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Enthalpic relaxation of convective desiccated trehalose–water glasses

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Abstract

Minimizing molecular mobility for desiccation preservation of biologics close to ambient temperature using trehalose glasses require quantitative characterization of their enthalpic relaxation at various end water contents. Differential scanning calorimetry (DSC) was used to characterize three different water contents: 0%, 1.5% and 10% over a wide range of aging temperatures. Results showed the characteristic time (τ) varies both with the water content and the aging temperature. τ increased with lowered aging temperature but showed a non-monotonous relationship as a function of water content. Fragility of trehalose glasses was analyzed using thermophysical parameters obtained from relaxation studies. The study showed trehalose to be a fragile glass former at all water contents, with 0% water samples showing a relatively stronger glass. A compromise between molecular mobility and glass fragility led to an optimal water content close to 1.5% and an aging temperature close to room temperature. This would ensure a τ value of 9000 h, which corresponds to a storage period of a year.

Keywords: Enthalpic relaxation; Fragility; Trehalose; Desiccation; DSC; Water content (WC)

1. Introduction

Desiccation preservation of biologics is an attractive alternative to cryopreservation because of the ease of storage near ambient conditions and simplicity of the logistics of handling and transportation. Trehalose, a non-reducing disaccharide, has been shown to be an effective excipient for desiccation preservation for many biologics including cells, membranes, enzymes and proteins [1–7]. The apparent high glass transition temperature for trehalose helps in maintaining a stable glassy environment at ambient conditions. A glassy environment is believed to reduce molecular mobility and, therefore, slow various harmful degradative events. Although molecular mobility is reduced by orders of magnitude below T_g (relaxation time constant, τ goes from seconds to hours from above to below glass transition), there may still be sufficient mobility below T_g to enable degradation over a long-time frame that may be anywhere from months to years. Therefore, the relaxation kinetics of trehalose glasses needs to be quantified in order to predict the long-term success of desiccated preservation.

Relaxation below glass transition is driven by the non-equilibrium state of the glassy system that is trying to relax to the equilibrium state [8]. This term is commonly referred to as aging [9]. As a supersaturated solution vitrifies, molecular relaxation becomes too slow to maintain equilibrium conformations and as a result the glass will have excess thermodynamic values of enthalpy, entropy, and free volume. If the glass is stored at a temperature slightly below T_g, low levels of molecular mobility in the glass allow relaxation to its equilibrium conformation, causing changes in these properties [10–13]. Molecular mobility, therefore, can be quantified by changes in these properties during relaxation.

Aging of trehalose glasses at different water contents is relatively unexplored in the literature. The plasticizing effect of moisture on trehalose is well documented [14], but quantitative data on molecular mobility below T_g for plasticized trehalose glasses is not available. Studies have shown that T_g may not be the best indicator of molecular mobility below T_g [15]. Trehalose–monoclonal antibody mixtures may have more molecular mobility at temperatures below T_g than

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sucrose–monoclonal antibody mixtures despite the fact that T_g of sucrose is substantially lower than that of trehalose [15]. Aging of glasses has been measured by many techniques, such as differential scanning calorimetry (DSC) [13,15,16], dielectric relaxation [15], thermomechanical analysis [13] and isothermal microcalorimetry [17,18]. Qualitative data on the enthalpic relaxation of frozen trehalose solutions and freeze-dried trehalose have been reported [12,19]. However, quantitative data of convectively dried trehalose–water glasses have not been reported. This type of desiccation is important because convective desiccation is a methodology being used for mammalian cell desiccation [14,20–22].

Unlike in pharmaceutics, storage at completely dry conditions may not be a choice in the desiccation preservation of biologics. Biologics require substantial amount of water for survival even in the desiccated state. On the other hand, too high a water content may render the glasses fragile. In addition to the temperature-induced structural changes and alterations in thermophysical properties, fragility has adverse effects on mobility. Fragile glasses can undergo catastrophic changes in the relaxation time and structure near T_g [15]. The goal of this study was to quantify the enthalpic relaxation kinetics of trehalose glasses with varying water contents in order to characterize the molecular mobility while taking into account the implications of fragility. Experiments were conducted by drying samples in humidity chambers to obtain different end water contents. The different final water contents varied from 0% for completely dry trehalose to 10%. Each sample was aged at 5 °C, 10 °C, 15 °C, 20 °C or 25 °C below its respective glass transition temperature (T_g).

2. Materials and methods

2.1. Sample preparation

Samples of amorphous trehalose with various water contents were prepared by controlled desiccation protocols. Ultra pure trehalose (α,α-trehalose dihydrate) used in this study was obtained from Ferro Pfanstiehl Laboratories (Waukegan, IL). A 0.5 M trehalose solution was prepared by dissolving trehalose dihydrate crystals in double distilled water. Sixty microliters of 0.5 M trehalose solution were put into high volume/pressure stainless steel DSC pans from TA Instruments (New Castle, DE). Completely dry amorphous trehalose samples were obtained by baking 0.5 M trehalose–water solutions at 100 °C for 72 h in an oven. Confirmation of the amorphous state was obtained through the use of the DSC. The samples exhibited glass transitions around 117 °C with no detectable crystal peaks. For trehalose–water samples, the trehalose solution was placed in humidity boxes at 1.5% and 10% relative humidity. The humidity chambers were prepared by providing a constant RH environment on plastic food storage containers placed inside the larger Polystyrene stackable desiccator cabinets. A saturated lithium chloride solution prepared in the plastic container gave a RH environment of 10%. Similarly, 1.5% RH was obtained by using drierite [23]. The samples were periodically weighed until equilibrium conditions were reached, typically over a period of 3 weeks. The end water contents were then determined gravimetrically and recorded as a function of relative humidity.

2.2. Aging experiments

All aging experiments were performed on a Q1000 DSC (TA Instruments, New Castle, DE) equipped with a refrigerated cooling system (RCS). High-pressure sample pans were used to prevent any moisture leakage during the heating scans or subsequent aging experiments. The DSC was calibrated by indium and sapphire samples. Table 1 presents the different steps that were followed to carry out the aging experiments. All the heating runs were performed at the rate of 5 °C/min. The cooling runs were performed at the rate of 15 °C/min. For the aging experiment, the samples were subjected to a pre-melt cycle before holding them isothermal at an aging temperature and followed by a double melt cycle. This process was carried out for t_a's of 10 min, 60 min, 240 min, 720 min, and 1440 min.

2.3. Data analysis

The enthalpy difference, between aged and the unaged samples is given by the following equation:

$$\Delta H(t_a, T_a) = \int_{T_\alpha}^{T_\beta} (C_p(\text{aged}) - C_p(\text{unaged}))\, dT \quad (1)$$

where T_α is a temperature sufficiently below the glass transition temperature that no effects of the glass melt are included and T_β is a temperature sufficiently above the glass transition temperature. Fig. 1 shows a typical plot of C_p versus temperature for aged and unaged samples. The integral in Eq. (1) represents the area between the two curves in the figure. This area can be integrated numerically by the TA Instruments data analysis software.

Table 1
Experimental protocol for the aging experiments

Step	Method	Description
1	Pre-melt run	Samples heated to an upper limit of at least 50 °C above T_g, held isothermally at the temperature for 5 min to erase any thermal history and then cooled down to the lower temperature limit of about 40 °C below T_g causing them to vitrify
2	Isothermal run	Samples brought to the aging temperature (T_a) and held isothermally for the experimental aging time (t_a)
3	Double melt run	Samples brought to the lower temperature limit and then subjected to the thermal cycle between the upper and the lower temperature limits twice, as specified in the pre-melt run. The second cycle served as a baseline to the first cycle, which gave the value of enthalpy relaxation
4		Sample subjected to steps 1–3 for the subsequent aging experiment with a different t_a

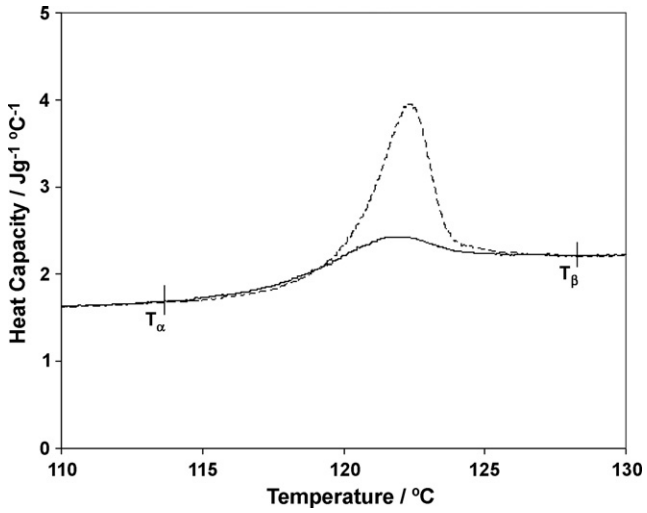

Fig. 1. Plot of C_p vs. temperature for an aged (– – –) and the corresponding non-aged (—) sample. The aged sample shows a large peak representing the relaxation process. The unaged sample shows a simple glass transition. T_β and T_α from Eq. (1) are labeled appropriately.

2.4. Statistical analysis

All the data values in figures and tables represent the average of $n = 2$ except for ΔT of 25 °C and 5 °C, which represent single experiments. The error value represents the standard deviation of the data set. A standard single factor ANOVA test has been used to test the statistical significance of the data sets. The calculated value of p has been used to evaluate the significance. Significance was assessed as $p < 0.05$.

3. Theoretical background

In order to quantify the molecular mobilities of different samples it is necessary to calculate the molecular relaxation time constants (τ) based on the experimental data of aging kinetics of trehalose–water glasses. An empirical equation describing the kinetics of enthalpic relaxation was proposed by Cowie and Ferguson [24].

$$\Delta H(t_a, T_a) = \Delta H_\infty(T_a)[1 - \Phi(t_a)] \quad (2)$$

ΔH_∞ represents the maximum recoverable enthalpy for a particular aging temperature, and the decay function for relaxation (Φ) is defined by the well-known two-parameter stretched exponential Kohlrausch–Williams–Watts (KWW) equation:

$$\Phi(t_a) = \exp\left[-\left(\frac{t_a}{\tau}\right)^\beta\right] \quad (3)$$

τ represents the characteristic relaxation time and β is known as the stretching parameter ($0 < \beta < 1$). β is a measure of deviation from the exponential behavior and physically represents the distribution of independently relaxing states, with a lower value of β suggesting a wider distribution of states [18].

The three unknown parameters $\Delta H_\infty(T_a)$, τ and β were determined from the enthalpic relaxation of the glasses. We calculated the maximum enthalpy recovery $\Delta H_\infty(T_a)$ for a given aging temperature using the following equation:

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

where T_g represents the glass transition temperature for the specific sample and ΔC_p represents the change in heat capacity at T_g. After determining the maximum enthalpic recovery using Eq. (4), Eq. (2) was used to calculate the decay function (Φ) based on the experimentally determined enthalpic recovery. The two unknowns τ and β were evaluated by using a non-linear curve fitting routine (MathCAD professional, 2001) and Eq. (3).

One of the important equations used to define the relaxation behavior of the glasses is the Vogel–Fulcher–Tammann (VFT) equation. It gives a measure of the non-Arrhenius variation in the relaxation time constant, τ, near the glass transition temperature. The VFT equation is given by

$$\tau = \tau_0 \exp\left(\frac{B}{T_a - T_0}\right) \quad (5)$$

where T_a is the aging temperature and τ_0, B, and T_0 are unknown constants. τ_0 represents the relaxation time at the high temperature limit. B is a coefficient inversely proportional to the glass fragility and T_0 is the temperature at which the relaxation time approaches infinity. The equation reduces to an Arrhenius equation as the value of T_0 approaches 0. Once τ has been determined, the constant parameters τ_0, B, and T_0 can be calculated using a non-linear curve fitting routine and Eq. (5).

4. Results

Fig. 2 shows representative DSC scans for completely dry trehalose glass aged for different times at a temperature 15 °C below its T_g. A clear trend of steeper enthalpic relaxation peaks can be seen as a function of aging time. The peak value of C_p progressively increased with aging time to a maximum of 5.5 J/g °C at 1440 min (24 h). Another feature observed during the succes-

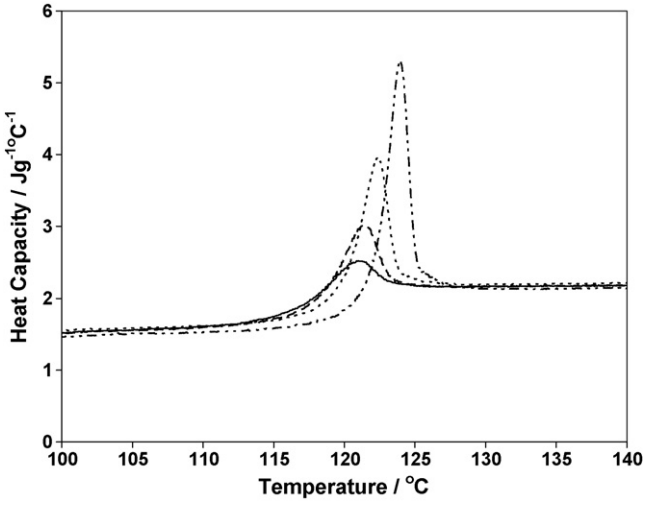

Fig. 2. Plot of C_p vs. temperature for samples aged at the same aging temperature for varying aging times. Solid line (—) represents aging of 10 min; dashed line (– – –) represents aging of 60 min; dotted line (- - -) represents aging of 240 min; dashed and double dotted line (– – – – –) represents aging of 1440 min.

Table 2
Specific heat capacity and the glass transition temperature of trehalose for different end water content and aging temperature

$(T_g - T_a)$ (°C)	ΔC_p (J g^{-1} °C^{-1})	T_g (°C)
0% WC		
10	0.63 ± 0.01	
15	0.59 ± 0.03	116.2 ± 0.7
20	0.62 ± 0.02	
1.5% WC		
10	0.65 ± 0.01	
15	0.65 ± 0.01	98.1 ± 2.7
20	0.65 ± 0.01	
10% WC		
10	0.79 ± 0.02	
15	0.81 ± 0.02	27.0 ± 0.1
20	0.81 ± 0.03	

sive aging experiments is that there was an increase in the value of T_g with increasing aging time. This was evidenced by the shift in the enthalpic relaxation peaks to the right in the figure. In summary, enthalpic relaxation increased with increasing aging time for a given $\Delta T(T_g - T_a)$.

Table 2 lists the measured T_g and ΔC_p values along with their standard deviations for the different aging temperatures as a function of moisture content. T_g decreases with an increase in water content. For 0%, 1.5% and 10% water content the values of T_g were 116.2 °C, 98.1 °C and 27.02 °C, respectively. On the other hand, the change in specific heat (ΔC_p) increases with water content. ΔC_p increased with increasing water content from 0.59 for ΔT of 15 °C for pure trehalose (no water) to a high of 0.81 for ΔT of 20 °C and 10% water content. However, no predictable trend was observed for ΔC_p as a function of aging temperature at specific water content.

The enthalpic relaxation kinetics at 1.5% water content as a function of aging time for the three different aging temperatures are shown in Fig. 3. The open circles represents the measured value for samples aged at 10 °C below T_g, open squares represents the sample aged 15 °C below T_g, and open triangles represent the enthalpic relaxation values for samples aged at 20 °C below T_g. The results show a clear trend of increasing relaxation with the time of aging for any aging temperature. The rate of relaxation is greatest in the first 2 h where the results indicate a steep climb in the enthalpic relaxation values. For aging at 20 °C below T_g, in 10 min the measured enthalpic relaxation is 0.9 J/g, in 60 min it climbs to 1.8 J/g and then to 2.5 J/g after 240 min of aging. After 24 h (1440 min) this value increases only moderately to 3.1 J/g. As expected, our results show enthalpic relaxation increases as a function of aging time (at a given aging temperature) and increased aging temperatures (smaller ΔT). This is true for amorphous trehalose and trehalose–water glasses at all the moisture contents we studied.

Table 3 shows the maximum recoverable enthalpy $\Delta H_\infty(T_a)$ as a function of water content. The value of ΔH_∞ increases with increasing water content. Except for ΔT of 15 °C between 0% and 1.5% water content, the increases in ΔH_∞ values were statistically significant ($p < 0.05$) as water content changed from

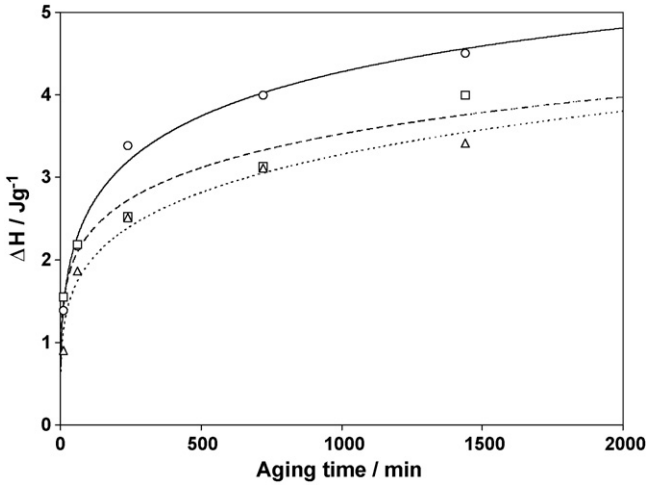

Fig. 3. Relaxation kinetics of trehalose glasses with 1.5% water. Open circles represent the values of enthalpic relaxation for $\Delta T(T_g - T_a) = 10$ °C; open squares represent the values for $\Delta T = 15$ °C; open triangle represent the values for $\Delta T = 20$ °C. The lines represent the "Williams–Watts equation" fit to the experimental. Solid line (—) represents the fit for the relaxation data of $\Delta T = 10$ °C; dashed line (– – –) represents the fit for the relaxation data of $\Delta T = 15$ °C; dotted line (- - -) represents the fit for relaxation data of $\Delta T = 20$ °C.

0% to 1.5% and from 1.5% to 10% for all aging temperatures, except in one case ($\Delta T = 15$ °C, 0–1.5% moisture).

Consistent with our previous results of the increase in ΔH, Φ decreases in an exponential manner with increasing aging time as shown in Fig. 4. This is because Φ was calculated by combining Eqs. (2) and (4), each of which gives a measure of the total enthalpic relaxation (ΔH) and the maximum recoverable enthalpy (ΔH_∞). The data points for a particular ΔT in the figure have been joined only to assist in visualizing the trend. Φ values decrease at a sharper rate for higher aging temperature (lower ΔT) indicating that the relaxation takes place quicker at the higher aging temperatures. For ΔT of 10 °C for 1.5% water content, the value of Φ (which is 1 by definition at time = 0) decreases rapidly to 0.81 in 10 min and down to 0.37 after 24 h. While the values of Φ for 0% water content samples are close to those of the 1.5% water samples for similar ΔT, the decay is less pronounced for 10% water content samples.

As shown in Table 4, there is a clear trend of increasing τ with increase in ΔT (lowering of the aging temperature). For $\Delta T = 5$ °C and 10% water content, the value of τ is of the order of 10–100 h. For $\Delta T = 15$ °C and 20 °C the τ values range between 100–500 and 400–2200 h, respectively, for all the moisture contents studied. For $\Delta T = 25$ °C and water contents of 0% and

Table 3
Values of ΔH_∞ at different water content and ΔT

ΔT (°C)	ΔH_∞ (J g^{-1})		
	0% WC	1.5% WC	10% WC
5			4.2
10	6.3 ± 0.0	6.6 ± 0.4	7.9 ± 0.2
15	9.0 ± 0.3	9.9 ± 0.1	12.2 ± 0.1
20	12.4 ± 0.0	13.1 ± 0.4	16.3 ± 0.5
25	15.8	16.2	

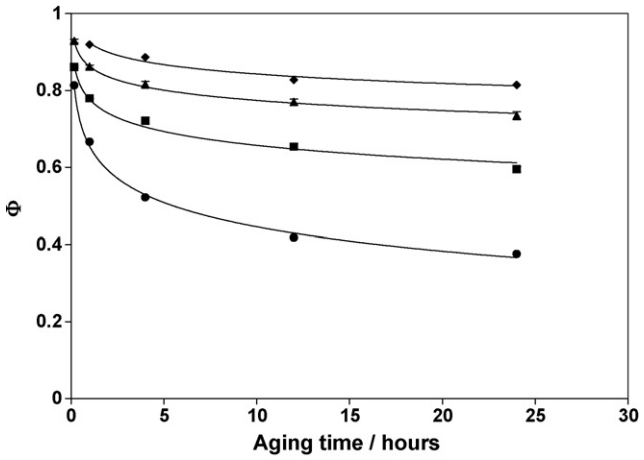

Fig. 4. Plot of aging time vs. Φ for 1.5% water content. Error bar represents the standard deviation. Filled circle represents the average value for $\Delta T = 10\,^{\circ}C$; filled square represents the average value for $\Delta T = 15\,^{\circ}C$; filled triangle represents the average value for $\Delta T = 20\,^{\circ}C$; filled diamond represent the value for $\Delta T = 25\,^{\circ}C$.

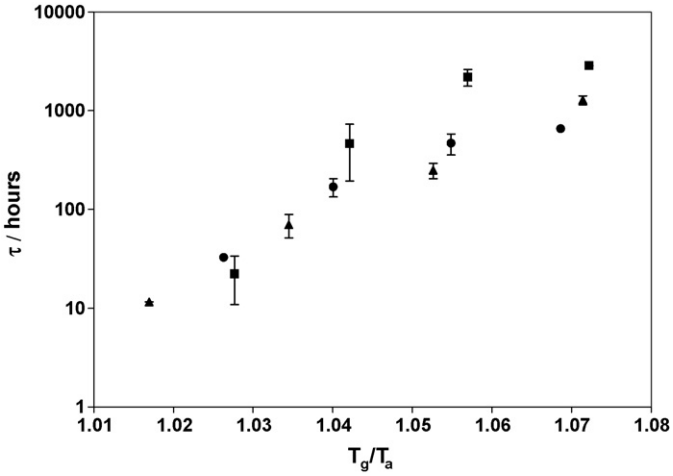

Fig. 5. Plot of the mean relaxation time constant (τ) vs. T_g/T_a. Error bar represents the standard deviation. Filled circle represents the average value of for 0% water; filled square represents the average value for 1.5% water; filled triangle represents the average value for 10% water.

Table 4
Variation of the mean relaxation time constant (τ) with water content and ΔT

ΔT (°C)	τ (h)		
	0% WC	1.5% WC	10% WC
5			12
10	33 ± 0	23 ± 11.41	70 ± 19
15	169 ± 36	463 ± 310	249 ± 45
20	467 ± 113	2193 ± 419	1280 ± 134
25	658	2859	

1.5%, the values of τ range between 600 and 3000 h. The values of τ are significantly different ($p < 0.05$) between $\Delta T = 10\,^{\circ}C$ and 15 °C for 0% water; $\Delta T = 15\,^{\circ}C$ and 20 °C for 1.5% moisture; $\Delta T = 10\,^{\circ}C$ and 15 °C and $\Delta T = 15\,^{\circ}C$ and 20 °C for 10% water. In summary, larger ΔTs did result in larger τ as expected.

The effect of water is less pronounced and more complex on τ compared to the aging temperature for the ranges explored in the current study. For a constant value of ΔT, there is a non-monotonous change in τ as a function of water content. For ΔT of 10 °C, the value of τ dips a bit with increase in water from 0% to 1.5%, although not significantly and then increases for further increase in water to 10%. However, for ΔT of 15 °C and 20 °C, the values of τ increases for increase in water from 0% to1.5% and subsequently decreases for 10% moisture. However, the statistical significance of this non-monotonous behavior is hard to establish. Differences in τ as a function of water content are not statistically significant ($p > 0.05$) for ΔT of 10 °C and 15 °C between 0–1.5% water, 1.5–10% water and 0–10% water. At ΔT of 20 °C, τ is significantly different for the change in water content from 0% to 1.5% and 0% to 10% water. However, it is important to note that the greatest value of τ we measured occurred at a water content of 1.5% not 0%.

The plot of τ versus T_g/T_a exhibits a non-Arrhenius behavior for trehalose glasses at all water contents, with the samples at 0% and 1.5% water content displaying more non-linearity than those at 10%. As seen from the Fig. 5, the non-linearity is due to a flattening out of τ as T_g/T_a reaches the highest values, which corresponds to an increase in ΔT from 20 °C to 25 °C in the 0% and 1.5% samples. It can thus be inferred that the temperature dependence of the relaxation time deviates from Arrhenius-like dependence as aging temperature deviates further from T_g. The values of activation energy and the steepness of the Arrhenius fit of the plot of ln(τ) versus T_g/T_a for different water contents are presented in Table 5.

Table 5 shows the values of the VFT parameters as well as the slope and the activation energy of the Arrhenius fit for trehalose equilibrated at 0%, 1.5% and 10% RH. The calculated values of T_0 were found to be approximately T_g-176 K, T_g-179 K and T_g-95 K for 0%, 1.5% and 10% water content, respectively. The corresponding τ_0 values were of the order of 10^{-6} h for 0% and 1.5% water content and 10^{-9} h for 10% water content. The

Table 5
VFT and Arrhenius parameters of trehalose glasses

	0% WC	1.5% WC	10% WC
τ_0 from VFT fit (h)	1.25×10^{-6}	9.55×10^{-7}	3.73×10^{-9}
B from VFT fit (K)	3050	3369	2037
T_0 from VFT fit (K)	213	192	203
T_0/T_g	0.55	0.52	0.68
Slope (m) from Arrhenius fit	70.6	108.0	84.6
Activation energy from Arrhenius fit (kJ/mol)	229	333	211

Table 6
Variation of the relaxation time distribution parameter (β) with water content and ΔT

ΔT (°C)	β		
	0% WC	1.5% WC	10% WC
5			0.372
10	0.339 ± 0.001	0.310 ± 0.029	0.359 ± 0.038
15	0.272 ± 0.023	0.242 ± 0.027	0.375 ± 0.018
20	0.342 ± 0.023	0.271 ± 0.009	0.381 ± 0.001
25	0.434	0.314	

B values were 3050 K, 3369 K, and 2037 K, respectively. The results show a similar range of values of the parameters for 0% and 1.5% water contents. However, for 10% water, there is a significant decrease in the value of τ_0 and B ($p < 0.05$). Similarly, the difference between the T_g and T_0 was less as compared to the lower water content samples. The values of T_0/T_g were calculated from VFT and T_g data. The largest value was reported at 0.68 for 10% water content and lowest of 0.52 for 1.5% water content.

The Arrhenius values were obtained by plotting the log of mean value of τ against T_g/T_a. The values of the activation energy were 229 kJ/mol, 333 kJ/mol and 211 kJ/mol for 0%, 1.5% and 10% water contents. The corresponding slopes of the fits (m) were 70.6, 108 and 84.6, respectively. The results show the highest value of the slopes and activation energies for 1.5% water content samples, followed by 10% water content and 0% water content.

As shown in Table 6, β does not vary as markedly as τ over the different aging temperatures at a particular water content but varies as a function of water content. Because these numbers are not significantly different ($p > 0.05$), it appears that at a particular water content, β essentially is constant over the range of aging temperatures studied. The values of β range from a mean value of 0.24–0.434 within the range of ΔT and water investigated. However, β seems to significantly vary as a function of water content ($p < 0.05$) for ΔT of 15 °C and 20 °C as the water content increased from 1.5 to 10%. The average values of β increased from 0.242 to 0.375 and 0.27 to 0.38, respectively, for these cases. Also the changes in β are statistically significant for $\Delta T = 20$ °C when water content changes from 0% to 1.5% ($p = 0.05$) and for $\Delta T = 15$ °C for water content change from 0% to 10%. We also noticed a general trend of decrease in the average value of β with an increase in the value of water content from 0% to 1.5% followed by an increase in the value of β as the water content increased to 10%. This indicates a non-monotonic change in β as a function of water content.

5. Discussion

Since trehalose–water glass studies in the current paper focus on regions below but close to T_g (within 25 °C), the systems we are dealing with are non-ergodic and, therefore, the thermal history associated with the formation of the samples is important [25]. The desiccation protocols of the current study were different from previous studies in the literature [13,15,16,18,19] because desiccation protocols used in cell preservation were followed. Our previous studies have shown that during natural convective desiccation, surface glass forms rapidly (60–120 min), but the trapped moisture gradually escapes over a long period of time [26]. Therefore, the process of equilibration by desorption is a complex process where the glass transition temperature of the entire solution is gradually lowered and parts (upper part) of the sample start aging within a day because a glass layer has formed on the top. There are no studies that have looked at the enthalpic relaxation of trehalose solutions that have been desorbed to equilibriated values of a given low water content (1–10%) where the entire solution is glassy at room temperature. In order to obtain 0% water we dried our trehalose solution in an oven for 72 h at 100 °C. This is different from other studies that have prepared samples by freeze-drying, and added moisture to the samples by adsorption in appropriate humidities [18,19].

Enthalpic relaxation of 0% water samples using our desorption protocol at $\Delta T = 20$ °C was significantly less than that reported by Surana et al. [19] for their desorption protocol. Whether this difference is a result of our drying technique or desorption technique is not clear. The enthalpic recovery values reported by Surana et al. [19] are 4.4 J/g, 6.4 J/g and 7.0 J/g at 8 h, 12 h and 20 h of aging. Our numbers are almost half of those values. Liu et al. [18] have shown that freeze-dried trehalose and quenched trehalose have T_g values close to each other (113 versus 115 °C, respectively), but widely varying enthalpic relaxation values (τ of 9.5×10^6 h as opposed to 1.5×10^6 h, respectively) at ΔT value of ∼65 °C.

Our studies for dried trehalose samples (0% moisture) yielded relaxation values of 32 h, 168 h, 468 h and 657 h for increasing ΔT values of 10 °C, 15 °C, 20 °C and 25 °C, respectively. These numbers are lower than those reported by Liu et al. [18] because aging took place at higher temperatures in our studies compared to their studies. Extrapolating (based on a log-linear approximation) for ΔT of 60 °C yields a τ value of 10^6, which is the same order as values reported by other studies. A closer comparison with the study of Liu et al. [18] can be made between freeze-dried samples that were quenched and then dehydrated to 2.7% water in a humidity chamber with our samples with 1.5% water content and ΔT of 20 °C. Our study yielded a τ value of 2192 h and theirs yielded $\tau = 937$ h. Therefore, while different methods of sample preparation (yielding very different thermal histories) result in different relaxation kinetics, they seem to result in relaxation times with the same orders of magnitude.

Our results indicate that the maximum recoverable enthalpy $\Delta H_\infty(T_a)$ increases with the aging time as well as water content. $\Delta H_\infty(T_a)$ is the difference in enthalpy between the aged sample at a particular temperature and the presumed 'equilibrium' value of the supercooled liquid [8,10,11,13]. More recent studies have characterized it as the excess enthalpy with respect to a metastable state [17]. The almost linear temperature dependence of $\Delta H_\infty(T_a)$ indicated an almost constant change in specific heat (ΔC_p) over the temperature range studied (Eq. (4)). A similar trend has been reported by Cowie and Ferguson [10,11] for polymers. This signifies a divergence between the non-equilibrium aging property line and the equilibrium (or metastable equilib-

rium) reference line as the temperature is progressively lowered below T_g. Our studies also show that the maximum recoverable enthalpy increases with the water content, which is primarily due to an increase in the ΔC_p value. It is unclear at this stage why an increase in water content would contribute to an increase in ΔC_p.

The present study shows a trend of lower enthalpic relaxation with lowering of aging temperature (or greater ΔT), which is consistent with other studies that have looked at relaxation of sugars or mixtures of sugars with other substances [13,15–19]. This is characterized by higher values of τ corresponding to lower temperatures below T_g. As aging temperature is lowered, molecular mobility is reduced and, therefore, it takes a longer time for the sample to relax back towards its equilibrium value.

5.1. Role of water in changing molecular mobility

Additives to sugars alter the enthalpic relaxation and it is expected that the mobility in amorphous solids will be strongly enhanced by residual water. Thus, one would expect that relaxation time should decrease strongly as residual water increases. For quenched trehalose, Liu et al. [18] have reported the relaxation time τ^β decreased approximately by a factor of 6 as the water content increased from nearly 0% to 2.7%. An increase in molecular mobility has also been found for lyophilized PVP and sucrose with an increase in water content [27]. Our results showed a non-monotonous effect of water content on relaxation, as indicated by the value of τ. For the higher values of ΔT studied, the greatest value of τ was obtained for 1.5% water and not for 0%. Although, not statistically significant for the lower ΔT, for ΔT of 20 °C, the τ values are significantly different with a maxima at 1.5%. The result suggests an intermediate water content at which molecular mobility is the lowest and optimal for long-term storage.

5.2. KWW equation: significance of parameters

The KWW expression has been used widely to quantify the relaxation processes of glassy systems and thereby predict long-term storage stability of pharmaceutical and biological products that are stored in a glassy matrix. Glass-forming systems do not relax in an exponential manner, therefore, the relaxation is quantified by two parameters: τ, which is the relaxation time and β, which is the stretching parameter. Although there are other multiparameter models that may be more accurate than the KWW, it appears to be the most economical and widely used function for quantifying relaxation processes in glass-forming liquids [26]. This equation has been used as a tool for predicting the long-term storage stability of pharmaceutical substances in the glassy state [13,15,16]. τ is a measure of the molecular mobility below T_g. It has been postulated based on empirical evidence that the relationship describing the mobility characterization of the relaxation process over a wide range of temperatures below T_g will enable the prediction of molecular mobility quantified by τ at any given temperature and time.

Our study indicated that τ increases with ΔT, meaning that at some aging temperature sufficiently below T_g, relaxation will not occur on any experimental time scale because viscosity and diffusivity are too low to allow significant molecular movement. This has effects on drying and dried storage. When a sample first vitrifies during a desiccation process ΔT is practically 0. However, as more water continues to leave the sample, T_g will continue to rise while the aging temperature remains constant, causing ΔT to increase. With this increase in ΔT, mobility will slow to a crawl and eventually become so low that any water remaining will be trapped. If the desired water content has not yet been reached, it will take a very long-time to reach that water content, if it can be reached at all. In storage, zero mobility is desired. Having knowledge of what temperature is needed to immobilize the glass would be very beneficial.

The stretching parameter, β is a measure of the extent of non-exponential behavior of the relaxation process [16]. An important significance of the value of β lies in its application to measure fragility of amorphous glasses [13]. A value of β close to unity signifies a strong glass whereas the lower values signify fragile glasses. We found β was much lower than 1 and essentially constant over the ranges of ΔT and water contents studied. The values of β varied from 0.24 to 0.43. This denotes a high non-exponentiality in the relaxation rate and at the same time indicates the formation of fragile glasses.

5.3. VFT equation: significance

The VFT equation is useful in explaining the degradation of the amorphous system and the fragility of the glass. Fragility of a glass is an important parameter in understanding the response of the samples to perturbations such as an increase in temperature [15]. Moreover, the VFT equation helps in predicting the value of τ at lower temperatures with the help of the data generated at higher temperatures. The value of VFT parameters for our samples showed similar results for 0% and 1.5% water and lower values for 10% water content. In the determination of the VFT parameters for trehalose–monoclonal antibody formation by Duddu et al. [15], the reported value of trehalose glass formation was 1.98×10^{-4} h for τ_0, 2169 K for B, and 185 K for T_0. This is different from the values that we have obtained (1.25×10^{-6} h, 3050 K, 213 K, respectively, for 0% water, 9.55×10^{-7} h, 3369 K, 192 K, respectively, for 1.5% water, 3.73×10^{-9} h, 2037 K, 203 K, respectively, for 10% water). The difference can be attributed to the presence of the antibody in the trehalose–glass mixture. The VFT parameter B has been related to the fragility of the glassy material [13]. As suggested by Walters [28], the coefficient is inversely proportional to the glass fragility. Our results showed a B value in the range of 3000–4000 K for samples stored at 0% and 1.5% water as compared to a value of the order of 2000 K for a 10% water sample. Thus, it can be inferred that higher water content enhances the fragility of trehalose glasses thus making it more perturbable to the degradation induced by temperature fluctuations.

5.4. Water content and fragility

The classification of fragility is based on a number of thermophysical properties. These include the change in ΔC_p at T_g,

values of the VFT parameter B, values of β, the ratio of T_0/T_g and the temperature dependence of relaxation time. Strong glasses exhibit higher values of B, lower values of ΔC_p, β values close to unity, values of T_0/T_g approaching zero and an almost linear dependence of the log of τ on T_g/T_a [13,15,28,29]. Moreover, strong glasses exhibit a smaller value of m (<16), the slope of Arrhenius fit in the plot of log τ versus T_g/T_a [15]. Our results show much lower values of β and high values of m (slope) for all water contents. This suggests that all the trehalose glasses are fragile. Determining the relative strength as a function of water content may be complex. Trehalose with 0% and 1.5% water contents exhibit lower values of ΔC_p and, T_0/T_g and higher values of B as compared to trehalose with 10% water content. These results suggest that the glasses formed at lower water contents are stronger than those at 10%. On the other hand, the values of β and the value of m are higher for trehalose with 0% and 10% water as compared to 1.5%. On the basis of all these parameters it can be inferred that trehalose forms stronger glasses at 0% water. Between 1.5% and 10% water, the parameters showed mixed results, with the values of ΔC_p, T_0/T_g and B signifying stronger glass at 1.5% water and the values of β and m signifying stronger glass at 10% water content. Studies by Hancock et al. [13] have shown similarly mixed results of fragility in their study of molecular nobilities of three amorphous pharmaceutical solids namely indomethacin, PVP and sucrose.

6. Conclusions

Knowledge of mobility in glasses makes it possible to optimize storage protocols. The results of the dependence of the characteristic time, τ on ΔT and water content show two distinct trends. τ increases with the increase in ΔT. Surprisingly, τ does not depend strongly on water content. Under certain conditions higher water content samples exhibited lower mobility than drier samples. In addition, our studies showed trehalose to be a fragile glass former. The results showed a relatively stronger glass formation at 0% water, but a more complex behavior for 1.5% and 10% water. The choice of optimal water content for long-term storage should be made considering the effect of fragility on molecular mobility. This is particularly significant given that we found mobility was not a strong function of water content. Our goal is to achieve a τ value of around 9000 h, which will ensure that a sample will not relax for a period of a year, and at the same time ascertain that the samples do not degrade over the storage period. Trehalose at 0% water forms relatively stronger glasses but does not yield the required τ value at near to ambient temperature and moreover, is not suitable for survival of stored biologics. On the other hand, a sufficiently high ΔT can reduce molecular mobility for high water samples, but then will be less stable. A relative humidity between 0% and 1.5% water content and ΔT close to 40 °C may help achieve our desired goal.

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