# Effect of Glass Transition Temperature on the Stability of Lyophilized Formulations Containing a Chimeric Therapeutic Monoclonal Antibody

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***Purpose.*** The purpose of this study is to highlight the importance of knowing the glass transition temperature, $T_g$, of a lyophilized amorphous solid composed primarily of a sugar and a protein in the interpretation of accelerated stability data.
***Methods.*** Glass transition temperatures were measured using DSC and dielectric relaxation spectroscopy. Aggregation of protein in the solid state was monitored using size-exclusion chromatography.
***Results.*** Sucrose formulation ($T_g \sim 59°C$) when stored at 60°C was found to undergo significant aggregation, while the trehalose formulation ($T_g \sim 80°C$) was stable at 60°C. The instability observed with sucrose formulation at 60°C can be attributed to its $T_g$ (~59°C) being close to the testing temperature. Increase in the protein/sugar ratio was found to increase the $T_g$s of the formulations containing sucrose or trehalose, but to different degrees.
***Conclusions.*** Since the formulations exist in glassy state during their shelf-life, accelerated stability data generated in the glassy state (40°C) is perhaps a better predictor of the relative stability of formulations than the data generated at a higher temperature (60°C) where one formulation is in the glassy state while the other is near or above its $T_g$.

**KEY WORDS:** glass transition temperature $T_g$; lyophilization; solid-state stability; proteins; monoclonal antibody; aggregation.

## INTRODUCTION

### Glass Transition During and After Lyophilization

Many lyophilized drug products are partially or completely amorphous, and exist in the glassy state below their glass transition temperature, $T_g$. In the context of lyophilization, a glassy material is formed following the crystallization of ice during the freezing process. As water changes into ice, freeze concentration takes place and the uncrystallized mixture gets concentrated and changes from a "syrupy viscous liquid" to a "brittle glassy" structure, i.e. the material undergoes glass transition. The temperature where this transition occurs in the maximally freeze-concentrated mixture is known as the "collapse temperature" (e.g. ~−32°C for sucrose-water system), and is of paramount importance in achieving a successful lyophilization cycle (1,2). Thus, non-crystallizing solutes, such as proteins, remain with the glassy matrix and become kinetically frozen-in.

Formation of glassy state is marked by a drop in the diffusional rates of molecules, an increase in viscosity to about $10^{13}$ poise and by a decrease in heat capacity at constant pressure ($C_p$) by 40% to 100% of the vibrational $C_p$ value, as the liquid degrees of freedom kinetically become inaccessible (3). As the secondary drying progresses, and more water is lost, the $T_g$ of the freeze concentrate increases to a higher temperature. For example, complete removal of residual water from the freeze concentrated glass of sucrose with a $T_g \sim -32°C$ (1) results in dry, glassy sucrose with a $T_g$ of ~77°C (4), an increase of 109 degrees. Depending on the residual water, the $T_g$ of the final lyophilized product changes, with products containing higher water content exhibiting lower $T_g$ values.

Since glass transition is a reversible phenomenon, heating a glassy lyophilized drug product (e.g. during accelerated stability testing) results in a decrease in viscosity and an increase in heat capacity, $C_p$, near $T_g$. Other important changes at $T_g$ include the molecular mobility, flow rate, mechanical and dielectric properties (5). Thus, storing a formulation above its $T_g$ for accelerated stability studies transforms the glassy formulation into a viscous liquid state, dramatically enhancing its mobility. As a result, degradation pathways requiring mobility would be expected to strongly depend on the storage temperature relative to $T_g$ (6–8).

The $T_g$ of a formulation is a function of the relative proportion of glass-forming components (e.g. the bulking agents, active component, buffers, additives) and the moisture content (9,10). Water acts as a plasticizer and depresses the $T_g$ of amorphous solids composed of higher molecular weight components (11). It is imperative that during formulation development, sensitivity of the formulation $T_g$ to moisture should be investigated. The final product specification for moisture content should be such that, even at the highest concentration of the residual moisture specified, the $T_g$ should be high enough to offer physical and chemical stability of the product.

### *Relevance of $T_g$ to the Accelerated Stability Studies on Lyophiles*

During early product development, accelerated stability studies carried out on solutions, and to some extent on crystalline drug products, may yield valuable information regarding their stability at lower temperatures, calculated according to the Arrhenius equation. However, for amorphous products, if the accelerated study is conducted near $T_g$ and the data are extrapolated to predict stability in the glassy state, then such a prediction could lead to a significant error since the kinetics in the glass transition region do not necessarily obey Arrhenius relationship (6,7). Therefore, knowledge of the $T_g$s of various potential formulations is extremely important during formulation screening process where formulation selection, in majority of the cases, is based on the relative stabilities of the formulations at elevated temperatures. It is important that such comparison be made at a temperature where all the formulations exist in the same physical state, viz., the glassy state, as they usually do during their shelf-life.

If a significant weight fraction of the formulation is in the amorphous form, then a visual cake collapse can be observed in the vial when it is kept at a temperature near its $T_g$. However, if the product is predominantly crystalline, then visual collapse may not be apparent. In such cases, the amorphous component of the product would still undergo glass transition as the temper-

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ature approaches its $T_g$ without being visually detected. If the active ingredient forms a part of the amorphous matrix, then the accelerated stability data generated at temperatures near $T_g$ can lead to wrong conclusions in the prediction of the stability of the dosage form at lower temperatures.

### *Background and Hypotheses*

Two potential lyophilized formulations, one containing sucrose and the other containing trehalose as a bulking agent (62.5 mg sugar/vial), of a chimeric monoclonal antibody (5 mg protein/vial) currently undergoing Phase II clinical trials were found to be stable at 40°C over a period of two months. However, significant aggregation of the protein was observed when the sucrose formulation was maintained at 60°C, while the trehalose formulation was stable at the same temperature. Interestingly, when the protein/sugar ratio was increased by 10 fold (i.e. 50 mg protein and 62.5 mg of sugar/vial), both sucrose and trehalose formulations exhibited comparable stability at 60°C. Since the low strength (5 mg protein/vial) sucrose formulation was unstable at 60°C, it would be expected that aggregation would be more extensive when the protein concentration is increased by a factor of ten. Interestingly, the results were found to be exactly the opposite.

The present study was carried out to test the hypothesis that the inferior stability of the sucrose low strength formulation at 60°C relative to both the trehalose formulations, and the high strength sucrose formulation, arises because the glass transition temperature of the low strength sucrose formulation is near or below 60°C whereas the other formulations have a glass transition temperature well above 60°C. Another objective of the work is to evaluate the validity of 60°C stability data as a predictor of the stability of low strength sucrose formulation at or below room temperature.

## MATERIALS AND METHODS

### Materials

Two ml of an aqueous solution containing the monoclonal antibody (5mg for low strength and 50 mg for high strength formulations), sucrose or trehalose (62.5 mg), 20 mM citrate buffer, 15 mM sodium chloride and 0.02 % w/w Tween 80 were filled into each vial and lyophilized. The lyophilization was carried out in a HULL lyophilizer. For trehalose formulations and placebo, the product was first frozen by maintaining the shelf temperature at −55°C for 3 hours, followed by primary drying at −5°C for 40 hours at 200 mTorr. The shelf temperature was then ramped at 5°/hr to 35°C, where secondary drying was performed for 6 hrs. For sucrose formulations and placebo, the product was frozen by maintaining the shelf temperature at −50°C for 3 hrs, and the shelf temperature was then ramped at a rate of 0.5°/min to −25°C while maintaining under a vacuum of 100 mTorr. The shelf temperature was held at −25°C for 12 hrs, and was raised to −10°C where it was held for an additional 20 hrs. The temperature was then ramped at 5°/hr to 30°C, where secondary drying was performed for 6 hrs. Immediately after the lyophilization, the vials were sealed and stored under specified temperatures for stability evaluation. Each of the above formulations were prepared at least in duplicate (i.e. two different lyophilization runs), and the moisture content for various formulations was found to be in the range of 1.5%–2.5% w/w. Slightly different lyophilization cycles were used for sucrose and trehalose formulations in order to yield products with similar moisture content.

The low and high strength formulations mentioned throughout this manuscript correspond to 5 and 50 mg, respectively, of protein per 62.5 mg of sugar. In case of sucrose, two separate formulations with different protein/sugar ratios, viz., 50 mg protein/31.2 mg sucrose and 50 mg protein/125.0 mg sucrose were also prepared. Placebo formulations were essentially the low strength (or the high strength) formulations without the protein, and were prepared to support the ongoing clinical trials.

### Methods

#### *Differential Scanning Calorimetry*

Samples (3–7 mg) in hermetically sealed aluminum pans were analyzed using a Seiko Instruments DSC120 Differential Scanning Calorimetry Analysis Module at a rate of 5°C/min under $N_2$ stream. Where glass transition was associated with enthalpic relaxation, samples were heated past $T_g$, cooled at ~5°C/min and reheated to measure the $T_g$ in the second scan. For the determination of dry glass transition temperatures, samples were heated in open pans under nitrogen stream at a rate 5°C/min up to the onset of $T_g$, cooled at 5°C/min and reheated again at the same rate. The dry $T_g$ was measured in the second scan. In all cases, the $T_g$ reported is the mid point of the baseline deflection in the DSC curve (or where the derivative of DSC exhibits a maximum).

#### *Dielectric Relaxation Spectroscopy (DRS)*

Dielectric scanning of the samples were performed using a Seiko DES100 Dielectric Module, as described earlier (12). Scanning was performed with a parallel plate electrode in the range of 10Hz–100 KHz while heating at a rate of 1°C/min. Data analysis was performed using a Seiko SSC/5200H Thermal Analysis System. In some instances, a shoulder was observed before the maxima when the dielectric loss, $\epsilon''$, or the dissipation factor, tan $\delta$, were plotted as functions of temperature. Upon cooling and reheating, the shoulder disappeared, with no change in the peak position. The appearance of the shoulder was not consistent and appears to be an artifact, perhaps related to the porosity and uneven packing of the sample. The fact that the peak position remains constant upon reheating suggests that in the dielectric set up mentioned here, and in the temperature range studied, moisture loss from first heat was found to be negligible. The maximum in tan $\delta$ *vs* temperature plot at the lowest frequency measured, 10 Hz, is reported as the $T_g$.

#### *Aggregation of the Protein in the Formulations*

A chromatographic technique was used to detect changes in the molecular size distribution caused by aggregation or fragmentation in formulations. The separation of aggregates, monomer, and fragments was performed with a TSK-GEL G3000 $SW_{xL}$ HPLC column (30 cm × 7.8 mm ID) using an isocratic mobile phase composed of 40 mM citrate and 200 mM sodium chloride at pH 6.0, with a flow rate of 1 mL/min. The detection was performed at 280 nm. The assay time for

**Table I.** Relative Stability of Low Strength (LS, 5mg Protein/Vial) and High Strength (HS, 50 mg Protein/Vial) Formulations Sucrose (S) and Trehalose (T) Containing a Therapeutic Monoclonal Antibody Stored at 5°, 40° and 60°C for 2 Months

| Formulation | 5°C %A | 40°C %A | 60°C %A |
|---|---|---|---|
| S-LS | 0.4 | 0.5 | 6.0 |
| T-LS | 0.7 | 0.6 | 1.1 |
| S-HS | 0.6 | 1.0 | 2.2 |
| T-HS | 0.8 | 1.1 | 2.3 |

Note: % A = % Aggregrates measured.

each sample was 15 minutes and the results are reported, to the tenth of a percent, as percent peak area of the aggregates, monomer or fragment peak relative to the total peak area including aggregates and fragments.

## Results and Discussion

### *$T_g$ of the Formulation and the Aggregation in the Solid State*

In the present study, the relative stability of the formulations was assessed by measuring the susceptibility of the protein to aggregation, as described in the Methods section. The stability data shown in Table I suggests that over a period of two months, the low strength sucrose formulation is as stable as the low strength trehalose formulation at 40°C. However, at 60°C, sucrose formulation was found to be unstable compared to the trehalose formulation. The $T_g$s of low strength sucrose (water content 1.6% w/w) and trehalose formulations (water content 1.7% w/w) were measured using DRS to be 60°C (59°C by DSC) and 81°C (80°C by DSC), respectively, as shown in Figure 1. It is clearly evident from Figure 1 that, when stored at 60°C, the low strength sucrose formulation would be near its glass transition, while the trehalose formulation would be ~20 degrees below its $T_g$. The enhanced mobility in the sucrose formulation near its glass transition may explain the significant aggregation observed with sucrose formulation at 60°C. However, at 40°C, both low strength formulations exhibited similar extents of aggregation over the time period studied, perhaps due to the fact that both formulations have considerably reduced molecular mobility as they both exist in glassy state. Thus, for low strength formulations, the degradation appears to be directly linked to the storage temperature relative to the $T_g$ of the formulation.

If the aggregation of the monoclonal antibody is not related to the $T_g$ of the formulation, then the aggregation in sucrose formulation at 60°C would be expected to increase with increasing protein concentration. However, the data in Table I suggest that higher strength sucrose formulation exhibits a superior stability at 60°C compared to the lower strength formulation. When analyzed by DSC, the higher strength formulations of sucrose (water content ~1.3% w/w) and trehalose (water content ~1.4% w/w) were found to have $T_g$s of 89°C and 100°C, respectively (Figure 2). The corresponding values using DRS were found to be 91°C and 101°C (Figure 3). Thus, the high strength sucrose formulation would be ~30 degrees below its $T_g$ when stored at 60°C. Therefore, it is very likely that the enhanced stability at 60°C of the higher strength sucrose formulation is due to an increase in its $T_g$ to 89°C. The higher strength formulations of both sucrose and trehalose exist well below their $T_g$s when stored at 60°C, and hence exhibit comparable stability. Thus, the hypothesis that the aggregation of the monoclonal antibody is directly linked to the $T_g$ of the formulation appears to explain the accelerated stability data obtained with both low and high concentrations of protein in the formulation.

### *Effect of Protein on the $T_g$ of the Glassy Matrix of Sucrose/Trehalose*

It is interesting that, at comparable water contents, a 10-fold increase in the protein/sugar ratio resulted in an increase in the $T_g$ of sucrose formulation by 30 degrees and that of trehalose by about 20 degrees. It is generally accepted that the glass transition temperature of a saccharide:protein formulation increases as the formulation becomes richer in protein. Non-crystallizing solutes can alter the collapse temperature of the system during lyophilization. Similarly, when translated to systems with low moisture content, it is possible that the $T_g$ of sucrose placebo can be altered by the presence of protein.

It is well known that moisture decreases the $T_g$ of a glass composed of a material which has a higher molecular weight than water itself (11). Further, different glasses are plasticized to different extents by water. Therefore, to investigate the effect

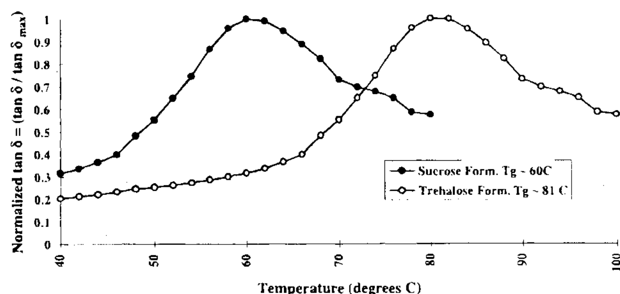


**Fig. 1.** Glass transition temperatures of lower strength (5 mg protein/ 62.5 mg sugar) sucrose and trehalose formulations of a monoclonal antibody measured using dielectric relaxation spectroscopy at a frequency of 10 Hz.

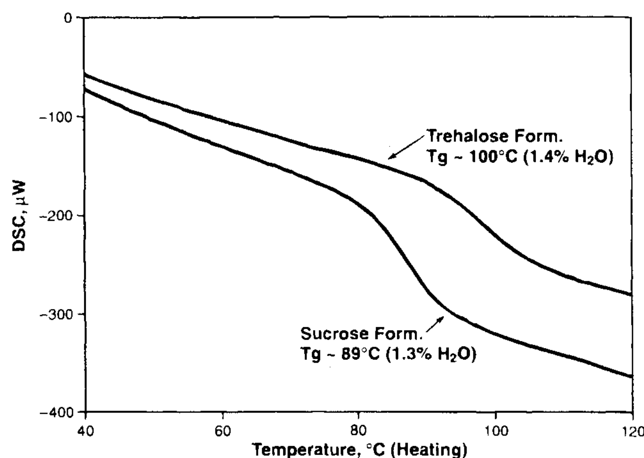


**Fig. 2.** Glass transition temperatures of higher strength (50 mg protein/ 62.5 mg sugar) sucrose and trehalose formulations of a monoclonal antibody measured using differential scanning calorimetry.

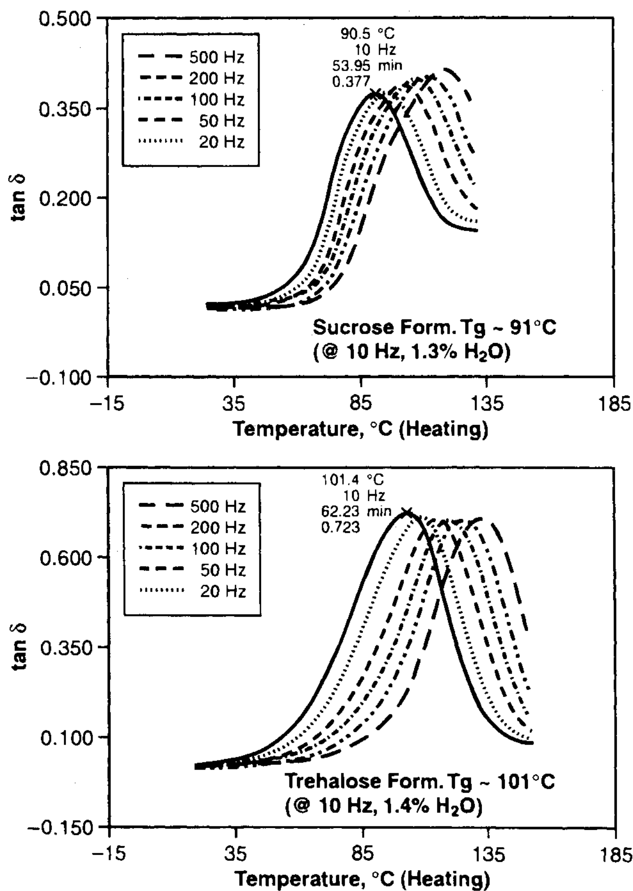


**Fig. 3.** Glass transition temperatures of higher strength (50 mg protein/62.5 mg sugar) sucrose and trehalose formulations of a monoclonal antibody measured using dielectric relaxation spectroscopy. Since the measurement of glass transition temperature, $T_g$, depends on the experimental time scale, higher frequencies measure $T_g$ at higher temperatures.

of protein on the $T_g$ of the placebo formulation, it is important that the variability due to different degrees of plasticization by water be minimized. In the present study, this was accomplished by measuring the dry $T_g$s of formulations containing various sugar/protein ratios. No thermal events were observed in DSC during the measurement of dry $T_g$s for any of the formulations, suggesting that no gross degradation of protein and/or sugar takes place when they are heated up to their respective dry glass transition temperatures.

As shown in Figure 4, the dry glass transition temperatures of sucrose and trehalose placebo formulations (obtained by lyophilizing an aqueous solution of 20 mM citrate buffer, 15 mM NaCl, 0.02% Tween 80 and 62.5 mg sugar/mL) determined by DSC were found to be 81°C and 119°C, respectively. Thus, the two placebos differ in their $T_g$s by 38 degrees. This difference is not surprising since the dry glass transition temperatures of pure sucrose and pure trehalose also differ by approximately the same magnitude ($T_g$ of dry sucrose = 77°C (4) and that of dry trehalose is 117°C (unpublished data from the authors)). The $T_g$s of sucrose placebos containing 1.9% and 2.5% w/w water were found to be 58°C (62°C by DRS) and 44°C (45°C by DRS), respectively. Thus, the presence of as little as 2.5% w/w of water was found to depress the $T_g$ of sucrose placebo

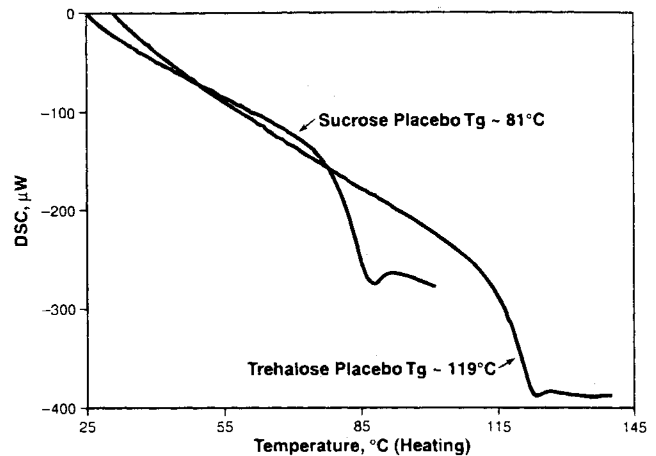


**Fig. 4.** Dry glass transition temperatures of sucrose and trehalose placebo formulations measured using differential scanning calorimetry.

by 40 degrees. The $T_g$ of trehalose placebo at a moisture content of 2.4% w/w was found to be 78°C, about 40 degrees lower than the dry $T_g$ of trehalose. Therefore, it appears that moisture plasticizes both sucrose and trehalose placebo formulations to approximately the same degree. The dry $T_g$s of low strength sucrose and trehalose formulations (weight fraction of protein ~0.07) were found to be 85°C and 120°C, respectively. Thus, the $T_g$s of the respective placebos were affected only to a minor, but to different, extents by the presence of 5 mg protein.

The dry $T_g$ of sucrose placebo changes from 81°C to 89°C as the weight fraction of protein changes from 0 to 0.29, as shown in Figure 5. Further increase in the weight fraction of protein to 0.62 raised the $T_g$ to almost 112°C. Two other intermediate ratios were tested but not shown in Figure 5 for clarity reasons. However, the data are presented in Table II. A similar increase in the weight ratio of protein was also found to raise the $T_g$ of trehalose, as presented in Table II. For a given protein weight fraction of 0.44 (corresponding to the higher strength formulations), the increase in the value of dry $T_g$ for sucrose formulation was ~19 degrees, while that for the trehalose formulation was 7 degrees. This increase in $T_g$ of ~19 degrees directly translates into the greater stability at 60°C for

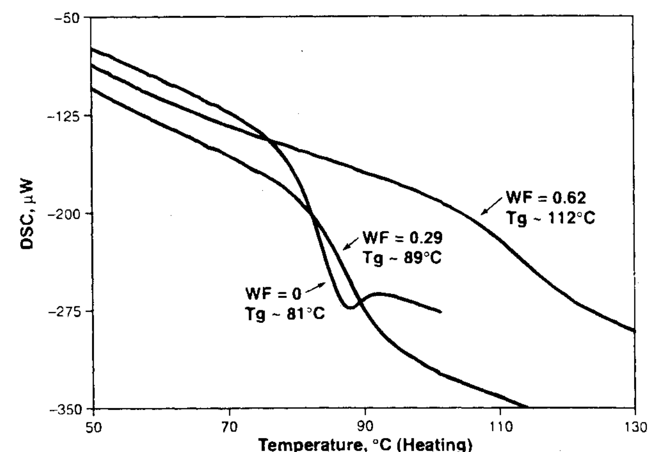


**Fig. 5.** The effect of the weight fraction ($W_f$) of monoclonal antibody on the glass transition temperature of sucrose placebo formulation.

**Table II.** The Effect of Weight Fraction of the Protein on the Dry Glass Transition Temperature of the Lyophilized Product

| $W_f$ protein | Sucrose formulations $T_g$ measured (°C) | Predicted $T_g$[a] of the protein |
|---|---|---|
| 0.00 | 81 | — |
| 0.07 | 85 | 148 |
| 0.29 | 89 | 110 |
| 0.44 | 100 | 127 |
| 0.62 | 112 | 134 |
| | Trehalose Formulations | |
| 0.00 | 119 | — |
| 0.07 | 120 | 145 |
| 0.44 | 126 | 138 |

[a] $T_g$ predicted from Fox Equation (Eq. 2. in the text).

the higher strength sucrose formulation compared to the lower strength formulation, as highlighted earlier.

The relationship between $T_g$ of a mixed amorphous system and the phase composition, in certain instances, can be described by the Gordon-Taylor equation (9,13,14), as shown below

$$T_{g\ mix} = [(w_1 \cdot T_{g1}) + (K \cdot w_2 \cdot T_{g2})]/[w_1 + (K \cdot w_2)] \quad (1)$$

where, K = the ratio of free volumes of the two components,

$$K = (\rho_1 \cdot \Delta\alpha_2)/(\rho_2 \cdot \Delta\alpha_1)$$

K is, in general, not easy to measure, especially for biological systems, since the change in thermal expansivity at $T_g$, $\Delta\alpha$, can be very difficult to determine. By assuming $\Delta\alpha \cdot T_g \sim$ constant (15), and that the densities ($\rho_1$ and $\rho_2$) of sucrose and the protein are nearly equal, we can arrive at a more simplified expression of the Gordon-Taylor equation, commonly referred to as the Fox equation (16),

$$1/T_g\ mix = (w_1/T_{g1}) + (w_2/T_{g2}) \quad (2)$$

Assuming that Equation 2 holds true for the placebo/protein systems in the present study, the $T_g$ value of the monoclonal antibody can be predicted from the $T_{g\ mix}$ composition data (see Table II). Considering the simplified nature of Equation 2 and the overwhelming assumptions, the predicted $T_g$ value for monoclonal antibody was found to be similar (range 127 to 148°C) in all but one composition for both trehalose and sucrose formulations. At a protein weight fraction of 0.29 in sucrose formulation, the predicted $T_g$ value was much lower than the others. When the solids containing various weight fractions of protein, described in Table II, were heated to nearly 180°C, only one glass transition was observed, suggesting that in all cases the amorphous matrix is perhaps present as a single phase. Considering the complex thermal behavior of proteins, it is also possible that the protein rich phase, if it exists in the matrix, may not exhibit a measurable $T_g$ under the current experimental conditions (viz., in a DSC at the heating rate employed).

## CONCLUSIONS

The aggregation of the monoclonal antibody in sucrose and trehalose formulations appears to be directly linked to the storage temperature relative to their respective glass transition temperatures. When maintained in the glassy state (e.g. at 40°C for the low strength and at 60°C for high strength formulations), both sucrose and the trehalose formulations exhibit comparable stability. Based on the glass transition measurements, it is imperative that the selection for the lower strength monoclonal antibody formulation should be based on the stability data obtained at 40°C, but not on the data generated at 60°C. The effect of protein/sugar ratio on the $T_g$ suggests that $T_g$ of the formulation can be increased by changing the ratio of the drug/excipient, or by using other excipients which may inherently have high glass transition temperatures. The complexities and underlying assumptions involved in interpretation of the stability data generated below $T_g$ are the subjects of our next communication.

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