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RESEARCH PAPER

Effect of pH and Excipients on Structure, Dynamics, and Long-Term Stability of a Model IgG1 Monoclonal Antibody upon Freeze-Drying

Jihea Park • Karthik Nagapudi • Camille Vergara • Ranjini Ramachander • Jennifer S. Laurence • Sampathkumar Krishnan

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ABSTRACT

Purpose To investigate the mechanism of IgG1 mAb stabilization after freeze-drying and the interdependence of protein structural preservation in the solid state, glassy state dynamics and long-term storage stability under different formulation conditions.

Methods IgG1 mAb was formulated with mannitol at pH 3.0, 5.0, and 7.0 in the presence and absence of sucrose and stability was monitored over 1 year at different temperatures. Physical and covalent degradation of lyophilized formulation was monitored using SEC, CEX, and light obscuration technique. Secondary and tertiary structure of the protein in the solid state was characterized using FTIR and fluorescence spectroscopy respectively. Raman spectroscopy was also used to monitor changes in secondary and tertiary structure, while SS-NMR ^{1}H relaxation was used to monitor glassy state dynamics.

Results IgG1 mAb underwent significant secondary structural perturbations at pH 3.0 and conditions without sucrose, while pH 5.0 condition with sucrose showed the least structural change over time. The structural changes correlated with long-term stability with respect to protein aggregate formation and SbVP counts. SS-NMR data showed reduced relaxation time at conditions that were more stable.

Conclusions Native state protein structural preservation and optimal solid-state dynamics correlate with improved long-term stability of the mAb in the different lyophilized formulations.

KEY WORDS IgG1 antibody lyophilization · protein formulation · protein structure · solid state NMR · solid state stability

ABBREVIATIONS

CEX	cation-exchange chromatography
FTIR	fourier transform infrared spectroscopy
HMWS	high molecular weight species
HPLC	high performance liquid chromatography
LMWS	low molecular weight species
mAb	monoclonal antibody
RWC	residual water content
SbVP	sub-visible particle
SEC	size-exclusion chromatography
SS-NMR	solid state nuclear magnetic resonance.
T_g	glass transition temperature
XRD	X-ray diffraction

INTRODUCTION

Many therapeutic proteins and other biotechnology products are not stable in aqueous solution due to degradation over shelf storage. Physical and chemical degradation of

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J. Park • C. Vergara • R. Ramachander • S. Krishnan
Amgen Inc., Process and Product Development
Thousand Oaks
California 91320, USA

K. Nagapudi
Amgen Inc.,
Small Molecule Process and Product Development
Thousand Oaks, California 91320, USA

J. S. Laurence
Department of Pharmaceutical Chemistry, University of Kansas
2030 Becker Drive, MRB 306
Lawrence, Kansas 66047, USA

Present Address:
S. Krishnan (✉)
Hospira Inc., 275 North Field Drive, Bldg H3-3N
Lake Forest, Illinois 60045, USA
e-mail: krishnan.sampathkumar@hospira.com

therapeutic biopharmaceuticals upon long term storage is of fundamental concern because of the potential impact on immunogenicity, toxicity, and efficacy *in vivo* (1,2). Freeze-drying or lyophilization has become the method of choice for long term storage stabilization of such proteins that are otherwise unstable in liquid (1,2). Also, the biotechnology industry is currently exploring freeze-drying as a mitigation strategy to inhibit proteinaceous particles for certain therapeutic fusion proteins and monoclonal antibodies which otherwise form particles in the liquid state. Although lyophilization is used to reduce the rate of chemical and physical degradation (2), proteins can be still unstable during lyophilization processes and/or during long term storage (3,4). Mannitol and glycine are often used as a bulking agents to provide cake structure in preparing lyophilized proteins, but do not effectively protect proteins against instability (1). Therefore, different stabilizers such as sugars and polyols are normally added and levels need to be optimized to protect proteins against degradation (5–7). However, the processes by which sugars or polyols stabilize proteins in the solid-state are not completely understood and need to be characterized for rational formulation development (1,5,8).

The effect of structural changes and molecular mobility on long term stability of proteins in solid state formulations is implicated in two different proposed mechanisms by which excipients are believed to stabilize proteins (2,5,6,9). Pikal *et al.*(10,11) and other research groups (12–16) have investigated these mechanisms of stabilization in the solid state. The glass dynamic hypothesis states that a good stabilizer of freeze-dried formulations forms a rigid, inert matrix into which the protein is molecularly dispersed and the motion of protein can be coupled to the motion of the matrix (3). α-relaxation of the glassy matrix occurs mainly due to translational motion, although rotational motions are considered to strongly influence the diffusion of reactive molecular species within the matrix (4–6). Therefore, protein unfolding and other degradation reactions that effect the physical stability of the protein would be expected to correlate with the molecular mobility within this matrix including α-relaxation phenomenon. Another proposed mechanism known as the water substitution or structural preservation hypothesis states that stabilizers such as sucrose or polyols hydrogen bond with the surface of proteins, thereby preserving the native structure when water is removed (1,2,9). The removal of water in the absence of a sugar, often results in a non-native protein structure in the dry state, as has been detected using spectroscopic techniques, and may lead to aggregation during reconstitution (5). In addition, it has been found that sugars provide a glassy matrix that reduces the rate of degradation during storage in the dried solid by preserving its native structure during lyophilization and storage (5,6,8).

Many studies have demonstrated that degradation of amorphous protein pharmaceuticals is related to some degree on molecular mobility (16–18). Therefore, reducing the molecular mobility of amorphous pharmaceuticals is considered to be a useful strategy for improving long term stability (16,17). However, a reduction in molecular mobility does not always result in improved stability. There are various factors such as solute concentration, pH changes, and temperature that contribute to storage stability. Understanding the interrelationships between chemical reactivity and molecular mobility of protein is critical for predicting storage stability (16,17). There are several examples in the literature where it has been shown that glass transition (Tg) and α enthalpic relaxation (time) are good predictors of lyophilized cake stability but not the long term storage stability of the protein (15–19). In such cases, determination of fast dynamics, as measured by Small Angle Neutron Scattering (SANS) & SS-NMR techniques, has been shown to play a useful role in determining storage stability (17–21).

Protein stuctural preservation during freeze-drying and long-term storage is also important as formulations that retain more native-like structure are expected to have better storage stability than formulations having less native-like structure (5). The preservation of secondary and tertiary structure of various proteins in the solid state has been investigated using a number of techniques including solid-state FTIR, Raman, NIR, fluorescence, phosphorescence spectroscopic techniques (22–28) as well as NMR over the past 30 years. One particular advantage of FTIR is that this technique allows for secondary structure analysis of protein in a wide variety of physical states. FTIR is frequently used to assess structure of lyophilized proteins in stability studies and to guide the choice of the optimal formulation. During the evaluation of lyophilized protein formulations, the spectrum of each formulation can be compared with an aqueous reference spectrum with the goal of determining quantitatively the degree of retention of native secondary structure (24). Raman spectroscopy can also be used to study protein conformation and to quantify the amount of protein secondary structure and, in some cases tertiary structure as well (22,23). Also, recent literature shows that solid-state fluorescence spectroscopy can be a useful for investigating the tertiary structure of proteins in solid powders and that tertiary structure correlates with long term stability (25–28). However, there have not been studies in the literature that systematically evaluate both secondary and tertiary structural changes with respect to effect on long term stability of proteins in the solid state.

The purpose of this study is to investigate the effect of pH and potential stabilizers such as sucrose and mannitol on structure, glassy state dynamics and long-term stability of anti-streptavidin IgG1 monoclonal antibody in the solid state after freeze-drying. Protein formulations were prepared at different pH and mannitol conditions in the presence and absence of sucrose as a stabilizer. The purpose was also to study the extent of conformational changes and instability caused at different pH conditions in the absence of stabilizer

(*i.e.* mannitol only conditions). The relationship between preservation of the protein's secondary and tertiary structure in the solid state and the long-term storage stability was obtained from examining data collected using spectroscopic techniques and by stability-indicating chromatographic as well as subvisible particle (SbVP) assays at different solution conditions. Also, SS-NMR ^{1}H relaxation time was used to monitor glassy state dynamics for the different formulations. The results of this study will help improve understanding of the role played by structural preservation and glassy state dynamics on long-term stability of therapeutic proteins. This will enable development of tools to predict and generate stable lyophilized protein therapeutic formulations.

MATERIALS AND METHODS

Materials and Preparation of Antibody Formulations

Purified monoclonal antibody anti-streptavidin IgG1 monomer (purity >98% determined by SEC, MW 142.2 kDa) was provided by purification group within Amgen, Inc. (Thousand Oaks, CA). The antibody was obtained at a stock concentration of 38.5 mg/mL bulk in 10 mM sodium acetate and 5% sorbitol, at pH 5.0. The antibody was dialyzed into corresponding formulation buffers and then diluted to a final concentration of 5 mg/mL at different pH values, with mannitol in the presence or absence of sucrose. The relatively lower antibody concentration utilized in the current study *versus* concentrations in marketed formulations is to present a worse case condition with respect to increasing interface related protein instability prevalent during freeze-drying and for reducing potential of antibody concentration related self-stabilization. The buffer conditions used for various formulations and corresponding sample code are shown in Table I. Sucrose was purchased from EMD (Gibbstown, NJ). Mannitol was purchased from Roquette (Keokuk, IA). Sodium citrate was obtained from JT Baker (Phillipsburg NJ). Each of the formulations was filled at 1 mL in 3 cc glass blowback vials (Amcor Type IA) with Daiko long stopper and lyophilized.

Table I Different Formulation Conditions along with Measured pH and Osmolarity Values Immediately after Lyophilization and Reconstitution

Sample code	Formulations (buffered with 10 mM sodium citrate)	Measured pH	Osmolarity (mOsm/L)
C3M	4% mannitol	3.1	295
C3MSu	4% mannitol + 2% sucrose	3.1	238
C5M	4% mannitol	5.0	267
C5MSu	4% mannitol + 2% sucrose	5.0	250
C7M	4% mannitol	6.9	251
C7MSu	4% mannitol + 2% sucrose	6.9	335

Lyophilization of Samples

The IgG1 mAb (5 mg/mL) was formulated in each of the formulation conditions described in Table I. One-milliliter aliquots of each protein formulation were added to vials and were lyophilized using a Virtis lyophilizer (SP Scientific, Stone Ridge, NY). Sample vials were loaded onto pre-chilled (4°C) shelves of the lyophilizer. Samples were held for 30 min at 4°C, cooled down to −45°C at 0.4°C/min, and held at −45°C for 180 min. Then, the samples were annealed by raising the shelf temperature to −15°C at 0.2°C/min and held at −15°C for 240 min. Finally, the temperature was lowered to −45°C at 0.2°C/min and held at that temperature for 120 min.

For primary drying, the shelf temperature was raised to −10°C at 0.15°C/min and held for 25 h while keeping the chamber vacuum at 120 mTorr. The condenser temperature was less than −50°C throughout drying. For secondary drying, the shelf temperature was raised to 25°C at 0.15°C/min while lowering the chamber pressure to 100 mTorr. Then, the shelf temperature was maintained at 25°C for 11 h at 100 mTorr. After all cycle steps were finished, the vials were stoppered inside the drying chamber. The vials were then removed and crimped before long-term stability studies.

Incubation Study of Formulations at Different Temperatures

Lyophilized samples were stored in incubators (Shel Lab, Cornelius, OR) equilibrated at different temperatures: 4°C, 25°C, 37°C, 50°C. Also samples were stored at −80°C as reference controls. Different time points were set up for different analysis: 0 month, 1 months, 3 months, 6 months, 9 months, and 12 months. Three vials of each lyophilized formulation were pulled at each time point. Just prior to analysis, one vial from each condition was reconstituted with 1 mL of milliQ water.

pH, Osmolarity, and Concentration Measurements

pH of the lyophilized formulations upon reconstitution was monitored using a MP200 Mettler Toledo pH meter (Columbus, OH). Samples were equilibrated at room temperature before the analysis. The instrument was calibrated using pH 4.0 and pH 7.0 standard buffers. Osmolarity was determined using an Advanced Instruments, Inc (Norwood, MA) Micro Osmometer, model 330. A 290 mOsm/kg standard was used as a reference standard and water as a control. The actual pH and osmolarity values of the formulations are shown in Table I. Concentration of mAb was measured using ND-1000 spectrophotometer Nano Drop (Thermo Scientific, Wilmington, DE). A280 nm method was utilized with 1.06 (mL. $mg^{-1}.cm^{-1}$) extinction coefficient for the anti- streptavidin IgG1.

Karl Fisher Analysis of Moisture in Lyophilized Product

Residual moisture content of all lyophilized formulations was determined using Karl Fisher coulometric titration (Aquastar AQC34 KF Coulometer. (EMD, Gibbstown, NJ)) using drying oven for introducing sample after temperature equilibration. The lyophilized cakes were crushed into powder prior to analysis in a dry glove box. 5% moisture containing lactose powder was used as a reference standard.

Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography was used to monitor monomer loss and high molecular weight aggregate formation using the Agilent 1100 HPLC system (Lexington, MA). The method employed one column (Tosoh G3000SWxl, 7.8 mm × 300 mm) attached with a guard column (TSKgel Guard Column SwXL 6.0 mm × 4.0 cm). Protein sample load of 20 μg was loaded onto the column and eluted isocratically at a flow rate of 0.6 mL/min with a mobile phase consisting of 100 mM sodium phosphate, 300 mM sodium chloride; pH 6.8 at 25°C.

The protein was monitored using UV detection at 215 nm. Peak areas in the chromatogram were used to quantify the amounts of monomer, high molecular weight species (HMW), and low molecular weight species (LMW).

Visual Inspection and SbVP Counting (HIAC)

HIAC light obscuration particle counting technique was used to monitor SbVP formation in the 2-25 μm range, and large aggregates (>125 microns) were monitored by manual visual inspections. Liquid particle counting was performed on a HACH Ultra (Grants Pass, OR) HIAC model 9703 system using the PharmSpec software. Prior to analysis, samples were degassed under vacuum for one hour based on the method developed for monoclonal antibodies. It has been shown that degassing under mild vacuum helps release air bubbles that otherwise artificially increase the particle count. The degassing condition for 1 h has not been found to cause physical damage for antibodies tested so far. Four 0.2 mL measurements were made for each sample and prior to sample measurements Millipore water was used to blank the system. The first run was discarded, and the last three runs were averaged to obtain the cumulative counts per milliliter. As per USP guidelines, particle sizes of ≥10 μm, and ≥25 μm were counted.

Cation-Exchange Chromatography (CEX)

CEX technique was utilized to monitor chemical degradation of the monoclonal antibody and was carried at 25°C using Dionex ProPac WCX-10 (4 × 250 mm) attached to a guard column (TSKgel Guard Column SwXL 6.0 mm × 4.0 cm) on an Agilent 1100 system (Lexington, MA). Protein sample load of 20 μg was loaded onto the column and eluted at the flow rate of 0.38 mL/min. Separation of various charged species was achieved through a gradient; mobile phase A was 10 mM $NaPO_4$, pH 7.1 and mobile phase B 10 mM $NaPO_4$, 250 mM NaCl, pH 7.0. The mobile phase mixture varied from 3% of mobile phase B at 8 min to 15% at 15 min, 17% at 20 min, 18.5% at 27.5 min, 21.5% at 42.5 min, 23% at 50 min, 40% at 53 min, 100% at 56 min to 58 min, and finally back to 0% from 60 min to 70 min. The protein was monitored using UV detection at 215 nm. The peak areas were integrated to quantify the amount of protein contained in the pre-peak (acidic variants), main peak and post peak (basic variants).

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy studies were conducted using Bomen TioTools MB-Series FTIR Spectrometer (ABB, Canada) to assess secondary structure in lyophilized solids. Lyophilized powder was mixed with KBr to form a disc pellet. The solid samples were prepared by pressing a ground homogeneous mixture of 400 mg KBr with approx 4 mg of dried protein formulation into a pellet using a mortar and pestle. This procedure has been shown to not cause any damage to proteins (24) let alone for the more stable monoclonal antibodies. The mixture was pressed with EZ-press approximately 5500 psi and the pressure was maintained for approximately 5 min. Then, spectra of samples in KBr were collected and analyzed using GRAMS software. A total of 64 scans were collected for each spectrum, and the scanning range was from 4,000 cm^{-1} to 700 cm^{-1}. The second derivative of the amide I region (1720–1580 cm^{-1}) of the spectra was baseline corrected and normalized to an area of unity. The intensity change of native β-sheet structure at 1640 cm^{-1} was monitored at different timepoints and temperatures to determine change in antibody secondary structure. The secondary structural determination using curve fitting procedure was not adopted due to inconsistency of the % β-sheet value determined using the method at different time points.

Solid State Fluorescence

All intrinsic tryptophan fluorescence measurements to monitor protein tertiary structural changes were made using a Cary-Eclipse spectrofluometer (Agilent, Santa Clara, CA). Studies were performed according to procedures described in the literature (27). The lyophilized cake from each vial was broken and mixed together thoroughly with a spatula, and the sample was loaded into the solid-state holder. The solid-state holder (Cary-Eclipse) consists of a powder receptacle which houses the silica disk and shell cavity into which the lyophilized

sample is placed. The silica disk in the shell cavity is completely covered with the sample to ensure reproducible measurement. A photo multiplier tube (PMT) voltage of 600 V was used for the measurements involving lyophilized samples. The emission was monitored at 280–450 nm utilizing 280 nm wavelength and bandwidth of 5 nm. Total of 10 scans were averaged for each data point measurement.

Raman Analysis

Raman analysis to monitor secondary and tertiary structure of protein was conducted using a Bruker Senterra (Billerica, MA). Lyophilized samples were prepared by pressing the sample on SpectRIM steel slide. A total of 100 scans were collected in the 2740 – 70 cm^{-1} range with grating 1, Aperture 50 × and 1000 μm as optical filter setting. The excitation wave length was 532 nm with laser power set at 20 mW. GRAMS software was used to analyze the samples. Due to the large protein signal compared to weak buffer signal in the spectral regions of interest, background subtraction was not applied. The data was processed using baseline subtraction, offset correction, and area normalization steps to enable ease of comparison of the spectra for different formulation and temperature conditions.

Solid State NMR Analysis

All SS-NMR measurements to monitor glassy state dynamics were conducted on a Bruker DSX spectrometer (Billerica, MA) operating at a ^{1}H resonance frequency of 600.23 MHz using a 4-mm double resonance MAS probe head. ^{1}H spectra were acquired using a single pulse experiment and ^{1}H T_1 values were measured using an inversion recovery sequence with the sample spinning at 14 kHz. ^{1}H 90° pulse length of 2.5-μs and a recycle delay of 240 s ($>5*T_1$) were used for all experiments. The data from inversion recovery experiment was fitted to either a mono or bi exponential using the Sigmaplot software. A cross polarization/magic angle spinning/dipolar decoupling (CP/MAS/DD) pulse sequence was used to generate ^{13}C data. A total suppression of spinning sidebands (TOSS) sequence was appended to the CP sequence to obtain spectra free of spinning sidebands. 3072 scans were collected for signal averaging at a recycle delay of 3 s with a contact time of 2-ms.

X-ray Powder Diffraction Analysis

The X-ray powder diffraction (XRPD) analysis was performed using a θ/θ diffractometer (X'pert MPD, Philips Analytical, Natick, MA) with Cu Kα radiation. All samples were analyzed with Bragg-Brentano geometry from 3–40° 2θ at a step size of 0.01° 2θ.

RESULTS

Residual Moisture Content and Cake Properties of Lyophilized Formulations

Increased residual water or moisture content in lyophilized drug product formulations can increase the deterioration rate of proteins and facilitate crystallization of formulation excipients. In our study, all lyophilized samples stored at 4°C had similar appearance with no visible signs of collapse. The final moisture level in all lyophilized samples was below the recommended 2% level, except for one sample stored at 4°C for 12 months. This sample had 2.46% residual water content (Table II), but had no visible sign of collapse and stability issues post reconstitution. No cake collapse was seen in all vials even during prolonged storage at 50°C.

Physical and Chemical Stability of IgG1 mAb on Long-Term Storage

All protein formulations stored at 4°C were clear upon reconstitution and no particulate matter was visible. The C3MSu formulation stored at 50°C for 6 months, however, appeared to undergo a color change, from clear to brown. It is known that at low pH sucrose may hydrolyze readily to form fructose and glucose, which can react with proteins *via* Maillard reaction (9,29) resulting in the browning of the cake or the liquid. Although this reaction was suspected to have occurred, the color change was not examined further.

Size-exclusion chromatography (SEC) was used to assess the physical stability of proteins upon reconstitution with respect to aggregation during storage. Loss of monomer was monitored as a function of time at different temperatures and increased loss was concomitant with increase of high molecular weight species (HMWS) and low molecular weight species (LMWS), presumably resulting from backbone hydrolysis (Fig. 1). It is well known that the pH of the protein solution before lyophilization often affects the stability of dried protein products during long-term storage (29). It was observed that

Table II Residual Water Content (RWC) % Measurement by Karl Fisher for Lyophilized Formulation Samples Stored at 4°C Over 12 Months

Sample code/ Conditions	RWC (%) 3 month	RWC (%) 6 month	RWC (%) 9 month	RWC (%) 12 month
STD (5% Lactose)	5.11	5.33	4.91	5.34
C3M_4°C	0.45	0.07	0.15	0.98
C3MSu_4°C	1.09	1.03	0.81	2.46
C5M_4°C	0.50	0.20	0.75	0.84
C5MSu_4°C	0.80	0.17	0.11	1.73
C7M_4°C	0.26	0.15	0.02	0.79
C7MSu_4°C	1.87	0.46	0.18	1.83

the loss of protein monomer (Fig. 2) was mainly due to formation of high molecular weight aggregates at all pH conditions and, to lesser extent, clip species formation at pH 3 conditions. The presence of sucrose, however, inhibited the loss of monomer at the pH 3 condition. More than 45% and 30% loss of monomer were observed at time zero for C3M and C3MSu, respectively. There was no significant difference in monomer loss for C5MSu and C7MSu formulations at the initial time point. Liquid samples stored at −80°C did not show any difference among any formulation along with time (data not shown). Long-term protein storage at 2–8°C and higher temperatures was performed to evaluate protein stability. The amount of monomer decreased with time and increasing temperatures for most formulations except for C5MSu and C7MSu. There were greater monomer losses for samples stored at higher temperatures over the period of 1 year (Fig. 2). Overall, monomer loss was greatest in the C3M condition, followed by C3MSu, then C5M, and C7M. The concomitant increase of soluble aggregate was more for both C3M and C3MSu followed by C5M and C7M (data not shown). There was no difference observed for C5MSu and C7MSu formulations, and these two conditions were the most stable. Not only did freeze-drying with sucrose/mannitol result in greater recovery of protein monomer compared to mannitol alone, but the presence of sucrose also improved stability at all pH conditions (Fig. 2). This is not surprising, as sugars such as sucrose have been shown to protect proteins from time-dependent denaturation (30) and resulting aggregation upon reconstitution.

Cation exchange chromatography was used to monitor charge related covalent changes happening in the mAb as a function of time, temperature and formulation conditions. The percent CEX main peak as well as the percent acidic species due to clips and isomerization and basic isoforms due to aggregation, deamidation, and formation of succinimide were evaluated.

An increase in the acidic peak was observed for low pH formulations due to clip formation. Overall post-lyophilized samples did show decreased main peak intensity at lower pH compared to the higher pH formulation mainly due to an increase in percent basic species. The presence of sucrose at pH 3, however showed protection of protein against degradation, and this condition showed no significant difference of percent main peak recovery over time (Fig. 3). In general, samples stored at 2–8°C for 1 year did not show significant changes in percent main peak *versus* −80°C samples except for pH 3 (Fig. 3).

SbVP Formation in Lyophilized Samples upon Reconstitution

SbVP were measured using HIAC for 2 μm, 5 μm, 7.5 μm, 10 μm, 15 μm, 20 μm, and 25 μm particle size bins (Fig. 4). The ≥2 μm cumulative particle counts were higher for pH 7.0 condition though this pH condition showed lower percent monomer loss by SEC. Following C7M, C5M showed the highest cumulative counts of particles. More particles were observed in the higher pH formulations than in lower pH formulations. At pH 7.0 and also for pH 5.0 conditions, the addition of sucrose helped to reduce the numbers of sub-visible particles than formulations containing only mannitol. Conversely, samples formulated at pH 3 did not show any difference between samples with and without sucrose. Particles ≥2 μm represent protein instability at this pH and demonstrate overall particle trend with respect to physical stability. Most of the formulations had particle counts below the USP guidelines (≤6000 particles per container for particles ≥10 μm and ≤600 particles per container for particles ≥25 μm) (31) for all formulations except for C7M conditions, which were above these limits.

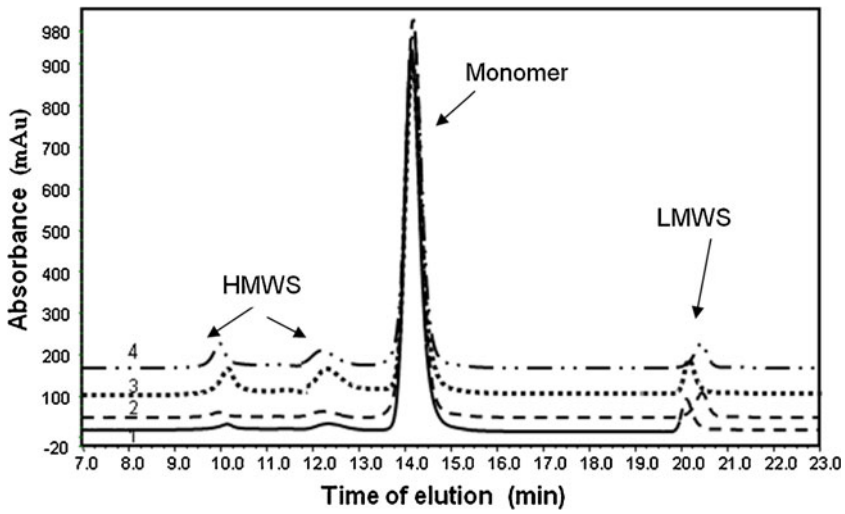

Fig. 1 SEC chromatogram of anti-streptavidin IgG1 monoclonal antibody showing the different species of monomer, HMWS and LMWS. Legend: (*1*) C5MSu 6 month at 25°C, (*2*) C5MSu 0 month at 4°C, (*3*) C5M 6 month 25°C, and (*4*) C5M 0 month 4°C.

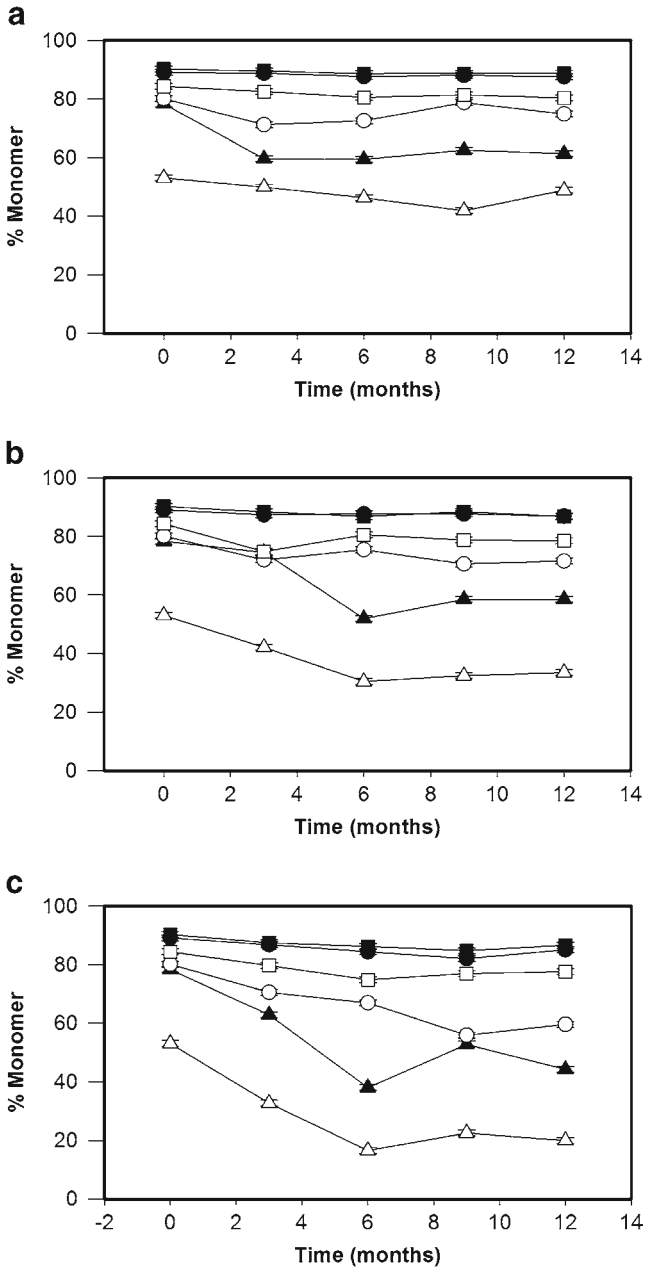

Fig. 2 Stability data of 5 mg/mL IgG1 lyophilized in different formulations at (**a**) 4°C, (**b**) 25°C and (**c**) 37°Cas measured by size exclusion chromatography (SEC). Scatter plot of SEC data. Legend: C3M (Δ), C3MSu (▲), C5M (○), C5MSu (●), C7M (□), and C7MSu (■). (Error bar is Std deviation based on average of 2 samples).

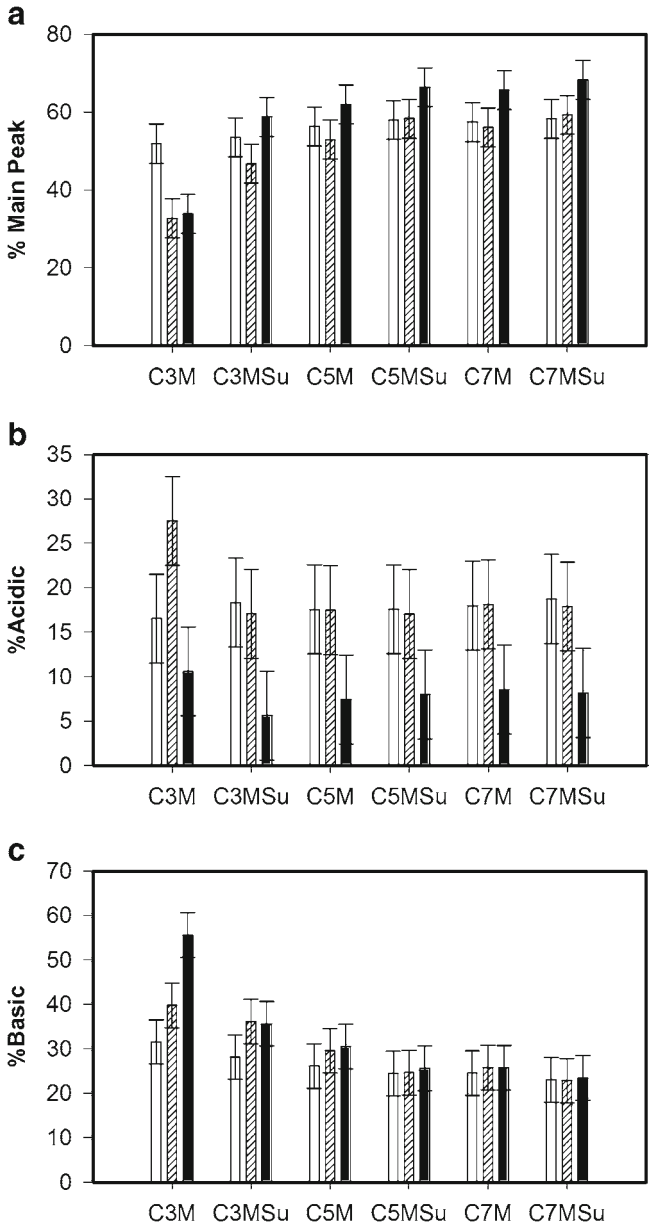

Fig. 3 Stability data of 5 mg/mL IgG1 lyophilized in different formulations at indicated accelerated storage conditions as measured by cation exchange chromatography (CEX). Legend: 12 month at -80°C (*open bar*), 6 month at 4°C (*dashed bar*), and 12 month at 4°C (*solid bar*) (Error margin based on method-induced variability is ±5% as determined from multiple sample analysis).

Effect of Lyophilization Storage in the Solid State on the Secondary Structure of IgG1 as Measured by FTIR and Raman Analysis

In our investigation, all FTIR spectral data were converted to second derivative peak signal for the amide I region (1580–1720 cm^{-1}), area normalized and base line corrected. The amide I band (amide C=O stretching) in this spectral region is commonly used to obtain protein structural information (5), as it is influenced by hydrogen bonding, which reflects the variation in secondary structure of the protein (9). The second derivative spectrum of hydrated native IgG1 antibody had two dominant bands at 1640 cm^{-1} and 1690 cm^{-1}. The bands at 1640 cm^{-1} are due to the native intramolecular β-sheet structure, and bands at 1615 cm^{-1} and 1690 cm^{-1} are due to intermolecular H-bonded anti-parallel β-sheet, which in this system is representative of aggregates in the solution state (32).

In the lyophilized mAb formulations, a more intense band at 1640 cm^{-1} means a higher degree of native structure in the

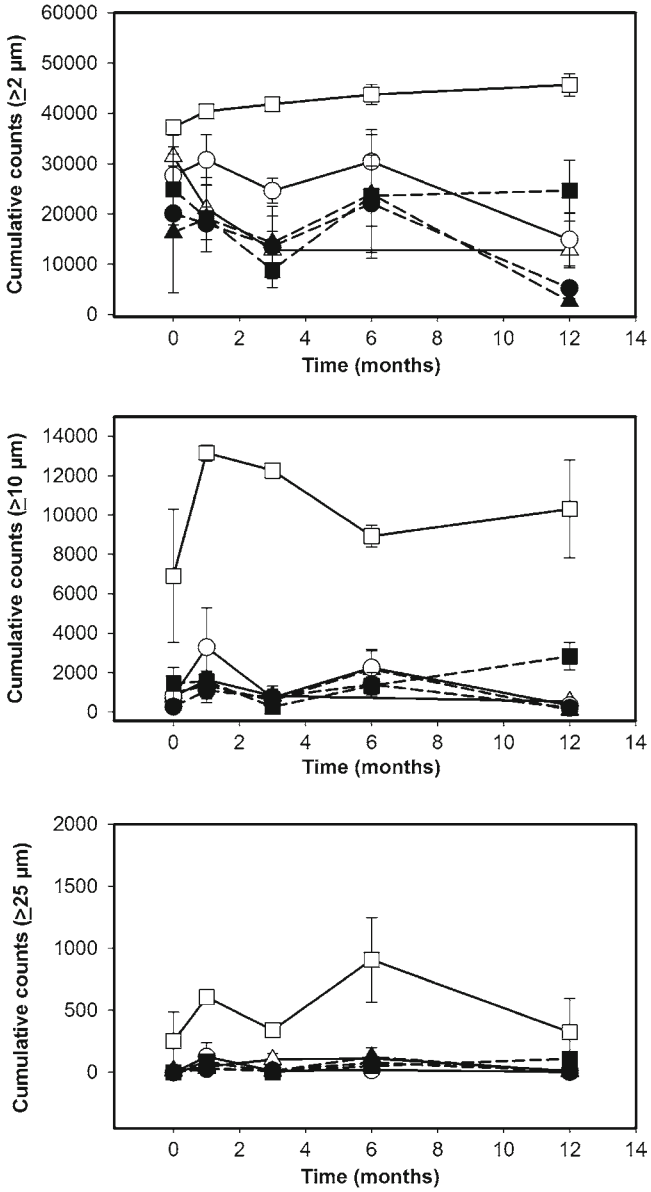

Fig. 4 Stability data of 5 mg/mL IgG1 lyophilized in different formulations at indicated accelerated storage conditions as measured by sub-visible particle by HIAC. 2 μm, 10 μm, and 25 μm cumulative counts per mL at 4°C. Legend: C3M (Δ), C3MSu (▲), C5M (○), C5MSu (●), C7M (□), C7MSu (■) (Error bar is Std deviation based on average of 3 samples).

sample (2,6,9). The band position agrees well with those reported for IgG1 molecules in general, as antibodies are mainly composed of intramolecular β-sheet structures with a small portion of α-helix (33). It is expected that the lyophilized formulation, which has protein spectra similar to the native protein in the liquid state, should have better storage stability (34).

First, the spectra of each formulation in the liquid and solid state were compared (Fig. 5). It was observed that the pH 3 liquid conditions has the lowest intensity of the 1638 cm^{-1} band compared to other pH conditions, indicating loss of native β-sheet structure (Fig. 5). Upon freeze drying minor conformational changes were observed. The β-sheet band shifted to 1640 cm^{-1} in the dried state, became broader and the intensity decreased as a result of freeze-drying stresses (Fig. 5). It was observed that immediately after lyophilization, pH 5 and sucrose-containing formulations retain most of their native structure, whereas pH 3 and pH 7 formulations do not. More native structure is retained in the pH 7 formulation compared to pH 3. Addition of sucrose to pH 3 and pH 7 conditions does help retain native secondary structure in the solid state compared to the same pH formulations lacking the stabilizer.

All formulations demonstrated a decrease in second derivative spectral intensity at 1640 cm^{-1} with time of storage for 12 months at all temperatures (Figs. 6 and 7). Also, the band intensity at high frequency 1690 cm^{-1} decreased, indicating a loss of β-turn with time. The spectrum showed a general broadening of the peaks with time, which likely indicates chain disordering or loosening of structure. Samples at pH 3 showed the greatest intensity loss when compared to other formulations. On the other hand, samples formulated at pH 5 and pH 7 maintained higher intensity and showed less change when compared to pH 3. Also, samples formulated with sucrose retained higher intensity than samples formulated without sucrose.

The C3M condition displayed the lowest intensity band corresponding to native β-sheet band while a shift of native β-sheet structure is observed for the C5M and C7M formulations after 12 months of storage. The C5MSu and C7MSu conditions maintained most of their native structure. The FTIR stability data at 25°C, 37°C showed similar trends as the 4°C samples (Fig. 7). Overall, sucrose containing protein formulations at pH 5 retained more native-like structure than that at pH 7. These data together indicate that sucrose improves the structural stability of this IgG1 regardless of pH, but that native-like structure is best preserved at pH 5.

Figure 8 shows the relationship between the amount of monomer lost, as observed using SEC (upon reconstitution) and percent native β-sheet intensity change using FTIR in the solid state for the different protein formulations at different time points. The percent change in monomer was calculated by comparing stability at 4°C, 25°C, and 37°C recorded at different time points post-reconstitution compared to the liquid state at the initial time point (pre-lyo control), time 0**.** Similarly, percent change in native β-sheet intensity in solid state was calculated by comparing the structure in solid state stability samples at different temperature and time points *versus* the liquid state pre-lyo control. The native β-sheet intensity change was less for samples containing sucrose/mannitol. The maximum loss of

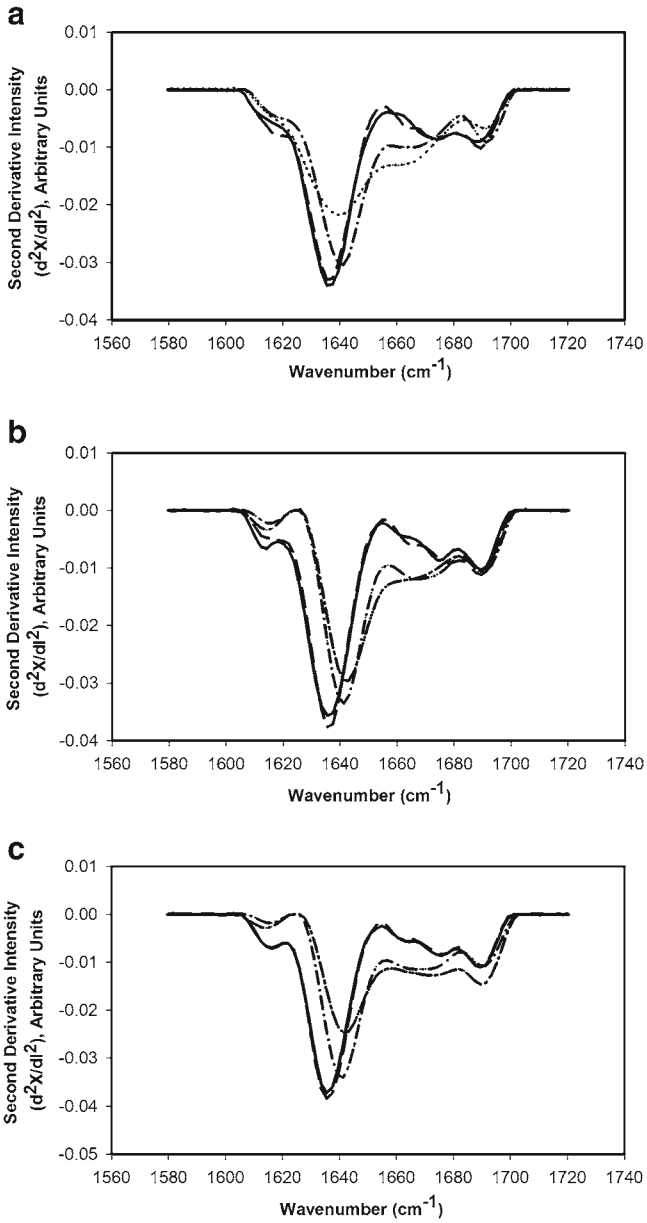

Fig. 5 Comparison of secondary derivative FTIR spectra of IgG1 in aqueous solution and solid, dried formulations at T=0 month in different formulations: C3M aqueous (*solid line*), C3MSu liquid (*dashed line*), C3M solid dried (*dotted line*), and C3MSu solid dried (*dashed dotted line*). Different conditions: (**a**) pH 3, (**b**) pH 5, (**c**) pH 7.

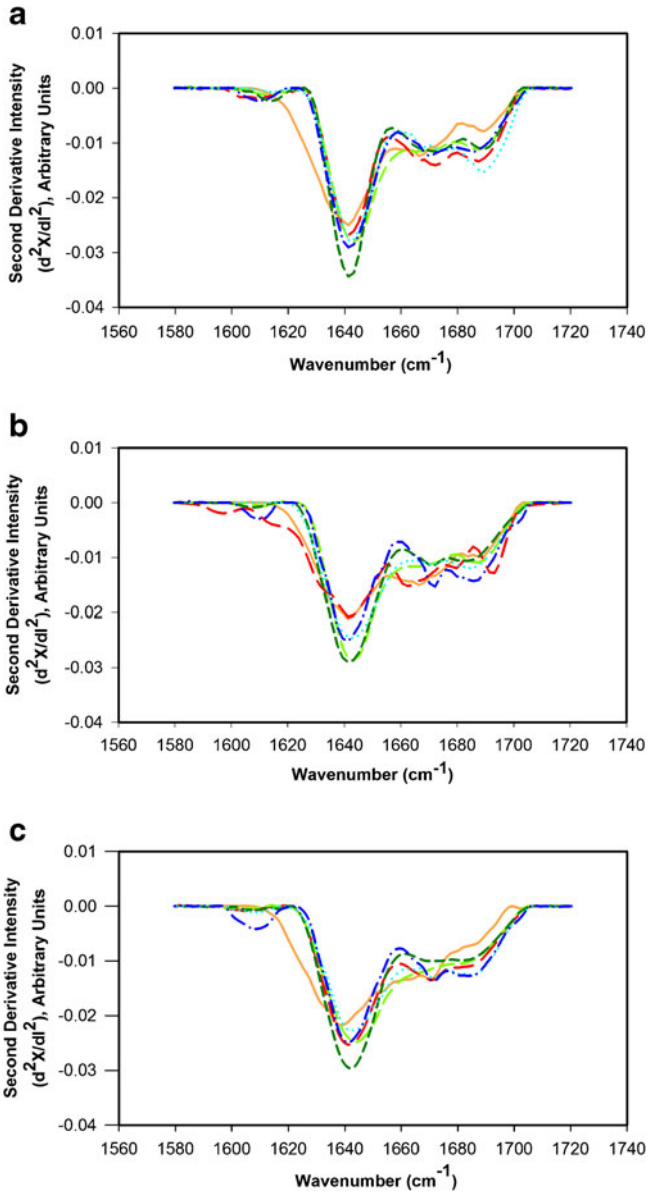

Fig. 6 Secondary derivative FTIR spectra of solid protein at (**a**) 4°C, (**b**) 25°C and (**c**) 37°C at T=1 month. Legend: C3M (*orange*); C3MSu (*red*); C5M (*green*); C5MSu (*dark green*); C7M (*light blue*); C7MSu (*blue*).

monomer was observed for pH 3 condition by SEC due to clip formation. Thus, the changes of monomer loss when compared to time zero were greatest for samples formulated at pH 3. There was greater change of monomer and percent β-sheet intensity change for formulations at pH 3 compared to pH 5 and pH 7 and with increasing temperature. There was a correlation between the degree of change in monomer loss and loss of percent native β-sheet intensity for each formulation (Fig. 8). The overall correlation plots showed an r^2 value of 0.78 for 4°C storage, and less correlation values of 0.44 for 25°C storage, and 0.55 for 37°C.

Tertiary Structure of Protein by Solid-State Fluorescence

The maximum fluorescence intensity observed in the emission scan was at 329–321 nm (data not shown). Typically, this spectral feature specific to tryptophan emission denotes that tryptophan is buried inside the protein in a hydrophobic environment (35). No shifts to lower or higher wavelength were detected for lyophilized samples on storage, but changes in intensity at the wavelength maxima were observed under all formulation conditions as a function of temperature and time.

The intensity of the intrinsic fluorescence spectra of lyophilized proteins in their respective buffers in the solid state

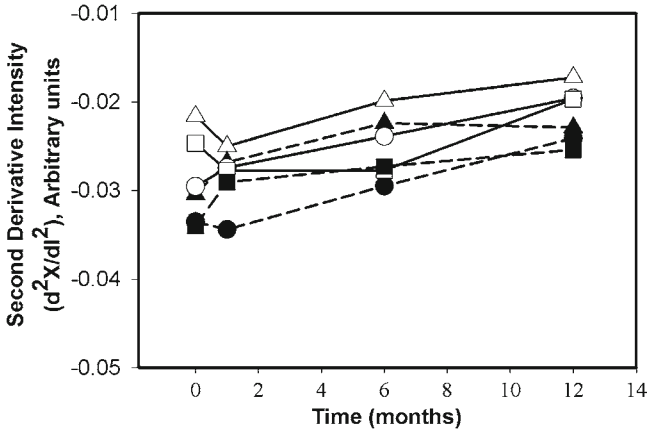

Fig. 7 Second derivative FTIR spectra of IgG1 formulations stored at 4°C. The intensity at 1640 cm^{-1} is plotted as a function of time. Legend: C3M (△), C3MSu (▲), C5M (○), C5MSu (●), C7M (□), C7MSu (■).

with time is shown in Fig. 9. The effect of storage temperature was not different for the mannitol only formulations compared to buffer only samples. Mannitol containing formulations showed some intensity changes at pH 3. Protein at pH 5 and pH 7 however, did not show differences in their fluorescence intensity with varied temperature and over the period of 12 months. For sucrose formulations, samples stored at higher temperatures had lower intensities at pH 3 and pH 5. By comparison, there was more change in intensity observed at different temperatures for C3MSu. Fluorescence intensity also changed for C5MSu formulation condition at different temperatures. Fluorescence intensity of sucrose containing formulations showed a decrease with increasing temperature and time which is inverse of the mannitol alone formulations.

The percent change in monomer was calculated as described in the FTIR section. Percent change in absolute value of fluorescence intensity in solid state was calculated by comparing the intensity in solid state stability samples at 25°C and 37°C at different time points with the corresponding intensity of 4°C samples. The changes of monomer loss when compared to time zero were greatest for samples formulated at pH 3. At 4°C, only C3M condition showed greatest fluorescence intensity changes. The degree of change in monomer loss and fluorescence intensity for each formulation increased with increasing temperature (data not shown). There was greater monomer loss for formulations at pH 3 compared to pH 5 and pH 7 as measure by fluorescence change. These results suggested that there may be more prominent changes in the conformation of the monomer around the environment of the tryptophan at pH 3 than at higher pH conditions. The increased degree of change may signify heterogeneity of tryptophan environment in the native to unfolded state ensemble of IgG1 that may in turn translate to the increased level of aggregates on reconstitution of the lyophilized samples at pH 3 condition. The overall correlation plots showed an r^2 value

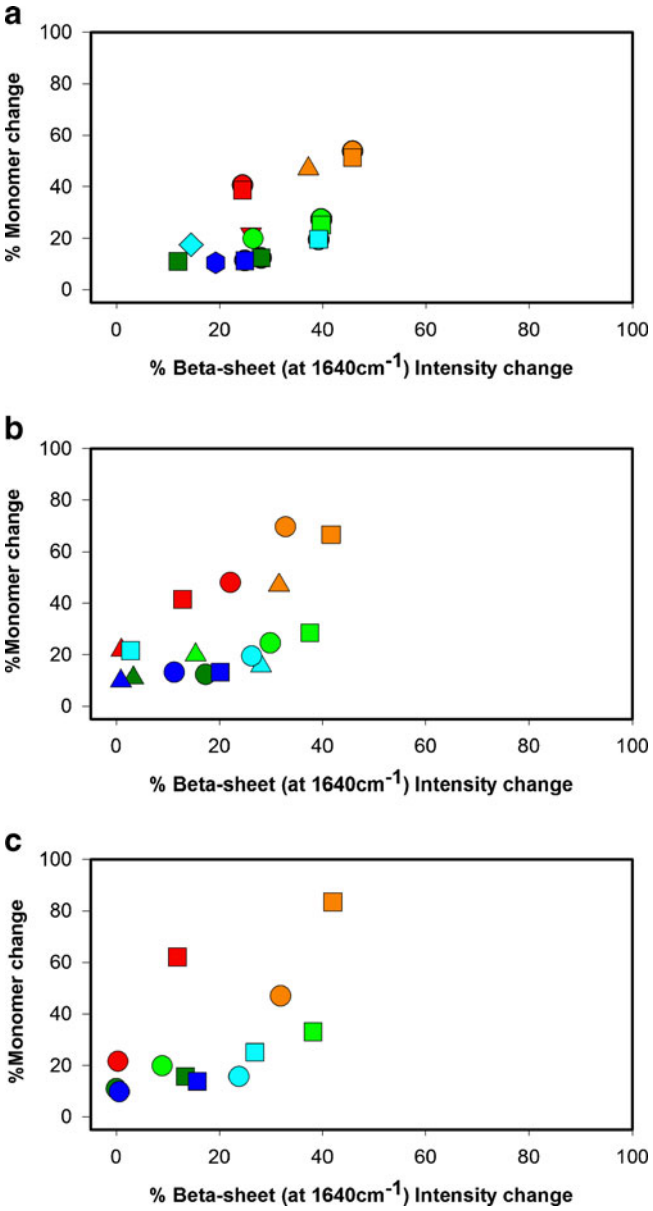

Fig. 8 Correlation plot showing percent monomer change after reconstitution as measured by SEC *versus*% native β-sheet intensity change at 1640 cm^{-1} in solid state at 4°C (**a**), 25°C (**b**), and 37°C (**c**). Legend: Samples pulled at 0 month (Δ): C3M (*orange*), C3MSu (*red*), C5M (*green*), C5MSu (*dark green*), C7M (*light blue*), C7MSu (*blue*); samples pulled at 6 month (○): C3M (*orange*), C3MSu (*red*), C5M (*green*), C5MSu (*dark green*), C7M (*light blue*), C7MSu (*blue*); samples pulled at 12 month (□): C3M (*orange*), C3MSu (*red*), C5M (*green*), C5MSu (*dark green*), C7M (*light blue*), C7MSu (*blue*).

of 0.56 for 4°C storage, 0.70 for 25°C storage, and 0.39 for 37°C.

Secondary and Tertiary Structural Changes as Monitored by Raman Spectroscopy

The amide I band from the Raman spectra was used to characterize the secondary structural changes in the protein

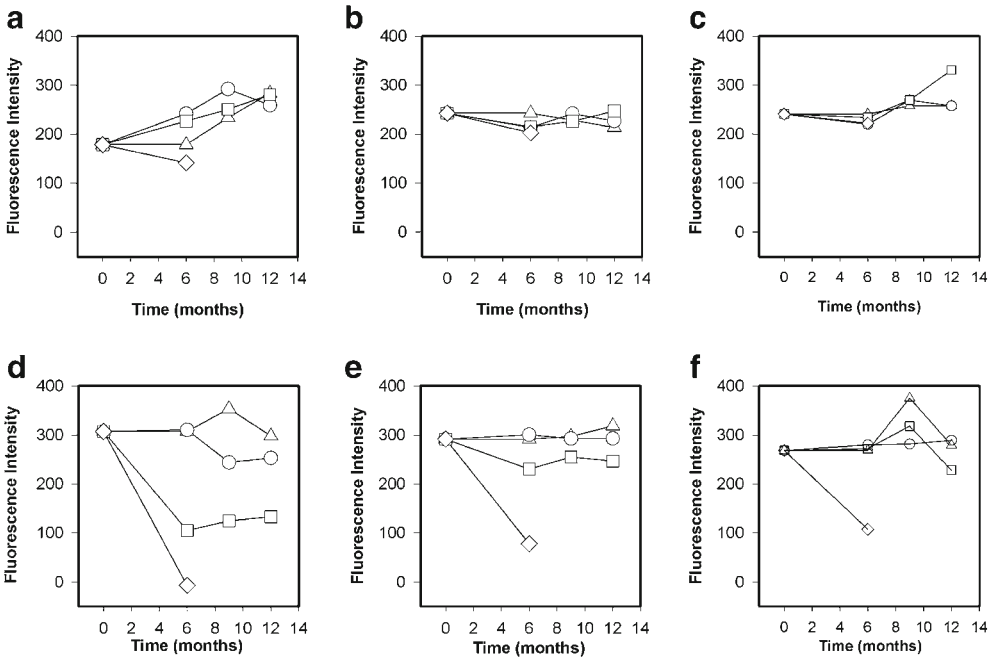

Fig. 9 Solid-state fluorescence intensity maximum at 329 nm for different formulations: C3M (**a**), C5M (**b**), C7M (**c**), C3MSu (**d**), C5MSu (**e**), C7MSu (**f**) along with time of incubation of the samples at different temperatures: 4°C (△), 25°C (○), 37°C (□), 50°C (◇).

and as an orthogonal technique to FTIR for tracking secondary structural changes in the solid state. Raman spectra in the 1500–1800 cm^{-1} region for lyophilized protein is shown in Fig. 10a.

The major band in the 1630–1720 cm^{-1} region is the amide I band, which is used to monitor secondary structure of the protein. The band at ~ 1670 cm^{-1} is characteristic of high β-sheet containing proteins. Samples formulated with mannitol only (C3M, C5M, and C7M) had broader bands than sucrose formulations (C3MSu, C5MSu, and C7MSu) (Fig. 10a). The broader amide I peaks on the higher wave number side of the spectra are usually associated with increased turn and unordered contents (24). In addition, C3M had the lowest peak intensity followed by C7M, and then C5M at ~1670 cm^{-1} region. The sucrose-containing formulations had higher intensity peaks than the mannitol only formulation.

The peaks in the 1550–1650 cm^{-1} region are due to the aromatic side chains (Fig. 10b). The 1615 cm^{-1}, intensity peak is associated with tryptophan, tyrosine, and phenylalanine. C5MSu condition showed the lowest intensity band for, and the highest intensity bands were observed for C3M and C5M. The 1604 cm^{-1} band is associated with phenylalanine and was most prominent for C5MSu formulation. The other formulations showed a decrease in peak intensity at 1604 cm^{-1} and in most cases showed a mere shoulder. Raman spectral peaks for mannitol formulations were less intense and shifted to 1552 cm^{-1}, whereas the sucrose containing formulations showed compact peaks at 1554 cm^{-1}. This shift is associated with ring vibrations due to the tryptophan (36–38).

At 4°C, C5MSu showed the least intensity at 761 cm^{-1}, and C3M the greatest (Fig. 10c). This region represents the cation-π interactions that are intrinsic to protein's structure (39) and has been shown to contribute to protein stability in the case of rh IL-1RA. At 37°C, a shift was observed for C3M and C3MSu. C5MSu showed the greatest intensity for this band (data not shown).

The amide I peak comparison at 37°C result was consistent with the results at 4°C. Samples formulated with mannitol only had broader bands than sucrose formulations (data not shown). The absence of sucrose led to significant loss in the β-sheet in the amide I region. C7M sample had the lowest intensity peak followed by C3M and then C5M.

The difference between the absence and presence of sucrose in the formulations was apparent and noticeable in the tryptophan peak at 1554 cm^{-1} at 4°C. However, the difference was not obvious in samples stored at 37°C. Peaks were all grouped together with the exception of C3M. The intensity of this peak in the C3M sample was the least. In the C3MSu spectrum this peak had the highest intensity of all the formulations.

There was no obvious correlation for 1604 cm^{-1} band at 37°C (data not shown) with 4°C data. Furthermore, the C5MSu peak resolution at 4°C samples was lost with 37°C storage. In addition, at 37°C, the C5MSu showed the lowest band at 1615 cm^{-1}, and C7M and C5M as the band with the sharpest peak. The basis for Raman spectral changes especially for monitoring tertiary structure have not been clearly understood compared to the other techniques (*e.g.* spectral changes for FTIR). Correlation plots were not calculated for Raman spectral intensity changes in the solid state *versus* loss of monomer by SEC (upon reconstitution) because only the time zero and three month data points were available for comparison. However, it should be noted

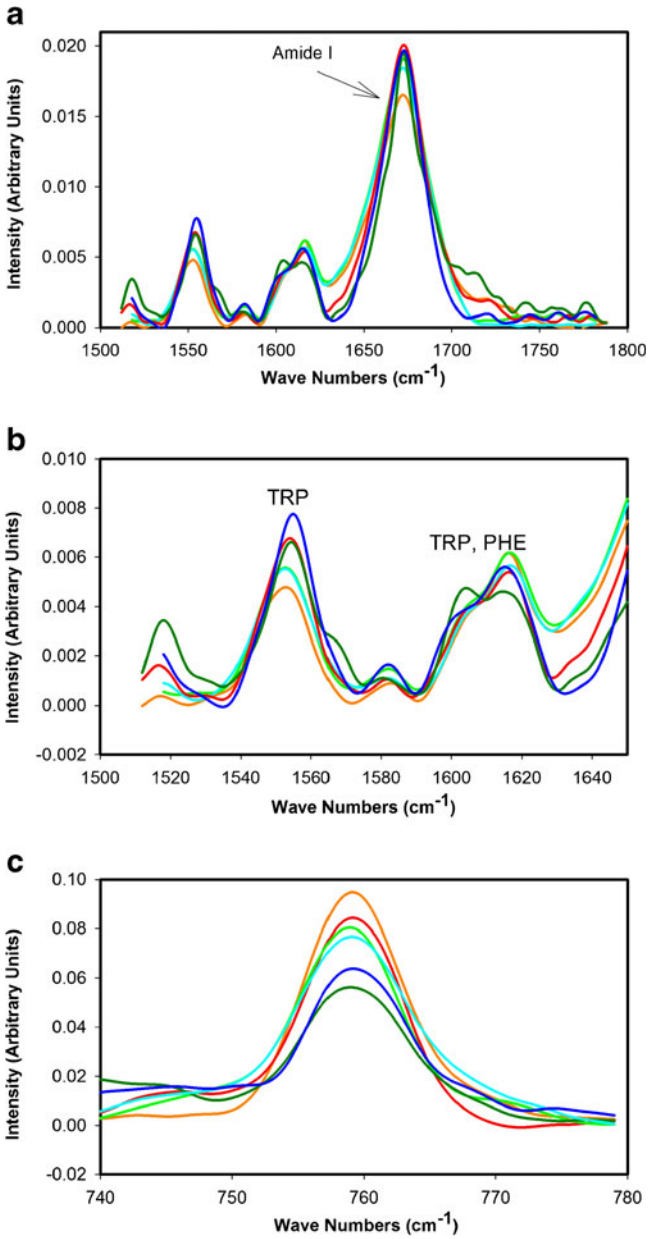

Fig. 10 Raman spectra of (**a**) Amide I region of IgG1 lyophilized formulation at 4°C at 3 months. (**b**) Tryptophan region of IgG1 lyophilized formulation at 4°C at 3 month. (**c**) Cation-π interaction region of IgG1 lyophilized formulation at 4°C at 3 month. Legend: C3M (*orange*); C3MSu (*red*); C5M (*green*); C5MSu (*dark green*); C7M (*light blue*); C7MSu (*blue*).

that the different Raman spectral regions of interest did show a trend with respect to intensity changes as described above and spectral changes showed correlation to ranking of formulations based on physical stability.

^{1}H NMR Relaxation Analysis of Formulation Buffer and Protein Samples

^{1}H SS-NMR T_1 relaxation constants were used to analyze the cake mobility/dynamics. ^{1}H T_1 measurements probe dynamics in the MHz regime or the ultra fast timescale. The ^{1}H relaxation rate was monitored as ^{1}H T_1 values tend to be phase specific due to spin diffusion. The normalized area of the peaks was plotted as function of time (Fig. 11a). For the placebos, the relaxation curve could be fitted using a single parameter inversion-recovery equation 1 shown below:

$$\textit{One parameter equation}: I = I(0) + a\left(1 - \exp\left(-\frac{t}{T_1}\right)\right) \quad (1)$$

where T_1 is the relaxation time constant. The T_1 value (in seconds) for the placebos was plotted as function of different formulations (Fig. 11b). Sucrose remained amorphous in the lyophilized cake, and mannitol crystallized to the same form in all samples. Crystallization of mannitol was more extensive in mannitol only buffers. On the other hand, crystallization was hindered (reduced ^{1}H T_1) when sucrose was added to the buffer (Fig. 11b). In addition, ^{13}C SS-NMR confirmed sucrose is amorphous in the formulation (data not shown). The above factors could lead to relaxation times in the two amorphous and crystalline phases being close thus resulting in one parameter exponential fit.

In the case of protein-containing lyophilized formulations, the relaxation curve followed more of a double exponential behavior (Fig. 12a) and fitted using the following equation 2:

$$\textit{Two parameter equation}: I = I(0) + a\left(w_a \exp\left(-\frac{t}{T_{1a}}\right) w_b \exp\left(-\frac{t}{T_{1b}}\right)\right) \quad (2)$$

Where w_a and w_b are the weight fractions and T_{1a} and T_{1b} are the relaxation time constants of components 1 and 2 respectively. Figure 12b shows the relaxation time (T_{1a} and T_{1b} in seconds) constants for different protein samples. The protein ^{1}H T_1 relaxation time is very short thus leading to a biexponential fit. In this case there are two components of the relaxation time reflecting T_1, one for the protein rich phase and another for the mannitol-rich phase. The component T_{1a} is associated with the mannitol-rich phase (longer T_1 corresponsing to crystalline material), and the component T_{1b} is associated with the protein-rich phase. The T_{1b} component remains nearly the same for all samples (2.3–3.6 s), with T_{1a} values showing the most change. It is assumed that the difference in T_{1a} values among samples is related to the extent of crystallization of mannitol as explained for the placebos.

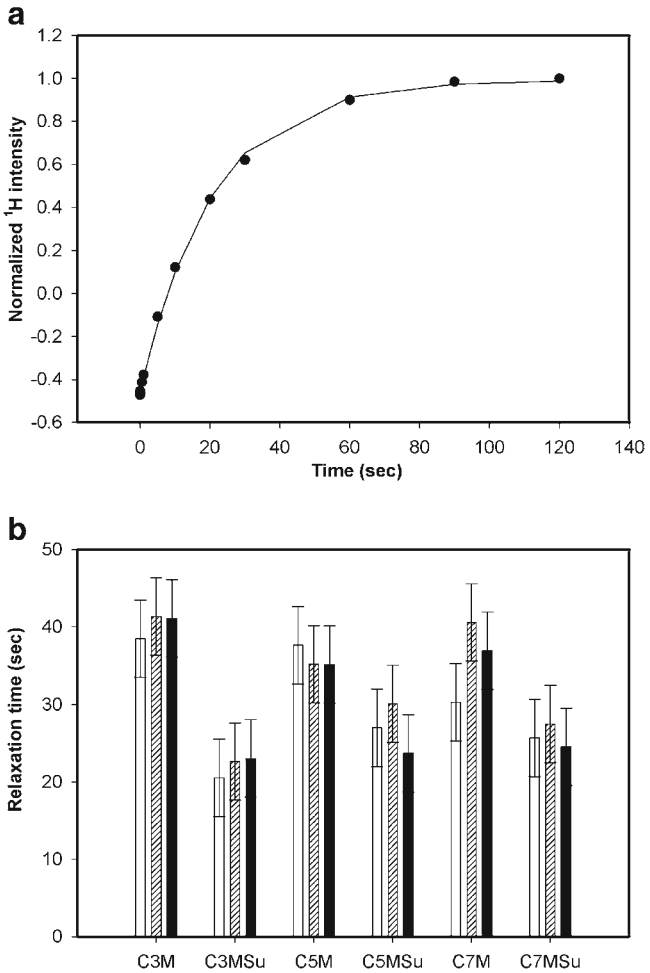

Fig. 11 (**a**) ^{1}H relaxation curve for C3M placebo showing peak intensity along with time of relaxation. (**b**) Relaxation time data for placebo samples calculated utilizing one parameter fit. Legend: 0 month (*open bar*), 3 month at 4°C (*dashed bar*), 3 month at 37°C (*solid bar*). (Error margin based on method-induced variability is ±5%).

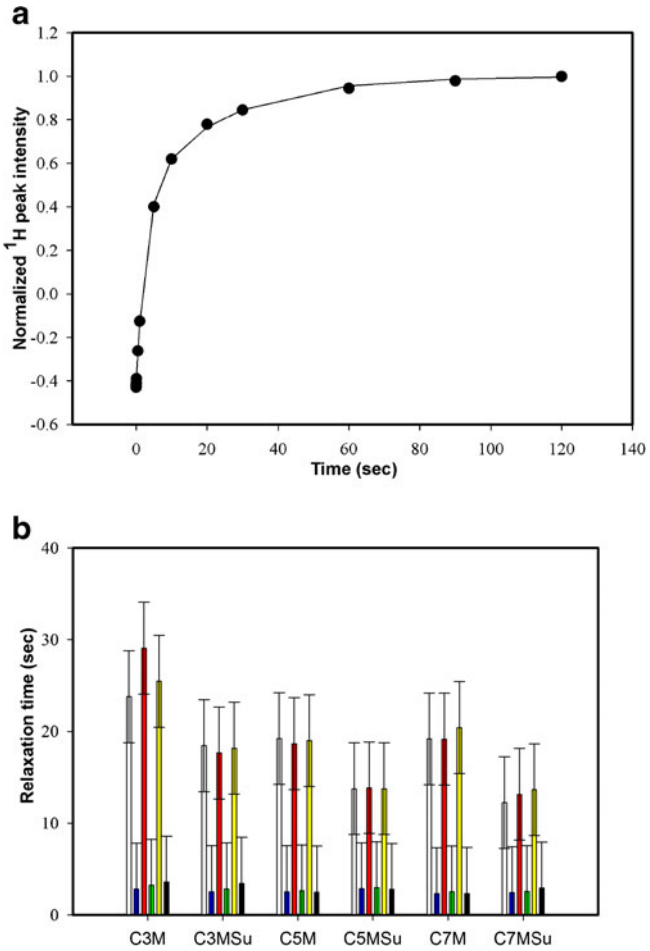

Fig. 12 (**a**) ^{1}H relaxation curve for C3M protein showing peak intensity along with time of relaxation. (**b**) Relaxation time data for protein samples calculated using two-parameter fit. Legend: 0 month T_{1a} (*white*), 0 month T_{1b} (*blue*), 3 month at 4°C T_{1a} (*red*), 3 month at 4°C T_{1b} (*green*), 3 month at 37°C (*yellow*), 3 month at 37°C T_{1b} (*black*) (Error margin based on method-induced variability is ±5%).

DISCUSSION

Effect of pH and Stabilizer on Solid-State Stability

Numerous studies have emphasized the importance of the physical state of buffer and excipients in stabilizing proteins during freeze-drying (25). Generally, a protein that retains native conformation is expected to be more resistant to degradation during storage (40). Proteins are often stable within a narrow pH range (32). It was found that at extreme pH values proteins have tendencies to unfold and likely increase the amount of aggregates (41). Here, it was found that there is an impact of pH on the stability and also biological activity of proteins as determined using *in vitro* assays. As such, possible changes in the acid–base characteristics of proteins should be considered since it can lead to destabilization of native structure during dehydration (32) affecting both physical and chemical stability.

In our stability study, the pH 3 formulation showed significant loss of monomer followed by pH 7, while pH 5 had the least monomer loss. Anti-streptavidin IgG1 has a pI of 8.8. It is well known that when the pH is close to the protein's pI, protein may self-associate, aggregate, and form particles (41). Although pH 5 and pH 7 showed similar stability data by SEC, more sub-visible particles were detected in pH 7 formulations, particularly C7M, which had the highest sub-visible particle count. This may be due to the different aggregation kinetics at higher pH of 7.0 *versus* pH 5.0 in the absence of sucrose as stabilizer, causing increased formation of sub-visible particles. However, the amount of monomer loss due to sub-visible particle formation involves small fraction of the protein based on the SEC results. Overall, it is observed that pH plays a dominant role in determining the physical stability of the IgG1 in the lyophilized state with pH 5.0 being the most stable followed by pH 7.0 and pH 3.0 conditions considering physical stability and particle formation. No chemical degradation was

observed during storage of samples for 1 year at 2–8°C and 25°C.

Results for the proteins that were freeze-dried only in the presence of mannitol showed higher amount of aggregation. Mannitol is often used as a bulking agent to provide cake structure in preparing lyophilized proteins, and it has been shown to crystallize upon freezing (25). Crystallization is thought to remove mannitol from the protein phase and result in the loss of molecular interaction with proteins (42,43). As such, maintenance of the amorphous state of mannitol or sucrose is important for imparting stabilization to proteins during storage (42,43). Several studies suggest that mannitol either facilitates sucrose-based stabilization of the protein or it directly stabilizes the protein when maintained in the protein phase during drying (amorphous form) (5). On the other hand, it is well known that sucrose assists in maintaining the native structure of proteins in the dried state (26). The hydrolysis of sucrose at low pH condition to glucose and fructose is expected to be significantly lower during storage stability in the solid-state *versus* in the aqueous state. Besides, the hydrolysis reaction is expected to result in glucose and fructose molecules and these molecules especially glucose have also been shown to stabilize protein in the solid-state (5,8,9). Although these monosaccharide sugar molecules can result in Maillard reaction (9,29), significant evidence of related protein degradants was not observed based on visual inspection, size-exclusion and ion-exchange chromatographic results during storage at different temperatures, except for samples stored at accelerated condition of 50°C. In fact, sucrose was shown to protect the protein from loss of monomer due to LMWS and HMWS formation for pH 3 condition at different temperatures as observed through SEC results though not as effective as pH 5 and 7 conditions potentially due to sucrose hydrolysis related events (*e.g. e.g.* loss of hydrogen bonding, phase separation, *etc.*). The current investigation also shows that the amount of monomer decreased with time and increasing temperature for most of the formulations except for C5MSu and C7MSu. The SEC data and particle analysis data for anti-streptavidin IgG1 show that under the optimal pH condition, the presence of sucrose drastically improves structural stability and mannitol alone is less effective at preventing aggregation. The above findings agree with previous observations reported in the literature (3,7).

Effect of pH and Stabilizer on Antibody Secondary and Tertiary Structure in Solid-State

A comparison of the results of the stability studies and spectroscopic analyses indicate a modest correlation exists between the retention of native structure in the lyophilized state and long term stability. The protein conformation as monitored by FTIR, solid state fluorescence and Raman analyses, was very sensitive to the pH of the formulation. Spectra obtained at different pH showed relative peak intensity differences as well as loss and gain of spectral features, which were indicative of conformational changes. Broadening of the individual amide I components in the spectra observed upon dehydration is indicative of general disorder of the protein backbone (24).

Based on the spectra, pH 3 showed altered structure compared to pH 5 and pH 7. The observed band at 1615 cm^{-1} and the enhanced absorbance near 1690 cm^{-1} for pH 3 formulation are most likely indicative of non-native β-sheet structure formed by unfolding and aggregation of the protein during lyophilization (2,9,44). Samples formulated at pH 5 and 7 had greater intensity bands at 1640 cm^{-1} region, indicating they better retained their native structure. Greater loss of intramolecular β-structure was observed at higher temperatures for all samples.

In the absence of sucrose, the protein structure was perturbed dramatically, as indicated by a large decrease in the intensity of the band at 1640 cm^{-1}. The FTIR studies have shown that many proteins undergo denaturation, often forming intermolecular β-sheet structures, upon lyophilization in the absence of stabilizers (2,9,24,44). With the addition of sucrose into the formulation, the extent of perturbation was moderated, as the protein retained more "native-like" structure with sucrose in the formulation. It is well known that sucrose assists in maintaining the native structure of proteins in the dried state (24). Freeze drying of the IgG1 mAb in the presence of sugars improved stability, resulting in spectra that are more native-like than in the absence of the sugar. The addition of sucrose maintained native structure better than mannitol only formulations at each pH. The native β-sheet band intensity loss showed a general correlation with monomer loss with time (SEC). However, correlation of the secondary structural changes with sub-visible particle formation was not observed and further investigations need to be conducted to understand the role of structural changes on protein sub-visible formation after reconstitution of the lyophilized protein.

Previous studies showed a correlation between fluorescence intensity and aggregation (27), which resulted from changes in the tertiary structure that may open up certain hydrophobic pockets (28). In our study, it was observed that formulation matrix as such can have an effect on the solid-state fluorescence spectra due to the effect of the excipients on fluorescence signal as observed for sucrose and mannitol containing formulation *versus* mannitol only formulations. The different trend for fluorescence data observed at low pH in the presence of sucrose may be attributed to sucrose hydrolysis and resulting effect on protein fluorescence spectra due to change in the matrix or local environment changes of tyrosine and tryptophan residues (*e.g.* loss of hydrogen bonding, phase separation *etc.*). Higher temperatures and longer time points show more tertiary structure change for pH 3 compared to other two pH conditions. Also, sucrose reduced magnitude of intensity change on the fluorescence spectra of the IgG1 at pH 3 may be by inhibiting heterogeneous conformational change, which may be a key

reason for protection against physical instability at this pH condition. At pH values of 5 and above, the fluorescence change was low at 4°C storage and correlated in general with lower loss of monomer peak.

In addition, Raman provided valuable information about conformational changes resulting from freeze-drying. Raman spectra provide information about both protein backbone and the orientation of the amino acid side-chains phenylalanine, tryptophan, and tyrosine as well as cation-π interaction (36,45). Sucrose-containing formulations had higher intensity peaks in the tryptophan region (1554 cm^{-1}) and amide I region, suggesting better conservation of secondary structure occurred even with increased storage temperature. There was a loss in the β-sheet content with a corresponding gain in the turn and unordered content. The results are consistent with FTIR data and show that sucrose protects IgG1 from degradation in the lyophilized state. The cation-π interactions are recognized as an important non-covalent biding interaction relevant to structural biology and likely contribute to protein stability (39) The peak ranking of cation-π interaction was as following: C3M, C3MSu, C5M, C7M, C7MSu, and C5MSu. The C5MSu showed least interactions by having the least intensity of peak (Fig. 10c). Conservation of residues involved in these interactions indicate structural stability for some proteins. These results support the conclusion that the mechanism of protein stabilization by additives during lyophilization depends on the maintenance of the native conformation in the dehydrated state (25).

Overall, a general correlation was observed between the secondary and tertiary structural data from FTIR, Raman and solid-state fluorescence in the solid state and long-term stability of the IgG1, as observed using SEC and particle analysis (upon reconstitution). Some exceptions were noted. For example, in this study, C5MSu and C7MSu did not have stability changes upon long-term storage based on SEC, but the FTIR and Raman data demonstrated minor changes and difference in structure between those two conditions (Figs. 5, 6 and 10). Structural results support the conclusion that the structural preservation hypothesis plays a major role in this protein's stability in the solid state. However, lack of strong correlation between structural change and instability may be due to the fact that glassy state dynamics also plays a significant role in determining stability .

Effect of Formulation Conditions on SS-NMR ^{1}H Relaxation Data for Glassy State Dynamics

A comparison of the ^{1}H T_1 analysis between placebo and protein containing samples shows differences in the state of mannitol. From the placebo data it is clear that more extensive crystallization is observed for buffers containing only mannitol compared to buffers containing mannitol and sucrose. As stated in the "Results" section, the protein ^{1}H relaxation data is best fit to a biexponential equation with two T_1 values (T_{1a} and T_{1b}). The second T_1 (T_{1b}) is assumed to be associated with the protein, as the protein was shown to be in an amorphous state at room temperature using ^{13}C SS-NMR measurements. Moreover, the T_{1b} value ranged between a narrow window of 2.3 to 3.6 s for all formulations investigated. Thus, the dynamics associated with the protein remains rather similar in all cases. However, major differences were observed in the slower T_1 (T_{1a}) associated with the mannitol. The T_{1a} value was found to be lower in the protein containing formulation when compared to similar T_1 values in placebo formulations. This shows that addition of protein further inhibits mannitol crystallization. This result is not surprising, as it well known that polymers inhibit the crystallization of small molecules. The extent of T_{1a} reduction was found to be the highest for C5MSu and C7MSu protein formulations indicating that these formulations had the highest levels of amorphous mannitol. Interestingly, the T_{1a} values for protein formulation C3MSu was similar to C5M and C7M but lower than C3M. The T_{1a} value follows the rank ordering C3M>C3MSu~C5M~C7M>C5MSu~C7MSu. Thus, the ^{1}H relaxation data based on the slower relaxing species correlated reasonably well with the long-term stability data at the different pH conditions and with the presence of stabilizer. The relation between ^{1}H relaxation time of different formulations observed through SS-NMR analysis and extent of mannitol crystallinity was confirmed by XRD results (Supplementary Material Figure S1). C5MSu and C7MSu protein formulation samples showed lowest degree of mannitol crystallinity among the different formulations as observed through XRD analysis. C3M condition showed high degree of crystallization into different polymorphs (α, β and δ forms) consistent with SS-NMR measurements . The absence of mannitol hydrate form was confirmed because its characteristic peaks (47) at 16.5, 17.9, 25.7 and 27.0° (2Theta) were not present in the XRD assayed samples. Mannitol in the amorphous state observed more in C5MSu and C7MSu conditions may potentially stabilize the protein along with sucrose in which is consistent with the reports in the literature (2,46). While more intensive SS-NMR analysis is required, analysis of glass state dynamics through ^{1}H relaxation behavior appears to be an useful tool to explain the gap observed for correlating structural changes to long-term stability. SS-NMR relaxation data sheds light on the role of excipients in protein stability. The relaxation data for the mannitol-rich phase and related stability of the formulation conditions shows that enhanced protein stability is imparted by the amorphous mannitol due to inability of mannitol to crystallize during freeze-drying potentially through hydrogen bonding interaction of the polyol to protein side-chains.

CONCLUSIONS

The relationship between solid-state protein structure, glassy state dynamics and long-term stability was systematically

investigated with a series of formulations using a model IgG mAb. It was found that antibody stability in solid state was sensitive to pH. Greater amounts of aggregates were present in low pH formulations and increased with increasing temperature. Also, more secondary and tertiary structure changes in the solid state were observed at low pH. The presence of sucrose in a formulation helped preserve the native structure of the protein in the solid state and inhibited physical instability during long-term storage across the pH range examined.

Both tertiary and secondary structural changes in the solid state generally correlated with long-term physical stability in the different formulations at different temperatures and time points. It has been demonstrated that retention of the native conformation (both secondary and tertiary) during lyophilization is an important determinant of stability. Also, the NMR ^{1}H relaxation data representing glassy state dynamics based on the slower relaxaing species correlated with the long-term stability data at the different pH conditions and with the presence of stabilizer. Understanding the relationship of physical stability, chemical reactivity, and molecular mobility of the protein is critical. Based on the major results of the current study native state protein structural preservation and optimal solid-state dynamics in the solid state correlate with improved long-term stability of the mAb in the different lyophilized formulations.

The results of this study strongly support the "structural preservation" and "glassy state dynamics" are equally important mechanisms dictating long term stability of protein in the solid state.

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