Drying-Induced Variations in Physico-Chemical Properties of Amorphous Pharmaceuticals and Their Impact on Stability (I): Stability of a Monoclonal Antibody

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ABSTRACT: The present study was conducted to investigate the impact of drying method and formulation on the storage stability of IgG_1. Formulations of IgG_1 with varying levels of sucrose with and without surfactant were dried by different methods, namely freeze drying, spray drying, and foam drying. Dried powders were characterized by thermal analysis, scanning electron microscopy, specific surface area (SSA) analysis, electron spectroscopy for chemical analysis (ESCA), solid state FTIR, and molecular mobility measurements by both isothermal calorimetry and incoherent elastic neutron scattering. Dried formulations were subjected to storage stability studies at 40°C and 50°C (aggregate levels were measured by size exclusion chromatography initially and at different time points). Both drying method and formulation had a significant impact on the properties of IgG_1 powders, including storage stability. Among the drying methods, SSA was highest and perturbations in secondary structure were lowest with the spray-dried preparations. Sucrose-rich foams had the lowest SSA and the lowest protein surface accumulation. Also, sucrose-rich foams had the lowest molecular mobility (both fast dynamics and global motions). Stability studies showed a log-linear dependence of physical stability on composition. Preparations manufactured by "Foam Drying" were the most stable, regardless of the stabilizer level. In protein-rich formulations, freeze-dried powders showed the poorest storage stability and the stability differences were correlated to differences in secondary structure. In stabilizer-rich formulations, stability differences were best correlated to differences in molecular mobility (fast dynamics) and total protein surface accumulation. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1983–2008, 2007
Keywords: composition heterogeneity; electron spectroscopy for chemical analysis; glass dynamics; incoherent elastic neutron scattering; molecular mobility; protein stabilization; protein secondary structure; structural relaxation time; specific surface area; surface coverage

INTRODUCTION

Drying proteins from aqueous solution to form a molecular dispersion of a protein in a glassy matrix has now become a standard practice in the pharmaceutical industry for stabilization.[1–4] Freeze drying is widely used for parenteral administration,[1–3] whereas spray drying is widely used for pulmonary delivery.[4,5] Other drying methods have emerged as alternate drying methods to both such as spray coating,[6] supercritical fluid technology,[7] and spray freeze drying,[8] a drying method that combines the atomization principle of spray drying and the freeze drying process to produce powders with different characters than those produced by either conventional freeze drying or spray drying. Drying methods based on the principle of foaming and rapid evaporation of water (foam drying) were developed in the 1960s for preservation of vaccines and bacteria.[9–12] Now, these methods or modifications of these methods are also being investigated as alternate drying methods.[13–15] Regardless of the drying method, the outcome is a dry amorphous formulation of mainly the protein and a stabilizer, hopefully with acceptable stability. However, systematic studies of the effect of processing variations on storage stability are lacking. Because of differences in process, including vast differences in thermal history, physical and chemical properties, including storage stability, are likely to vary with preparation method. For example, recent evidence has shown that thermal history differences may impact degradation rates in small molecule pharmaceutical systems.[16,17]

Physical and chemical stabilization upon long term storage is of fundamental concern to the pharmaceutical scientist because of potential alterations in immunogenicity, toxicity, and efficacy.[18] The choice of stabilizer is usually limited to a small molecular mass sugar such as trehalose or sucrose, or a mixture of a small molecular mass sugar and a polymeric excipient such as dextran.[1,2,19] There are two different proposed mechanisms by which these excipients are believed to stabilize proteins in the amorphous state, one is thermodynamic and the other is kinetic. The water substitution hypothesis proposes that stabilization results from native structure preservation by hydrogen bonding between the stabilizer and specific sites at the surface of a protein, thereby substituting for the thermodynamic stabilization function of water lost during drying.[1,2,20] It seems unlikely that this effect, being a thermodynamic stabilization mechanism, would vary dramatically with the process. The glass dynamics hypothesis proposes that stabilization results from suppression of global motions, also known as α-relaxations, that may be relevant to protein instability via the formation of a rigid, glassy matrix.[21,22] α-relaxations occur mainly due to translational and rotational motions (rotation of the entire molecule), and therefore strongly influence the diffusion of reactive molecular species.[23] Degradation involving diffusion is strongly affected by changes in molecular mobility around glass transition temperature.[24] Since global relaxation is impacted by thermal history,[17] one expects process variations that provide significantly different thermal histories to impact stability.

Studies have been interpreted in terms of both the "water substitute" concept and in terms of glass dynamics. Cleland et al.[25] explained the log-linear composition dependence of stability of a monoclonal antibody in terms of the water substitution hypothesis. The authors observed that the physical stability of the lyophilized monoclonal antibody was correlated to the retention of secondary structure in the solid state, and it was assumed that the "water substitute" role of the sugar was responsible for stabilization of the native structure. On the other hand, susceptibility to aggregation has also been interpreted in terms of to the glass dynamics theory. Duddu et al.[26] found a correlation between aggregation of a monoclonal antibody in lyophilized formulations and molecular mobility, as measured by enthalpy relaxation time. In another study, Yoshioka et al.[27] found that aggregation of bovine serum albumin (BSA) in the solid state increased as molecular mobility measured by proton nuclear magnetic resonance (^{1}H NMR) increased.

There are several examples in the literature where neither the glass dynamics nor the water substitution hypothesis separately or together can fully explain stabilization.[28] For example, no correlation was observed between stabilization and matrix mobility in dried formulations of insulin and poly(vinyl)pyrrolidone,[29] or in several enzyme systems.[30,31] Furthermore, in these enzyme systems, excellent preservation of enzyme activity was observed even above the glass transition temperature (T_g).[30,31] This is surprising since above T_g, global motions are relatively high and one would expect high instability. Therefore, other factors seem to be responsible for stabilization.

Some of the problem may involve the assumption that it is the global relaxation process, which is linked to viscosity, which is most relevant to the degradation dynamics of interest. It has been proposed that dynamics on a smaller time scale than global motions may be important for protein stabilization in glasses.[23,32,33] β-relaxations are examples of such fast dynamics which generally involve localized motion of specific portions of the molecule.[34] They can occur over a broad range of timescales, anywhere between vibrational and rotational motion timescales.[23,32–34] It is argued that β-relaxations influence diffusion of small molecules such as gases in systems of larger molecules, and possibly control small amplitude protein motions that can result in protein aggregation.[23,32,33] Therefore, one would expect minimal contribution of global motions in reactions (chemical or physical) controlled mainly by β-relaxations.[24,34] Cicerone et al.[23] showed that the addition of small amounts of glycerol to an amorphous matrix of horse radish peroxidase (HRP) and poly(vinyl)pyrrolidone (PVP) resulted in increased stability of HRP, even though T_g of the system was lowered. Improvement in stability was consistent with a decrease in mobility associated with β-relaxation (as measured by elastic incoherent neutron scattering). A similar effect was observed with yeast alcohol dehydrogenase. Therefore, it appeared that glycerol, a plasticizer for global motions, acted as an antiplasticizer for the β-motions. Furthermore, if β-motions are relevant to stability, suppressing such β-motions would be the key to stabilization. Therefore, a complete analysis of stability in terms of "glass dynamics" needs to also consider the possible role of "fast dynamics."

Specific surface area is one material property that is likely to vary greatly with preparation method.[35,36] A few studies have shown that formulations with a high specific surface area (SSA) had inferior storage stability relative to the same formulations with lower SSA prepared by other drying methods.[35,36] For example, Sane et al.[35] observed that the storage stability of a freeze dried antibody formulation was better than that of the same formulation spray dried. Protein secondary structure, as measured by Raman spectroscopy, was the same in both formulations but SSA of the spray-dried formulation was higher. Carpenter et al.[36] studied the storage stability of spray freeze dried and freeze dried recombinant human interferon-γ (rhIFN-γ) formulation with trehalose in the presence and

absence of Tween 20. Second derivative FTIR spectra showed that protein secondary structure was similar in all preparations. The spray freeze-dried formulation without Tween 20 had the highest aggregation rate constant, consistent with its highest SSA and highest protein surface concentration (as measured by electron spectroscopy for chemical analysis, ESCA). The lyophilized formulation with Tween 20 had the lowest aggregation rate constant, consistent with its low SSA and lowest protein surface concentration. None of these studies, however, provided a mechanistic interpretation of these trends. It is becoming well known[36–43] that there may be a significant separation of stabilizer from protein during spray drying. The surface region becomes rich in protein and the interior therefore must become richer in stabilizer.[36–43] The use of a surfactant has been shown to moderate the accumulation of protein at the surface for spray dried samples.[36,37,39–43] Such an effect, which is compositional or chemical heterogeneity, refers to the separation of chemical components during drying without phase separation.[36,38–43] Development of compositional heterogeneity from a solution initially uniform in composition requires molecular mobility, at least at some stage of the process. That is, one must have the molecular mobility to support the mutual diffusion needed to create separation of components. Such heterogeneity could arise during freezing for a freeze-drying process and during much of the drying process for a spray dried material. Although there is spatial variation in composition in freeze dried materials, it does appear that composition variation in spray dried materials can be an order of magnitude larger[39] thus, both SSA and protein accumulation at the surface are dependent on drying method, and both factors would be expected to lead to inferior stability for spray dried powders.

Now the question is "What can be the relationship between chemical heterogeneity and storage stability?" It is becoming established that storage stability of proteins in amorphous pharmaceuticals show a log-linear dependence on composition.[25,28,44–46] Assuming that the measured or observed rate constant for degradation ($k_{obs.}$) is a summation of contribution of stability from both surface and bulk protein, we may write:

$$k_{obs} = k_S F_{PS} + k_B F_{PB} \qquad (1)$$

where k_S is the rate constant for decomposition of surface protein, k_B is the rate constant for decomposition of bulk protein, F_{PS} is the fraction

of the surface protein and F_{PB} is the fraction of bulk protein. Thus, in a formulation with a large protein fraction and little stabilizer fraction at the surface, the contribution of $k_S F_{PS}$ to degradation will increase compared with an identical formulation with lesser protein surface coverage. Moreover, if stability has a log-linear dependence on composition, simple numerical calculations[47] show that the loss of stability in the region depleted of stabilizer is greater than the gain in stability in the core region enriched in stabilizer, the effect being greater in importance for systems rich in stabilizer. Thus, in general, the overall degradation rate would be higher for those samples that have a higher degree of component separation. Evidence, therefore, suggests that SSA and possible protein surface accumulation should be considered as additional factors in protein stabilization.

The main objective of this study was to evaluate the impact of variations in drying process on storage stability for selected formulations. Formulations contained varying levels of sucrose as stabilizer, with or without surfactant. We assessed the impact of drying method on surface composition, specific surface area and molecular mobility (both slow and fast dynamics), and the correlation of these physical properties with stability. Here, a monoclonal antibody was used as a model protein.

MATERIALS AND METHODS

Materials

Purified recombinant humanized monoclonal antibody against integrin $\alpha_2 V_\beta$ (rhuMAb) or Medi-522, an IgG_1, (1.76% mass fraction aggregate, 0.25% mass fraction fragments) was supplied as 100 mg/mL stock solution in 10 mM phosphate buffer (potassium phosphate monobasic and dibasic) pH 6.0 by the MedImmune Purification Development Group (Santa Clara, CA). Chemicals and excipients were all used as supplied. Sucrose powder was purchased from Pfanstiehl Laboratories (Waukegan, IL). Pluronic F68 (a hydrophilic nonionic surfactant with an HLB value >25 and a CMC of ≈ 8 μmol/L) was purchased from Sigma. 13 mm 2 mL and 20 mm 5 mL type I borosilicate clear tubing glass vials, as well as V2 F451 13 mm and V10 F451 20 mm single-vent Flurotec® (Daikyo Seiko) lyophilization stoppers were purchased from West Pharmaceuticals (Lititz, PA).

Preparation and Drying of Medi-522 Solutions

Using a stock solution of 80% by mass sucrose, the ratio of rhuMAb to sucrose was adjusted to 4:1, 2:1, 1:4, and 5:95 by mass. The total solids content in solution was adjusted for each drying method for optimum drying by diluting with de-ionized water. Final pH was 6.1 ± 0.1 and phosphate buffer concentration was 1 mM after dilution. Solids content for solutions to be freeze dried and spray dried were 5% by mass. Solids content for solutions to be foam dried were (11 to 12)% by mass for protein-rich formulations and 25–30% by mass for sucrose-rich formulations. Formulations with a higher mass ratio of protein to stabilizer are labeled "protein rich" (i.e., 4:1 and 2:1), while formulations with a higher mass ratio for stabilizer are labeled "stabilizer-rich" or "sucrose-rich" (i.e., 1:4 and 5:95). The 4:1 and 1:4 formulations of rhuMAb with sucrose were also prepared with Pluronic F-68. The concentration of surfactant was adjusted to 0.2% by mass in the final solution (well above critical micelle concentration). The ratio of surfactant to protein, however, differed according to solids content in solution. In 4:1 protein:Sucrose formulation with surfactant, the ratio of surfactant-to-protein was 1:20 in solutions to be spray and freeze dried and was 1:38 in solutions to be foam dried. In 1:4 protein:Sucrose formulation with surfactant, the ratio of surfactant-to-protein was 1:5 in solutions to be spray and freeze dried and was 1:25 in solutions to be foam dried.

Spray Drying

Solutions were spray dried with a Buchi 190 Mini Spray Drier (Flawil, Switzerland) operating inside an enclosure to maintain low humidity and good temperature control. The Buchi atomizer nozzle was replaced by a custom designed atomizer nozzle designed to produce high pressure effervescent atomization, a technology borrowed from the diesel industry.[48,49] In short, the idea is to bubble a small amount of the atomizing gas (in this case, nitrogen) into the stream of solution to be spray dried inside the atomizer nozzle. As the atomizing gas and solution mix is discharged from the orifice of the atomizer, the gas phase expands, breaking the liquid into small droplets of uniform droplet size distribution. Smaller droplet sizes result from this method, compared to those from pressure atomization (i.e., the normal Buchi

nozzle). There are claims that this method provides a high process yield without significant reduction in product purity.[49] The solution feed rate was 2 mL/min, the inlet temperature was 87°C, the outlet temperature ranged from 50 to 60°C, relative humidity in the chamber was maintained below 5%, the atomizing gas was nitrogen and the atomizing pressure was 12.4 MPa (1800 psi). Spray dried powders were subjected to a post drying time of 30 min in the collector tube at 60–63°C to reduce moisture content.

Freeze Drying

Solutions were freeze-dried using a FTS Systems Lyostar II (Stone Ridge, NY). Solutions were lyophilized in 5 mL serum vials with a fill volume of 1.5 mL. Solutions were placed on shelves precooled at 5°C. Shelf temperature was cooled at a rate of 2.5°C/min to −40°C and was held for 90 min to freeze the solutions. Shelf temperature was raised to −30°C at a rate of 1.3°C/min for carrying out primary drying (below the collapse temperature). Collapse temperatures were about −34°C or greater, depending on formulation. Shelf temperature was held at −30°C for 48 h during primary drying while maintaining a chamber pressure of 66 Pa (50 mTorr) (for sublimation of ice). Average product temperature during primary drying was −37°C to −38°C. The end of primary drying was marked by a rise in product temperature above the shelf temperature with a concurrent decrease in dew point to a constant value. For secondary drying (evaporation of water in the amorphous phase), shelf temperature was heated to 10°C at a rate of 0.1°C/min. Temperature was maintained at 10°C for 280 min. Pressure during secondary drying was maintained at 66 Pa (50 mTorr). While 10°C is not a typical secondary drying temperature for proteins, it is our experience that using higher temperatures in secondary drying usually leads to very low moisture content in the final product (<0.5% by mass). This temperature was used in our studies to control the final moisture content to the desired range for all processes, ≈1–2% by mass.

Annealing Followed by Freeze Drying

Some rhuMAb solutions were annealed in the frozen state prior to primary drying. The purpose of this step was to produce samples of lower specific surface area than the regular freeze drying cycle. This variation also allowed a test of the concept, which states that maintaining the protein above a glass transition temperature during the process leads to instability. Annealing was performed at −8°C for 48 h (above glass transition temperature of the freeze concentrate—$T_{g'}$. Highest $T_{g'}$ was ∼−10°C). Solutions were re-frozen to −40°C after annealing. Conditions for primary and secondary were the same as under "*Freeze drying*."

Cakes produced upon freeze-drying (with and without annealing) were elegant with excellent retention of cake structure.

Foam Drying

Solutions were foam dried using a Virtis Genesis freeze dryer equipped with a condenser model 25EL (Gardiner, NY). Solutions were foam dried in 2 mL serum vials with a fill volume of 0.3 mL. Vials containing solutions were placed on shelves at 15°C and kept for 15 min to equilibrate. Foaming was initiated by reducing the chamber pressure to 66 Pa (50 mTorr) at a shelf temperature of 15°C. Chamber pressure used is significantly lower than vapor pressure of water at the prevailing temperature to obtain foaming. Product temperatures initially decreased to ≈−20 to 5°C, depending on the formulation composition, and remained at this temperature during most of primary drying. None of the solutions froze, presumably due to the very high solute content. The drying process is considered to be composed of two drying phases: primary drying and secondary drying. Primary drying was done at a shelf temperature of 15°C and a chamber pressure of 66 Pa (50 mTorr) for 1 h. This step is a much shorter step than primary drying in a freeze-drying cycle and water is removed by evaporation. Secondary drying, a step to remove remaining moisture in amorphous phase, was done by raising shelf temperature to 33°C at a rate of 1°C/min and maintained at that temperature and at 33 Pa (25 mTorr) for 72 h. The unusually long secondary drying time required for foam drying is probably attributable to the very low specific surface area of the foams (results presented later).

Adjusting Moisture Content in Dried Powders

Because the types of powders produced were quite different in their secondary drying

characteristics, the drying processes led to large variations in moisture content (results not shown). In order not to convolute the effects of moisture variation on storage stability with the effects due to variations in process and formulation, moisture contents in all formulations were adjusted to be in the range of $\approx 1–2\%$ by mass. Variability of moisture content within this range was associated with minimal variations in stability of IgG_1 (results not shown,[28]). Powders with less than 1% by mass moisture, as measured by Karl Fischer titration, were re-hydrated in 10% relative humidity for 30–45 min and sealed under nitrogen. Powders with more than 2% by mass moisture were subjected to further vacuum drying. Vacuum drying was performed in a Virtis Genesis freeze dryer equipped with a condenser model 25EL (Gardiner, NY) at a shelf temperature of $35^\circ C$ and a chamber pressure of 33 Pa (25 mTorr) for 1 h. Vials were sealed under nitrogen at the end of the drying cycle. With sucrose-rich foams (where initial moisture content was >2% by mass), additional drying times for up to 48 h were employed due to the very low SSA of the dried foams.

Karl Fischer Moisture Determination

Residual moisture content of all formulations was measured by direct injection using coulometric Karl Fischer titration (Denver Instrument Company, Denver, CO). Powders were weighed and filled into vials in a glove bag where a low relative humidity (RH) was maintained (<2%) by flushing with dry nitrogen. Powders were dissolved/dispersed in 2 mL of low moisture formamide and 0.5 mL of the solution was injected. Blank corrections were applied. Standard deviation from replicate measurements was not more than 0.1% H_2O.

Scanning Electron Microscopy (SEM) studies

SEM was utilized to characterize morphology of selected spray-dried, freeze-dried, and foam-dried preparations. Samples were mounted on silicon wafers mounted on top of double-sided carbon tape on an aluminum SEM stub. The sample was then sputter coated in a Denton sputter coater for 60–90 s at 100 Pa (75 mTorr) and 42 mA with gold:palladium. Images were taken with a Philips XL30 ESEM operated in high vacuum mode using an Everhart-Thornley detector to capture secondary electrons for the image composition.

Accelerating voltage was set at 20 kV using a LaB_6 source. Working distance was 5–6 mm.

Modulated Differential Scanning Calorimetry (MDSC) Studies

MDSC studies were performed to determine the glass transition temperature (T_g) of the different dried formulations with and without surfactant to aid in proper design of storage stability studies and to characterize thermal behavior of the different preparations. A Q-1000 Differential Scanning Calorimeter equipped with a DSC refrigerated cooling system, TA instruments, Inc. (New Castle, DE) was used. The powdered sample (5–15 mg) was filled and pressed into compact pellets, placed in aluminum DSC pans and hermetically sealed using a sample encapsulation press. The entire process was performed in a glove box, with RH maintained under 2%. Nitrogen was used as a purge gas for MDSC at $30 \text{ cm}^3/\text{min}$. All MDSC measurements were done in triplicate or more. The MDSC protocol was as follows:

- Equilibrate at $0^\circ C$.
- Modulate $+/- 1^\circ C$ every 120 s.
- Ramp $2^\circ C/\text{min}$ to $100^\circ C$.

Powder Density Measurements

The density (ρ) (g/cm^3) of dried powders was measured with nitrogen pycnometry (Micromeritics AccuPyc 1330 Gas Pycnometer, Norcross, GA). Powders were weighed into 1-cm^3 sample aluminum sample cells and the cells were mounted into the analysis chamber. The number of purges was 10, the purge fill pressure was adjusted to 134 kPa (19.5 psig), the number of runs was adjusted to 5, the run fill pressure was adjusted to 134 kPa and the equilibration rate was set to 34 Pa/min (0.005 psig /min). The gas pyconometer was tested for precision, accuracy, and inter-day variability using crystalline sodium chloride. The published ρ value for crystalline NaCl is 2.17 g/cm^3.[50] Our measurements gave a ρ value of 2.182 ± 0.026 g/cm^3 ($n = 5$ replicates on 5 separate days). Additionally, reproducibility (inter-vial variability) of ρ measurements on four different amorphous samples (different sucrose-based formulations labeled A, B, C, D) gave 1.039 ± 0.008 g/cm^3 (A), 1.590 ± 0.019 g/cm^3 (B), 1.433 ± 0.004 g/cm^3 (C), and 1.570 ± 0.011 g/cm^3 (D).

Specific Surface Area (SSA) Measurement

BET (Brunauer Emmett Teller) Specific surface area (SSA) analysis was performed using a Micromeritics, FlowSorb II 2300 BET surface area analyzer (Norcross, GA). Sample size was at least 100 mg. Powder samples were degassed for at least 3 h in the Flow Prep oven at $25°C$ using krypton purge prior to measurement. Single point calibration was performed prior to taking surface area measurements using 0.1 mol Kr gas. Percent relative standard deviation (%RSD) for SSA measurements (replicate samples) ranged between 1% and 7% for preparations with SSA ≥ 0.5 m^2/g, but was higher for preparations with very low SSA of ≤ 0.1 m^2/g (Tab. 2).

Electron Spectroscopy for Chemical Analysis (ESCA) Studies

ESCA was used to probe elemental composition of the surfaces of dried formulations, with an analysis depth of ≈ 50 Å. Survey spectra were collected with a VG ESCALAB MK II series Spectrometer (pass energy = 60 eV). An $Al_{K\alpha}$ radiation (emission current of 11 kV anode voltage and 34 mA emission current) from $Al_{K\alpha}$ X-ray source was directed onto a circular region of approximate diameter of 3 mm. Pressure was maintained in analysis chamber at less than 10^{-5} Pa ($<10^{-8}$ Torr). Measurements were done on triplicate samples. The peak areas from the spectra were converted to elemental atomic concentrations on the surface of each sample. %RSD for ESCA measurements (replicate samples) did not exceed 4% for protein-rich systems and ranged between 4% and 13.5% for sucrose-rich systems (Tab. 2). Details of data analysis are described elsewhere.[47,51]

Fourier Transform Infra Red (FT-IR) Spectroscopy Studies

FT-IR spectroscopy studies were performed using a Nicolet Magna IR 760 Spectrometer (Thermo-Nicolet, Madison, WI). Studies were performed on both rhuMAb in solution (i.e., native conformation) $\approx 10–15$ mg/mL and in all dried powders according to procedures described in the literature.[20,52–56] A total of 256 scans and a resolution of 4 cm^{-1} were used for each spectrum. The scanning range was 4000 to 400 cm^{-1}. The spectrum for native, aqueous rhuMAb (used as a control) was obtained by placing a 15 mg/mL solution between 2 CaF_2 windows separated by a 6 μm Mylar spacer. The spectrum of rhuMAb in solution was corrected by subtracting the spectra of liquid water and water vapor as described elsewhere.[57] The solid samples were prepared by pressing a ground homogeneous mixture of 100 mg KBr with 2–3 mg of the dried protein formulation into a pellet (disk) using a stainless steel die. Effects of water vapor and carbon dioxide in the chamber were eliminated by subtracting the background spectrum. The second derivative IR spectra of the samples were obtained using Omnic software version 6.0a (Nicolet, Madison, WI). The amide I region 1600 to 1700 cm^{-1} of the spectra (smoothed by seven point smoothing) were baseline corrected and area normalized, then overlaid to compare the spectra. Conformational changes of rhuMAb in dried samples were characterized by measuring the magnitude of change in secondary structure of rhuMAb in amide I region relative to that in solution. To quantitatively compare between the spectra of the different dried preparations, the β-sheet ratio was calculated from the two bands at 1690 cm^{-1} and 1640 cm^{-1} that presumably correspond to different types of β-sheet configurations.[56,58] Additionally, the spectral correlation coefficient (r) was determined to quantify spectral similarity between spectra of dried formulations and that of aqueous solution,[52,58] defined by the following formula:

$$r = \frac{\sum (x_i y_i)}{\sqrt{\sum x_i^2 \sum y_i^2}} \qquad (2)$$

where x_i and y_i are the intensity of the spectra for solid samples and the spectra of protein in solution (native structure) at wave number (i), respectively. %RSD for determination of r from FTIR studies (replicate samples) did not exceed 2.4% (Tab. 1).

Molecular Mobility

Measurement of Structural Relaxation Time by Isothermal Microcalorimetry

Isothermal microcalorimetry studies were performed to measure directly the rate of enthalpy relaxation during aging experiments.[59,60] A thermal activity monitor (TAM) (Thermometric, Sweden), was employed to measure the rate of enthalpy relaxation of some

Table 1. FTIR Studies: Measures of "Native-Like" Structure in the Solid State

Formulation	Treatment	Correlation Coefficient (r)**	β-Sheet Ratio ± SD*
Solution (Reference)			0.28
Medi522:Sucrose 4:1	LYO	0.79	0.81 ± 0.02
	ANNLYO	0.80	0.72 ± 0.02
	Spray dried	0.86	0.60 ± 0.04
	Foam dried	0.82	0.68 ± 0.00
Medi522:Sucrose 4:1 (with Pluronic F68)	LYO	0.82	0.68 ± 0.02
	ANNLYO	0.82	0.72 ± 0.02
	Spray dried	0.89	0.50 ± 0.02
	Foam dried	0.83	0.62 ± 0.02
Medi522:Sucrose 2:1	Spray dried	0.88	0.49 ± 0.01
	Foam dried	0.84	0.61 ± 0.01
Medi522:Sucrose 1:4	LYO	0.90	0.48 ± 0.03
	ANNLYO	0.89	0.49 ± 0.01
	Spray dried	0.93	0.41 ± 0.01
	Foam dried	0.91	0.64 ± 0.08
Medi522:Sucrose 1:4 (with Pluronic F68)	LYO	0.92	0.43 ± 0.01
	ANNLYO	0.90	0.48 ± 0.01
	Spray dried	0.95	0.38 ± 0.01
	Foam dried	0.85	0.61 ± 0.03
Medi522:Sucrose 5:95	LYO	0.94	0.32 ± 0.02
	ANNLYO	0.96	0.38 ± 0.01
	Spray dried	0.99	0.31 ± 0.00
	Foam dried	0.96	0.39 ± 0.02

A high correlation coefficient and/or a low β-sheet ratio means high native-like structure content.

*SD, Standard deviation for multiple measurements.

**Standard deviation for r values did not exceed 0.02.

Spectral correlation coefficients and β-sheet ratios, defined by

$$\beta\text{-sheet ratio} = \frac{\text{Depth of band ca.1690 cm}^{-1}}{\text{Depth of band ca.1640 cm}^{-1}}$$

representative spray dried, foam dried, and freeze dried formulations. TAM experiments were performed at 50°C for at least 60 h. Samples were loaded into stainless steel cylinders in a dry bag purged with dry nitrogen to minimize moisture uptake during loading. Glycine of roughly the same mass as the sample was used as an inert reference for the solid samples to minimize noise. The sample and reference containers were lowered to a thermal equilibrium position in the calorimeter and allowed to equilibrate with the calorimeter for about 30 min. The sample and reference were then slowly lowered into the measuring zone of the calorimeter and power was recorded (μW/g) as a function of time (h). Structural relaxation times were determined for the tested formulations from the power-time data. Structural relaxation times are reported here as

ln τ^β (ln of stretched time) since τ^β is a more robust measure of a relaxation time constant.[59,60] Details of data analysis are described in literature.[59,60]

Fast Dynamics from Incoherent Elastic Neutron Scattering

Elastic incoherent neutron scattering measurements were performed on seven selected preparations at the National Institute of Standards and Technology (NIST) Center for Neutron Research on the High Flux Backscattering Spectrometer (HFBS) on the NG2 beam line.[61] A beam of cold neutrons with a kinetic energy of 2.08 meV ± 50 μeV and a wavelength of 6.27 Å was focused onto the sample of interest. The accessible momentum transfer (Q) range was 0.25–1.75 Å^{-1}. Elastic scattering intensity was recorded as a

Table 2. Surface Properties of Powders and Density Measurements

Formulation	Treatment	Powder Density (g/cm^3)	SSA (m^2/g) $\pm$ SD	Protein Surface Coverage (% w/w) $\pm$ SD	Estimated % of Total Protein on Surface $\pm$ SE*
Medi522:Sucrose 4:1	LYO	1.338	1.22 ± 0.02	87.8 ± 0.5	1.0 ± 0.01
	ANNLYO	**	1.64 ± 0.03	89.3 ± 2.4	1.2 ± 0.01
	Spray dried	1.385	3.04 ± 0.13	78.5 ± 1.7	2.1 ± 0.08
	Foam dried	1.352	1.46 ± 0.03	87.5 ± 0.1	1.1 ± 0.02
Medi522:Sucrose 4:1	LYO	1.349	1.03 ± 0.01	78.8 ± 0.7	0.7 ± 0.01
(with Pluronic F68)	ANNLYO	1.528	1.12 ± 0.04	67.9 ± 1.3	0.8 ± 0.02
	Spray dried	**	3.94 ± 0.18	47.1 ± 0.0	1.7 ± 0.06
	Foam dried	1.337	1.41 ± 0.06	71.3 ± 2.8	0.9 ± 0.04
Medi522:Sucrose 2:1	Spray dried	1.341	2.53 ± 0.02	70.7 ± 0.9	1.8 ± 0.02
	Foam dried	1.384	1.10 ± 0.03	80.3 ± 1.8	0.9 ± 0.03
Medi522:Sucrose 1:4	LYO	1.436	1.33 ± 0.08	45.8 ± 2.0	2.2 ± 0.12
	ANNLYO	1.475	0.60 ± 0.03	52.0 ± 2.1	1.2 ± 0.06
	Spray dried	1.470	2.06 ± 0.11	60.7 ± 2.7	4.6 ± 0.24
	Foam dried	**	0.04 ± 0.01	60.1 ± 2.3	0.1 ± 0.02
Medi522:Sucrose 1:4	LYO	1.577	1.21 ± 0.02	16.3 ± 0.8	0.8 ± 0.03
(with Pluronic F68)	ANNLYO	**	0.81 ± 0.06	21.3 ± 2.9	0.7 ± 0.08
	Spray dried	1.423	1.96 ± 0.07	12.0 ± 0.7	0.9 ± 0.05
	Foam dried	**	0.04 ± 0.01	23.2 ± 1.7	0.03 ± 0.01
Medi522:Sucrose 5:95	LYO	1.573	1.25 ± 0.06	33.9 ± 3.9	6.4 ± 0.58
	ANNLYO	1.598	0.50 ± 0.03	52.3 ± 3.7	4.2 ± 0.27
	Spray dried	1.494	1.68 ± 0.05	51.8 ± 1.0	13 ± 0.34
	Foam dried	1.577	0.17 ± 0.02	54.8 ± 2.0	1.4 ± 0.26

All values based on replicate measurements ($n = 2$–3). An estimate of the total protein surface accumulation on the powder surface is also given, calculated from density, SSA and protein surface coverage (as described in the text).

*SE, standard error calculated from propagation of errors.

**For regions marked, values not determined due to insufficient sample availability. However, rough estimates were made for densities of these powders for calculation of estimated % of total protein on the surface, based on average density values for the different other preparations of the same formulation.

function of Q while the sample was heated at 0.5 K/min from 40 K to 333 K (below the glass transition temperature of the tested formulations). The Q-dependence of the incoherent elastic scattering $I_{\text{inc.}}(Q, \omega = 0)$ was analyzed in terms of the Debye-Waller factor (a harmonic oscillator model), and the hydrogen-weighted mean-square atomic displacement $\langle \mu^2 \rangle$ was determined at different temperatures. Here, we report $\langle \mu^2 \rangle$, in units of Å^2, at 40°C and 50°C. In a number of cases, it has been demonstrated that the amplitude of the mean square atomic displacements scales inversely with log(τ) where τ is the characteristic time for protein degradation in glassy systems.[32,33]

The HFBS accesses wave vectors covering $0.25 \, \text{Å}^{-1} > Q > 1.75 \, \text{Å}^{-1}$ (i.e., length scale range of 3.6 Å to 25 Å). Motions seen at lower Q (length scale range of 3.6 Å to 9 Å) are not well-fit by a harmonic approximation. The concept of atomic motions being represented by a harmonic oscillator is better founded in the high Q range (length scale ranges $>$9 Å). Scattering events that arise from motions occurring on a time scale of approximately 5 nanoseconds are preferentially detected by the HFBS instrument. Only elastic events are directly observed, but total scattering is conserved, so inelastic scattering intensities are easily inferred. Fundamentals of this technique and details of data analysis are described in the literature.[23,32,33,61]

Stability Studies and Assays

HPLC Assay Methodology

Total covalent and noncovalent IgG$_1$ soluble aggregates were measured by size exclusion chromatography (SEC). The LC system consisted of a HP1100 HPLC pump equipped with a UV multiple wavelength detector, an automatic sampling system and an in-line degasser. The LC column used was a TosoHaas TSK Gel Analytical

Column G3000SWXL (5 μ, 7.8 mm × 300 cm I.D.) preceded by a TSK Gel Guard Column SWXL column (7 μ, 6 mm × 4 cm I.D.). The mobile phase consisted of 0.1 mol/L dibasic sodium phosphate, 0.1 mol/L sodium sulfate, and 0.05% by mass sodium azide. pH was adjusted to 6.8 with 1 N HCl. The method was run isocratic at 0.8 mL/min. The wavelength for detection was 280 nm, the column temperature was ambient, and the injection volume was 25 μL. Samples were run in duplicate. The SEC method was tested for reproducibility (inter-vial variability and inter-day variability) and precision (for measuring aggregate formation). %RSD for precision test (multiple injections from the same vial) was not more than 2.7%. %RSD for inter-vial variability was not more than 3%. Additionally, inter-day variability was assessed by storing two different samples (treated as control samples) after initial SEC assay at −20°C for 9 months after which the samples were re-assayed (samples were labeled LYOSB and ANNSE). Assay results for initial time points for the control samples were 1.74 ± 0.03 (#LYOSB) and 1.77 ± 0.04 (#ANNSE). Assay results after 9 months at −20°C for the control samples were 1.70 ± 0.04 (#LYOSB) and 1.66 ± 0.02 (#ANNSE).

Stability Studies and Characterization of Degradation Rates

Aggregate levels (% aggregates) in all formulations were measured by the SEC-HPLC method initially and at three different time points during a 6-month storage stability study. Stability studies were conducted in stability ovens at 40 ± 1°C and at 50 ± 1°C. Two to three vials of each preparation were assayed at each time point.

RESULTS AND DISCUSSION

Moisture Content and Glass Transition

Final adjusted moisture level in all dried preparations was in the range 1–2% by mass, as measured by Karl Fischer titration. None of the dried preparations showed birefringence when examined using polarized light microscopy indicating purely amorphous character.

The drying method did not have a significant impact on glass transition behavior. In protein-rich formulations (i.e., 2:1 and 4:1 IgG$_1$:Sucrose), no glass transition event could be detected by MDSC, consistent with what was observed by others.[28,62,63] In sucrose-rich systems, two major

transitions were observed in the presence and absence of surfactant; a glass transition event with an associated weak enthalpy recovery followed by an exothermic transition most probably due to crystallization (Fig. 1). Glass transition temperatures (midpoint) (T_g) were found in the range ≈55–66°C, the exact value depending on formulation and preparation method and averaged 61.0 ± 4.5°C for all formulations. Differences in heat capacity change at T_g (ΔC_P) were minimal between different drying processes, except for foams. ΔC_P in the foam dried preparations averaged 0.46 ± 0.05 J/(g.°C), but for all other preparations the average was 0.64 ± 0.06 J/(g.°C). The lower ΔC_p at T_g would imply a higher heat capacity in the glassy state for the foam-dried material since the heat capacity above T_g should be the same for all materials. At the present time, we do not have an explanation for this observed effect.

The sucrose-rich spray dried preparation with surfactant showed multiple transitions (Fig. 2) indicative of phase separation, one of the phases being a pluronic-rich phase. The first two transitions coincided with the same transitions of pure pluronic (MDSC thermogram of pure pluronic not shown), followed by a T_g with significantly higher ΔC_P. In the second heat cycle, only one transition (the main glass transition event seen in the 1st heat cycle) was observed. Although MDSC thermogram of a protein-rich spray dried system of 4:1 IgG$_1$:Sucrose did not show a glass transition for a sucrose/protein phase, it did show an endothermic transition possibly originating from pluronic.

Effect of Drying Method (Preparation Method) and Formulation on Particle Morphology (SEM)

In this section and those that follow, the term formulation is used to denote a sample of given chemical composition in the solid, and "preparation" is used to denote a given drying method.

Powders displayed different morphologies, depending mainly on the method of drying and on the disaccharide content. Freeze dried powders were composed of thin plates (Fig. 3A and B). Surfactant-free foam dried powders with high sucrose content were composed of very large thick plates (Fig. 3C). Morphology of the same powders with surfactant was similar (figures not shown). Surfactant-free foam dried powders with high protein content, on the other hand, were composed of much thinner and smaller flakes (Fig. 3D), suggesting a larger SSA. Morphology of the same
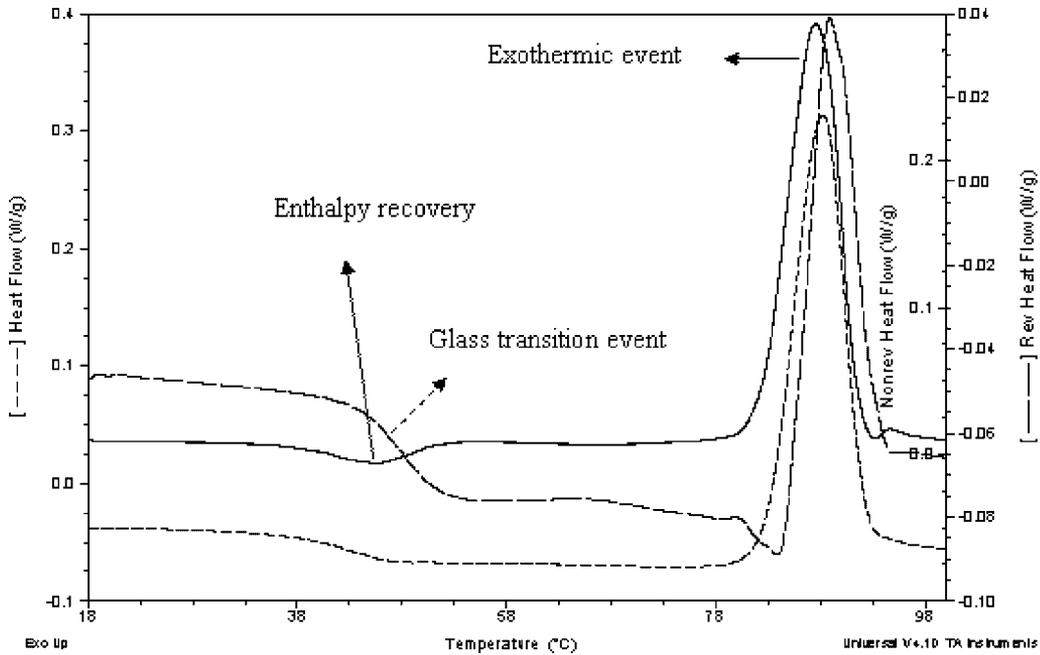
Figure 1. A typical MDSC thermogram for a dried sucrose-rich IgG_1 formulation (freeze dried, spray dried or foam dried) showing three signals (total heat flow, reversing heat flow, and nonreversing heat flow signals).
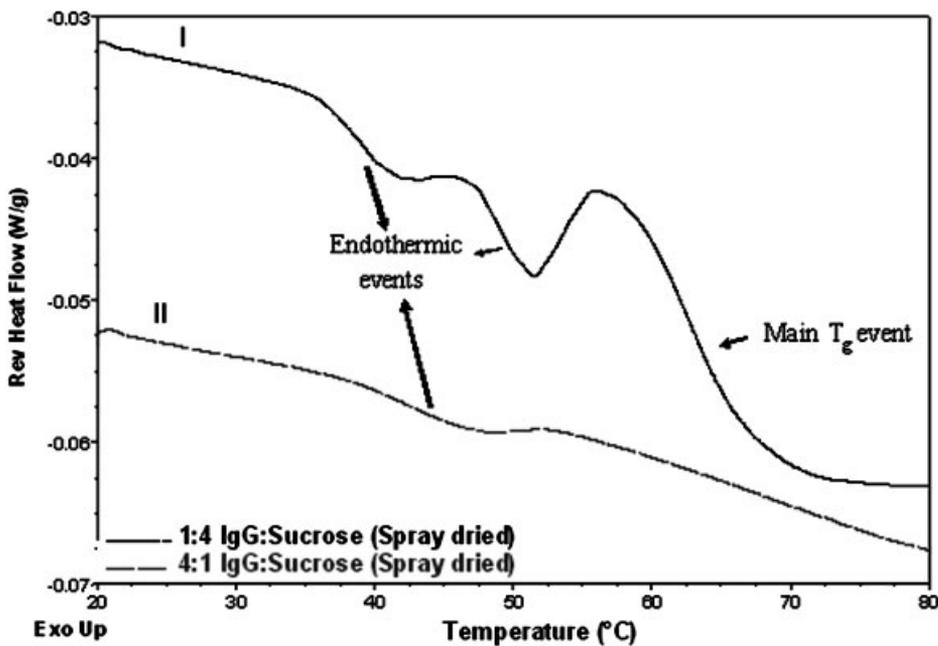
Figure 2. Reversing heat flow signal for spray dried formulations with surfactant: (I) Spray dried 1:4 IgG_1:Sucrose with pluronic—1st heat cycle shows two endothermic events corresponding to surfactant before the main T_g. The two endothermic events were absent in the 2nd heat cycle. (II) Spray dried 4:1 IgG_1:Sucrose with pluronic—1st heat cycle shows an endothermic event attributable to surfactant.
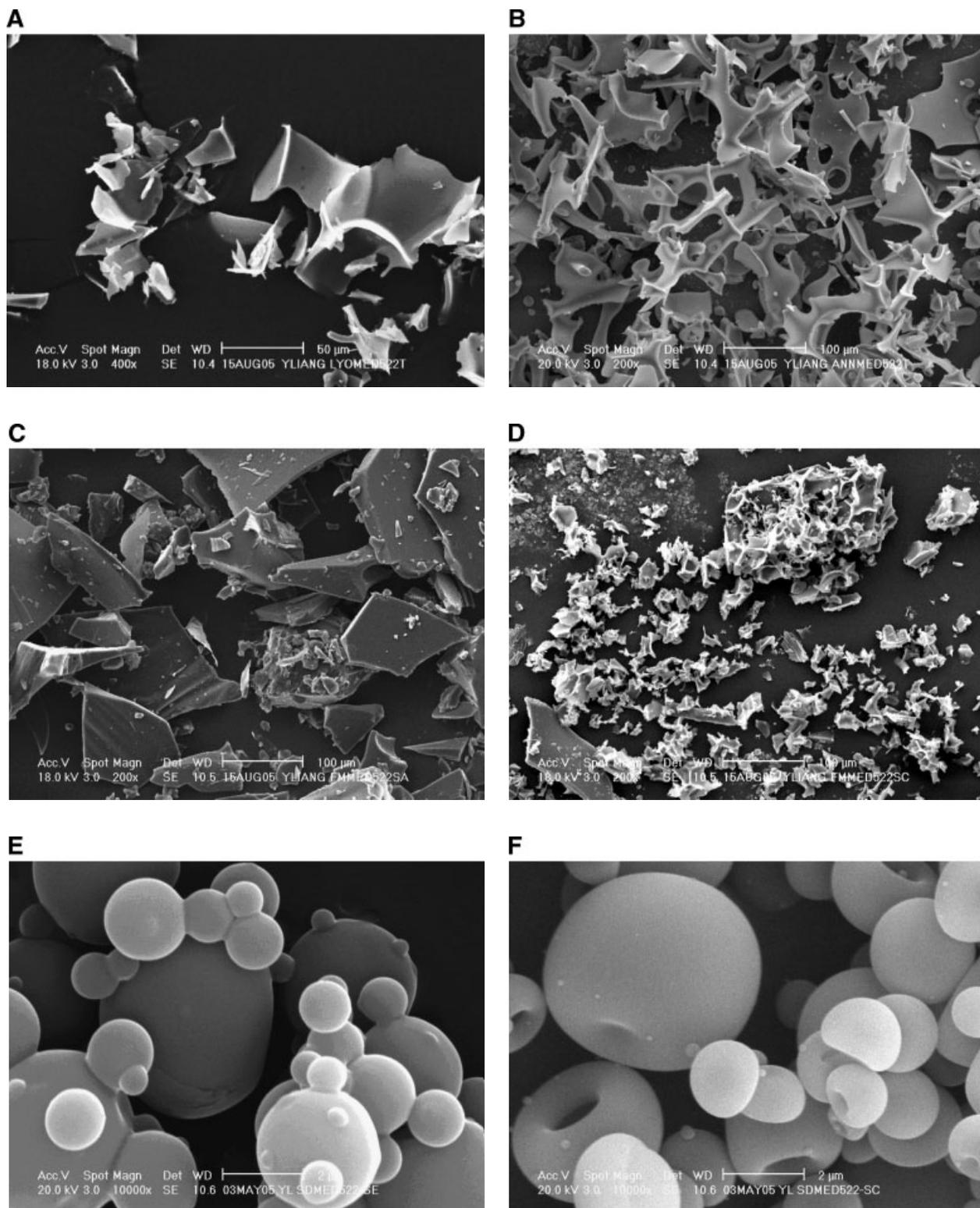
Figure 3. Morphology of dried formulations as observed by SEM (A), A surfactant-free saccharide-rich freeze dried preparation (B), A surfactant-free saccharide-rich freeze dried preparation annealed in the frozen state (C), A surfactant-free saccharide-rich foam (D), A surfactant-free protein-rich foam (E), A surfactant-free saccharide-rich spray dried preparation (F), A surfactant-free protein-rich spray dried preparation. Magnification for each image is given on the SEM panel.

powders with surfactant was similar (figures not shown). The influence of disaccharide content on particle morphology was also apparent with spray-dried powders. Surfactant-free, sucrose-rich spray dried particles were spherical with no dimples. Larger particles were wrinkled and many particles were fused together (Fig. 3E). Particle fusion increased dramatically with a large increase in sucrose content. This "fusion" phenomenon may contribute to a reduction in the measured SSA of the powder. Morphology of the corresponding powders (1:4 IgG$_1$:Sucrose) with surfactant was similar (figures not shown). Surfactant-free, protein-rich spray dried particles were spherical with an abundance of dimples (Fig. 3F). Morphology of these powders did not change upon the addition of surfactant (figures not shown).

## Effect of Drying Method and Formulation on Protein Secondary Structure in the Solid State

Figure 4A and B shows examples of FT-IR spectra of IgG$_1$ in different dried formulations compared to the spectrum of IgG$_1$ (control) in aqueous solution. All data represent area normalized base line corrected second derivative FT-IR spectra in the amide I region 1600–1700 cm^{-1}. The amide I band arises primarily due to the C=O stretching vibration of the peptide bonds of the backbone structure, which is sensitive to changes in protein conformation and secondary structure. In solution, the FTIR spectrum of the rhMAb in the amide I region is dominated by a strong band at 1639 cm^{-1}, the marker band for IgGs arising from the native β structure.[25,28,45,58] Another band at 1690 cm^{-1} is also due to the β structure, while bands at 1665 and 1680 cm^{-1} are due to turn structure.[58] Upon drying IgG solutions, conformational changes (i.e., perturbation in secondary structure) occur, manifested by a decrease in the intensity and broadening of the band at 1639 cm^{-1}, and a shift in the high frequency β-sheet band to ≈1693 cm^{-1} accompanied by an increase in the area of this band.[1,2,20,36,58,64] No intermolecular β-sheet structure at 1622 cm^{-1} was observed, consistent with the findings of Cleland et al.[25] Secondary structure by FTIR is significant because formulations with more native-like structure are expected to have better storage stability than formulations with less native-like structure.[1,2,65]

A good correlation was observed between protein secondary structure and composition of the formulation. As expected,[25,28] preparations with higher sucrose levels showed less perturbation in secondary structure, as evidenced by "more native" appearing spectra (Fig. 4), as well as higher spectral correlation coefficient values (Tab. 1), regardless of the drying method (Tab. 1).

The β-sheet ratio was more sensitive to changes caused by variation in drying method than were the correlation coefficients (Tab. 1). A high β-sheet ratio is indicative of less native structure, and vice versa. Therefore, in our discussion of secondary structure, we will focus on β-sheet ratio to compare small structural differences due to the drying method. The impact of drying method on structure can be summarized as follows:

(1) Regardless of the composition and of the presence of surfactant, spray dried preparations showed more native-like structure than the other preparations.

(2) Regardless of the composition and of the presence of surfactant, annealing in the frozen state did not impact the secondary structure of freeze dried formulations, suggesting that even 48 h of annealing was not sufficient for a significant fraction of the protein to undergo unfolding due to the ice-water interface.

(3) In a protein-rich formulation of 4:1 IgG$_1$:Sucrose, more perturbation of secondary structure was observed with the freeze-dried preparations, as evidenced by higher β-sheet ratios. Upon addition of surfactant to this system, slight improvements in the secondary structure were observed, suggesting the surfactant offered some protective effect against the ice interface. A more significant improvement was observed, however, in the secondary structure of the spray dried preparation. However, given the relatively low fraction of protein on the surface and the limited sensitivity of FTIR to detect minor populations, it is debatable whether the observed correlation between 1690:1639 cm^{-1} ratio and storage stability can be interpreted wholly in terms of structural alteration at the ice:aqueous interface or air:aquous interface. Detection of conformationally altered subpopulation in the surface region would likely require that the surface region be two to three molecules deep rather than one molecule deep as assumed by our reported calculation of "%Total protein on surface" (Tab. 2).
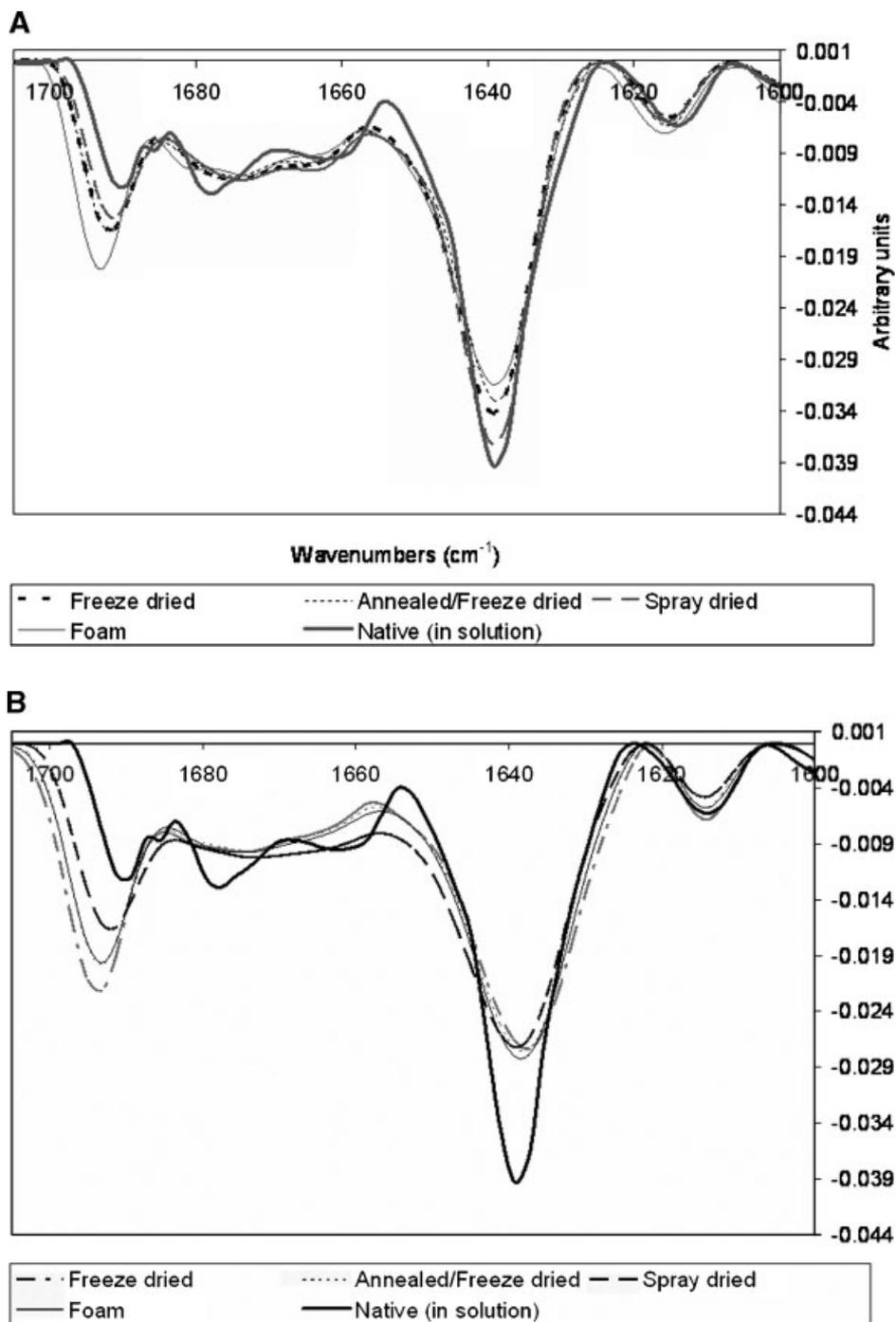
Figure 4.   Second derivative FT-IR spectra in the amide I region for preparations of the 1:4 IgG$_1$:Sucrose formulation (Fig. 3A) and the 4:1 IgG$_1$:Sucrose formulation (Fig. 3B).

While possible, we have no evidence for a surface region several molecules deep.

(4) In a sucrose-rich formulation of 1:4 IgG$_1$: Sucrose, the foam dried preparation showed the greatest perturbation in secondary structure. Upon addition of surfactant to this system, little improvement in the secondary structure was observed and the order for the conformational changes remained the same (Foam dried > All freeze

dried > Spray dried). In a 5:95 IgG_1:Sucrose system, differences between freeze dried and foam dried preparations were small.

Effect of Drying Method and Formulation on Specific Surface Area (SSA), Surface Composition, and Powder Density

Specific Surface Area (SSA)

An effect of sucrose level on SSA was observed only with foams and spray dried powders: increasing SSA was associated with an increase in IgG_1 level (Tab. 2). It is possible that particle fusion in spray dried powders (Fig. 3E) contributed to the reduction in the SSA with increasing sucrose level.

Drying method also had a large impact on SSA (Tab. 2). Regardless of the composition and of the presence of surfactant, spray dried preparations had the largest SSA.

We note that contrary to expectations, an annealing treatment during freezing did not significantly reduce SSA in a protein-rich system of 4:1 IgG_1:Sucrose. While there is no report in the literature on the effect of annealing on the SSA of protein-rich formulations, there are reports on SSA changes upon annealing stabilizer-rich systems[66–69] ling normally allows larger ice crystals to grow at the expense of small ones in a process called Ostwald ripening, thereby resulting in a product with a significantly lower SSA than the same formulation with no annealing treatment[66–69] in protein-rich systems where foam dried powders had SSA similar to freeze dried powders; the SSA of foam dried stabilizer-rich powders was extremely low compared to the other preparations. Annealing in the frozen state prior to primary drying resulted in a lowering of the SSA of the final freeze-dried powder (Tab. 2). Small changes were observed in SSA of all preparations of the 1:4 IgG_1:Sucrose system upon addition of surfactant, and the order of decrease in SSA remained the same.

Protein Surface Coverage

From an analysis of the relative amount of the different elements in the pure components and in a multi-component powder, one can determine the molecular composition in the surface composition region (i.e., surface coverage for each component).[37,41,42,47,51] ESCA measurements showed that pure IgG_1 is composed of approximately 70% by mass C, 15.2% by mass N, and 14.8 mass % O. Both sucrose and pluronic contain only

C and O. The N peak, therefore, is a direct measure of the presence of IgG_1 in the outer 50 Å surface region,[36,51] and hence it is possible to determine the protein surface coverage, also termed protein surface concentration.[37,41,42,51] Surfactant-free formulations with 4:1, 2:1, 1:4, and 5:95 IgG_1:Sucrose are loaded with 80, 66.7, 20, and 5% by mass IgG_1, respectively. Thus, it follows that the protein surface coverage is ≈ 80, 67, 20, and 5 mass % IgG_1 if IgG_1 were homogeneously distributed in the corresponding formulations. Similarly, the surface layer of 4:1 and 1:4 IgG_1:Sucrose formulations with surfactant should contain approximately 77% by mass and 19% by mass IgG_1, respectively.

Protein surface coverage was impacted strongly by formulation composition and the presence of surfactant (Tab. 2). As expected and as is consistent with literature, protein surface coverage increased as protein weight fraction increased,[37–39,51] irrespective of the drying method. Protein surface coverage significantly decreased upon the addition of surfactant, similar to what was observed previously.[40–42] Additionally, protein surface coverage showed either a surface excess (i.e., more protein than would be expected for a homogeneous sample) or surface deficiency (i.e., less protein than would be expected for a homogeneous sample). Therefore, it appears that component separation occurred during drying, and that the most surface active component in solution (assumed to be protein in surfactant-free formulations, and surfactant in formulations containing pluronic) enriched the surface of the drying solutions and hence the dried powders.[37,39–42,70,71]

Drying method did not have a strong impact on protein surface coverage. The main impact of drying method could be seen when comparing sucrose-rich freeze dried formulations annealed in the frozen state versus no annealing treatment. Annealing in the frozen state resulted in an increase in protein surface coverage. Similar results were obtained by Fureby et al.[39]

Total Protein Surface Accumulation

Total protein surface accumulation reflects how much of the total protein (mass fraction) added to the formulation has accumulated at the surface of the powders (Tab. 2). Total protein surface accumulation is an important parameter since this environment is potentially a more reactive state for the protein. ESCA studies

have traditionally only reported protein surface coverage. Surface coverage is a good indicator of the degree of homogeneity but does not measure the fraction of the protein in the sample, which has accumulated at the surface. For example in a formulation with high protein surface coverage but very low SSA, the fraction of protein at the surface would be very small and the stability implications would therefore be small.

Total protein surface accumulation was impacted mainly by drying method. Regardless of the composition and of the presence of surfactant, spray dried preparations had the largest total protein surface accumulation, largely as a result of their high SSA. In protein-rich systems (with and without surfactant), differences in total protein surface accumulation between powders were modest. Drying method had more of an impact on total protein surface accumulation in surfactant-free sucrose-rich formulations, where total protein surface accumulation decreased in the following order: Spray dried > Freeze dried powder (no annealing treatment) > Freeze dried powder annealed prior to lyophilization ≫ Foam dried powder. Unlike protein-rich formulations, the total protein surface accumulation was lowest with sucrose-rich foam dried powders, as a direct result of their very low SSA. Addition of surfactant in the 1:4 IgG_1:Sucrose preparations decreased total protein surface accumulation. Annealing sucrose-rich systems in the frozen state prior to primary drying resulted in a lower SSA and lower total protein surface accumulation, even though annealing caused an increase in protein surface coverage.

Effect of Drying Method and Formulation on Molecular Mobility

Relaxation times (τ^β at 50°C) and the amplitude of fast motions ($\langle\mu^2\rangle$ at 40°C and 50°C—temperatures for storage stability studies) for some selected formulations are shown in Table 3. Formulation did not have a measurable impact on global (slow) motions in the systems studied, but did have a significant impact on the amplitude of local motions (i.e., fast dynamics) (Tab. 3). Relaxation time—τ^β (h)—may be regarded as proportional to the inverse of molecular mobility for global motions.[22,72] The higher the τ^β value, the lower the molecular mobility and vice versa. The neutron scattering parameter, $\langle\mu^2\rangle$ (in units of Å^2), is a measure of the amplitude of fast motions. The higher this value is, the greater

the mobility for motions on the nanosecond time scale.

Global Motions

Figure 5 shows the power data from isothermal microcalorimetry experiments at 50°C as a function of time for preparations of the 1:4 IgG_1:Sucrose formulation. Note that with the saccharide-rich foam, an initial endothermic response was observed that dissipated after approximately 4–5 h followed by a normal exothermic response that decayed with time. The same was observed with the 5:95 IgG_1:Sucrose foam. We are not certain of the origin of this initial endothermic response and did not include this range of data for estimation of τ^β. Data were analyzed starting at the 5 h time point for sucrose-rich foams. Additionally, different portions of the exothermic response starting at several time points ≥5 h were analyzed to determine consistency of τ^β values obtained by the analysis. τ^β values were essentially the same for all data sets. For example, with a 5:95 IgG_1:Sucrose foam: τ^β value from $t = 5$–60 h was 14.9 h, from $t = 8$–60 h was 14.7 h, from $t = 10$–60 h was 15.5 h and from $t = 15$–60 h was 15.2 h. The endothermic response was not observed with protein-rich foams.

Within the uncertainties in the data and given the composition range explored, composition did not have a significant impact on τ^β values for spray dried and freeze dried formulations ($P > 0.1$ from Bonferroni test). Chang et al.[28] reported a maximum value for τ^β at ≈ a 1:1 ratio of sucrose to protein (recombinant human serum albumin and a monoclonal antibody). However, we did not work with sugar-protein compositions in the middle of the composition spectrum, so it is possible that the lack of composition effect in our data was simply due to the fact that we did not have data in the range of the maximum. On the other hand, composition did impact τ^β values for foams. Sucrose-rich foams showed significantly higher τ^β values than the protein-rich foam.

The impact of drying method on τ^β values depended on composition. No significant differences were observed between τ^β values of the different preparations of 4:1 IgG_1:Sucrose. In sucrose-rich systems, however, the foams had the highest τ^β values, followed by spray-dried powders and were lowest with the freeze-dried preparations. Annealing during freezing did not impact the τ^β values for the 1:4 IgG_1:Sucrose freeze-dried preparation. In the presence of surfactant, the

Table 3. Molecular Mobility Studies: (1) TAM Experiments (for Global Motions) to Evaluate τ^β (h) of Some Lyophilized (LYO), Lyophilized Annealed in the Frozen State Prior to Primary Drying (ANNLYO), Spray Dried and Foam Dried Preparations of Different Formulations

Formulation	Treatment	Global Motions (from TAM Studies at 50°C)	Fast Dynamics (from Neutron Scattering Studies)		
		$T_g - T^*$	τ^β (h) $\pm$ SD	$\langle \mu^2 \rangle$ (Å)^2 at 40°C	$\langle \mu^2 \rangle$ (Å)^2 at 50°C
IgG1 (pure)***	LYO	X**	1.22		
Medi522:Sucrose 4:1	LYO	X**	1.64 ± 0.36	0.221	0.232
	Spray dried	X**	1.80 ± 0.21		
	Foam dried	X**	1.52 ± 0.03		
Medi522:Sucrose 1:4	LYO	14.3	0.97 ± 0.04	0.131	0.138
	ANNLYO	6	0.96 ± 0.03	0.127	0.135
	Spray dried	15.5	1.97 ± 0.24	0.155	0.164
	Foam dried	14.4	17.2 ± 2.8	0.079	0.083
Medi522:Sucrose 1:4 (with Pluronic F68)	LYO	10.3	0.89 ± 0.02		
	Spray dried	13.5	9.18 ± 0.26		
	Foam dried	13.9	7.65 ± 1.24		
Medi522:Sucrose 5:95	LYO	12.1	0.86 ± 0.05	0.108	0.125
	Spray dried	15.5	2.29 ± 0.34		
	Foam dried	8.4	16.6 ± 1.4	0.138	0.151
Sucrose	LYO	14		0.109	0.121
	Spray dried	12	2.40 ± 0.42		

β values for foam dried preparations (sucrose-rich) was in the range of 0.7–0.9. For all other preparations, β values ranged from (0.2 to 0.3) (including the protein-rich foam). (2) Incoherent elastic neutron scattering (NS) studies (for fast amplitude motions) to evaluate $\langle \mu^2 \rangle$ (Å)^2 at 40°C and 50°C.

(For gray shaded areas, values not determined).

*T is the temperature at which TAM experiments were performed (i.e., 50°C).

**It was not possible to measure a T_g for these protein-rich systems using MDSC. Using thermally stimulated current (TSC), a T_g of ~80°C was obtained for the 4:1 IgG$_1$:Sucrose system (1% moisture). Therefore, $T - T_g$ ~30°C is a good first approximation for protein-rich systems.

***Moisture content of pure freeze dried IgG$_1$ was 0.51% w/w (i.e., AT LEAST 0.5% lower moisture content than all other freeze dried formulations). Also reported τ^β value of the pure freeze dried IgG$_1$ is for a single run.

1:4 IgG$_1$:Sucrose foam had lower τ^β values than the spray dried preparation, an unexpected observation. However, we note that DSC evidence for phase separation was observed for the latter preparation. Perhaps phase separation had an effect on τ^β, although the mechanism for this postulated effect is far from obvious.

Fast Dynamics

Figure 6 shows $\langle \mu^2 \rangle$ values as a function of temperature for different preparations of the 1:4 IgG$_1$:Sucrose formulation. Note that, as expected, $\langle \mu^2 \rangle$ increases with increasing temperature. At 313 and 323 K, $\langle \mu^2 \rangle$ values increase as protein fraction increases in freeze dried formulations (Tab. 3). Just as formulation impacted fast dynamics, so did the drying method. In a 1:4 IgG$_1$:Sucrose formulation, the order of decrease of $\langle \mu^2 \rangle$ values at both 313 K and 323 K was: Spray dried > Freeze dried ≈ Freeze dried annealed in the frozen state > Foam. Therefore, since the molecular mobility of the 1:4 IgG$_1$: Sucrose foam is lowest by both measures, one would expect this foam to have superior stability relative to the other preparations, provided mobility differences dominate stability differences. Note that this prediction is opposite to the predictions made earlier using FTIR results. It is most curious that for the 5:95 IgG$_1$:Sucrose system, the foam had faster local dynamics at both 313 K and 323 K than the same formulation freeze dried.

Relationship between Global Motions and Fast Dynamics

Overall, there was not a reasonable correlation between fast dynamics and global motions, either
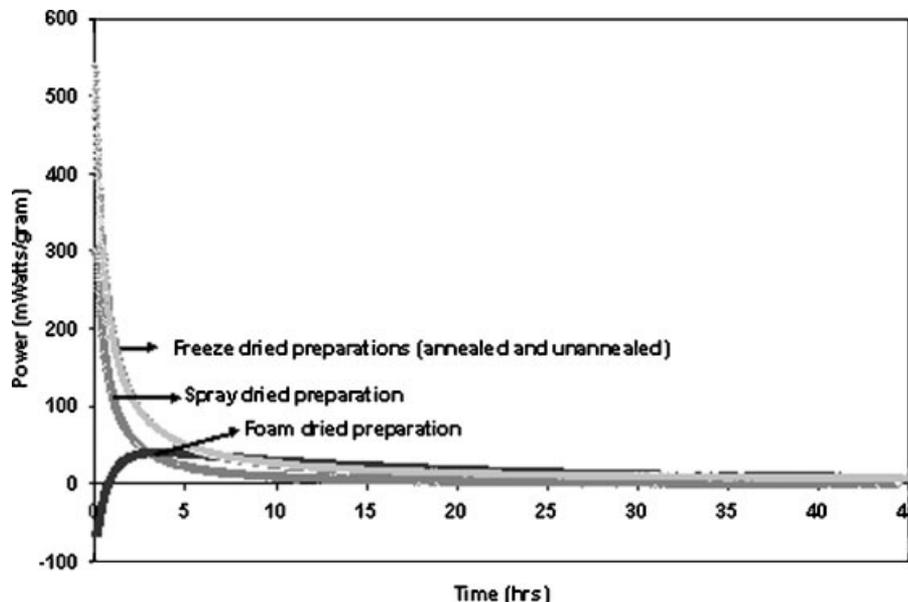
Figure 5. Plot from TAM experiments at 50°C for preparations of the 1:4 IgG_1:Sucrose formulation showing power (mw/g) as a function of time (h).

with the variable being composition (at least in freeze dried powders, Figure 7) or drying method (Tab. 3). In fact, the data in Figure 7 show that as protein fraction increases, amplitude of fast dynamics increases (i.e., greater mobility) but global mobility (i.e., reciprocal of τ^{β}) apparently goes through a minimum. Clearly, fast dynamics and global dynamics are at least partially decoupled for these samples. The study of different types of molecular mobility and their impact on stability is currently attracting much interest.
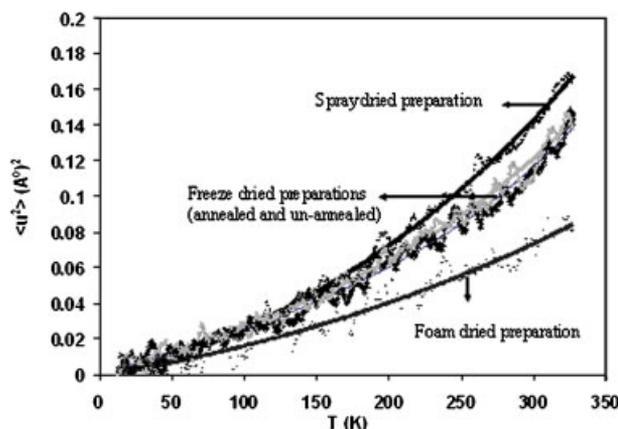
Figure 6. Plots for Debye-Waller factors from incoherent elastic neutron scattering for preparations of the 1:4 IgG_1:Sucrose formulation showing $\langle \mu^2 \rangle$ as a function of temperature (K).

Even though different types of mobility have been studied[22,73] and some studies have examined the correlation between a given type of mobility and storage stability[23,33,34], data that examine the variation of both types of mobility and stability in a controlled series of samples is unique to this research.

In-Process Stability

The effect of drying method on initial physical aggregation of IgG_1 (i.e., In-process physical stability), as assayed by size exclusion chromatography, is summarized in Table 4. In-process aggregation showed only a small correlation to composition. Protein-rich formulations (4:1 and 2:1 IgG_1:Sucrose), regardless of the drying method used, showed slightly higher aggregate levels after drying than sucrose-rich formulations. Spray drying, a process known to be damaging to many proteins vulnerable to the air–water interface,[36,74] did not seem to be damaging to the IgG_1, consistent with the preservation of secondary structure as measured by FTIR noted earlier.

Storage Stability (Physical Stability) and Physico-Chemical Properties

Degradation kinetics in amorphous pharmaceuticals is often described by "square root of time"
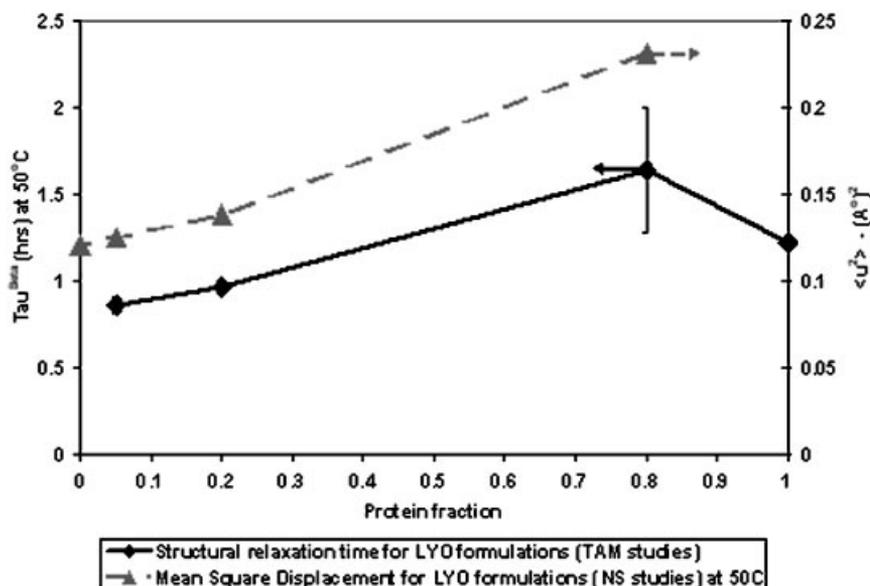
Figure 7. The correlation of protein fraction with global motions at 50°C (in freeze dried formulations and spray dried formulations) and fast dynamics at 50°C (in freeze dried formulations).

Table 4. In-Process Degradation of IgG$_1$ in All Formulations and Rate Constants for Physical Aggregation—k (%P/month$^{0.5}$)—after storage stability studies at 40 and 50°C. Initial aggregate level in solutions pre-dried was (1.72 ± 0.04)%.

Formulation	Treatment	Initial Levels of Aggregation (In-Process) $\pm$ SD*	k at 40°C $\pm$ SE** (%P/$\sqrt{month}$)	k at 50°C $\pm$ SE** (%P/$\sqrt{month}$)
Medi522:Sucrose 4:1	LYO	2.08 ± 0.01	6.4 ± 0.2	12.9 ± 0.2
	ANNLYO	2.00 ± 0.01	6.4 ± 0.2	13.2 ± 0.2
	Spray dried	2.62 ± 0.03	5.5 ± 0.2	12.1 ± 0.2
	Foam dried	2.26 ± 0.03	5.5 ± 0.2	11.5 ± 0.2
Medi522:Sucrose 4:1 (with Pluronic F68)	LYO	1.91 ± 0.01	5.4 ± 0.4	11.4 ± 0.4
	ANNLYO	1.91 ± 0.03	5.0 ± 0.4	10.7 ± 0.4
	Spray dried	2.02 ± 0.01	5.4 ± 0.5	12.6 ± 0.4
	Foam dried	2.57 ± 0.46	5.5 ± 0.4	10.7 ± 0.4
Medi522:Sucrose 2:1	Spray dried	2.03 ± 0.01	2.7 ± 0.2	6.8 ± 1.3
	Foam dried	2.21 ± 0.01	2.2 ± 0.2	7.9 ± 1.3
Medi522:Sucrose 1:4	LYO	1.79 ± 0.00	0.11 ± 0.01	0.31 ± 0.02
	ANNLYO	1.82 ± 0.01	0.05 ± 0.01	0.27 ± 0.02
	Spray dried	1.74 ± 0.01	0.08 ± 0.01	0.29 ± 0.02
	Foam dried	1.85 ± 0.05	≤ 0.01	0.20 ± 0.02
Medi522:Sucrose 1:4 (with Pluronic F68)	LYO	1.74 ± 0.03	0.09 ± 0.01	0.24 ± 0.06
	ANNLYO	1.74 ± 0.02	0.07 ± 0.01	0.32 ± 0.06
	Spray dried	1.69 ± 0.03	0.22 ± 0.02	1.13 ± 0.07
	Foam dried	1.78 ± 0.01	0.04 ± 0.02	0.22 ± 0.07
Medi522:Sucrose 5:95	LYO	1.79 ± 0.03	≤ 0.01	0.02 ± 0.01
	ANNLYO	1.77 ± 0.04	≤ 0.01	0.08 ± 0.01
	Spray dried	1.71 ± 0.01	0.15 ± 0.02	0.20 ± 0.02
	Foam dried	1.74 ± 0.02	≤ 0.01	0.05 ± 0.02

Overall, the time dependence of degradation was more consistent with square-root-of-time kinetics. The F value for first order kinetics was 304 versus 1063 for square-root-of-time kinetics at 40°C and 165 versus 1018 at 50°C.

*SD, Standard deviation from two or three vials.

**SE: Standard error for k value (%P/month$^{0.5}$) estimated from multiple linear regression using a GLM model.

or "stretched exponential" kinetics[24,28,59,75,76], similar to the time dependence shown by relaxation kinetics. For our data, a statistical analysis based on F-values showed that the model with square-root of time kinetics fit the IgG_1 stability data more appropriately than did first order kinetics (Tab. 4). The percent aggregates, $\%P$, followed the linear equation[75]:

$$\%P = P_0 + k\sqrt{t} \qquad (3)$$

where P_0 is the initial level of aggregates and k is the apparent rate constant for physical aggregation on the stretched time scale. Multiple linear regression was done using a general linear model (GLM) to plot and fit the assay data to the model (Eq. 3). Bonferroni tests were performed to determine if differences between physical aggregation rate constants due to drying methods are statistically significant. Additionally, paired t-tests were performed to test if differences due to the presence or absence of surfactant are statistically significant.

Results for physical storage stability studies are summarized in Table 4. Overall, differences in stability between preparations of the same formulation due to varying drying method were small. Additionally, it is obvious that in most cases 40°C was not high enough a temperature to reveal small stability differences between different preparations of the same formulation. On the other hand, more conclusive information was obtained from the 50°C data.

Effect of Formulation

As expected and as is consistent with literature, stability of IgG_1 in the solid state showed a log-linear dependence on composition.[25,28,44] Regardless of the drying method, stability of the formulations increased with sucrose weight ratio. In freeze-dried formulations, the storage stability of IgG_1 significantly improved (at both 40°C and 50°C) as the weight fraction of stabilizer increased. This trend correlates with an improvement in protein secondary structure in the solid state, a decrease in mass fraction of protein at the surface of the formulations, a decrease in molecular mobility as measured by fast dynamics and an increase in density in sucrose-rich systems (Fig. 8, Tab. 2). The latter is the inverse of specific volume, which is directly related to free volume. Therefore, one would expect a lower free volume as sucrose weight fraction increases, assuming mean atomic masses are the same (calculated to be approximately 7.6 for pure sucrose and approximately 8 for pure IgG_1). Moreover, one would expect stability to improve with a decrease in the free volume. There was no correlation between improvement in stability and global
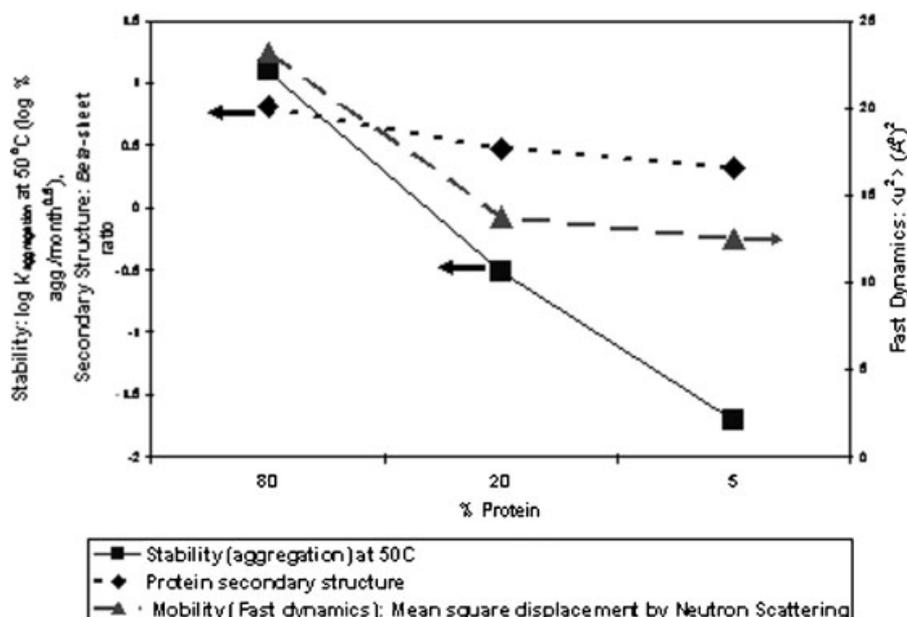
Figure 8. Relationship of stability (aggregation rate constants) in freeze dried systems of IgG_1:Sucrose at 50°C to physico-chemical properties: Stability correlated with molecular mobility (fast dynamics) and protein secondary structure.

motions, unlike what has been observed with other protein systems,[26,28] but this lack of sensitivity to global motions may be a result of the limited number of compositions investigated and the existence of a minimum in global mobility at a concentration we did not study.[28] A similar stability trend was observed with both spray-dried and foam-dried preparations.

The effect of surfactant varied. In a protein-rich system of 4:1 IgG$_1$:Sucrose, the stability of both freeze dried preparations improved with surfactant which coincided with a slight improvement in secondary structure, as well as a significant decrease in protein surface coverage. However, there was no significant change in the stability of the spray dried or foam dried preparations even though surfactant addition was associated with significantly lower protein surface coverage and improved secondary structure. Molecular mobility in these systems was not measured. In a sucrose-rich system of 1:4 IgG$_1$:Sucrose, the stability of the foam and both freeze dried preparations were not significantly different than the same preparations without surfactant. The spray dried preparation with surfactant showed inferior stability, as compared to the same preparation without surfactant, perhaps as a result of phase separation.

Effect of Drying Method

Drying method impacted protein storage stability in different ways, depending on composition. An important finding from storage stability studies on all systems studied with and without surfactant is that there were, at most, minor differences observed between the storage stability of freeze dried formulations annealed in the frozen state and those with no annealing treatment. Note that annealing caused an increase in protein surface coverage, possibly due to increased molecular mobility during annealing above T'_g, thereby permitting more protein molecules (which are surface active) to migrate to the ice−water interface. Therefore, it is expected that conditions become more favorable for protein unfolding due to higher mobility above T'_g and exposure to the ice surface for the duration of annealing. However, FTIR studies showed that annealed formulations did not undergo a significant change in secondary structure during annealing. Moreover, one must also recognize that annealing produces lower SSA, resulting in a lower total protein surface accumulation, which

should stabilize. A study by Tang et al.[77] showed a relatively high coupling between system viscosity and unfolding rate, especially as stabilizer ratio increased. That is, as viscosity increases, unfolding slows greatly. In the freeze concentrated systems studied, even well above T'_g, protein unfolding was very slow on the time scale of freeze drying. Calculations showed that even at 20°C above T'_g, the estimated half-life for protein unfolding of the two proteins studied was on the time scale of years. Other studies have also reported that annealing did not have a damaging effect on protein secondary structure.[36,78] Here, we show that not only did annealing not alter protein secondary structure, but also annealing did not cause appreciable deterioration in storage stability.

In a 1:4 IgG$_1$:Sucrose system, the foam was the most stable preparation at both 40°C and 50°C, even though foam dried material underwent more perturbation in secondary structure than the other preparations. This improvement in stability correlated with a decrease in molecular mobility on both scales (global motions and fast dynamics), along with a significant reduction in both SSA and total protein surface accumulation (Fig. 9A). Similar stability for spray-dried and freeze-dried preparations correlates somewhat better with fast dynamics (i.e., similar amplitudes for spray dried and freeze dried samples) than global dynamics (i.e., higher mobility for freeze dried than spray dried samples).

In the 5:95 IgG$_1$:Sucrose system, the composition at which chemical heterogeneity effect is expected to be large,[79] there was a rough correlation between storage stability and total protein surface accumulation (Fig. 9B). Based on structural relaxation time values, one would expect the foam (which had the highest τ^β value) to be the most stable followed by the spray-dried preparation followed by both freeze dried preparations (the un-annealed freeze dried preparation the lowest τ^β value). Based on protein secondary structure, one would expect the spray-dried preparation to be the most stable. In fact, the spray dried preparation was the least stable preparation at both 40°C and 50°C. Lower stability with the spray-dried preparation correlated with its higher total protein surface accumulation. While total protein surface accumulation was higher for the freeze-dried preparations than the foam, stability differences between these three preparations were small. Fast dynamics studies suggest that the freeze-dried preparation (no annealing) had
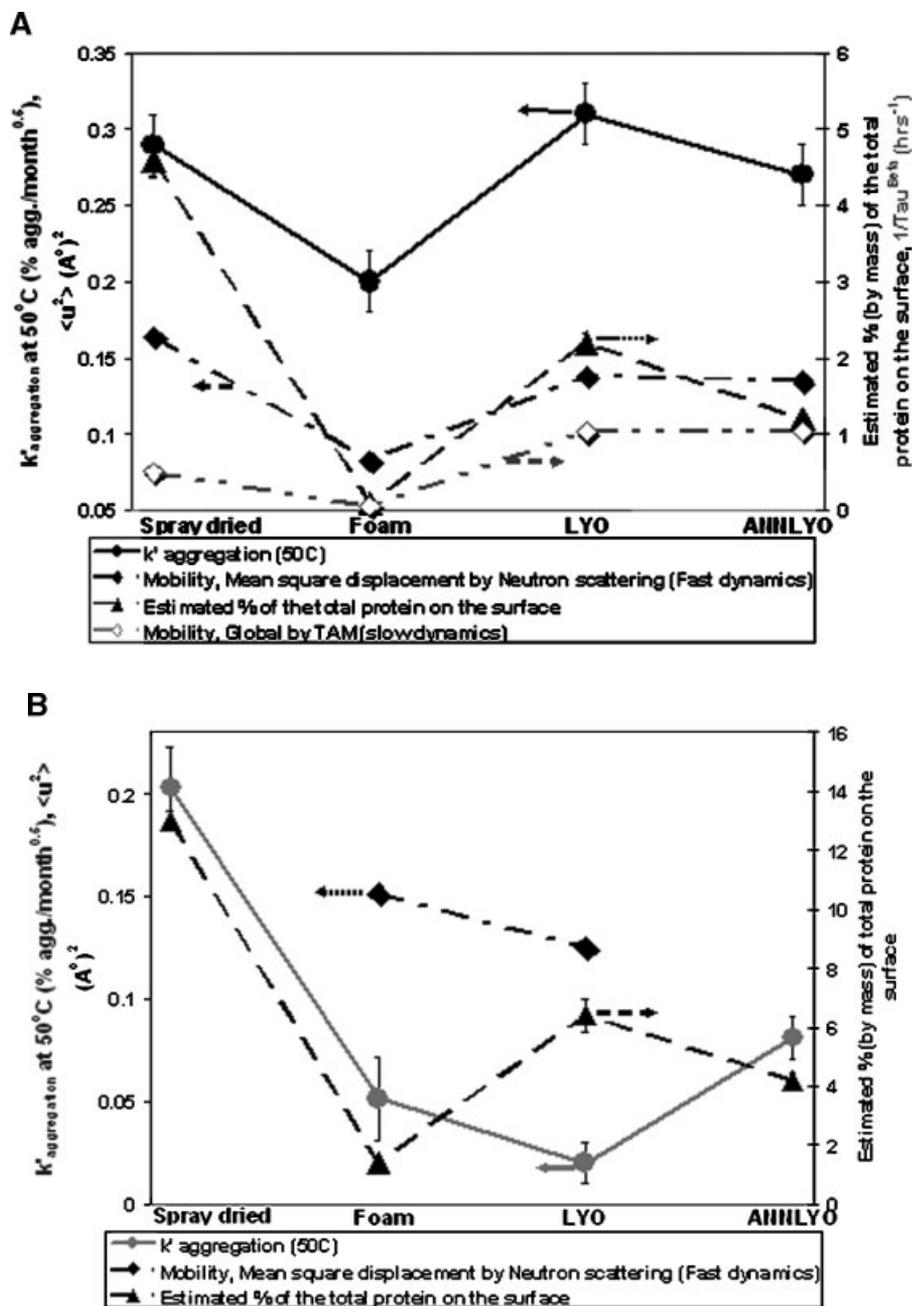
Figure 9. Relationship of stability (aggregation rate constants) at 50°C to physico-chemical properties: Stability correlated with (A) molecular mobility (global motions and fast dynamics) and total protein surface accumulation in 1:4 IgG$_1$:Sucrose and (B) molecular mobility (fast dynamics) and total protein surface accumulation in 5:95 IgG$_1$:Sucrose. (The connecting lines in A–B are for the reader's convenience only and do not represent interpolations between points).

slightly less molecular mobility than the foam. The preceding observation suggests that fast dynamics, which is not always well correlated to the viscosity of the glass, represents an additional dimension to consider in the relationship between

mobility and stability. In the 4:1 IgG$_1$:Sucrose protein-rich system, both lyophilized preparations showed poor stability relative to the spray-dried and foam-dried preparations. This poorer stability did correlate with greater perturbation

in protein secondary structure (Tabs. 2 and 4), but did not correlate with molecular mobility and total protein surface accumulation (Tabs. 3 and 4).

In both the 1:4 and 4:1 IgG_1:Sucrose systems with surfactant, the spray dried preparation was the least stable, but differences in stability between all 3 other preparations were small. Lower stability correlated roughly with a higher total protein surface accumulation, but it is curious that the higher instability for spray-dried materials was not the observation in systems without surfactant. One would expect the addition of surfactant would have the greatest stabilization effect for spray dried material, and therefore if stability for spray dried material is comparable without surfactant, addition of surfactant should lead to spray dried material being the most stable.

CONCLUSIONS

Formulation and drying method both influenced the physical stability of IgG_1. Stability of IgG_1 improved as sucrose fraction increased, regardless of the drying method. The impact of drying method on stability varied depending on composition. In general, annealing during freezing did not have a significant impact on storage stability of freeze-dried samples. In a protein-rich system, differences in stability between preparations correlated with protein secondary structure. In sucrose-rich systems, differences in stability between preparations correlated with differences in molecular mobility from fast dynamics (rather than molecular mobility associated with global motions) and also correlated roughly with total protein surface accumulation.

ACKNOWLEDGMENTS

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