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MEASUREMENT OF T_g IN LYOPHILIZED PROTEIN AND PROTEIN EXCIPIENT MIXTURES BY DYNAMIC MECHANICAL ANALYSIS

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The glass transition of lyophilized materials is normally measured by conventional or temperature modulated differential scanning calorimetry (TMDSC). However, because of the weakness of these transitions when protein concentrations are high, these techniques are often unable to detect the glass transition (T_g). High ramp rate DSC, where heating rates of 100 K per min and higher are used, has been shown to be able to detect weak transitions in a wide range of materials and has been applied to these materials in previous work. Dynamic mechanical analysis (DMA) is also known to be much more sensitive to the presence of relaxations in materials than other commonly used thermal techniques. The development of a method to handle powders in the DMA makes it now possible to apply this technique to protein and protein-excipient mixtures. HRR DSC, TMA and DMA were used to characterize the glass transition of lyophilized materials and the results correlated. DMA is shown to be a viable alternative to HRR DSC and TMA for lyophilized materials.

Keywords: *amorphous, DMA, DSC, excipients, glass transition, lyophilized, proteins, TMA*

Introduction

Due to inadequate stability in aqueous solution, many therapeutic proteins are developed as lyophilized products. An important requirement for long-term stability of proteins in a dried formulation is storage at a temperature below its glass transition temperature (T_g) [1, 2]. Therefore, determination of a formulation's T_g is a critical component of formulation development and testing. Differential scanning calorimetry (DSC) is often used to measure T_g, as well as the heat capacity change at T_g(ΔC_p) [3–5]. However, many components in pharmaceutical dosage forms have either low amorphous content and/or weak thermal signals at the glass transition, which often prevents the measurement of the T_g with conventional DSC. For example, the T_g of freeze-dried protein often cannot be detected due to a small heat capacity change at T_g arising from significant microstructural heterogeneity and a broad distribution of relaxation times [6]. Therefore, T_g values for proteins have been estimated by extrapolating to zero excipient concentration using T_g values measured for binary mixtures of protein and another glass-forming excipient such as sucrose over a range of excipient concentrations [7, 8].

Also, with many polymeric excipients (e.g., hydroxyethyl starch, HES), the thermal changes associated with T_g are often difficult to detect [9, 10]. In addition, in the study of organisms that can withstand drying, such as bacterial spores, it is often of interest to determine whether components of the organism form glasses upon drying, but measuring the T_g values of these systems is also problematic (unpublished observations).

Recently new approach to study samples with amorphous content is to use high ramp rate differential scanning calorimetry (HRR DSC), which is also called high heating rate differential scanning calorimetry, fast scan DSC, where very high heating rates (from 100 K min^{-1}) are employed. The Diamond DSC employed in this study (PerkinElmer, Shelton CT) uses an ultra low-mass furnace with small dimensions to ensure that the system is under adequate control, even when making measurements at high heating rates [11, 12]. The increased sensitivity from fast scan DSC is due to the fact that, as the heating rate is increased, the same heat flow occurs over a shorter time period, increasing the observable signal from the thermal event. This allows low-energy transitions (i.e., those with small ΔC_p at T_g) to be detected and measured. Application of the HRR DSC approach to very weak thermal transitions has been reported previously for spray dried lactose [12], themoplastics [13], and thermosets of epoxy [14, 15]. Recently work on the application of high ramp rate DSC to samples lyophilized protein formulations and bacterial spores for which T_g could not be detected using conventional DSC has been published [16].

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In developing that approach, it was found that mechanical methods, well known to be more sensitivity to the presence of weak transitions than DSC, were an excellent tool for confirming these transitions. Katayama's work [16] used thermomechanical analysis (TMA) with a dilatometer to measure the glass transition. With the development of techniques like the Material Pocket™, a stainless steel envelope developed to hold powders and other samples incapable of supporting themselves, the greater sensitivity of dynamic mechanical analysis (DMA) can be used on powdered pharmaceutical samples like those discussed above. DMA using a material pocket has the advantages of being more sensitive to the presence of amorphous material than either HRR DSC or TMA as well as being easier to prepare than TMA samples. The use of material pockets to successfully measure amorphous content in both lactose [17] and celecoxib [18] has been reported. In this work, the technique is applied to proteins, protein–excipient mixtures, and hydroxyethyl starch.

Experimental

Materials

Hen egg white lysozyme, bovine pancreatic ribonuclease A, ovalbumin and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer salts were purchased from Fisher Scientific (Fairlawn, NJ). Sucrose and trehalose were purchased from Pfanstiehl Laboratories (Waukegan, IL). Hydroxyethyl starch (HES; Viastarch) was purchased from Fresenius (Graz, Austria) and had a mean molecular mass of 200 kDa [19]. Aluminum DSC pans, the indium standard, and the material pockets were purchased from PerkinElmer LAS.

Methods

Freeze-drying method

Protein formulations were freeze-dried in an FTS Durastop microprocessor-controlled freeze-drier equipped with a Dura dry MP condenser unit (Stone Ridge, NY). Samples were placed into 2 mL vials (13 mm ID) obtained from the West Company (Lionsville, PA) and partially sealed with gray butyl stoppers (West Company). Samples were placed on the lyophilizer shelf at room temperature. Samples were frozen by cooling the shelves to 233 K at a rate of 2.5 K min^{-1}. After samples were at this temperature for 9000 s, primary drying was started by reducing the chamber pressure to 13.3 Pa, and continued by maintaining the shelf temperature at 233 K for 172800 s. Secondary drying (13.3 Pa chamber pressure) was initiated by increasing the shelf temperature to 298 K at a rate of 0.1 K min^{-1}. This temperature was maintained for 28800 min. Samples were then stored in seal vials under nitrogen at 273 K.

Differential scanning calorimetry (DSC)

All samples were analyzed on a PerkinElmer Diamond DSC equipped with an Intercooler II system for cooling. The data were analyzed on Pyris 7.0 software. The DSC was calibrated *vs.* indium as a temperature and enthalpy standard. 10 and 1 mg samples were heated at 20 and 100 K min^{-1}, respectively in 50 μL aluminum pans with a 20 cc min^{-1} of nitrogen purge. Glass transitions temperatures (T_g) are reported as the onset temperature as calculated by the Pyris 7.0 software using the derivative to indicate the initial baseline and midpoint for the calculation. All samples were weighed and hermetically sealed in aluminum pans in a dry air purged glove box. Samples were run through a heat–cool–heat cycle from 298 to approximately 40 K above the glass transition, cooled to 298 K at 200 K min^{-1}, and reheated at the same rate. Transitions measured on the second heat. All samples were run in triplicate and the results averaged.

Thermomechanical analysis (TMA)

Samples were run using a PerkinElmer Diamond TMA running on Pyris 7.0 software, using a quartz expansion probe and sample tube with nitrogen purge of 40 mL min^{-1} and air cooling. The instrument was calibrated using indium and zinc as temperature standards. Approximately 50 mg of sample was loaded into a quartz dilatometer tube of 10 mm diameter and packed by tapping for 5 min under dry nitrogen in a glove box. Each sample was then run under a 5 mN load at 5 K min^{-1} from 298 K min^{-1} to approximately 30 K above the T_g. The T_g was calculated as the onset of the drop in probe position [22] using the onset function in the Pyris 7.0 software. Points were chosen using the derivative to indicate the initial baseline and midpoint. All samples were run in triplicate and the results averaged.

Dynamic mechanical analysis (DMA)

DMA was run on a PerkinElmer DMA 8000 with the DMA 8000 software. Samples were handled in dry nitrogen as much as possible to reduce the chance of moisture [19] lowering the temperature of the glass transition. Approximately 25 mg of materials was loaded into a material pocket, which was then closed using pliers in a glove bag continuously purged with nitrogen. Figure 1 shows the method of loading the material pocket. The pocket was then loaded into the

single cantilever geometry as shown in Fig. 1 b. Samples were run while applying 50 micron deformation at multiple frequencies of 1, 10 and 50 Hz with a 2 K min^{-1} heating rate from 298 to 30 K above the glass transition. The samples were purge for 900 s before heating. The T_g was calculated by the drop in the storage modulus as described in the literature [20]. Samples were run in triplicate and the values averaged. Variation is reported as the range.

For the humidity experiments, the same conditions were used except the DMA was connected to the Triton Humidity Controller (Triton Technology, Keyworth, UK). The sample was loaded as above into the DMA and the relative humidity set at 80%. The sample was held isothermally for 1 h to allow the sample to equilibrate and then it was heated to 343 K at 2 K min^{-1}. The glass transition temperature was calculated as above.

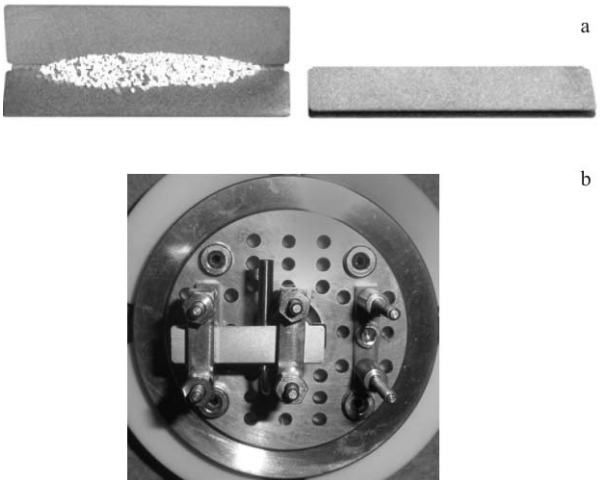

Fig. 1 The process of loading a material pocket is shown; a – shows the method of loading a material pocket, b – shows the material pocket loading in a single cantilever fixture in the DMA 8000 before closing the furnace

Results and discussion

It was found early on that samples had to be handled in dry atmosphere to reduce the variability of the runs. This is a known problem with protein-excipient mixtures [16] and exposure to humidity reduces the glass transition dramatically. An early attempt to investigate this by running sucrose samples under both dry conditions and at 80% relative humidity showed a change in the T_g from ~349 to ~305 K. Based on these results and those reported in the literature [21], all work was done under as dry conditions as possible.

Glass transitions are very easy to detect in the DMA, as shown in Fig. 2 for lysozyme, as either the drop seen in *E*' or as the peak of tan. Values were taken by using the drop in *E*' as discussed in the literature [20]. Average T_g values are reported for samples run in Table 1. Overall, there was good agreement of the onset of the T_g of the dried pure proteins as measured by DSC, TMA and DMA. The differences in the value of the T_g are what one would expect between the three techniques, since the heat capacity

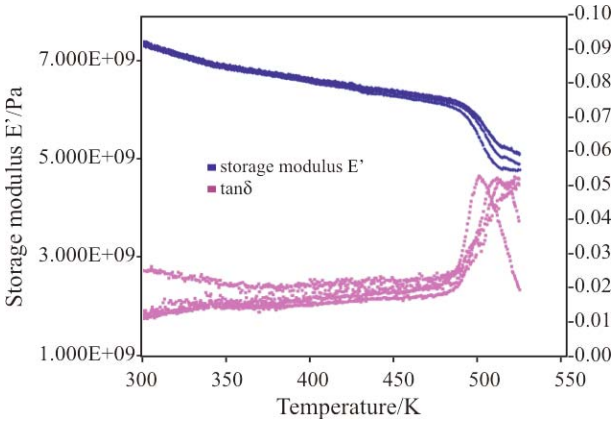

Fig. 2 DMA run on lysozyme in a material pocket. The T_g was found to be 485.6 K for 1 Hz by the drop in *E*' and 293.2 by the peak of the tanδ. The shift to higher temperatures as frequency increases is clearly seen

Table 1 Results from DSC, TMA and DMA runs

Sample	DSC		TMA		DMA	
	average	range	average	range	average	range
Lysozyme	472.7	0.4	482.1	1.4	484.9	0.9
Ovalbumin	480.8	0.5	480.8	1.2	487.5	0.8
RNA A	456.5	0.4	467.1	1.3	479.4	1.1
Bovine serum albumin	467.9	0.6	476.7	1.5	489.1	0.9
Hydroxyethyl starch	498.0	0.3	513.9	1.4	515.1	1.2
Lysozyme–sucrose (20:100)	325.1	0.8	328.0	1.0	331.7	0.9
Sucrose–PVP	361.7	0.3	363.5	1.1	369.1	0.7
Lysozyme–trehalose (20:150)	332.2	0.5	397.2	2.1	340.4	0.9
(phase separated)	394.4	0.9			399.5	1.1

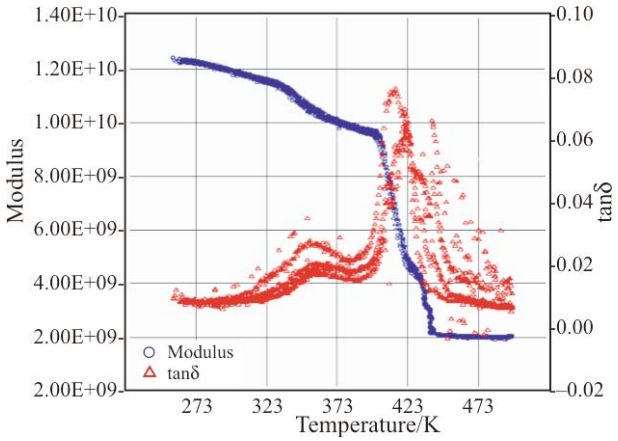

Fig. 3 Phase separation seen in the DMA. Phase separation appears as the two T_g seen here, corresponding to a sugar rich and protein rich phase, respectively. Depending on the degree of separation, more T_gs may also be present

change, expansion, and loss of thermomechanical rigidity at the glass transition may not perfectly coincide [22–24]. The change is the T_g value with frequency is expected and for the sample in Fig. 2 is approximately 9 K per decade. This is in the range expected for the glass transition [20, 22].

On problem encountered in lyophilization is the separation of the material into sugar rich and protein rich phases. In a production environment, this means the batch is un-useable and one of the goals of formulation development is to develop formulations and drying cycles where this does not occur. Some of the protein-excipient mixtures were found to be phase separated in this work. The phase separation was detected in both the HRR DSC runs as well as the DMA studies. An example is seen in Fig. 3 for a lysozyme–trehalose mixture. Interestingly, the TMA did not detect the phase separation for these materials. It may be the volumetric change of the phase separated material was small enough in this small as to be undetected. A sample with a greater amount of phase separation may be detectable and more work would be required to confirm this. The greater sensitivity of the DMA to phase separation makes it a more useful tool for this application.

Conclusions

Results indicate that DMA is a useable technique for determining T_g values in these materials and that it may actually be more sensitive to phase separations than DSC. The increased sensitivity of the TMA over DSC may not be as useful as it appears to be unable to detect the phase separation as easily as does DSC or DMA.

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