The Relationship Between Protein Aggregation and Molecular Mobility Below the Glass Transition Temperature of Lyophilized Formulations Containing a Monoclonal Antibody

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Purpose. To find out if the physical instability of a lyophilized dosage form is related to molecular mobility below the glass transition temperature. Further, to explore if the stability data generated at temperatures below the glass transition temperature can be used to predict the stability of a lyophilized solid under recommended storage conditions.

Methods. The temperature dependence of relaxation time constant, τ, was obtained for sucrose and trehalose formulations of the monoclonal antibody (5 mg protein/vial) from enthalpy relaxation studies using differential scanning calorimetry. The non-exponentiality parameter, β, in the relaxation behavior was also obtained using dielectric relaxation spectroscopy.

Results. For both sucrose and trehalose formulations, the variation in τ with temperature could be fitted Vogel-Tammann-Fulcher (VTF) equation. The two formulations exhibited difference sensitivities to temperature. Sucrose formulation was more fragile and exhibited a stronger non-Arrhenius behavior compared to trehalose formulation below glass transition. Both formulations exhibited <2% aggregation at t/τ values <10, where t is the time of storage.

Conclusions. Since the relaxation times for sucrose and trehalose formulations at 5°C are on the order of 10^8 and 10^6 hrs, it is likely that both formulations would undergo very little (<2%) aggregation in a practical time scale under refrigerated conditions.

KEY WORDS: sucrose; trehalose; molecular mobility below glass transition; protein aggregation; Vogel-Tammann-Fulcher (VTF) equation; fragility of glasses.

INTRODUCTION

Molecular Relaxations in the Glassy State

The glass transition of supercooled fluids (e.g. freeze concentrates during lyophilization) is characterized by a number of kinetic phenomena. As glass is formed from a fluid during cooling, the relaxation time of the constituents of the solid increase in a non-Arrhenius fashion by several orders of magnitude over a very narrow temperature range. While the mean relaxation time constant, τ, at T_g is on the order of 10–100 sec, its magnitude 10–20 degrees below glass transition temperature is typically in the order of tens to hundreds of hours, depending on the system (1,2). The relaxation time constant itself can be obtained from the measurement of a time dependent response to a perturbation, which typically follows a stretched exponential form, shown in equation 1

$$\Phi(t) = \exp[-(t/\tau)^{\beta}] \quad (1)$$

where Φ is the relaxation function and $0 < \beta < 1$ is the stretching exponent. A value of unity for β indicates a single relaxation time. Near the glass transition, many systems usually respond non-exponentially to perturbations (3–4), and β value deviates from unity. Typical values for β range from 0.3 to 0.8 for various systems (4), suggesting that different systems have different degrees of non-exponentiality in their relaxation behavior. It has been suggested that the non-exponentiality in relaxation and the deviation of the relaxation behavior from Arrhenius behavior are probably related (4). The non-Arrhenius variation in relaxation time constant, τ, near T_g is better described by the Vogel-Tammann-Fulcher (VTF) equation

$$\tau = \tau_0 \exp(B/(T - T_0)] \quad (2)$$

where τ_0, B and T_0 are constants (5). As it can be seen, when $T_0 = 0$, the above equation reduces to the more common Arrhenius equation. The VTF equation and the stretching exponential functions were successfully applied to describe the behavior of not only pure homogeneous glasses, but also to heterogeneous glasses containing more than one component, e.g. 15% ethylene glycol in propylene glycol (5).

Strong and Fragile Glasses

Angell classified amorphous materials as "strong" and "fragile", based on a number of properties such as heat capacity, C_p, and τ (6,7). The temperature dependence of τ of strong glasses exhibits an Arrhenius behavior and the relaxation itself tends to be exponential (i.e. $\beta \sim 1$ in equation 1). Further, strong glasses exhibit a small change in C_{p_p} at T_g. Fragile materials, on the other hand, show a large change in C_p at T_g, and exhibit strongly non-Arrhenius behavior in their relaxation behavior with a β value much less than unity (4). Thus, a plot of log τ Vs T_g/T is almost linear for strong glasses and exhibits significant curvature for fragile glass formers near T_g (4). The steepness (m) of the log τ Vs T_g/T plot near a value of $T/T_g = 1$ can be used as a measure of the fragility of the system. Fragile systems have higher m values (The lower limit of m = 16, for strong glass formers).

Strong glasses have a built in resistance to a structural change, while fragile glasses, with little provocation from thermal excitation, reorganize to structures that fluctuate over a variety of orientations (3). Catastrophic changes in the relaxation time (and structure) occur near T_g for fragile glasses. At least two carbohydrates, sorbitol and sucrose, used in lyophilized pharmaceutical products yield fragile glasses (2,4). Sorbitol was reported to have an m value of 93 and a β value of 0.53 (4). The extent of the fragility of various glasses varies with the structure and composition of the glassy matrix. Bohmer *et al.* (4) suggested that the fragility (m) and the non-exponentiality in relaxation (β) are related to each other by a general empirical equation

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$$m = m_0 - s\beta \quad (3)$$

with $m_0 = 250$ and $s = 320$. Thus, the non-exponentiality in relaxation (β) can give us a rough estimate of the fragility of the system.

Fragility of Glasses in Relation to Accelerated Stability Testing

Relaxation time constant (τ) is a measure of the mobility of the system. Hence, the variation of τ with temperature is a measure of the mobility of the system with temperature. Our previous work (8,9) indicated a relation between enhanced molecular mobility achieved during glass transition and chemical degradation. Recently, Hancock et al. (2) suggested the use of molecular mobility measurement below T_g in the prediction of shelf-lives of amorphous drugs and excipients, assuming a direct correlation between the molecular mobility and the degradation of the product. Two main questions related to the accelerated stability testing procedure for amorphous materials have to be addressed in this context:

1. At what temperature relative to T_g should the testing be performed (i.e. T/T_g of 0.5 or 0.6 or 0.9 etc.)?
2. Under what circumstances can the data be treated according to Arrhenius equation, and when do we have to use a more complex VTF equation?

If the product is very strong (i.e. low m value ~ 20, and a $\beta \sim 1$, materials such as silicon dioxide), then τ varies with temperature according to the Arrhenius equation. In such a case, Arrhenius kinetics may be utilized to predict the stability of the dosage form. However, if the variation of τ with temperature is strongly non-Arrhenius, then the degradation process can be potentially non-Arrhenius. For fragile liquids, the τ value dramatically changes near T_g during cooling and this trend continues even below (2). Therefore, more complex VTF equations may have to be used to explain the degradation of these systems. Therefore, the fragility of a glass may be an important parameter in understanding the response of the lyophilized product to such perturbation as an increase in temperature, i.e. thermally induced degradation. In this study, we tried to explore the relation between the extent of aggregation undergone by a protein embedded in a glassy matrix over an experimental time scale and the time scale of relaxation process undergone by the system.

MATERIALS AND METHODS

Materials

Two ml of an aqueous solution containing a chimeric monoclonal antibody (5 mg) 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, as described earlier (9). The residual moisture content of the lyophilized solids was found to be approximately 1.6%–1.7% w/w. The % increase in aggregation (referred to as % aggregation) during storage of sucrose and trehalose formulations under various temperature conditions were determined according to the method described earlier (9).

Enthalpy Relaxation Studies

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 gas stream. Samples were stored to different temperatures below their respective glass transition temperatures (at 5, 22, 30, 40 and 45°C for sucrose (Tg ~ 59°C) and at 5, 22, 40, 50 and 60°C for trehalose (Tg ~ 81°C) formulations. Typical enthalpic recovery curves for sucrose formulation are shown in Figure 1. The enthalpy relaxation was obtained by calculating the area between the DSC curve of the aged sample and that of the super cooled liquid baseline (1,2). The maximum enthalpic recovery at a given storage temperature, T, was obtained using the formula

$$\Delta H_\infty = (T_g - T) \cdot \Delta C_p \quad (4)$$

where T_g is glass transition temperature. The relaxation function ($\Phi(t)$) is related to the extent of relaxation under a given condition and is fitted to the Wiiliams-Watts equation (10)

$$\Phi(t) = [1 - (\Delta H/\Delta H_\infty)] = \exp[-(t/\tau)^\beta] \quad (5)$$

the parameters β and τ were obtained by non-linear regression. The values of τ obtained at various temperatures were plotted against temperature fitted to the VTF equation (Equation 2).

Dielectric Relaxation Spectroscopy (DRS)

Dielectric scanning of the samples were performed using a Seiko DES100 Dielectric Module. Samples were prepared according to the method described earlier (9). Scanning was performed with a parallel plate electrode in the range of 10Hz–100 KHz between 25 to 130°C, in steps of 1degree, while holding the sample isothermally at each step. Data analysis was performed using a Seiko SSC/5200H Thermal Analysis System.

The Cole-Cole plots at $T/T_g \sim 1.05$ were generated by plotting the ϵ'' vs ϵ'. The value of β (in Equation 1) was calculated from the Cole-Cole plots according to the method described by Havriliak-Nagami (11). The dielectric loss spectra at each temperature were plotted as a function of log frequency, and the loss maximum was located in each case by fitting the data to a gaussian function. The half width of the peak was

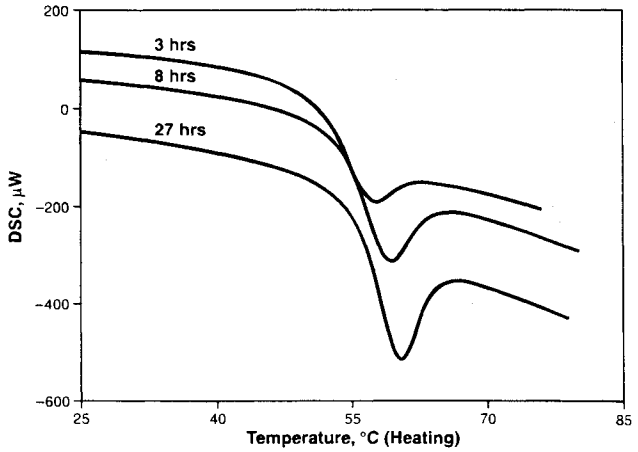

Fig. 1. Time dependence of enthalpic recovery of sucrose formulation stored at 40°C (~Tg-19 degrees).

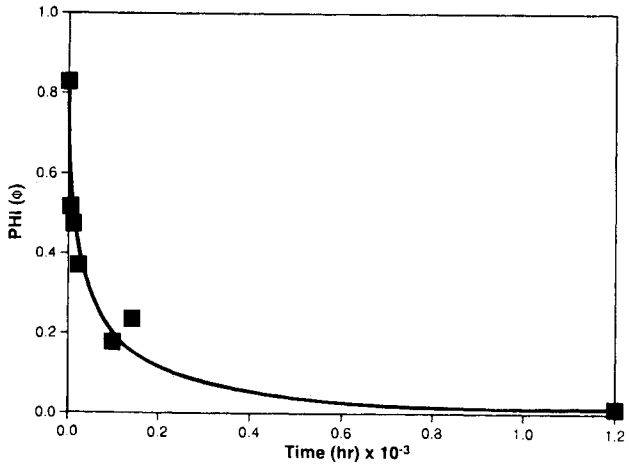

Fig. 2. Φ vs time plot for the sucrose formulation at 40°C (~Tg-19 degrees) generated using enthalpic relaxation studies.

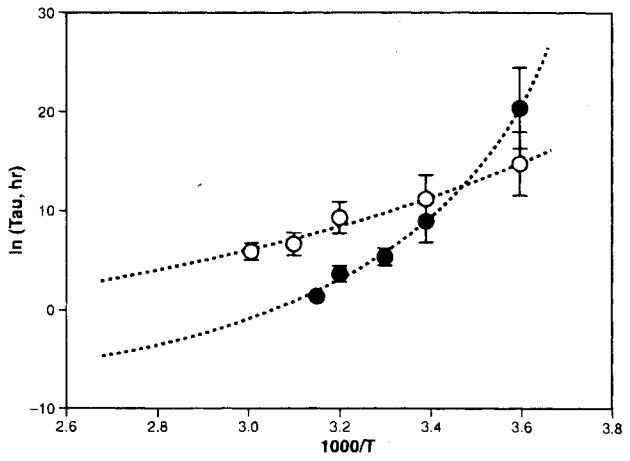

Fig. 4. Temperature dependence of relaxation time constant, τ, for sucrose and (closed circles) trehalose (open circles) formulations. The dashed lines represent the best-fit lines to the VTF equation.

measured at each temperature and the stretched exponential parameter (β) in Equation 1 was calculated according to the Dixon's equation (12)

$$(1 - \beta) = 1.047(1 - \omega^{-1})$$

where, ω is the full width at half maximum at various temperatures of the ϵ'' vs log (frequency) plots. The β value obtained by this method was compared to that obtained using the Cole-Cole plot.

RESULTS AND DISCUSSION

Changes in the area under the relaxation enthalpy curve are typified by Figure 1 for sucrose formulation at 40°C. The Φ vs time plot of the sucrose formulation at 40°C generated from the enthalpy relaxation data is shown in Figure 2. Non-linear regression (*Scientist* Program, Micromath) using iterative least squares minimization was employed to estimate the parameters β and τ, as shown in Figure 2 for sucrose at 40°C, and in Figure 3 for the trehalose formulation at 60°C. Initial estimates for the values of β were obtained from the dielectric relaxation spectroscopy studies (discussed later). For both formulations, τ increased significantly with a decrease in temperature in a non-linear fashion, suggesting some degree of fragility. Further, the data could be fitted to VTF equation in both cases, as shown in Figure 4. The best fit VTF parameters estimated using the fitting program in Origin (Microcal, Northampton, MA) are given in Table I. The T_0 values predicted were found to be approximately T_g-80 for sucrose and T_g-169 for trehalose. The τ_0 values were found to be on the order of 10^{-4} hrs for trehalose and 10^{-6} hrs for sucrose. The B values found were consistent with those reported in the literature for mannitol and sorbitol (5).

An interesting trend can be seen in Figure 4. The τ for sucrose formulation varies in a much more non-Arrhenius fashion than for the trehalose formulation, suggesting that sucrose formulation is perhaps more fragile than the trehalose formulation. At a comparable T/T_g value of 1.05, the values of stretching exponent, β, obtained using Cole-Cole plots of their dielectric relaxation behavior were found to be 0.38 and 0.44 for sucrose and trehalose formulations, respectively. Similar values (0.37 for sucrose and 0.48 for trehalose formulations) were also observed at the same T/T_g values using the asymmetry of the dielectric loss plots (Dixon's equation). The β values obtained by non-linear regression of ϕ versus time plots generated using the enthalpy relaxation studies were higher for the trehalose than for the sucrose formulation, typically in the same range as those obtained using the dielectric relaxation studies. According to the relation between the non-exponentiality parameter, β, and the fragility value m described earlier (Equation 3), m values of approximately 100 and 125 were obtained for trehalose and sucrose formulations. Thus, the log τ vs temperature data, and the empirical equation relating β and fragility,

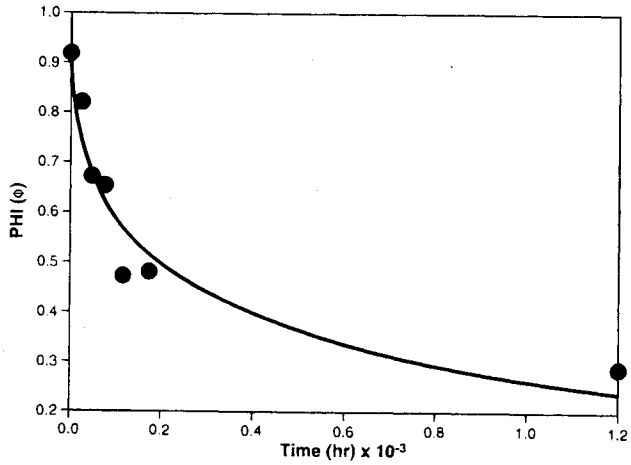

Fig. 3. Φ vs time plot for the trehalose formulation at 60°C (~Tg-20 degrees) generated using enthalpic relaxation studies.

Table I. The VTF Parameters for the Sucrose and Trehalose Formulations Obtained by Non-Linear Regression from Figure 2

Formulation	τ_0 (hr)	B	T_0 (K)
Sucrose	1.88×10^{-6}	1088	246
Trehalose	1.98×10^{-4}	2169	185

both predict sucrose formulation to be more fragile than trehalose formulation.

The differences in the fragilities of these two sugars translate into a very interesting and complex phenomena. Although sucrose formulation has a lower T_g than the trehalose formulation, since temperature dependence of τ for sucrose formulation exhibits much more non-linearity, it intersects the τ versus temperature curve for trehalose formulation at approximately 12°C (Figure 4). Thus, at lower temperatures (<12°C) the molecular mobility in the sucrose formulation appears to be lower than that of the trehalose formulation, despite the fact that sucrose formulation has a lower T_g compared to the trehalose formulation. Similarly when heated, since the τ value for sucrose formulation decreases in more non-Arrhenius fashion compared to that of trehalose formulation, it reaches the characteristic experimental time scale at a lower temperature compared to the trehalose formulation, i.e. sucrose formulation exhibits a lower glass transition temperature than the trehalose formulation. Therefore, it is possible that a formulation with a higher T_g may not necessarily have a lower molecular mobility at temperatures $<T_g$, compared to a different formulation with a lower T_g, because of differences in their fragilities. In general, for systems with comparable fragilities, the greater the T_g, the lower is the molecular mobility at any temperature $<T_g$. As an initial estimate, the β values determined using DRS may give a rough comparison of the relative fragilities of formulations.

For both sucrose and trehalose formulations, the aggregation of monoclonal antibody was found to depend on the temperature of storage relative to the T_g, and the time of storage. Since sucrose and trehalose formulations have different sensitivities to thermal stress (i.e. different T_gs and different fragilities), a comparative evaluation of their stability should be ideally performed under identical molecular mobilities. This comparison was accomplished (as shown in Figure 5) by plotting the percent aggregation as a function of reduced time, t/τ, where the time of storage at a given temperature was divided with the relaxation time measured at that temperature. This way, the time dependence of a dynamic process (e.g. aggregation) can be defined

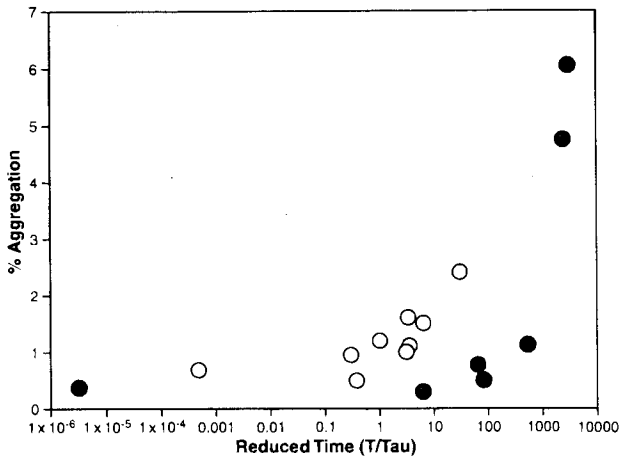

Fig. 5. Plot of % aggregation as a function of reduced time, t/τ, where t is the storage time at a given temperature and τ is the corresponding relaxation time constant at the same temperature. The closed and open circles represent, respectively, the sucrose and the trehalose formulations.

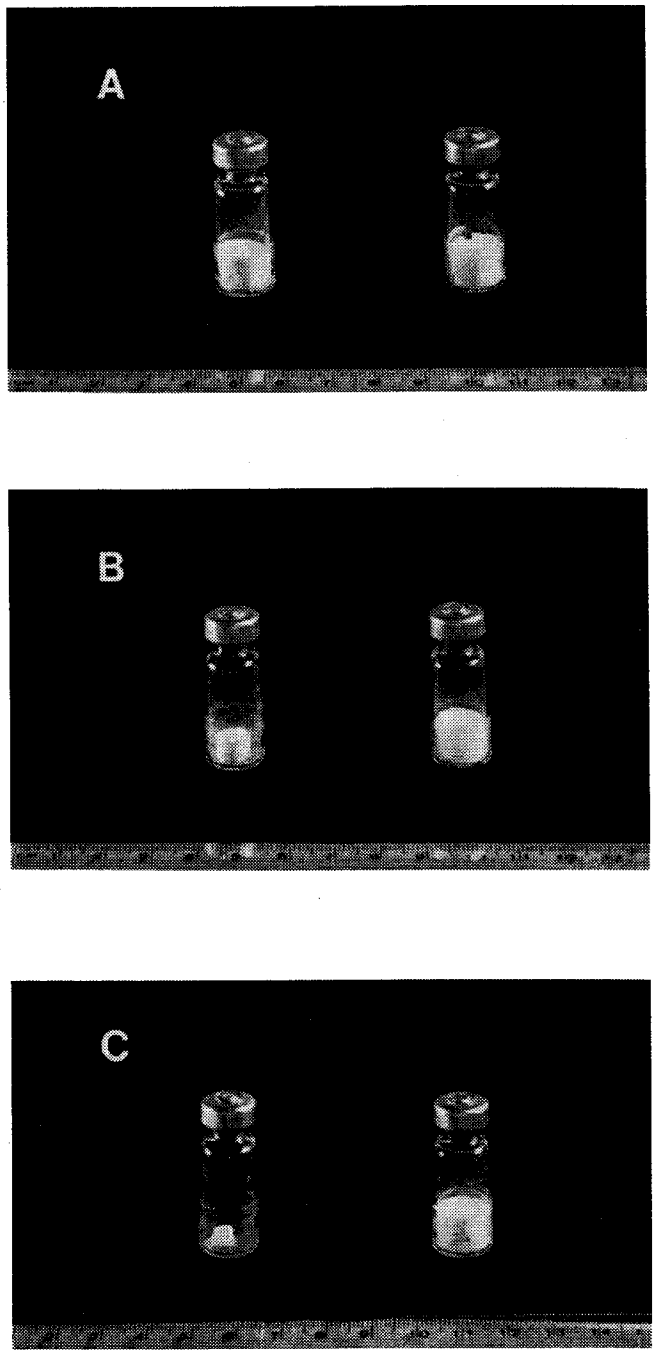

Fig. 6. Volume relaxation in sucrose and trehalose formulations over a one month storage at (A) 40°C; (B) 50°C and (C) 55°C. In all three photographs, the vial on the left-hand side contains the sucrose formulation and the one on the right-hand side contains the trehalose formulation. Storage for one month at the three temperatures (40, 50 and 55°C) correspond to the following t/τ values for the two formulations; (A) sucrose (t/τ = 35), trehalose (t/τ = 0.16); (B) sucrose (t/τ = 286), trehalose (t/τ = 0.55); (C) sucrose (t/τ = 675), trehalose (t/τ = 0.95).

in terms of a relation between the experimental time scale and the time frame of relaxation process undergone by the system. As it can be seen from the Figure 5, both formulations have <2% aggregation at t/τ values <10. Since the relaxation times for sucrose and trehalose formulations at 5°C were found to be in the order of 10^8 and 10^6 hrs, respectively, a t/τ value of 10

for these systems translate into approximately 10^7 and 10^9 hrs, i.e. several years. Therefore, it is likely that both formulations would undergo <2% aggregation in a practical time scale under refrigerated conditions. For sucrose formulation, significant aggregation (5–6%) was observed at t/τ values on the order of thousands, as shown in Figure 5.

Figure 6 represents the physical appearance of sucrose and trehalose formulations when stored for 1 month at 40°C, 50°C and 55°C. When the t/τ value is less than 35, no significant volume relaxation was observed, as shown in Figure 6. However, at higher t/τ values (on the order of several hundreds), significant volume relaxation was observed, suggesting that over a time scale greater than $\sim 100\tau$ the system can undergo significant volume change, along with an increased tendency for the protein to aggregate.

The determination of τ at temperatures much lower than the T_g value typically take several months, and cannot be measured very accurately due to technical difficulties involved in the measurement of very small enthalpic relaxations, even using such advanced techniques as the modulated differential scanning calorimetry. However, VTF equation permits us to predict τ at lower temperatures from the data generated at higher temperatures. The temperature dependence of τ (measured at $T < T_g$) may yield important information regarding the storage temperature below which the product is expected to be stable over the desired shelf-life. There are only limited literature data available on pharmaceutical systems (especially none on multicomponent systems prior to this report) where the applicability of VTF equation at $T < T_g$ has been demonstrated (2). Interestingly, at comparable t/τ values (<100), the aggregation observed with sucrose formulation was consistently lower by a small percentage than the trehalose formulation (Figure 5), suggesting that sucrose formulations are perhaps more stable than the trehalose at low t/τ values.

CONCLUSIONS

It may be possible, at least in certain instances, to obtain valuable information regarding the long term stability of lyophilized solids from the temperature dependence of molecular mobility below T_g. Clearly, more work in this area needs to be done before any routine practical utility of these measurements can be claimed. Comparative stability studies involving several amorphous formulations at arbitrary temperatures may provide a relative stability of the two formulations at that temperature. However, one formulation may exhibit a superior stability compared to the other, simply due to a lower molecular mobility at that temperature. Enhanced stability due to a direct chemical stabilization by a formulation ingredient (e.g. stabilization of a protein in the lyophilized state by a sugar) may be claimed only when the systems are compared at similar molecular mobilities.

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