical resistance increases as the solution conductance decreases. The thermal events in ER results were indicated at the crossing points of tangents in the curves.

Freeze-Drying Microscopy Observation

The freeze-drying events of TNF-MAb solution were microscopically observed on a freeze-drying microscope stage. The microscope was equipped with thermoelectric modules, thermocouples and attached to a vacuum pump. TNF-MAb solution (10 μl) was placed on the sample stage, frozen to –45°C and warmed up at a given rate. The changes of morphology at the freeze-drying front were observed and photographed. The set-up of freeze-drying microscopy and experimental procedures were described in detail elsewhere (15) and the experiments were performed in Dr. Nail's laboratory.

Moisture Sorption Study

The moisture sorption isotherm was determined by measuring water content in the lyophilized product equilibrated under different relative humidity conditions. The product was stored in desiccators with solid salt or saturated salt solutions for 48 hours equilibration at an ambient temperature. The salts included phosphorus pentoxide, lithium chloride, potassium acetate, magnesium chloride, potassium carbonate, and sodium chloride, which generated relative humidities of, approximately, 0, 11%, 23%, 33%, 43%, and 75%, respectively. The vials were sealed immediately after equilibration. The moisture in the lyophilized product was determined by a gravimetric method, which was based on the principle of loss-on-drying (19). Samples were placed in a vacuum chamber (less than 200 mTorr) with acetone in the jacket, and then heated at 60°C for 6–8 hours. The residual moisture was quantified by the loss of the sample weight. The moisture content was plotted against relative humidity to generate the water adsorption curve.

Moisture Optimization

The lyophilized product was challenged under different relative humidity conditions as described above. The sealed samples with different moisture contents were then stored at 40°C and the percent monomer of TNF-MAb, determined by HPLC-SEC, was analyzed at predetermined time intervals up to 6 weeks.

RESULTS

The study focused on how to characterize a protein formulation for the purpose of freeze-drying cycle development. Approaches used in this work were DTA/ER probe, freeze-drying microscopy, moisture sorption isotherm, and moisture optimization.

Freezing/Thawing Behavior by DTA/ER

A series of three separate and consecutive studies were conducted under different freezing and thawing conditions. The freezing process can be conveniently divided into two categories: regular freezing and freezing with a thermal treatment.

Regular Freezing

Aqueous TNF-MAb solution was frozen at –1°C/min to –75°C. The frozen sample was then warmed at a constant rate of 3°C/min through 0°C. Figure 1 shows DTA thermogram during cooling, revealing a spontaneous nucleation of ice starting at approximately –14°C, and a subsequent crystallization of solute at –24°C which was attributed to glycine T_x. Similar to the finding by DTA, electrical resistance curve in Figure 2 (lower curve) demonstrated the nucleation of ice with an abrupt rise in resistance due to crystallization in the same temperature range. Concurrently, the ER curve revealed two glass transitions, primary T_g' at –36°C and secondary T_g'' at –60°C (Figure 2).

DTA results during warming exhibited a well characterized but moderate exothermic event in the range of –36°C to –25°C, suggesting the further and probable completion of glycine crystallization during the warming (Table I). The endothermic sliding curve implied that the frozen sample began to melt even before glycine crystallization was completed. The ER during the same warming (Figure 2, upper curve) copied the behavior of the cooling curve (Table I), but in the opposite direction of the lower curve, except for one region from approximately –30°C to –22°C where decrease of resis-

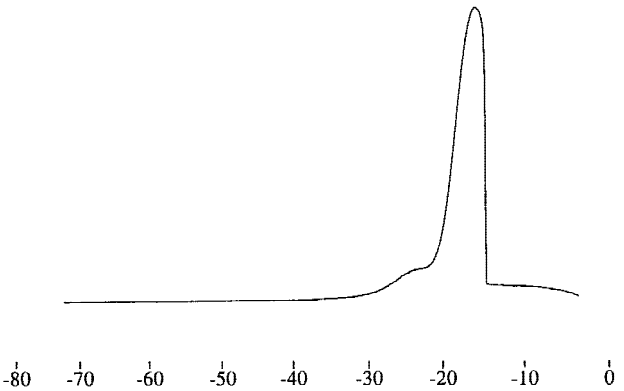

Fig. 1. DTA thermgram of TNF-MAb formulation during slow cooling at 1°C/min. Y-axis: differential temperature (an upward peak represents an exothermic transition).

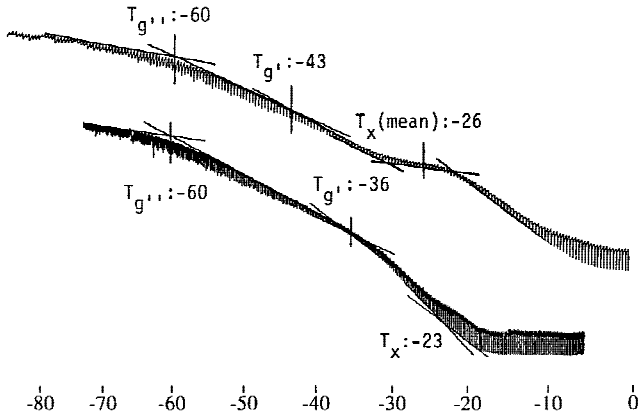

Fig. 2. Electrical resistance of TNF-MAb formulation during cooling at 1°C/min and subsequent warming at 3°C/min. Lower curve: electrical resistance during cooling; Upper curve: electrical resistance during warming. Y-axis: Electrical resistance (log_{10} ohms).

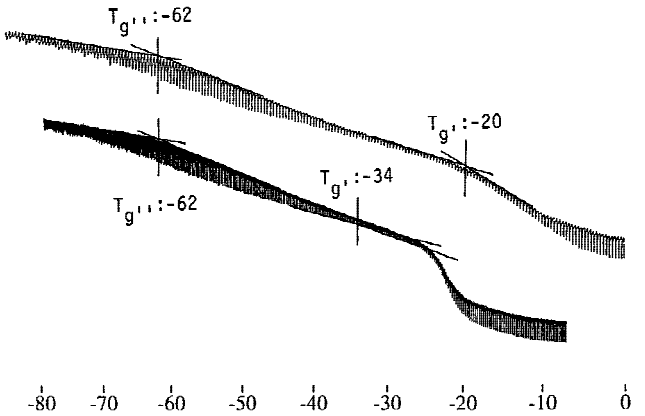

Fig. 3. Electrical resistance of TNF-MAb formulation during cooling with "sub-zero holding" and subsequent warming at 3°C/min. Lower curve: electrical resistance during cooling; Upper curve: electrical resistance during warming. Y-axis: Electrical resistance (log_{10} ohms).

tance was slowed, due to glycine crystallization. The combined findings by DTA/ER in Figures 1 and Figure 2 suggested that the freezing at the rate of 1°C/min occurred with only partial glycine eutectic crystallization and the crystallization process was further completed during the warming process.

Freezing with Thermal Treatments

Freezing with High Subzero Temperature Holding. The TNF-MAb sample was frozen to –4°C at –1.5°C/min, held at a high subzero temperature, –4°C for 1 hour, and then cooled from –4°C to –80°C at –1.5°C/min. The frozen sample was then warmed at a constant rate of 3°C/min through 0°C. DTA during cooling demonstrated a well-determined ice crystallization at approximately –10°C and glycine eutectic crystallization at approximately –22°C (data not shown). As shown in the lower curve of Figure 3, ER during the cooling depicted a more rapid rise in electrical resistance occurring in the glycine eutectic crystallization temperature range, which implied a much more complete glycine crystallization during freezing with the high subzero holding (–4°C) than the regular freezing.

In contrast to the data from the regular freezing study, DTA during warming after the thermal treatment did not exhibit any eutectic exothermic transition. The results further confirmed the completion of glycine crystallization during the freezing where the ice was allowed to nucleate at a high subzero temperature. The upper curve of ER result in Figure 3 suggested that T_g' of remaining solution was shifted to a much higher temperature, –20°C after glycine was completely crystallized out from the solution. This is reasonable because glycine has a relatively low T_g' at approximately –70°C (20).

Freezing with Low Subzero Temperature. Two consecutive cooling and warming cycles were conducted to ensure completion of glycine crystallization in the first cycle. The first

Table I. Summary of the Thermal Transition Temperatures During the Warming After Cooling with Different Treatments[a]

Treatments	Results	T_g'' (°C) during warming[b]	T_g' (°C) during warming[c]	T_x (°C) during warming[d]	T_{im}, (°C) during warming[e]
Regular cooling at 1°C/min	DTA	—[a]	–43	–31	–16
Regular cooling at 1°C/min	ER	–60	–43	–26	–18
Thermal treatment #1: cooling at 1.5°C/min, held at –4°C	DTA	—	–26	—	–18
Thermal treatment #1: cooling at 1.5°C/min, held at –4°C	ER	–62	–20	—	–15
Thermal treatment #2: First cycle: cooling at 3°C/min, then warm up to –15°C	DTA	–71	–48	–28	—
Thermal treatment #2: First cycle: cooling at 3°C/min, then warm up to –15°C	ER	–70	–42	–26	—
Thermal treatment #2: Second cycle after annealing: cooling at 3°C/min, then warm up	DTA[f]	–61	–26	—	–12
Thermal treatment #2: Second cycle after annealing: cooling at 3°C/min, then warm up	ER[f]	–59	–21	—	–12

[a] — represents "not observed" or "not applicable."
[b] T_g'' represents a second order phase transition identified with the onset motion of small molecules, i.e. water, which is not directly related to the "collapse phenomenon."
[c] T_g' identifies the glass transition of maximum frozen concentrates, which is closely related to the collapse phenomenon in freeze-drying.
[d] T_x represents the eutectic crystallization temperature.
[e] T_{im} is onset of ice dissolution into a softened, amorphous solute concentrate.
[f] Data from the second warming.

freezing was conducted at –3°C/min from 5°C to –160°C. A first warming was made at 3°C/min but was stopped at –15°C after which the sample material was cooled again to –165°C. A second warming was conducted at 3°C/min through 0°C.

It was evident that the first freezing at –3°C/min proceeded with no glycine crystallization, but the pronounced exothermic peak in the DTA trace during the first warming or annealing to –15°C implied that glycine was crystallized completely (data not shown). Because glycine has a relatively high T_e (approximately –4°C) (18), warming the sample up to –15°C in the first warming cycle did not permit any eutectic melting of the glycine eutectic crystals. Since glycine was completely crystallized during the first warming, there was no further occurrence of the glycine crystallization during the second cooling and warming cycle. More importantly, ER curve from the second warming showed that the T_g' of the remaining solution was elevated from low –42°C to –21°C due to completion of glycine crystallization during annealing (the first freeze-thawing cycle) (Table I). It is noteworthy that when the freezing rate was –3°C/min, the crystallization of glycine was not induced during the first freezing. This implied that an initial freezing as slow as –3°C/min might be too fast to permit the development of nucleation and growth of glycine crystals during the freezing.

Freeze-Drying Microscopy

Freeze-drying microscopy provides real-time image of freezing, melting, crystallization, collapse, and melt-back during the freeze-thawing and freeze-drying processes. After the TNF-MAb formulation was initially frozen at –45°C, annealed to –20°C and then returned to –45°C, the microscope then monitored the morphology changes of the drying front during the drying. As shown in Figure 4, the cake structure was maintained when the frozen TNF-MAb solution was freeze-dried at a product temperature up to –25°C. It was apparent that the cake structure progressively disappeared at product temperature between –20° to –10°C due to collapse of amorphous structure at the product temperature (T_p) above the T_g' of the remaining frozen solution. As expected, glycine eutectic melting took place and cake structure totally disappeared as the product temperature rose above –7°C (Figure 4).

Moisture Adsorption Properties of Lyophilized Cake

Water adsorption characteristics of a freeze-dried formulation provide information on the affinity of water for a dried product. The water adsorption isotherm at 22°C was obtained by plotting water content in the TNF-MAb dry cake as a function of the relative humidity to which the samples were exposed. Figure 5 illustrates the water adsorption isotherm of TNF-MAb lyophilized sample at 22°C. The adsorption isotherm reflected a progressive increase in water content as the relative humidity was increased. The TNF-MAb lyophilized product could adsorb up to 10% of moisture at the 75% relative humidity level. The results implied that TNF-MAb formulation was moderately hygroscopic and secondary drying

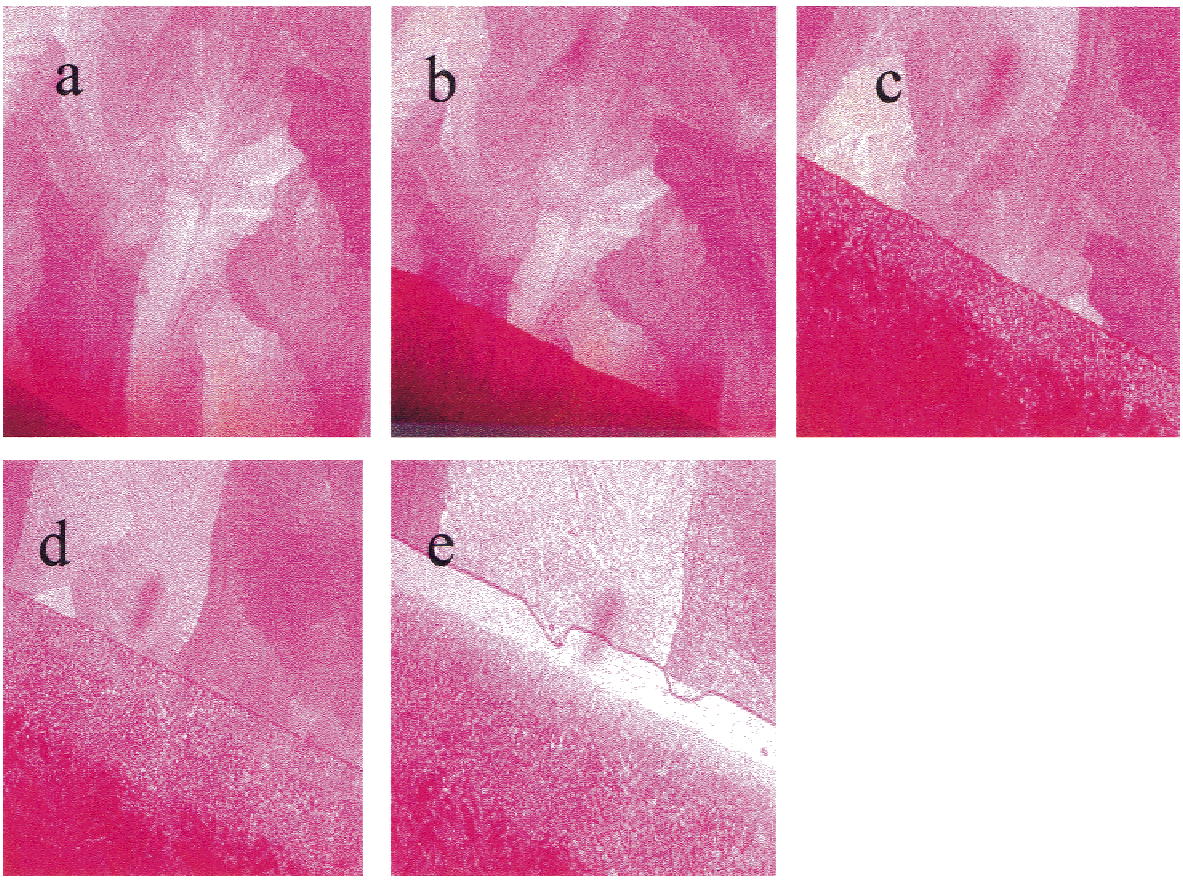

Fig. 4. Freeze-drying microscopy of TNF-MAb structure during primary drying after freezing with annealing treatment: (a) T_p = –30°C; (b) T_p = –25°C; (c) T_p = –20°C; (d) T_p = –10°C; (e) T_p = –7°C.

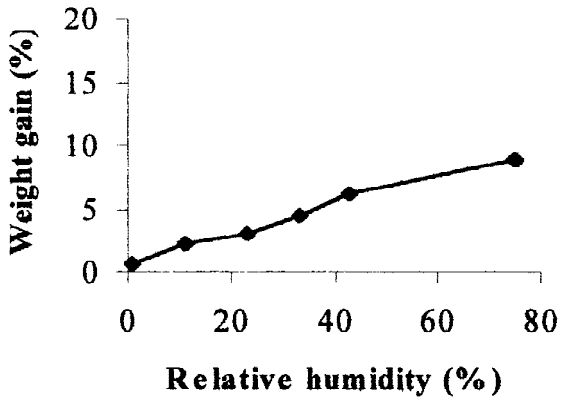

Fig. 5. TNF-MAb water adsorption isotherm at 22°C.

with a sufficient time length was necessary in the freeze-drying cycle.

Moisture Optimization

Samples were exposed to different relative humilities for 48 hours to allow them to adsorb various amount of moisture. An accelerated stability program was then conducted at 40°C. The effect of water content on aggregation of the freeze-dried TNF-MAb was evaluated. The results presented in Figure 6 indicated that the concentration of protein monomer drastically decreased and, consequently, aggregation increased during storage as the moisture content was increased from 0.5% to 8%. The data clearly demonstrated that the stability of the freeze-dried TNF-MAb was increased with a decrease of moisture content. Over all, the results support the use of a secondary drying cycle that makes the freeze-dried TNF-MAb product as dry as possible.

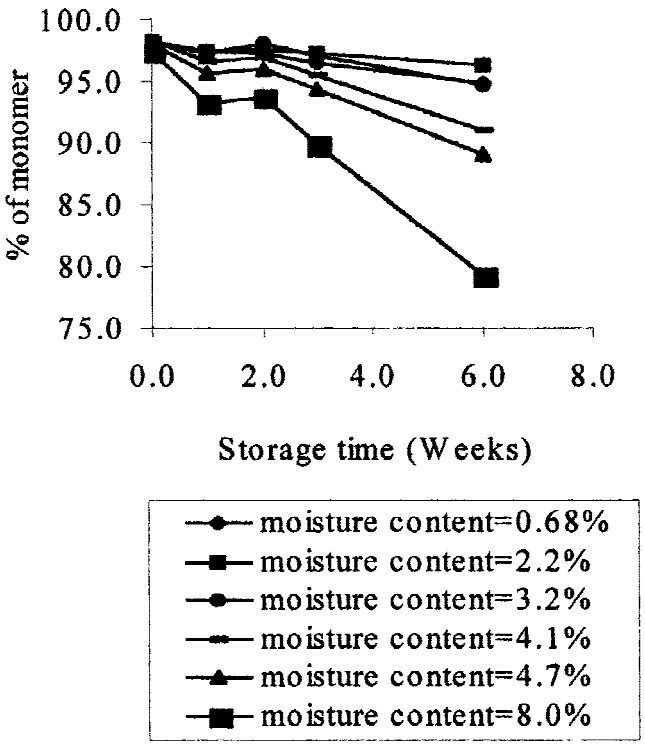

Fig. 6. Percent of TNF-MAb monomers measured by HPLC-SEC for samples stored at 40°C

DISCUSSIONS

A pharmaceutical protein drug formulation can consist of many different excipients. Sugars such as sucrose are usually used to stabilize protein conformation against denaturation due to water removal. Polymers and other proteins such as human albumin serum also function similarly. These excipients usually remain amorphous. On the other hand, bulking agents such as mannitol and glycine are commonly used for providing cake structure and appearance. Such excipients are usually expected to be in crystalline states after lyophilization. For pharmaceutical protein products, a formulation termed "crystalline matrix" is widely used. In such a formulation, crystallizable components are added at a relatively high level so that a crystalline matrix is formed for the amorphous components to collapse upon. In such a way, the crystalline component provides excellent cake appearance, good reconstitution characteristics, and ease in lyophilization. Meanwhile, the amorphous component stabilizes proteins during processing and storage. The lyophilization of such a formulation allows partial collapse (also termed Micro-collapse) without affecting cake appearance. As a result, the product can be lyophilized at a relatively higher product temperature during primary drying if protein activity is not compromised.

There are several basic principles for design and development of a freeze-drying cycle. First, as discussed above, it is desirable to have both crystalline and amorphous components in a formulation matrix during and after lyophilization. Therefore, the knowledge of the eutectic crystallization temperature, $T_{x,}$ of excipients (especially crystalline bulking agent, such as glycine, alanine, mannitol) in a particular formulation is necessary before the freeze-drying cycle development. A well-designed formulation and an appropriate freeze-drying process can achieve the balance of amorphous and crystalline structures in a formulation. Second, during the primary drying phase, T_p needs to be below one or more of the T_e's of any crystallized solutes. T_p should also be kept below T_g' for the solution with a non-crystalline phase to prevent collapse and melt-back effects if no crystalline matrix supports the cake structure (21). Third, the product storage temperature needs to be lower than the glass transition temperature (T_g) of the lyophilized final product. Therefore, it is desirable to have a higher T_g for a lyophilized product. Since water is known as a plasticizer to reduce the $T_{g,}$ effectively, in practice, moisture content in the lyophilized product should always be controlled to an optimal range (17,22).

Table 1 compares the thermal transition temperatures during warming after freezing with different treatments. The results from the DTA/ER work demonstrated the tendency of the TNF-MAb formulation to undergo a "noneutectic" freezing at a cooling rate of –3°C/min. The data in Figure 3 showed that glycine eutectic crystallization was delayed until the system was warmed above the devitrification temperature (T_d) where the metastable glass components were crystallized. It was typically the case when freezing was very fast, as with liquid nitrogen, but it was somewhat surprising that the same behavior was seen even with a freezing rate of –3°C/min. It has to be mentioned though that the freezing rate of –3°C/

min is still too fast for most practical freeze-drying processes. Nevertheless, it was clear that the probability of glycine crystallization during an initial freezing was quite low in the TNF-MAb formulation. As shown in Figure 1, a further reduction in freezing rate to –1°C/min would allow a nucleation of glycine during freezing. Partial crystallization occurred at the freezing rate of –1°C/min without sub-zero temperature holding (Figure 1). The completion of the glycine eutectic crystallization with subsequent warming suggested a borderline freezing rate of –1°C/min, that was slow enough to allow a nucleation of solute, but fast enough to retard crystal growth. When supercooling was controlled and limited by a high subzero temperature holding and deliberate nucleation of ice at –4°C, glycine eutectic crystallization was completed during freezing with no further residual crystallization during the subsequent warming (Figure 3).

Based on the results described above, it was clear that glycine was not able to crystallize completely during the initial cooling at –1°C/min, resulting in a very low T_g', –42°C. From a practical point of view, it was obvious that the T_g' with the regular freezing treatment was too low for TNF-MAb to be freeze-dried. The treatment involves the warming of the glassy solution through and above the devitrification temperature or eutectic crystallization temperature of a given solute to induce crystallization. The data from freezing with the treatments demonstrated that either a high subzero temperature holding or annealing through the devitrification temperature could promote the maximum crystallization of the glycine, which would dramatically increase the T_g' of the remaining solution (Figure 3 & 4). Therefore, an annealing treatment should be implemented in the freeze-drying cycle in order for TNF-MAb to be dried at a higher product temperature.

Although DTA and DSC have been widely used to measure thermal properties in the frozen temperature range, those methods sometimes fail to detect the glass transition, T_g', in frozen solution due to a too low sensitivity. Freeze-drying microscopy offers the opportunity to directly observe physical phase change of the drying front during the primary drying. The freeze-drying microscope photographs (Figure 4) further manifested that, after annealing, glycine was crystallized and the cake structure was fully maintained at a T_p below –25°C during freeze-drying. When product temperature, T_p, reached the range –20° to –10°C, the cake structure at the drying front collapsed progressively, which confirmed the results from DTA/ER analysis.

The glass transition temperature of a dry product, T_g, is usually considered as a critical property of a protein formulation during long term storage. If the storage temperature exceeds the product T_g for an extended period, the protein may undergo degradation or aggregation due to the molecular high mobility (23). It is also known that for a fixed formulation, the lower the moisture content, the higher the T_g. Therefore, the removal of bound water from amorphous solids and the maintenance of relatively low residual moisture are necessary. The former is usually completed in the secondary drying phase, the latter by an effective container closurer. In this study, the combined results indicated that the TNF-MAb formulation was moderately hygroscopic (Figure 5) and a drier cake provided less aggregation during storage (Figure 6). The information will guide us for the development of secondary drying phase. The moisture content in the final product should be controlled as low as possible upon the completion of the cycle. The moisture content in the TNF-MAb formulation as function of shelf temperature, chamber pressure and drying time should be carefully evaluated in the development of secondary drying phase to ensure the moisture content achieving a desirable low level.

CONCLUSIONS

This study characterized protein formulation for freeze-drying in general, but the emphases were on thermal properties at sub-freezing temperatures measured by DTA/ER and observed by freeze-drying microscopy, and moisture sorption properties. We have determined the thermal properties, such as T_x, T_g' and T_{im} for TNF-MAb formulation, investigated the moisture adsorption isotherms and optimized the moisture content. As a result, the following process parameters for the freeze-drying cycle development were recommended: 1) An annealing treatment: freezing the TNF-MAb formulation to –45°C, slowly warming to –25°C, then freezing the product back to –45°C. The annealing step was necessary in the freezing phase to induce glycine crystallization and, consequently, allowed us to elevate the product temperature during the primary drying phase. 2) After glycine crystallized, the highest allowable product temperature was –25°C during primary drying. 3) A secondary drying phase should be implemented to make the TNF-MAb product as dry as possible.

The purpose of the study was to characterize the TNF MAb formulation. The resulting information was used to develop a specific freeze-drying cycle for this protein. Generalization of the information collected for this protein to other proteins was out of the scope of this study. However, the analytical tools and strategies of protein characterization used in this study can be applied to other protein characterization work. Better understanding of protein pharmaceutical formulations and rational design of freeze-drying cycles accordingly will significantly reduce trial and error experiments in freeze-drying cycle development.

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REFERENCE

1. M. Pikal. Freeze-drying of proteins. Part I: Process Design. *BioPharm*. **9**:18–27 (1990).
2. J. Carpenter, M. J. Pikal, B. Chang, and T. W. Randolph. Rational design of stable lyophilized formulations: some practical advice. *Pharm. Res*. **14**:969–975 (1997).
3. F. Franks. Freeze-drying: From empiricism to predictability. The significance of glass transitions. In J. May and F. Brown (eds.), *Developments in Biological Standardization,* Karger, Basel, Vol 74, 1991, pp. 9–38.
4. M. Pikal. Freeze-drying of proteins. Part II: formulation selection. *BioPharm*. **10**: 26–30 (1990).
5. T. Jennings. Thermal-analysis instrumentation for lyophilization. *MD&DI*. **11**: 49–57 (1980).
6. A. MacKenzie. Factors affecting the transformation of ice into water vapor in the freeze-drying process. *Ann. N.Y. Acad. Sci,* **125**: 522–547 (1965).
7. L Rey. Thermal analysis of eutectics in freezing solutions. *Ann. N.Y. Acad. Sci*. **85:** 510–534 (1960).
8. L. Rey. Study of the freezing and drying of tissues at very low temperatures. In A. Parkes and A. Smith (eds.), *Recent research in freezing and drying,* Blackwell, Oxford, 1960, pp. 40–62

9. P. DeLuca and L. Lachman. Lyophilization of pharmaceuticals, I: effect of certain physical-chemical properties. *J. Pharm. Sci.* **54**: 617–624 (1965).
10. R. H. M. Hatley. The effective use of differential scanning calorimetry in the optimization of freeze-drying processes and formulations. In J. May and F. Brown. (eds.), *Developments in Biological Standardization,* Karger, Basel, Vol 74, (1991), pp. 105–136.
11. A. MacKenzie. Changes in electrical resistance during freezing and their application to the control of the freeze-drying process. In *"Refrigeration Science and Technology"* 1985, pp. 155–163.
12. A. MacKenzie. Collapse during freeze-drying- qualitative and quantitative aspects. In S. Goldblith, L. Rey and W. Rothmayr (eds.), *Freeze-drying and advanced food technology,* Academic Press, 1975, pp.277–307.
13. A. MacKenzie. A current understanding of the freeze-drying of representative aqueous solutions. In *"Refrigeration Science and Technology"*. 1985, pp. 21–34.
14. L. Rey. Dispositif pour l'examen microscopique aux basses temperatures. *Experientia.* XIII: 201–202 (1957).
15. S. L Nail, L. Her, C. P. B. Proffitt, and L. L. Nail. An improved microscope stage for direct observation of freezing and freeze drying. *Pharm. Res.* **11**: 1098–1100 (1994).
16. P. Meredith, A. M. Donald, and R. S. Payne. Freeze-drying: *In situ* observations using cryoenvironmental scanning electron microscopy and differential scanning calorimetry. *J. Pharm. Sci.* **85**: 631–637 (1996).
17. C. C. Hsu, C. A. Ward, R. Pearlman, H. M. Nguyen, D. A. Yeung, and J. G. Curley. Determining the optimum residual moisture in lyophilized protein pharmaceuticals. In J. May and F. Brown. (eds.), *Developments in Biological Standardization,* Karger, Basel, Vol 74 (1991), pp. 255–271.
18. D. H. Rasmussen and A. P. MacKenzie. The glass transition in amorphous water. Application of measurements to problems arising in cryobiology. *J. Phys. Chem.* **75**:967–973 (1971).
19. E. B. Seligmann Jr. Review of United States requirements for residual moisture in drugs. In *Developments in Biological Standardization.* Karger, Basel, Vol 36 (1977), pp. 175–179.
20. F. Franks. Solid aqueous solutions. *Pure & Appl. Chem.* **65**:2527–2537 (1993).
21. F. Franks. Improved freeze-drying: an analysis of the basic scientific principles. *Proc. Biochem.* **2**: iii–vii (1989).
22. J. C. May, R. M. Wheeler, N. Etz, and A. D. Grosso. Measurement of final container residual moisture in freeze-dried biological products. In J. May and F. Brown (eds.), *Developments in Biological Standardization,* Karger, Basel, Vol 74 (1991), pp. 153–164.
23. S. Duddu, and P. Monte. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimerical therapeutic monoclonal antibody. *Pharm. Res*. **14**:591–595 (1997).