

# The Impact of Thermal Treatment on the Stability of Freeze-Dried Amorphous Pharmaceuticals: II. Aggregation in an IgG1 Fusion Protein

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**ABSTRACT:** The objective of this research was to investigate the impact of thermal treatment on storage stability of an IgG1 fusion protein. IgG1 protein formulations were prepared by freeze-drying the protein with sucrose. Some samples were used as controls, and others were subjected to a further heat treatment (annealing). The protein structure was investigated with Fourier transform infrared spectroscopy (FTIR), and protein aggregation was monitored with size exclusion HPLC. Enthalpy recovery was studied using DSC, and global mobility represented by the structural relaxation time constant ( $\tau^*$ ) was characterized by a thermal activity monitor (TAM). The local mobility of the protein system was monitored by both <sup>13</sup>C solid-state NMR and neutron backscattering. Annealing increased the storage stability of the protein, as shown by the smaller aggregation rate and less total aggregation at the end of a storage period. The structural relaxation time constant of an annealed sample was significantly higher than the unannealed control sample, suggesting a decrease in global mobility of the protein system upon annealing. However, annealing does not significantly impact the protein secondary structure or the local mobility. Given the similar protein native structure and specific surface area, the improved stability upon annealing is mainly a result of reduced global molecular mobility. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 93:683–700, 2010

**Keywords:** annealing; physical aging; freeze drying; lyophilization; molecular mobility; global dynamics ( $\alpha$  relaxation); local dynamics; enthalpy relaxation; pre- $T_g$ ; endotherm; enthalpy recovery; protein structure; pharmaceutical stability; protein aggregation; IgG1 protein

## INTRODUCTION

Stabilization of protein therapeutics is important for efficacious treatments for human diseases and conditions. One of the most effective methods for stability improvement is to freeze dry these biologicals with a stabilizer such as sucrose or trehalose,<sup>1,2</sup> and the obtained products may have a shelf life of 18–24 months, which is typically required for economic viability.<sup>3,4</sup> However, the cold chain requirement of storage condition may

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limit the availability of biopharmaceuticals to developing countries. Also the bioshield project (<http://www.whitehouse.gov/infocus/bioshield/>) proposes a longer shelf life (~10 years) for "next-generation" drugs including vaccines. In order to meet these requirements, strategies other than the traditional formulation methods are desired to further improve the stability of biopharmaceuticals.

Recently, it was reported that the stability of amorphous solids is also dependent on the process used to prepare the material, with "thermal history" being one critical factor.<sup>6-7</sup> Thus, the drying processes with different thermal histories may impact the stability of pharmaceuticals.<sup>8,9</sup> Glass dynamics have been proposed as an important factor in the stabilization effect of amorphous excipients on proteins during drying and storage.<sup>10,11</sup> This mechanism states that the excipient forms a rigid matrix in which the protein is molecularly dispersed, and the limited mobility in the high viscosity glass slows down the protein mobility which is necessary for protein degradation. According to the Stokes-Einstein equation, the diffusion coefficient in supercooled liquid is inversely proportional to the viscosity of the system.<sup>12</sup> Structural relaxation time, a measure of molecular mobility, is also related to the diffusion coefficient.<sup>11,12</sup> Thus, chemical reactions that require diffusion can have a dependence on molecular mobility or structural relaxation time of the system.

DEU and other calorimetry studies suggest that a correlation does exist between chemical instability and the structural relaxation time of the amorphous system.<sup>13-15</sup> For example, Duddu et al.<sup>17</sup> reported the correlation of aggregation of an IgG1 antibody with the reduced time ( $t^*$ ) below  $T_g$ . Shamblin et al.<sup>18</sup> reported strong coupling between the dimerization rate of ethacrynate sodium (ECA) and structural relaxation time in formulations cryophilized with sucrose, trehalose, and PVP. Recently, it was found that there is a correlation between the rate of degradation and structural relaxation time in human growth hormone (hGH) protein systems (lyophilized with several sugars.<sup>19</sup> However, it is also noticed that there are reports in pharmaceutical literature that provide examples of a poor correlation. The contribution of molecular mobility was found to be small in insulin degradation in trehalose formulations under high humidity conditions,<sup>20</sup> as well as with PVP.<sup>21</sup> Therefore, while there are numerous examples of a correla-

tion between structural relaxation and reactivity, the correlations may not always be strong.

Thermal treatment of dried glass results in an increase in the structural relaxation time (or decrease in global mobility). A glass is in a non-equilibrium state and it exhibits higher enthalpy, free energy, and entropy than the corresponding equilibrium supercooled liquid. Due to its higher energy, a glass will relax toward the equilibrium curve in an experimentally accessible timescale if there is sufficient mobility. The process of relaxation toward equilibrium is known as physical aging or annealing.<sup>22</sup> In this article, we use "annealing" to describe a process wherein an amorphous material is kept at a temperature below  $T_g$  for a period of time sufficient to allow significant relaxation toward the equilibrium state. During the annealing period, some physical and mechanical properties of amorphous solid will change with time. For example, the time-dependent changes in glassy polymers upon annealing include increase in density, yield stress, and decreases in stress relaxation rate, free volume, and enthalpy.<sup>23,24</sup> For our interests, the primary effect of annealing of amorphous glass is the increase in structural relaxation times and thus reduction in global mobility.<sup>8,22,25</sup>

If chemical/physical stability and structural relaxation time are coupled in a glass, then a sample with a larger structural relaxation time resulting from annealing should have better stability. That is, stabilization against pharmaceutical degradation could be achieved by annealing for a short period of time. There are several studies supporting the concept of "stabilization by annealing" in both the food and the pharmaceutical literature. The first demonstrated example of stabilization of pharmaceuticals by annealing involved an antibacterial (moxalactam disodium) formulation cryophilized with 12% (w/w) mannitol.<sup>26</sup> Drying of the antibacterial system (with a  $T_g$  about 121°C) using 80°C instead of 40°C during the secondary drying process gave samples of essentially identical residual moisture but the higher temperature drying protocol gave a systematic improvement in storage stability that averages about 20%. This system was further investigated, and the stabilization effect was correlated to the reduced molecular mobility.<sup>25</sup> Hill et al.<sup>6</sup> studied the annealing effect in food systems, and the rate of Maillard reaction between lysine and glucose was found to be about 20% slower in the aged glassy matrix after aging for 8 weeks at a temperature of 30°C below  $T_g$ .

Luthro et al.<sup>25</sup> reported that rate of cyclization degradation reaction in aspartame/disaccharide glass decreases significantly when the freeze-dried sample was heat treated postlyophilization in an oven. Recently, it was found that annealing resulted in the decrease in dimerization rate of ECA, and thus annealing can also stabilize intermolecular degradation reactions that involves mobility on longer length scales such as dimerization processes.<sup>27</sup> While these observations show that thermal treatment may be used to increase the long-term stability, evidence for this stabilization effect on amorphous pharmaceuticals is still limited. Furthermore, these annealing studies only focused on small molecule pharmaceuticals, and the consequence of annealing on delicate large protein molecules is unknown. Given the desire to improve the stability of biologics, it will be very important to determine if protein biopharmaceuticals can also be stabilized by annealing.

In this article, the impact of annealing on the stability of an IgG1 fusion protein is investigated. Previous studies showed that the stabilization effect from sugar is dependent on the disaccharide level, and a formulation with higher sucrose level generally exhibited much better storage stability.<sup>19,28,29</sup> However, a protein formulation with higher sugar level is not always practical due to limits imposed by the disease state (such as diabetes) or manufacturing constraints (such as long lyophilization cycle due to high solid content associated with high sucrose level). Here, low sugar content formulations would be preferred if sufficient stability can be obtained, for example, by annealing. We have chosen two formulations with low-to-moderate sugar level (sucrose/IgG1 protein mass ratio of 0.5 and 1) in this study and have conducted an extensive investigation of the impact of annealing on selected physical properties, protein structure in the solid, molecular mobility, and aggregation stability.

## EXPERIMENTAL

### Materials

A recombinant humanized IgG1 fusion protein with a molecular weight of 185 kDa was provided by Wyeth Biopharma (Andover, MA). The protein drug substance was ultrafiltered through a regenerated cellulose membrane filter (Billerica, MA, area of 50 cm<sup>2</sup>, 30 kDa molecular weight cutoff

value).<sup>1</sup> The *L*-histidine, sodium chloride, poly-L-lysine, 2-hydroxypropyl- $\beta$ -cyclodextrin, pluronic F68 (a hydrophilic nonionic surfactant), polysorbate-80, and glycine were purchased from Sigma (St. Louis, MO).  $\alpha$ -Sucrose with reduced heavy metal (<5 ppm) content was purchased from Calbiochem of EMD Biosciences (San Diego, CA). These reagents were of the highest grade available and were used without further purification.

### Lyophilization Procedures

Ten milligrams per milliliter IgG1 protein solutions were prepared after dialysis against two buffer solutions: one buffer was 1 mM histidine buffer (pH 6.5), and another was 1 mM histidine buffer and 40 mg/mL of sucrose (pH 6.5). By mixing two stock solutions at different ratios, two protein formulations with sucrose/IgG1 protein mass ratios of 0.5 and 1 were prepared. The formulation with sucrose/IgG1 protein mass ratios of 0.5 and 1 is defined as P10S5 and P10S10, respectively, where P represents the protein, S represents sucrose, and the number is the concentration (mg/mL) of the component in the formulation.

Protein solutions (1 mL) were filled into 2 mL tubing glass vials and were lyophilized in a Genesis 25 EL (SP Industries, Gardiner, NY) freeze-dryer. The samples were first dried at a shelf temperature of -25°C with a chamber pressure of 6.6 Pa (50 mT) for 30 h, and then the samples were further dried at about 4 Pa (30 mT) with a shelf temperature of -25°C for 35 h. Primary drying was conducted below the glass transition temperature of freeze-concentrate ( $T_g$ ) of both formulations. Secondary drying was carried out for 6 h at 25°C. After the drying process was finished, vials were stoppered under dry nitrogen with Daikyo Flurotec stoppers (Weet Pharmaceutical, Latitz, PA) and capped with aluminum seals. All the samples were amorphous after freeze-drying as evidenced by the absence of birefringence with polarized light microscopy (PLM; Linkam, McCrone, Chicago, IL).

### Residual Water Measurement

The water content for each formulation after freeze-drying was determined using Mettler

<sup>1</sup> Certain commercial equipment and materials are identified to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified is necessarily the best available for this purpose.

Karl Fischer coulometer (Riverview, FL). At least two vials of each sample were used. Freeze-dried sample vials were reconstituted in 2 mL dried methanol and 0.5 mL of the solution was injected into the titrator. Blank corrections were applied. Standard deviation from replicate measurements was less than 0.2% (w/w).

#### Specific Surface Area Measurement

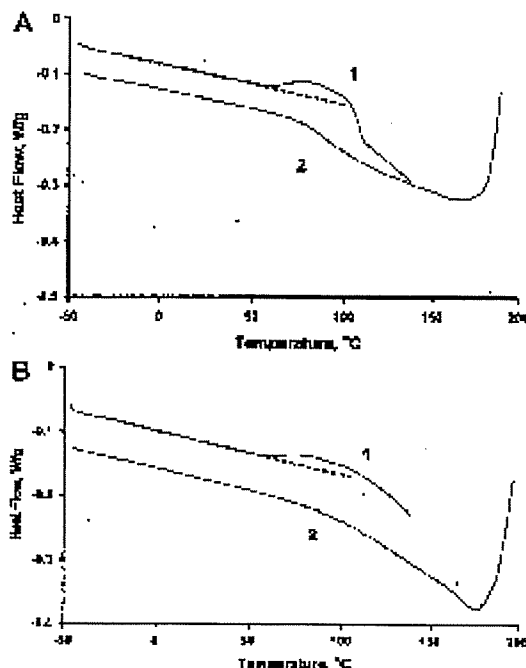
Specific surface area (SSA) analysis was performed using a FlowSorb II 2300 surface area analyzer (Micromeritics, Norcross, GA). A protein sample of about 100 mg was loaded into the sample holder in the glove bag with controlled humidity (<2%). Single point calibration was performed prior to taking surface area measurements using 1 mL Krypton gas. Powder samples were degassed for at least 3 h at 35°C in the Flow Prep oven, and surface areas were measured using krypton (Kr) as an adsorbate and helium as carrier.

#### Differential Scanning Calorimeter (DSC) Measurements

Modulated DSC (Q1000; TA Instruments, New Castle, DE) was used to measure the glass transition temperature ( $T_g$ ) and the change in heat capacity at  $T_g$  ( $\Delta C_p$ ) of protein samples. The samples were run at a 2°C/min heating rate, modulated with a  $\pm 1^\circ\text{C}$  amplitude every 120 s. Fresh protein samples with sucrose/protein mass ratios of 0.5 and 1 give  $T_g$  of 106 and 101°C (mid-points), respectively.

Enthalpy recovery ( $\Delta H$ ) of the annealed sample was measured using standard (nonmodulated) DSC following the previously described procedure.<sup>27</sup> The scan rate used was 10°C/min. Briefly, the heating curve of the first DSC scan was used to obtain the area of the endothermic recovery peak by integration using Universal Analysis Software (TA Instruments). The thermal history of the annealed sample was then erased by holding the sample at a temperature above  $T_g$ , the sample was cooled, and a second DSC curve was obtained, representing zero annealing time. The second scan curve is given in Figure 1. We define the enthalpy recovery ( $\Delta H$ ) as the enthalpy difference between the annealed sample and the sample with erased thermal history.

The relaxation enthalpy at infinite time ( $\Delta H_\infty$ ) at a given annealing temperature ( $T_a$ ) was



**Figure 1.** DSC of a fresh IgG/sucrose sample showing the exothermic pre- $T_g$  peak in the P10S10 formulation (A) and P10S5 formulation (B). The dashed line in the first scan (curve 1) is added to guide the eye to differentiate the pre- $T_g$  event. The second scan after eliminating the thermal history (curve 2) showed the disappearance of pre- $T_g$  event. The lines were offset for clarity.

calculated using the following equation:<sup>30,31</sup>

$$\Delta H_\infty = (T_g - T_c) \times \Delta C_p \quad (1)$$

A relaxation function ( $\Phi$ ) served as a measure of the extent of enthalpy relaxation during annealing and was calculated as follows:<sup>31–35</sup>

$$\Phi = 1 - \frac{\Delta H}{\Delta H_\infty} \quad (2)$$

where  $\Delta H$  is the enthalpy recovery measured from DSC and  $\Delta H_\infty$  is the corresponding value at time infinity as obtained from Eq. (1).

#### Structural Relaxation Time Measurement by Thermal Activity Monitor

Isothermal microcalorimetry was used to measure the rate of enthalpy relaxation of the sample at the storage temperature (50°C) using a Thermometric, Z277 Thermal Activity Monitor (Thermo-

metric, Jarfalla, Sweden). The experimental procedure and data analysis using Kohlrausch-Williams-Watts (KWW) curve fitting is similar to that described in a previous report.<sup>37</sup>

#### Studies of Protein Aggregation by Size Exclusion Chromatogram

Assay for chemical degradation was not routinely performed because the protein samples did not show sufficient chemical degradation to allow a quantitative analysis of chemical instability. Size exclusion chromatography (SE-HPLC) was used to assess the physical aggregation of the protein during storage. The initial SE-HPLC assays were run on both fresh samples (nonannealed) and on samples exposed to different annealing conditions. The rest of the sample vials were then transferred to an oven at 60°C. After storing for preset times (0.5, 1, 3, and 8 months), the samples were reconstituted with 1 mL of purified water. All solutions obtained were visually clear after reconstitution, and the soluble protein aggregate was analyzed with HPLC.

A column of YMC DL30S05-3008WT (Waters, Milford, MA) was used with a flow rate of 1 mL/min. The mobile phase consisted of 0.03 mol/L  $\text{NaH}_2\text{PO}_4$  and 0.2 mol/L NaCl (pH 7.2). UV absorption was monitored at 280 nm. The dimer eluted at about 8.2 min and the monomer peak appeared at about 6.8 min. The standard error in the percentage of aggregate for all sample replicates was <0.5%.

To evaluate the effect of variable diluent composition on the measured aggregation, reconstitution of lyophilized protein formulations was also carried out using reconstitution media containing 0.5% (w/w) additives such as phosphate buffer, dextran sulfate, 2-hydroxypropyl- $\beta$ -cyclodextrin, pluronic F-68, and polysorbate-80. Then, the amount of aggregate in the reconstituted sample was measured by SE-HPLC analysis.

#### Protein Structure Characterization by FTIR

Fourier transform infrared spectroscopy (FTIR) studies were conducted using a Nicolet Magna-IR 560 spectrometer (Thermo Electron, Madison, WI). The study was carried out using transmission mode on the freeze-dried protein solids according to procedures in the literature.<sup>36,35</sup> A total of 128 scans and 4  $\text{cm}^{-1}$  resolution were used for each spectrum. About 2 mg of solid protein sample was mixed with 200 mg dried KBr powder, and the

mixtures were then pressed with a Carver press at 11,000 psi for about 2 min. The obtained transparent pellet was put into the sample holder and then loaded into a dried air-purged measurement chamber. The protein spectrum was collected and the absorbance signal was obtained using the corresponding background. Second derivative spectra were then obtained using an OMNIC software. The second derivative of the amide I region (1700–1600  $\text{cm}^{-1}$ ) of the spectra was baseline corrected and area normalized. The peak height of the major band was used to compare the similarity between spectra.

#### Fast Local Dynamics from a Neutron Backscattering Spectrometer

The fast local dynamics with timescales of nanoseconds was studied with a high flux backscattering (HFBS) spectrometer at the Center for Neutron Research on the NG2 beam line at the National Institute of Standards and Technology (NIST).<sup>38</sup> Only those motions with timescales shorter than 5 ns can be measured with this instrument.<sup>37</sup> The details of the experimental procedure and data analysis have been published elsewhere.<sup>37,39,38</sup> A larger mean square of displacement of H-atom ( $\text{msd}$ ,  $\langle u^2 \rangle$ ) means a higher local mobility of the system.

#### Determination of $T_{1\rho}$ of Protein and Sucrose by $^{13}\text{C}$ Solid-State NMR

The rotating-frame spin-lattice relaxation time ( $T_{1\rho}$ ) of the IgG1 carbonyl carbon in lyophilized protein/sucrose formulations was determined at 25°C using a UNITY plus spectrometer (Varian, Inc., Palo Alto, CA) operating at a proton resonance frequency of 400 MHz. Spin-locking field was equivalent to 19 kHz. The rotor size was 7 mm and spinning speed was 4 kHz. Peak height at about 180 ppm due to IgG1 carbonyl carbon and 80 ppm from sucrose were followed with different delay times.<sup>40</sup> Similar measurement of  $T_{1\rho}$  was performed for annealed protein samples. The data were fit to a biexponential decay model to determine the two  $T_{1\rho}$  as reported elsewhere for freeze-dried systems.<sup>40,41</sup> Since the shorter  $T_{1\rho}$  value was the same for all samples, the longer  $T_{1\rho}$  values were used for comparison between samples annealed at different conditions.

## RESULTS AND DISCUSSIONS

## Impact of Annealing on Physical Properties of IgG1 Protein Samples

Annealing appeared to have no impact on the physical form or the SSA of the protein samples we studied. The fresh unannealed and annealed samples were confirmed to be amorphous by PLM. Even upon storage at 60°C for 3 months, we observed no crystallization of sucrose by PLM. Sample SSA was also unaffected by annealing. For the fresh unannealed sample, the SSA was approximately  $1.7 \pm 0.2 \text{ m}^2/\text{g}$ , which is similar to that reported for other protein pharmaceuticals.<sup>6,48</sup> After annealing at 70°C for 20 h, the most aggressive annealing protocol we used, the SSA of the aged sample was essentially unchanged at  $1.9 \pm 0.1 \text{ m}^2/\text{g}$ . This result was consistent with previous reports that aging did not lead to perceptible changes in morphology or particle size in a trehalose system.<sup>43</sup>

The impact of annealing on residual moisture of the protein sample was determined with Karl Fischer titration (Tab. 1). The fresh protein sample immediately after freeze drying showed low moisture level (about 0.5%, w/w). Upon annealing of protein samples at high temperatures for 10 or 20 h, the residual water increased by 0.5–1%. In addition, upon storage at 60°C for 3 months, all samples, fresh and annealed, showed a moisture level of about 2% (w/w). The increase in the moisture upon annealing or storage is believed due to the transfer of moisture from the stopper to the dried cake.<sup>44–46</sup> Even though there are changes in moisture during annealing, these small changes are not expected to significantly impact enthalpy relaxation and stability. In

similar protein systems, changes in moisture from 0% to 3% showed only very slight changes in stability.<sup>47</sup> Also in previous peptide/sugar and ECA/sugar systems where there were large total sample amounts in a vial, no obvious changes in moisture were observed upon annealing, even though annealing caused significant changes in enthalpy relaxation.<sup>26,27</sup> In short, there is significant precedent for annealing causing increases in relaxation time and stabilization, even if not in protein systems, so the results obtained in this study are consistent with previous results. In addition, we note that the structural relaxation time increases sharply upon annealing as shown below from the TAM experiments, so the water plasticization effect on global molecular mobility is obviously overwhelmed by the annealing effect itself, so the impact of water content variations such as those shown in Table 1 on relaxation dynamics and stability is not expected to be significant.

DSC Investigation of Pre- $T_g$  Event and Enthalpy Recovery

Pre- $T_g$  exothermic events have been reported for freshly freeze-dried glasses,<sup>27,48–50</sup> and here we investigated this phenomenon by DSC. The IgG1/sucrose formulations were scanned from about -50°C to a temperature above  $T_g$ . Figure 1 shows the DSC curves of fresh P10S10 ( $T_g$  of 101°C) and P10S5 ( $T_g$  of 106°C) formulations, which were dried for 6 h at 25°C in secondary drying. Notice that pre- $T_g$  exothermic peaks were observed at about 60°C in both protein formulations in the first scan. To investigate the source of the pre- $T_g$  peak in the fresh sample, the DSC sample was kept at a temperature of about 30°C above  $T_g$  for 5 min to erase the thermal history of the glass, and then cooled down to -50°C and rescanned. The second scan showed a typical glass transition, with a clear absence of a pre- $T_g$  exothermic peak in both formulations.<sup>48,50</sup> This suggests that the pre- $T_g$  event in the freshly lyophilized sample is a result of the thermal history it experienced during the normal freeze-drying cycle.

The pre- $T_g$  event in annealed protein samples was also investigated using DSC. The impact of annealing on DSC behaviors of P10S10 and P10S5 is shown in Figure 2. Upon annealing at low temperature, the pre- $T_g$  exothermic peak converted to pre- $T_g$  endothermic peak for both samples. Increasing the annealing temperature resulted

Table 1. Percent Residual Water Content (%) in P10S6 Sample upon Annealing at Different Conditions

Sample	Initial Sample (%)	Stored at 60°C for 3 Months
Fresh	$0.5 \pm 0.06$	$2.0 \pm 0.2$
An60d10h	$1.0 \pm 0.06$	$2.4 \pm 0.2$
An60d20h	$1.0 \pm 0.21$	n/d
An70d10h	$1.4 \pm 0.06$	$2.3 \pm 0.03$
An70d20h	$1.6 \pm 0.32$	n/d
An80d10h	$1.4 \pm 0.07$	$2.4 \pm 0.10$

n/d, not determined.

Standard error shown is based on three replicates. The term "An60d10h" means that the sample was annealed at 60°C for 10 h.

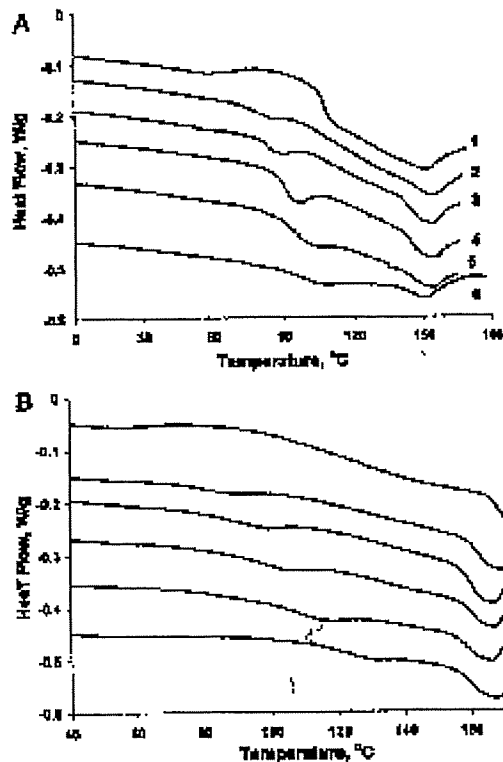


Figure 2. DSC curves of unannealed fresh IgG1/sucrose sample (curve 1) and samples annealed for 20 h at different temperatures: (A) P10S10 formulation annealed at 60°C (curve 2), 65°C (curve 3), 75°C (curve 4), 85°C (curve 5), and 95°C (curve 6); (B) P10S5 formulations annealed at 60°C (curve 2), 70°C (curve 3), 80°C (curve 4), 90°C (curve 5), and 105°C (curve 6). The endothermic thermal events around 150°C (P10S10) and 170°C (P10S5) are likely denaturation endotherms while the thermal events at lower temperatures represent  $T_g$  and relaxation events.

in the shift of the endothermic peak to a higher temperature, and finally the peak appeared as a  $T_g$  overshoot. The physical basis of the pre- $T_g$  events is not well understood even though these events have been reported elsewhere.<sup>47,48,51-53</sup> Our studies show that annealing significantly impacts pre- $T_g$  events, but as discussed below, we observe no impact of annealing on  $\beta$  relaxation, either as measured by neutron scattering or by NMR. Thus, it seems that pre- $T_g$  event is not a result of  $\beta$  relaxation motions. Presumably these

peaks are associated with a very broad distribution of the structural relaxing populations.<sup>4,48,50-52,54</sup> It is possible that some relaxing species may have relaxation times shorter than the annealing time at the annealing temperature, and thus they have relaxed during the annealing step.<sup>46</sup> Upon reheating the sample during the DSC scan, these fast relaxing species may regain the lost energy which then results in the formation of a pre- $T_g$  endotherm.<sup>27</sup>

The enthalpy relaxation upon annealing was studied as a function of annealing time and temperature. For relaxation studies using DSC, it is assumed that the enthalpy relaxation that occurred during the annealing process is identical to the enthalpy recovery measured during the heating scan in DSC. Here, enthalpy recovery was determined by integration of the endothermic peak with a sigmoidal baseline feature, as discussed in the Experimental Section. Figure 3 shows the impact of annealing temperature on the enthalpy recovery of IgG1/sucrose formulations. The magnitude of enthalpy recovery increased upon increasing the aging temperature initially. However, a maximum enthalpy recovery was observed at about 65°C for the P10S10 formulation, and further increases in annealing temperature produced a decrease in the enthalpy recovery. Similarly, a maximum enthalpy recovery is observed at about 90°C for the P10S5 formulation. Thus, both formulations give a maximum

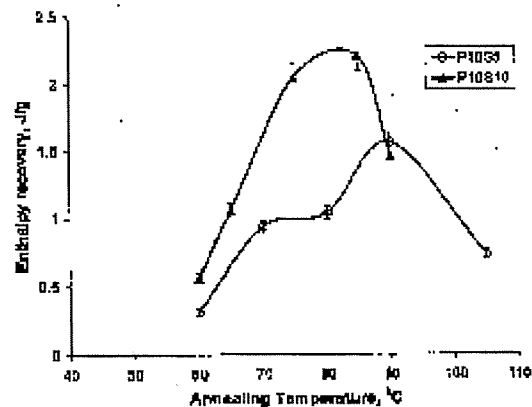


Figure 3. Impact of annealing temperature on the enthalpy recovery of IgG1/sucrose formulations after annealing for 20 h. Note the enthalpy recovery reaches a maximum for both protein formulations at about 15°C below  $T_g$ . Error bars are standard errors for 2–3 replicates.

enthalpy recovery at about 15°C below their  $T_g$ , which is expected based on previous studies.<sup>27,48</sup>

The impact of annealing time on enthalpy recovery was also investigated. The enthalpy recovery was found to be proportional to the log of annealing time (results not shown), which is consistent with previous report on small pharmaceutical molecules.<sup>26,27</sup> The logarithmic dependence of enthalpy on annealing time means that the enthalpy relaxes much more rapidly in the short time range than at longer times. Density studies from this laboratory (unpublished) on the pure trehalose system also showed that the density increase (or free volume decrease) is most pronounced early in the annealing process (for about 5–10 h). Thus, for the application of annealing to lyophilization cycle development, the annealing time has been selected to be 10 or 20 h in our study.

Figure 3 shows that P10S10 give larger enthalpy recovery than P10S5 upon annealing at the same conditions (60°C for 20 h). Also the P10S10 sample annealed at 75°C exhibited higher enthalpy recovery than the P10S5 sample annealed at 60°C for the same periods of time. Therefore, it seems that the protein formulation with higher sucrose fraction shows higher enthalpy recovery than the protein formulation with lower sucrose level. This observation is a direct result of Eq. (1) and the larger  $\Delta C_p$  for a system richer in sucrose. As a strong glass, a pure protein exhibits a very broad  $T_g$  with a very small heat capacity change,  $\Delta C_p$ , which makes it difficult or impossible to measure the  $T_g$  of pure proteins with DSC.<sup>50</sup> In contrast, sucrose is a fragile glass and exhibits large  $\Delta C_p$  at  $T_g$ , and thus the enthalpy relaxation in sucrose is much larger than in a protein, according to Eq. (1).

#### Investigation of the Impact of Annealing on the Storage Stability of IgG1

In order to determine if the reconstitution process will impact the measured aggregation, the effect of reconstitution media on the amount of protein aggregate measured was studied. Table 2 shows the aggregate level in the IgG1 protein formulation upon reconstitution in fresh and annealed samples. We note that the addition of surfactant or polyanions did not significantly impact the amount of aggregation measured. Similar observations have been made for some other proteins.<sup>39,40</sup> Thus, for the systems studied in this research, the reconstitution process did not significantly impact aggregate formation. We thus conclude that stability differences of the IgG1 protein upon annealing are mainly the result of the bimolecular aggregation events that actually occur in the solid-state during storage. That is, development of aggregation that correlates with storage time and conditions could reflect a bimolecular aggregation event that occurs during storage or could reflect time-dependent protein structural alteration during storage, with the actual aggregation event occurring mostly during reconstitution when molecular mobility becomes very high. For our systems, the role of aggregation during reconstitution is, at best, minimal.

The time dependence of IgG1 aggregation upon storage at 60°C was studied for both protein formulations. As shown in Figure 4A, the fresh sample of the P10S10 formulation showed less degradation than the annealed sample at time 0, as expected because some degradation will occur during annealing. However, upon storage for about 1 month, these samples showed almost identical aggregate level, and further storage for

**Table 2.** Effects of Reconstitution Medium on the Amount of Protein Aggregation Measured in the P10S6 Formulation upon Lyophilization and Storage at 60°C

Reconstitution Medium	Immediately After Freeze Drying	Anneal at 60°C for 7 Days
Water	5.5 (0.32)	6.8 (0.17)
Phosphate buffer	5.6 (0.27)	6.0 (0.14)
Dextran sulfate	5.8 (0.14)	6.1 (0.18)
2-hydroxypropyl- $\beta$ -cyclodextrin	5.8 (0.16)	5.9 (0.04)
Pluronic F-68	5.5 (0.18)	5.8 (0.12)
Tween-80	5.3 (0.20)	5.7 (0.11)

0% additives unless specified otherwise

Values in brackets represent standard deviation ( $n = 6$ ).



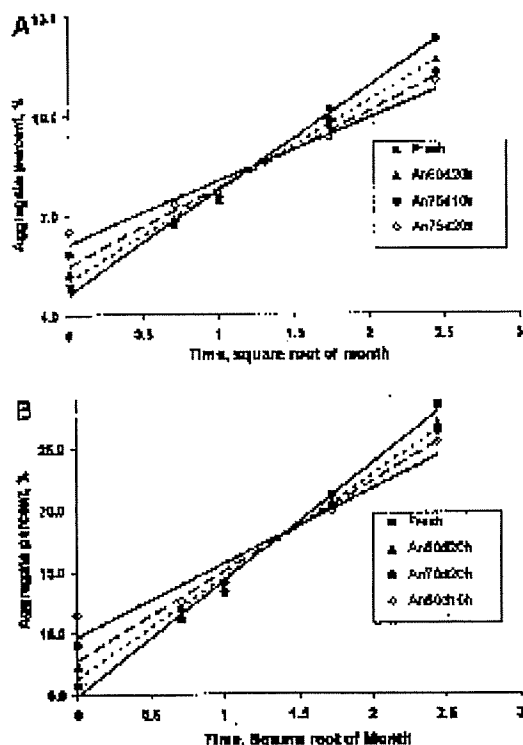


Figure 4. Time dependences of IgG1 aggregation upon storage at 50°C for P10S10 (A) and P10S5 formulation (B). Note the linear relationship between the protein aggregation and square root of time in the x-axis. The correlation coefficients for P10S10 are 0.996, 0.991, 0.987, and 0.968 with increasing annealing temperature. The regression coefficient for P10S5 is 0.993, 0.989, 0.979, and 0.937 with increasing annealing temperature. The slope of the best-fit straight line is used as the degradation rate constant. The error bars are about the size of the symbol.

longer times resulted in the annealed sample having less aggregation. That is, the annealed sample not only showed a lower degradation rate during storage (i.e., smaller slope), but in spite of the degradation suffered during annealing, actually exhibited less total degradation at the end of storage than the fresh sample. These data (Fig. 4A) are an excellent example of large differences in stability that can result from annealing. Similar to the P10S10 formulation, annealing also stabilizes the protein-rich P10S5 formulation as shown in Figure 4B. Again, annealed samples exhibited lower total aggregate

levels after storage for more than about 2 months. Thus, our postulate that protein systems can be stabilized by annealing is valid for both IgG1 formulations.

The protein aggregation kinetics in freeze-dried solids were evaluated and these stability data were found to fit square root of time model best, as measured by a high correlation coefficient.<sup>27</sup> The correlation coefficients, as shown in Figure 4, are still relatively high enough even though they do seem to decrease upon increasing annealing temperature. Since the fit to square root of time model was better than the first-order kinetics even for the annealed samples, the square root of kinetics was used throughout the article for consistency when comparing the storage stability of different samples. This square root of time degradation kinetics is commonly observed in glassy materials.<sup>18,26-27,31,33</sup> Therefore, the aggregation rate constants were obtained as the slope of the straight line in Figure 4. Table 3 shows the aggregation rate constants and initial aggregate levels of IgG1 protein at 50°C upon annealing at different conditions. The annealed P10S10 formulation had only slightly higher initial degradation in the annealed samples relative to the unannealed system (with initial aggregate range from 4.8% in fresh to 6.5% maximum in annealed), which is due to degradation occurring during the thermal treatment. In contrast, the P10S5 formulation gives a relatively large change in initial aggregate upon annealing at high temperatures. Also, the P10S5 formulation gave a higher rate of degradation as compared to the P10S10 formulation with the same thermal history. The better stability in P10S10 than P10S5 is consistent with previous findings that a higher amount of sucrose in protein formulations can afford better protection to the protein in the glassy solid.<sup>19,20,32</sup> It was found that P10S10 formulation showed lower molecular mobility, lower free volume, and lower degree of native structure perturbation than P10S5,<sup>30</sup> all of which are consistent with the better stability in P10S10 than P10S5 upon annealing and storage.

The general trend in the physical stability data shows that as annealing temperature increased, long-term stability improved (i.e., the rate constant decreased). Therefore, annealing below  $T_g$  can slow down the aggregation rate of the protein. In spite of increased initial aggregation after the annealing process, annealed samples actually showed less aggregation after storage for 3 and 6 months (see Fig. 4). For P10S10, the control

**Table 3.** Initial Aggregation Level and Aggregation Rate Constants of IgG1 Protein Sucrose Formulations at 50°C upon Annealing at Different Conditions

Annealing Temperature (°C)	Annealing Time (h)	Initial % Aggregation	k at 50°C
P10 S10 control (fresh)		4.8 ± 0.1	3.1 ± 0.1
60	10	5.1 ± 0.1	2.9 ± 0.1
60	20	6.3 ± 0.2	2.7 ± 0.1
65	10	5.4 ± 0.3	2.8 ± 0.2
65	20	6.6 ± 0.4	2.6 ± 0.2
75	10	5.8 ± 0.3	2.9 ± 0.2
75	20	6.6 ± 0.3	1.9 ± 0.2
P10 S5 control (fresh)		6.8 ± 0.2	9.4 ± 0.6
60	10	6.9 ± 0.3	5.8 ± 0.6
60	20	7.4 ± 0.3	8.2 ± 0.6
70	10	7.9 ± 0.4	7.6 ± 0.4
70	20	9.1 ± 0.3	7.2 ± 0.6
80	10	11.5 ± 0.3	8.0 ± 0.8
80	20	13.3 ± 0.4	5.6 ± 0.6

Uncertainties given for the rate constants are standard errors as provided by the regression analysis based on the square root of time kinetics.

(fresh) sample and sample annealed at 75°C for 20 h give degradation rate constants of 3.1 and 1.9, respectively. Thus, annealing results in a decrease in degradation rate constant of about 40% for IgG1 protein. Also the P10S5 sample annealed at 80°C gives similar improvement in stability. These data (Tab. 3 and Fig. 4) clearly demonstrate that annealing improves long-term stability of the IgG1 protein.

In order to further address the question "Does annealing significantly impact aggregation of IgG1 protein?" a statistical test using the general linear model (GLM) procedure was performed on the rate constant data to determine the effect of both annealing temperature and time. The GLM results for P10S5 data showed a significant contribution of both annealing time ( $p = 0.058$ ) and annealing temperature ( $p < 0.001$ ) on the differences observed in aggregation rate between control (fresh) and annealed samples. Similarly, annealing significantly improved the stability of the P10S10 sample. Therefore, both annealing time and temperature significantly impact the stability.<sup>27</sup> It should be pointed out that initial degradation level may need to be considered to address product quality in a broader sense, as, for example, if there is potential toxicity and/or immunogenicity due to the degradation product produced during annealing. In the current study, the initial aggregate level upon annealing is

relatively high due to the protein drug substance, formulations, and annealing conditions used, but it should be emphasized that the total aggregate level of the annealed sample is less than that for the nonannealed sample after about 2 months into the stability study. Moreover, the amount of degradation during annealing is protein, formulation, and process dependent. For example, our unpublished results on rHSA systems showed that the initial aggregate upon thermal treatment at 65°C for 20 h was still <0.5%, and a similar stabilization effect was observed upon annealing. In this example, the annealing approach is obviously a practical procedure for improving stability.

#### Impact of Annealing on Protein Native Structure

Figure 5 shows the impact of annealing on the protein structure in the solid as determined by area-normalized FTIR spectra of the IgG1 formulations. The spectra are characteristic of IgG1 protein which has a predominance of  $\beta$ -sheet structure at 1641 and 1693  $\text{cm}^{-1}$ . Figure 5 shows the FTIR spectra of P10S10 after annealing at 70°C for different periods of time. After annealing at 70°C for 10 h, the peak height at 1641  $\text{cm}^{-1}$  was essentially the same as the fresh sample. Even a sample annealed at 70°C for up to 40 h showed no

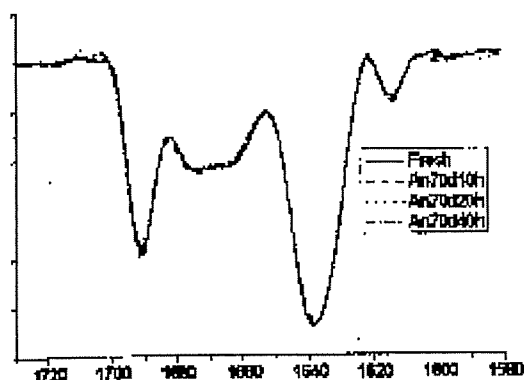


Figure 5. Area-normalized second derivative FTIR spectra of IgG1 P10S10 formulations after annealing at different conditions. Notice that no significant change in protein structure was observed upon high-temperature annealing. The data in the curve represent the average of three repetitions.

obvious change in the amide I band. The same qualitative result has been observed for the P10S5 formulation (data not shown). These results indicate that no significant differences in the secondary structure of the protein are produced upon annealing for either formulation, at least within the sensitivity of the technique. The tertiary structure of the IgG1 upon reconstitution was also studied using fluorescence spectroscopy, and it was found that there was no significant shift in the emission peak maximum for both tyrosine and tryptophan residues upon annealing of P10S10 at 65°C for 10 h (data not shown). Of course, this does not mean changes in tertiary structure did not exist in the solid but simply "refolded" upon reconstitution. Since methodology suitable for characterization of tertiary structure in the glassy is not available, we studied secondary structure by FTIR. As indicated above, no changes in secondary structure upon annealing were observed. These results are consistent with recent observation that thermal denaturation of hGH in a glassy solid occurs only well above  $T_g$ .<sup>55</sup> FTIR, HPLC, and DSC results indicated that brief exposure of hGH to temperatures slightly less than the onset denaturation temperature in dried solid (i.e., below 150°C) did not cause significant damage to the protein.<sup>55</sup> These results suggest that thermal treatment of dry solids at a temperature well below  $T_g$  does not cause significant change of protein native structure.

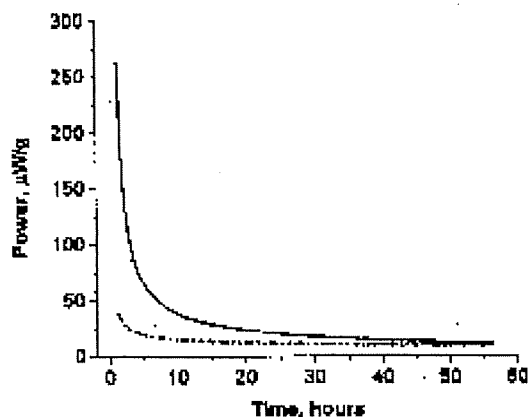


Figure 6. Power-time data at 50°C for a P10S10 sample annealed at 75°C for 10 h (dashed line) and fresh control sample (solid line). Note the higher power in the fresh sample than the annealed sample at same time, which indicates that annealed sample has a much lower molecular mobility.

#### Impact of Annealing on the Global Mobility

The rate of enthalpy loss was monitored using isothermal microcalorimetry. Figure 6 compares the thermal activity versus time curves at 50°C for control and annealed P10S10 samples. The thermal activity of the both samples decreases rapidly in the first 10 h, and then decreases slowly. This relaxation behavior is normally observed and is due to structural heterogeneity in the system.<sup>56,57</sup> It is widely accepted that a glass consists of a collection of "substates" with different sizes and entropies, each relaxing independently at different rates, giving rise to a broad distribution of structural relaxation times.<sup>58,59,60</sup> Also note (Fig. 6) that the annealed protein sample shows a very small heat flow signal compared to the

Table 4. Structural Relaxation Time Constant ( $\tau^2$ ) of P10S10 Sample at 50°C With Different Thermal Histories

Annealing Temperature (°C)	Annealing Time (h)	
	10	20
Fresh control	2.3 ± 0.6	
60	42 ± 3	49 ± 9
65	48 ± 5	41 ± 5
75	45 ± 4	52 ± 6

The  $\tau^2$  value was calculated based on fitting of TAM data to the KWW model. The uncertainty is the standard error based on 2–3 repetitions.

unannealed control sample. The effect of annealing conditions on the structural relaxation parameters of P10S10 is shown in Table 4. All annealed samples showed a large decrease in molecular mobility, as shown by the large increase in  $\tau^g$  value from 2.3 for the control sample to about 40–50 after annealing. This decrease in global mobility upon annealing is caused by the post-lyophilization annealing process. The substrates with small relaxation times are depopulated during annealing, leaving behind the populations with longer relaxation times. Therefore, annealing results in the increase in the relaxation time constant (i.e., a decrease in molecular mobility), as expected.

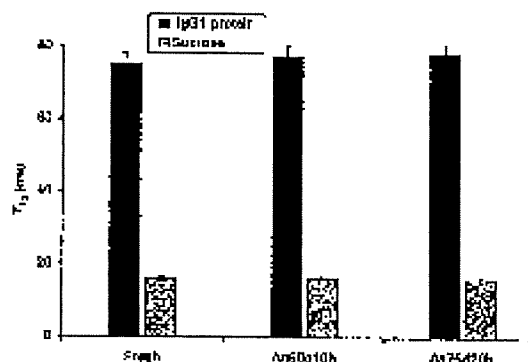
#### Local Dynamics Measurement by Neutron Scattering and Solid-State NMR

The effects of annealing on local motions with timescales much shorter than global motions were also investigated. The fast local mobility with a timescale of <5 ns was investigated with neutron backscattering, and the mean-squared displacement ( $\langle u^2 \rangle$ ) of the hydrogen atomic motion was recorded at temperatures ranging from 40 to 330 K. Table 5 shows the  $\langle u^2 \rangle$  of the P10S10 samples with different thermal histories at 25, 40, and 50°C.  $\langle u^2 \rangle$  values increase monotonically with temperature for both samples, which reflects the higher mobility of the sample at higher temperature. However, there is no significant difference in the  $\langle u^2 \rangle$  of the control (fresh) sample and the sample annealed at 60°C for 10 h at any temperature studied. Thus, it seems that the annealing does not significantly impact the fast local mobility in the protein/sucrose system, at least for the annealing conditions studied. This finding is consistent with the previous neutron backscattering study on ECA system with ECA/sugar mass ratio of 1:10.<sup>27</sup> Even upon moderate to strong

**Table 5.** Fast Local Mobility ( $\langle u^2 \rangle$ ) of the P10S10 Sample Studied With Neutron Backscattering at Temperature of 25, 40, and 50°C

T (°C)	Fresh	An6010h
50	0.297 ± 0.005	0.285 ± 0.006
40	0.284 ± 0.006	0.273 ± 0.005
25	0.264 ± 0.006	0.253 ± 0.005

Standard error shown is the uncertainty in the best fit of data to a Debye-Waller harmonic model.



**Figure 7.** Impact of annealing on the rotating frame spin-lattice relaxation times ( $T_{1\rho}$ ) of the IgG1 protein and sucrose matrix in the P10S10 formulation studied by solid state  $^{13}\text{C}$  NMR at 25°C. Error bars represent the uncertainty from the best fit of data to the model.

annealing of ECA/sugar formulations, no systematic differences in  $\langle u^2 \rangle$  were observed for either ECA/sucrose and ECA/trehalose samples.

The local molecular motion in the protein sample was also studied with high-resolution solid-state  $^{13}\text{C}$  NMR. Due to the specificity of the technique, it is possible to separate the motions in the protein molecule at 180 ppm due to the IgG1 carbonyl carbon from motions of sucrose at 80 ppm. Figure 7 shows the spin-lattice relaxation times ( $T_{1\rho}$ ) in the protein and the sucrose molecules upon annealing at different conditions. Annealing at 60°C for 10 h did not cause a significant change in  $T_{1\rho}$  relaxation time in either protein or sucrose molecules. Strong annealing of the protein sample at 75°C for 20 h still did not produce an obvious change in relaxation time for either protein or sucrose molecules. Thus, it seems that even though the global molecular mobility was significantly impacted by these annealing conditions, the local dynamics studied by both neutron scattering and  $^{13}\text{C}$  NMR were not affected by annealing in the IgG1 protein/sucrose system. Since motions monitored by neutron scattering and  $^{13}\text{C}$  NMR have different time and length scales, these different techniques may detect different parts of the local motions in the system. Given the consistent results by both techniques, we conclude that, contrary to the impact of annealing on both global mobility (i.e., TAM measured) and stability, annealing does not significantly affect local motions of the protein system, at least for the annealing conditions investigated.

However, the impact of annealing on local mobility does seem to be variable, as documented in the literature.<sup>11,26-33</sup> Thus, the impact of physical aging on the local mobility (beta relaxation) may depend on the nature of the sample, aging conditions, and/or detection techniques.<sup>34</sup> Large protein molecules are quite different from small organic molecules in that they have unique internal protein motions such as partial unfolding transitions,<sup>53,54</sup> thus different motions may be measured in protein systems than in synthetic polymers or small molecule organic systems, even when using the same techniques.

**Correlation of Stability With Protein Structure and Molecular Mobility**

FTIR structure of the protein does not change significantly upon annealing for either protein formulation. However, the long-term stability clearly increases upon annealing as shown by the decrease in the aggregation rate constant. Obviously, changes in protein secondary structure are not the origin of the improved stability upon annealing, and thus we look to changes in dynamics as the mechanism to account for the stability trends.

We note that in our system, the protein stability does not correlate with glass transition temperature. It has been reported that the degradation rate of a pharmaceutical correlates with  $T-T_g$ , at least for several systems,<sup>51,54</sup> where formulation with a higher  $T_g$  may show better stability. However,  $T_g$  of the IgG1 protein formulations is not significantly impacted by the annealing, as noticed by DSC. Also the formulation of P10S10 gives much better stability than that of P10S5 even though P10S10 has a lower  $T_g$  than P10S5. Thus, a formulation with high  $T_g$  does not necessarily mean better stability, and  $T-T_g$  is not a good predictor of protein stability, at least at temperature well below  $T_g$ .<sup>5,25,29</sup>

Figure 8 shows the correlation of stability of P10S10 with global mobility at different annealing temperatures. For 10h annealing (Fig. 8A), the increase in the annealing temperature caused a decrease in the aggregation rate constant and the global mobility ( $1/\tau^g$ ). Thus, the improved stability upon annealing correlates qualitatively with reduced global mobility. Also, in order to evaluate the suitability of using DSC to correlate with stability, the trend of the relaxation function ( $\Phi$ ) upon annealing, calculated using Eq. (2), is also given in the graph. It is noticed that the

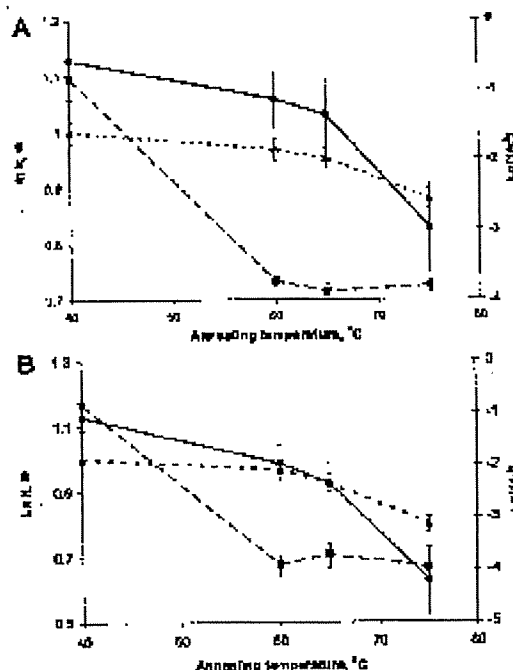


Figure 8. The relationship between physical stability ( $\bullet$ ,  $\ln k$ ), structural relaxation time ( $\blacksquare$ ,  $\ln(1/\tau^g)$ ), and initial relaxation function ( $\blacktriangle$ ,  $\Phi$ ) for the P10S10 formulation after annealing for 10h (A) and 20h (B) at different temperatures. Error bars represent standard errors.

relaxation function decrease monotonically with increasing annealing temperature, which correlates with the decrease in the global mobility and aggregation rate constant. Therefore, similar to global mobility, the relaxation function obtained from a simple and fast DSC study may also be predictive of the protein stability. Further, the quantitative correlation with stability is better with  $\Phi$  than with  $1/\tau^g$ . That is, the major change in  $1/\tau^g$  occurs between the fresh sample and the sample annealed at 60°C, but for both the rate constant and  $\Phi$ , the major change occurs between the samples annealed at 60 and 75°C. Figure 8B shows the same plot for the samples annealed at 20 h. Similar to 10 h annealing, the rate constant, global mobility, and relaxation function decreases with increase in the annealing temperature, but the quantitative correlation is best between stability and  $\Phi$ . Figure 9 shows the correlation of aggregation rate constant with relaxation

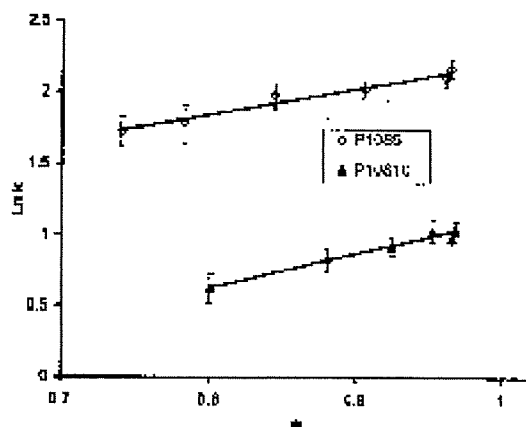


Figure 9. Correlation of protein aggregation rate with initial relaxation function ( $\Phi$ ) for both protein formulations. Error bar represents standard errors in the protein stability.

function for both protein formulations. Aggregation rate constants decreases with a decrease in relaxation function, showing an excellent linear correlation between  $\ln(k)$  and  $\Phi$ . Similar observations were made in a small molecule system (ECA/sugar system).<sup>27</sup> Therefore, it seems that the stability trend upon annealing may be well predicted using DSC data that is quick to obtain, uses little sample, and is quite precise relative to the structural relaxation time data obtained with isothermal calorimetry.

The better correlation of stability with  $\Phi$  than with  $1/\tau^2$  may be explained by the following factors. First, both TAM and DSC can be used to probe the enthalpy relaxation; however, these techniques may not measure exactly the same motion. It was found that the enthalpy relaxation obtained from the TAM was much higher than the enthalpy recovery obtained from DSC, which is consistent with previous observations that relaxation time constants obtained from DSC were larger than that from TAM for several systems.<sup>69</sup> This difference may be related to the much longer timescale for TAM experiment (several days) as compared to DSC experiment (minutes to hours), and it is possible that the TAM could measure some faster kinds of motion in addition to those motions related to enthalpy recovery (i.e., those that are mobilized only at or near the glass transition).<sup>65</sup> Second, it may be difficult to resolve small differences in  $\tau^2$ , especially at a

high annealing temperature. In the nonlinear curve fitting procedure for the calculation of  $\tau^2$  using the KWW equation,<sup>27,48</sup> the fitting parameters have an uncertainty associated with both  $\tau$  and  $\beta$ . The KWW equation assumes that the relaxation time is a constant. However, aging occurs during the calorimetric experiment, and  $\tau$  constantly increases with time. The impact of this in-process aging on the values of  $\tau$  and  $\beta$  has been studied,<sup>37-39</sup> and it was found that this experimental procedure often gave  $\tau$  values much larger than the initial value of the sample but also gave  $\beta$  values that were too small. However, the structural relaxation time constant,  $\tau^2$ , was found to be relatively constant and representative of the constant for the initial material (i.e., before the TAM run). Therefore,  $\tau^2$  is usually considered a more reliable measure of global molecular mobility. However, this parameter is still subject to significant experimental error. The errors associated with  $\tau$  in annealed protein formulations were about 10–15%, as given from the nonlinear regression analysis. The results reported in Table 4 are the average  $\tau^2$  of 2–3 replicates, where the uncertainty shown (generally ~10–20%) reflects standard error of the mean from the replicates. We clearly do not have the accuracy to observe small differences in  $\tau^2$ , especially at high annealing temperatures. All of the above factors may contribute to the different correlations of stability with  $1/\tau^2$  and with  $\Phi$ .

In the present study, an attempt has been made to address the question "What kind of mobility is relevant for stabilization: global, local or both?" It has previously been found that stability differences between protein formulations cannot be attributed solely to differences in global mobility,<sup>13,68,67</sup> and local mobility may also play an important role in protein stability. Recent studies on several proteins also showed that aggregation stability correlates well with local mobility but not with either global mobility or FTIR structure over the whole range of sucrose concentration studied (mass fraction range from 0 to 0.8).<sup>33</sup> Thus, fast, local motions may dominate the protein stability trends at temperatures well below  $T_g$ . In the present study, we find that annealing does not significantly impact the local dynamics of the protein, formulation, as measured by either NMR or neutron backscattering, and this finding is consistent with previous observations with the small molecule ECA systems studied.<sup>27</sup> The results obtained here suggest that it is global mobility rather than either local

mobility or FTIR structure that correlates with the improved protein stability upon annealing. Thus, with due consideration of both previous observations and the results of this study, it seems that both global and local motion are relevant for protein stabilization, even though the relative importance of each kind of mobility for stabilization could vary depending on the system and experimental conditions such as temperature, and the variable being explored. The differences chemically and physically between the samples being investigated in a stability study of the effect of annealing do differ from differences typical of the samples in a stability study. Traditional stability studies focus on variations between different formulations that generally have large differences in chemical composition, glass transition temperature, fragility, and glass strength among the formulations. However, when annealing is the variable in a stability study, the chemical composition and  $T_g$  are constant, and variation in most physical properties is minimal. Although one might expect global mobility to dominate stability trends when differences in  $T_g$  and other bulk glass properties differ greatly, and local mobility to dominate stability trends when most bulk properties differ little, we actually observed the opposite behavior. When annealing is the stability variable, it seems that global mobility is the dominant factor for long-term stability.

## CONCLUSIONS

This study demonstrated, for the first time, that drying process with different thermal histories (i.e., annealing) can have a significant impact on protein stability, and thus the details of the drying process need to be defined to control product stability. Moreover, the effect of thermal history on stability may be used to further stabilize fragile protein therapeutics through the use of appropriate annealing protocols. Annealing below  $T_g$  stabilized IgG1 protein formulations by decreasing the degradation rate such that not only was the degradation rate decreased by annealing but also the unannealed sample actually exhibited lower total aggregation than the control (fresh) sample after a short storage time. Thus, the concept of "stabilization by annealing" is valid for the IgG1 protein system. Annealing does not significantly impact many properties, including the SSA,

protein native structure, and local mobility; however, the enthalpy recovery and structural relaxation time constant increase upon annealing, and the improved stability upon thermal treatment is quantitatively correlated to the magnitude of relaxation function, and thus simple DSC studies may be used to guide the optimization of annealing conditions and predict stability.

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## Feasibility of $^{19}\text{F}$ -NMR for Assessing the Molecular Mobility of Flufenamic Acid in Solid Dispersions

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The purpose of the present study was to clarify the feasibility of  $^{19}\text{F}$ -NMR for assessing the molecular mobility of flufenamic acid (FLF) in solid dispersions. Amorphous solid dispersions of FLF containing poly(vinylpyrrolidone) (PVP) or hydroxypropylmethylcellulose (HPMC) were prepared by melting and rapid cooling. Spin-lattice relaxation times ( $T_1$  and  $T_{1\rho}$ ) of FLF fluorine atoms in the solid dispersions were determined at various temperatures (–20 to 150 °C). Correlation time ( $\tau_c$ ), which is a measure of rotational molecular mobility, was calculated from the observed  $T_1$  or  $T_{1\rho}$  value and that of the  $T_1$  or  $T_{1\rho}$  minimum, assuming that the relaxation mechanism of spin-lattice relaxation of FLF fluorine atoms does not change with temperature. The  $\tau_c$  value for solid dispersions containing 20% PVP was 2–3 times longer than that for solid dispersions containing 20% HPMC at 50 °C, indicating that the molecular mobility of FLF in solid dispersions containing 20% PVP was lower than that in solid dispersions containing 20% HPMC. The amount of amorphous FLF remaining in the solid dispersions stored at 60 °C was successfully estimated by analyzing the solid echo signals of FLF fluorine atoms, and it was possible to follow the overall crystallization of amorphous FLF in the solid dispersions. The solid dispersion containing 20% PVP was more stable than that containing 20% HPMC. The difference in stability between solid dispersions containing PVP and HPMC is considered due to the difference in molecular mobility as determined by  $\tau_c$ . The molecular mobility determined by  $^{19}\text{F}$ -NMR seems to be a useful measure for assessing the stability of drugs containing fluorine atoms in amorphous solid dispersions.

**Key words**  $^{19}\text{F}$ -NMR; molecular mobility; stability; crystallization; solid dispersion

Amorphous solid dispersions are used for improving the dissolution rate and solubility of poorly soluble drugs. However, drugs in amorphous form are generally less stable than crystalline drugs because of their higher energy state and higher molecular mobility. It is well known that polymeric excipients can reduce the crystallization rate of many amorphous drugs.<sup>1–12</sup> This stabilization by poly(vinylpyrrolidone) (PVP) is partly attributable to its ability to decrease molecular mobility, as indicated by increases in the glass transition temperature ( $T_g$ ).<sup>9</sup> Therefore, it is of great interest to estimate the molecular mobility of drugs in solid dispersions. Although  $^{13}\text{C}$ -NMR relaxation measurements are useful for assessing the molecular mobility of drugs in solid dispersions,<sup>13</sup> the low sensitivity of  $^{13}\text{C}$  because of its low natural abundance is a drawback of  $^{13}\text{C}$ -NMR. In contrast to  $^{13}\text{C}$ ,  $^{19}\text{F}$  has very favorable sensitivity in NMR experiments, since it is present in 100% natural abundance, is second only to the proton in its resonance frequency (except  $^1\text{H}$ ) and has a spin quantum number of 1/2. The receptivity for  $^{19}\text{F}$  is 83% of that for  $^1\text{H}$  and 4700 times of that for  $^{13}\text{C}$ .<sup>14</sup> Many drugs containing fluorine atoms are listed in The Japanese Pharmacopoeia. In contrast, almost all pharmaceutical excipients do not contain fluorine atoms.  $^{19}\text{F}$ -NMR may therefore have an advantage over  $^{13}\text{C}$ -NMR or  $^1\text{H}$ -NMR for selectivity and sensitivity when assessing the molecular mobility of drugs containing fluorine atoms in pharmaceutical dosage forms such as solid dispersions.

The orientations and molecular mobility of flufenamic acid (FLF)<sup>15</sup> and  $^{19}\text{F}$ -labeled  $\alpha$ -tocopherol<sup>16</sup> in a lipid bilayer were studied using  $^{19}\text{F}$ -NMR. Structures and molecular mobility of  $^{19}\text{F}$ -labeled peptides and proteins in biological membranes were also investigated.<sup>17–20</sup> To the authors' knowledge, application of  $^{19}\text{F}$ -NMR to studies of drug molecular mobility in solid dispersions has not been reported.

This paper describes the feasibility of  $^{19}\text{F}$ -NMR for assessing the molecular mobility of FLF in PVP or hydroxypropylmethylcellulose (HPMC) solid dispersions, and discusses the effect of polymer excipients on the crystallization tendency of FLF in solid dispersions in terms of differences in molecular mobility.

### Experimental

**Materials** FLF (Fig. 1) was purchased from Wako Pure Chemical Industry (Osaka), and PVP and HPMC were from Sigma (St. Louis, MO, U.S.A.). FLF solid dispersions with PVP or HPMC were prepared by melting and cooling of mixtures of FLF with PVP or HPMC. The solid dispersions obtained were confirmed to be amorphous from microscopic observation under polarized light.

**Nuclear Magnetic Relaxation Measurements**  $^{19}\text{F}$ -NMR measurements were carried out using a model JNM-MU25 pulsed NMR spectrometer (JEOL DATUM, Tokyo) operating at a resonance frequency of 25 MHz. Time profiles of spin-spin relaxation of the  $^{19}\text{F}$  atoms of FLF were measured using the "solid echo" pulse sequence to overcome the dead time of the instrument. Spin-lattice relaxation time in the laboratory frame ( $T_1$ ) was measured using the inversion recovery pulse sequence. Spin-lattice relaxation time in the rotating frame ( $T_{1\rho}$ ) was measured at spin locking intensity of 10 G.

**DSC Measurements**  $T_g$  of FLF-PVP and FLF-HPMC solid dispersions was measured by DSC using a model 2920 differential scanning calorimeter and a refrigerator cooling system (TA Instruments, Newcastle, DE, U.S.A.). Approximately 5 mg of each solid dispersions was put into an aluminum sample pan and then sealed hermetically.  $T_g$  was measured at a heating rate of 20 °C/min. Temperature calibration of the instrument was carried out using indium.

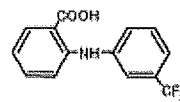


Fig. 1. Structure of FLF

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## Results and Discussion

**Molecular Mobility of FLF as Measured by  $^{19}\text{F}$ -NMR Spin-Lattice Relaxation Time  $T_1$  and  $T_{1\rho}$**  of fluorine atoms of FLF in PVP and HPMC solid dispersions were measured using a pulsed NMR spectrometer in the temperature range from  $-20$  to  $150^\circ\text{C}$ .  $T_1$  is sensitive to the molecular motion on the time scale of the resonance frequency (MHz order). On the other hand,  $T_{1\rho}$  is sensitive to the molecular motion with a frequency equivalent to the intensity of spin locking field (typically mid kHz order).<sup>21</sup> The temperature dependence of  $T_1$  and  $T_{1\rho}$  exhibits minimum at a specific temperature at which the molecules of interest have molecular motion with MHz time scale or mid kHz time scale predominantly. The resonance frequency of 25 MHz, lower than that of a conventional high resolution NMR spectrometer, was used to observe  $T_1$  minimum in the temperature range studied. Figure 2 shows the temperature dependence of  $T_1$  and  $T_{1\rho}$  of FLF fluorine atoms in PVP and HPMC solid dispersions. For FLF-PVP solid dispersions (7:3), the minimum of  $T_1$  or  $T_{1\rho}$  was observed at about  $90^\circ\text{C}$  and  $60^\circ\text{C}$ , respectively (Fig. 2A). When the PVP content decreased to 20% (w/w),  $T_1$  and  $T_{1\rho}$  of FLF at temperatures above  $70^\circ\text{C}$  could not be determined due to rapid crystallization. Similar temperature dependence of  $T_1$  or  $T_{1\rho}$  was observed for the FLF-HPMC solid dispersions (Fig. 2B). The temperature difference between  $T_1$  and  $T_{1\rho}$  minimum is considered to be due to the difference in the time scale of molecular motion reflected on  $T_1$  (MHz order) and  $T_{1\rho}$  (mid kHz order). Since the molecular motion on MHz time scale becomes predominant at higher temperature than molecular motion on mid kHz time scale,  $T_1$  minimum is observed at higher tempera-

ture than  $T_{1\rho}$  minimum.

We made following assumptions in order to estimate the molecular mobility of FLF from  $T_1$  and  $T_{1\rho}$  of FLF fluorine atoms: first, we assumed that FLF fluorine atoms in the solid dispersions relaxes mainly *via* dipolar interaction, and that the contribution of the spin-rotation interaction mechanism<sup>21</sup> is negligible. While relaxation *via* the spin-rotation interaction mechanism has been reported for liquid sample,<sup>22-24</sup> complete domination of dipolar interactions has been reported for fluorine atoms for polycrystalline van der Waals molecular solid.<sup>25</sup> We also made an assumption that the contribution of the cross-relaxation between fluorine and proton atoms can be considered small. It is known that relaxation is not intrinsically single-exponential when cross-relaxation between fluorine and proton atoms takes place.<sup>14</sup> However, we assumed small contribution of the cross-relaxation, because the relaxation of FLF fluorine atoms in the solid dispersions was exponential within experimental uncertainty. In studies of molecular motions, a large number of models describing molecular motions have been proposed for calculation of the spectrum density function.<sup>26</sup> We used a simple model that the molecular motion reflected on  $T_1$  or  $T_{1\rho}$  is represented by single correlation time for the purpose of comparing the mobility of FLF in the PVP and HPMC solid dispersions. According to the above assumptions,  $T_1$  and  $T_{1\rho}$  are described by Eqs. 1 and 2.<sup>21</sup>

$$\frac{1}{T_1} = \frac{6}{20} \frac{\gamma^4 \hbar^2}{r^6} \left\{ \frac{\tau_c}{1 + \omega_0^2 \tau_c^2} + \frac{4\tau_c}{1 + 4\omega_1^2 \tau_c^2} \right\} \quad (1)$$

$$\frac{1}{T_{1\rho}} = \frac{3}{20} \frac{\gamma^4 \hbar^2}{r^6} \left\{ \frac{3\tau_c}{1 + 4\omega_0^2 \tau_c^2} + \frac{5\tau_c}{1 + \omega_1^2 \tau_c^2} + \frac{2\tau_c}{1 + 4\omega_1^2 \tau_c^2} \right\} \quad (2)$$

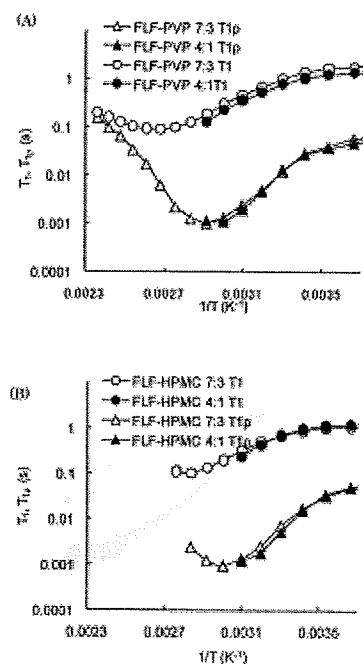


Fig. 2. Temperature Dependence of  $T_1$  and  $T_{1\rho}$  of FLF Fluorine Atoms in PVP (A) and HPMC (B) Solid Dispersions

where  $\tau_c$  is the correlation time that characterizes molecular reorientations, and  $\omega_0$  and  $\omega_1$  are the resonance frequencies of fluorine atoms in the static magnetic field and spin locking field, respectively.  $\gamma$ ,  $r$  and  $\hbar$  are the gyromagnetic ratio of fluorine, the distance of neighboring fluorine atoms, and the Plank constant divided by  $2\pi$ , respectively. Equations 1 and 2 infer that  $T_1$  and  $T_{1\rho}$  become minimal when  $\omega_0 \tau_c$  is approximately 0.62<sup>27</sup> and  $\omega_1 \tau_c$  is approximately 0.52,<sup>21</sup> respectively. When the minimum of  $T_1$  or  $T_{1\rho}$  is observed, we can calculate the unknown value,  $r$ , in Eqs. 1 and 2. If  $r$  is known, the  $\tau_c$  value can be calculated from the observed  $T_1$  or  $T_{1\rho}$  value, assuming that  $r$  does not change with temperature.

The values of  $r$  calculated from the  $T_1$  and  $T_{1\rho}$  minimum observed for the FLF-PVP solid dispersion (7:3) were 2.3 and 2.4 Å, respectively, and similar  $r$  values were obtained for the FLF-HPMC solid dispersion (7:3). These values are comparable to the reported value (2.174 Å) for 3-(trifluoromethyl)phenanthrene,<sup>23</sup> indicating that dipole interaction between neighboring fluorine atoms can be considered the predominant relaxation mechanism of FLF fluorine atoms in the solid dispersions. The difference between the  $r$  values obtained in this work and the reported value suggests that the possibility of the spin-rotation interaction mechanism and/or dipole interaction between fluorine and proton atoms cannot be excluded as a relaxation mechanism of FLF fluorine atoms.

Figure 3 shows the temperature dependence of  $\tau_c$  calculated from  $T_1$  and  $T_{1\rho}$  for FLF fluorine atoms in the solid dis-