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

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Received December 24, 1996; accepted February 11, 1997

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, $\tau$, 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, $\beta$, in the relaxation behavior was also obtained using dielectric relaxation spectroscopy.

Results. For both sucrose and trehalose formulations, the variation in $\tau$ 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 $t/T$ values <10, where $t$ is the time of storage.

Conclusions. Since the relaxation times for sucrose and trehalose formulations at $5^\circ$C are on the order of $10^8$ and $10^9$ 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, $\tau$, 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

$$\Phi(t) = \exp[-(t/\tau)^\beta]$$

(1)

where $\Phi$ is the relaxation function and $0 < \beta < 1$ is the stretching exponent. A value of unity for $\beta$ indicates a single relaxation time. Near the glass transition, many systems usually respond non-exponentially to perturbations (3−4), and $\beta$ value deviates from unity. Typical values for $\beta$ 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, $\tau$, at $T_g$ is better described by the Vogel-Tammann-Fulcher (VTF) equation

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

(2)

where $\tau_0$, $B$, and $T_0$ are constants (5). As it can be seen, when $T=0$, 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 $\tau$ (6,7). The temperature dependence of $\tau$ 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 $C_p$ 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 $\beta$ value much less than unity (4). Thus, a plot of log $\tau$ 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 $\tau$ 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 $\beta$ 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 ($\beta$) are related to each other by a general empirical equation

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\[ m = m_0 - s\beta \]  

(3)

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

**Fragility of Glasses in Relation to Accelerated Stability Testing**

Relaxation time constant (\( \tau \)) is a measure of the mobility of the system. Hence, the variation of \( \tau \) 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 \( \sim 20 \), and a \( \beta \sim 1 \), materials such as silicon dioxide), then \( \tau \) 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 \( \tau \) with temperature is strongly non-Arrhenius, then the degradation process can be potentially non-Arrhenius. For fragile liquids, the \( \tau \) 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 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 N₂ 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 \sim 59°C) \ and \ at \ 5, \ 22, \ 40, \ 50 \ and \ 60°C \ for \ trehalose \ (Tg \sim 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

\[ \Delta H_m = (T_g - T) \cdot \Delta C_p \]  

(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 Williams-Watts equation \((10)\)

\[ \Phi(t) = \exp[-(t/\tau)^\beta] \]  

(5)

the parameters \( \beta \) and \( \tau \) were obtained by non-linear regression. The values of \( \tau \) 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 1 degree, 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 \( \varepsilon'' \) vs \( \varepsilon' \). The value of \( \beta \) \((in \ Equation \ 1)\) was calculated from the Cole-Cole plots according to the method described by Havriliak-Negami \((11)\). 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