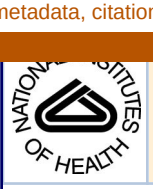


Published in final edited form as:

J Pharm Sci. 2015 February ; 104(2): 495–507. doi:10.1002/jps.24242.

Characterization of the Physical Stability of a Lyophilized IgG1 mAb After Accelerated Shipping-like Stress

Srivalli Telikepalli[1], **Ozan S. Kumru**[1], **Jae Hyun Kim**[1], **Sangeeta B. Joshi**[1], **Kristin B. O'Berry**[2,3], **Angela W. Blake-Haskins**[2,4], **Melissa D. Perkins**[2,5], **C. Russell Middaugh**[1], and **David B. Volkin**[1,*]

[1]Department of Pharmaceutical Chemistry, Macromolecule and Vaccine Stabilization Center, University of Kansas, Lawrence, KS 66047

[2]Department of Drug Product Sciences, GlaxoSmithKline Inc, Rockville, MD 20850

Abstract

Upon exposure to shaking stress, an IgG1 mAb formulation in both liquid and lyophilized state formed subvisible particles. Since freeze-drying is expected to minimize protein physical instability under these conditions, the extent and nature of aggregate formation in the lyophilized preparation was examined using a variety of particle characterization techniques. The effect of formulation variables such as residual moisture content, reconstitution rate, and reconstitution medium were examined. Upon reconstitution of shake-stressed lyophilized mAb, differences in protein particle size and number were observed by Microflow Digital Imaging (MFI), with the reconstitution medium having the largest impact. Shake-stress had minor effects on the structure of protein within the particles as shown by SDS-PAGE and FTIR analysis. The lyophilized mAb was shake-stressed to different extents and stored for 3 months at different temperatures. Both extent of cake collapse and storage temperature affected the physical stability of the shake-stressed lyophilized mAb upon subsequent storage. These findings demonstrate that physical degradation upon shaking of a lyophilized IgG1 mAb formulation includes not only cake breakage, but also results in an increase in subvisible particles and turbidity upon reconstitution. The shaking-induced cake breakage of the lyophilized IgG1 mAb formulation also resulted in decreased physical stability upon storage.

Keywords

proteins; protein aggregation; particles; monoclonal antibody; IgG; stability; particle size; freeze drying/lyophilization

[*]Corresponding author: D.B. Volkin, Multidisciplinary Research Building, 2030 Becker Dr., Lawrence, KS 66047. Phone: (785) 864-6262; volkin@ku.edu.

[3]Current address: Department of Formulation Sciences, MedImmune, Gaithersburg, MD 20878

[4]Current address: Drug Delivery and Device Development, MedImmune, Gaithersburg, MD 20878

[5]Current address: Department of Manufacturing Science & Technology, Hospira, McPherson, KS 67460

Supporting Information Available: This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com.

Introduction

When a protein therapeutic is appropriately lyophilized with stabilizing excipients, the solid dosage form typically displays increased physicochemical stability during storage and shipping, compared to a liquid formulation counterpart, resulting in a longer shelf life including a lower propensity toward aggregation.[1-3] Many proteins, including monoclonal antibodies (mAbs), have been shown to be more stable during exposure to elevated temperatures in the lyophilized than in the liquid state[1,4-7], although there are some exceptions to this general rule.[6] Despite protein lyophilization being a relatively well-established formulation technology, there remains numerous challenges both in terms of developing an improved understanding of protein degradation pathways in the solid state as well as better optimizing lyophilization process design and scale-up [1,5,8,9].

The lyophilization process typically consists of freezing, primary and secondary drying, and reconstitution steps, each of which may structurally damage proteins[10-14]. Freezing of bulk water in a protein solution can cause cryoconcentration of protein and excipients, pH changes, and/or adsorption of protein to the surface of ice crystals[10,13,14]. Primary drying removes the frozen bulk water and further concentrates the protein and stabilizers, allowing the possibility of unfavorable interactions [5,11]. Protein instability is also possible during secondary drying, during which the non-frozen water bound to the protein or excipients is removed. The composition of the reconstitution medium and it's rate of addition may also affect the stability of a lyophilized protein upon reconstitution back into the liquid state[15]. For example, if the reconstitution medium is added too rapidly, the dried protein may not be given sufficient time to rehydrate and assume its native conformation, and the presence of this improperly rehydrated protein may lead to aggregation. [6,16]. The aggregation of monoclonal antibodies in the lyophilized state, and/or upon reconstitution, has also been correlated with formation of non-native intermolecular disulfide bonds[17] as well as the appearance of aggregates of different sizes including an increased number of subvisible particles[18].

A freeze-dried cake's physical structure and moisture level are typically optimized as part of protein lyophilization development, since either can potentially affect the extent to which a protein may aggregate in the solid state [6,19-21]. These parameters can be interrelated since changes in residual moisture content may affect not only protein structure, but also the physical integrity of the lyophilized cake itself (i.e., a change from a viscous to a rubbery state where molecular mobility increases[7]). Some lyophilized protein preparations with high moisture content have shown increased chemical degradation due to increased mobility and the ability of water to participate in chemical reactions.[22,23] However, a bell shaped relationship between moisture content and physical stability (aggregation) has also been observed, e.g., lyophilized recombinant human albumin displayed maximum aggregation at ~50% moisture content. [24] In terms of the effect of the physical integrity of a freeze-dried cake on protein stability, it has been shown that when a lyophilized cake of an IgG1 mAb is physically collapsed to different extents by using different amounts of stabilizers and bulking agents during the freeze-drying process, the mAb can still remain stable in the different preparations.[19-21] The method of cake collapse, however, either during the freeze-

drying cycle or during storage at elevated temperatures, has been shown to potentially be an important factor in determining protein stability during subsequent storage.[21]

The effect of mechanical stress on the stability of lyophilized proteins has not been as widely examined. In this work, to examine the potential of shipping stress to cause protein aggregation, we implemented a stress shipping test based on considering guidelines in the vibration testing document D999 proposed by ASTM International Standard Test Methods for Vibration Testing of Shipping Containers[25]. Currently, there are no guidance documents available that are specific for pharmaceutical products that outline the testing criteria to use when evaluating product quality impact of shipping-related stress. Initially, we examined the physical stability of an IgG1 mAb formulation in both a liquid and lyophilized state after exposure to shaking stress with the expectation that the protein in the lyophilized state would be more stable in terms of aggregation and particle formation. Surprisingly, the lyophilized preparation also displayed mAb physical instability (upon reconstitution).

The focus of this work was to better understand the effect of shaking stress, used to mimic extreme shipping conditions, on the physical stability of a lyophilized mAb preparation. A wide variety of analytical characterization techniques were used to size and count protein aggregates and particles across a wide size range including size-exclusion chromatography (SEC), dynamic light scattering (DLS), Nanoparticle Tracking Analysis (NTA), Resonant Mass Measurement (Archimedes), Microflow-Imaging (MFI), and turbidity. We also used data visualization tools (e.g., radar plots) to display and compare the number and size of protein particles formed under different conditions. The composition of the protein found in particles was further examined using SDS-PAGE and FTIR Microscopy. We determined how formulation variables such as residual moisture content, reconstitution speed, and composition of the reconstitution medium affected the formation of subvisible particles and solution turbidity when the lyophilized mAb was exposed not only to shaking stress, but also to subsequent storage for three months at various temperatures.

Materials and Methods

Materials

Lyophilized IgG1 mAb samples prepared at 0.6% and 6.8% moisture content, and their corresponding matching placebos without protein, were supplied in stoppered 20 mL glass vials by Human Genome Sciences (currently GlaxoSmithKline). Upon reconstitution with 5 mL of deionized water from Water Pro PS Station (Labconco, Kansas City, MO), the target concentration was approximately 30 mg/mL protein, in a formulation consisting of 0.08 mg/mL citric acid monohydrate (Avantor Performance Materials, JT Baker 0115, Center Valley, PA), 1.6 mg/mL sodium citrate dihydrate (Avantor Performance Materials, JT Baker 3647), 11 mg/mL glycine (Avantor Performance Materials, JT Baker 0581), 3 mg/mL sucrose (Avantor Performance Materials, JT Baker 4005), and 0.12 mg/mL polysorbate-80 (Croda International, SR48833, England) at pH 6.5. Lyophilized samples were stored at 4 °C unless otherwise indicated. The IgG1 mAb has a pI of ~8.4 and protein concentrations were determined by UV spectroscopy at 280 nm with an extinction coefficient of $\varepsilon^{0.1\%} = 1.58\ (g/100mL)^{-1}\ cm^{-1}$.

Methods

Shaking stress studies—Glass vials containing lyophilized and liquid IgG1 protein samples and corresponding placebo controls were taped horizontally inside of a lightweight, cryogenic box (13 × 13 × 5 cm), which was then taped to the cuphead of a 4.9 mm orbit Fisher Scientific Analog Vortex Mixer (Waltham, MA) and shaken vigorously at 3200 rpm for different periods of times at ambient temperature. Depending on the experiment, the shake-stressed samples were either in the lyophilized (solid) state or in the reconstituted (liquid) state. Unstressed and stressed samples of lyophilized protein and placebos were reconstituted with 5 mL of deionized water (Labconco) over 10 s, unless otherwise noted. To prepare the liquid samples, the lyophilized protein formulation was reconstituted with 5 mL of deionized water (Labconco) prior to shaking.

To study the effect of reconstitution medium type and addition rate, shake-stressed (for 24 h) and unstressed lyophilized IgG1 mAb samples were reconstituted by adding 5 mL of four different diluents (deionized water, 150 mM NaCl, 150 mM NaCl + 0.05% polysorbate 80, and 500 mM NaCl) at two rates (5 mL injected over 10 s and 5 mL injected over 2.5 min). To study the effect of shake-stress on subsequent storage stability, lyophilized mAb samples in stoppered glass vials were shaken for 2.5 min or 24 h and placed at 4, 40, or 55°C for up to 3 months at ambient humidity. Intact, unstressed samples (no cake breakage) were also stored up to 3 months at these temperatures. At time zero in the stability study, intact cake samples, 2.5 min shaking, and 24 h shaking samples were analyzed immediately (no storage). Placebos were also analyzed after 3 months storage at 55°C.

Turbidity—To monitor solution turbidity of samples, a HACH 2100 AN turbidimeter (HACH, Loveland, CO) was used. Prior to analyzing the experimental samples, StableCal calibration standards (Hach, Loveland, CO), ranging from <0.1 to 4000 Nephelometric Turbidity Units (NTU), containing hexamethylenetetramine and demineralized water, were used for generating a standard curve. The method is based on comparing intensity of light scattered by a sample under defined conditions with the intensity of light scattered by a standard reference suspension. Samples were not centrifuged or diluted for analysis.

Size-exclusion HPLC (SE-HPLC)—A Shimadzu UFLC HPLC system equipped with a diode-array detector and a Tosoh Bioscience (Tokyo, Japan) TSK-Gel G3000SW_{XL} (7.8 mm ID × 30.0 cm, 5μm) and the corresponding guard column (TSK-Gel Guard Column $SW_{XL,}$ 6.0 mm ID × 4.0 cm, 7μm) were used to monitor for the presence of soluble aggregates (< 100 nm). Prior to sample runs, the columns were rinsed for 60 min with deionized water followed by equilibration at 30°C for 1 h using mobile phase (10 mM sodium phosphate, 450 mM sodium chloride, pH 7.4) at a flow rate of 0.5 ml/min. Molecular weight standards (Biorad Laboratories, Hercules, CA) were run to test for efficacy of separation and resolution. Samples were centrifuged at 16,000 × g for 5 min and 10μL of supernatant was injected for analysis and monitored at 280 nm for each 35 min sample run. Aggregates, monomers, and fragment peaks were quantified using the LC Solutions data analysis software provided with the instrument.

Nanoparticle Tracking Analysis—Submicron sized particles (50-1000 nm) were measured using a Nanosight LM-14 (Nanosight, Amesbury, UK) with a CCD camera. Stressed samples and controls were centrifuged at 16,000 × g for 5 min, to separate larger aggregates outside the instrument sizing range. The supernatant was diluted 100 fold in formulation buffer (η=1.08 mpa. S for formulation buffer and η=1.36 mpa. S for the unstressed sample). Three hundred microliters of the 100 fold diluted supernatants were injected into the sample holder. Three 30 s movies were taken at ambient temperature for each sample. All samples were corrected for dilution. Data analysis was performed using the NTA 2.3 software, provided with the instrument, with detection threshold of 16, a screen gain of 7, and a minimum expected particle size of 50 nm.

Dynamic Light Scattering (DLS)—DLS measurements were performed to monitor small nanometer sized particles (1-1000nm) using a DynaPro™ Plate Reader (Wyatt Technologies, Santa Barbara, CA). Prior to analysis, the protein samples and controls were centrifuged at 16,000 × g for 5 min to remove large aggregates. The supernatant was separated and 30μL of the supernatant was loaded into a clear bottom 384 well assay plate (Corning Incorporated, Corning, NY). The plate was then centrifuged at 1177 × g for 3 min to remove air bubbles. Measurements were performed at 20°C with auto attenuation using the globular protein model and with the viscosity values determined using an Anton Parr Stabinger Viscometer 3000 (Anton Parr Inc., Ashland, VA). The data were collected using the Dynamics V 7.1.6 software, provided with the instrument, and analyzed using multimodal analysis.

Resonant Mass Measurements—Analysis of 0.25 to 3 micron sized particles was accomplished using an Archimedes particle metrology system (Affinity Biosciences, Santa Barbara, California). The instrument was first calibrated with NIST standard 1 μm polystyrene beads prior to analyzing experimental samples. To prevent clogging of the Hi-Q micro sensors, samples were centrifuged at 16,000 × g for 5 min and supernatants were analyzed. Triplicates of each sample were allowed to run until 500 particles were counted to obtain statistically significant data. Particle Lab software, provided with the instrument, was used to obtain particle size and concentration.

Micro-flow Digital Imaging and Radar Chart Analysis—Micron sized subvisible particles (2-100 μm) were analyzed and imaged using an MFI DPA-4200 (Protein Simple, Santa Clara, CA). See the method described in Telikepalli et al. 2014[26] for further details. Protein containing samples were diluted 100 fold prior to analysis and this dilution factor was accounted to determine particle concentration. MFI's MVAS 1.3 software was used to collect particle imaging data, which was then analyzed by radar plots to assess the particle size and concentration distribution for the unstressed and stressed samples using in-house software (Middaugh Suite) as described in detail elsewhere[26-28]. Additional radar plot analysis was performed in which the particle concentrations in each size bin for the stressed samples were normalized relative to its “control,” an unstressed sample that is similar in all other parameters. This normalization helps in visualizing and rank ordering the relative impact of a particular formulation parameter (e.g., stress) on the relative extent and size distribution of particle formation.

SDS-PAGE—Samples were mixed with 4× NuPAGE LDS sample buffer (Life Technologies, Carlsbad, CA) with and without, 50mM dithiothreitol (BioRad Laboratories, Hercules, CA) and incubated at 80°C for 90 s. Approximately 10 μg of each sample was separated on a 3-8% Tris-Acetate gel using Tris-Acetate Buffer (Life Technologies, Carlsbad, CA). Hi-Mark Unstained Protein Standard (Life Technologies, Carlsbad, CA) was used as a molecular weight ladder. Protein bands were visualized by staining the gels with Bio-safe Coomassie Blue G250 stain (BioRad Laboratories, Hercules, CA).

FTIR—Unstressed lyophilized mAb, after being reconstituted with 5 mL deionized water over 10 s, was analyzed for overall secondary structure content as a function of temperature by Fourier transform infrared spectroscopy (FTIR) using instrumentation and methodology presented in Telikepalli et al. 2014[26].

FTIR Microscopy-15× Objective-Reflectance mode—The method from Telikepalli et al. 2014[26] was used to prepare and isolate protein particles from stressed samples, and then to perform overall secondary structure analysis of proteins within individual isolated particles using a Bruker Hyperion FTIR Microscope (Bruker Biosciences, Billerica, MA) with a 15× objective in reflectance mode to image individual particles on gold coated filters (Pall Corporation, Port Washington, NY).

Results

Comparison of the physical stability of an IgG1 mAb formulation in the solid and liquid state during shaking

Samples of the IgG1 mAb were shaken (to simulate extreme shipping stress conditions) in the same formulation in the liquid or solid state for 5 min, 2h, 6h, and 15 h. Upon shaking, the solid state lyophilized cake increasingly turned into a finer, broken down powder after each of these shaking conditions. The physical stability of the mAb was assessed by a combination of analytical techniques including SE-HPLC, DLS, NTA, MFI, and turbidity. These techniques were used to determine differences in the aggregation behavior of protein from lyophilized and liquid shake-stressed samples across a wide aggregate size range of nanometers to hundreds of microns[29]. Aggregate formation in the size range of 1-1000 nm (detectable by a combination of SE-HPLC, DLS, and NTA) was minimal with similar results in both types of stressed samples (data not shown). For example, with SE-HPLC, approximately 99% monomer and 1% aggregate was noted at time zero with no change after shake-stress. Additionally, no changes in the total area of the SEC peaks were observed, suggesting no detectable change in the total protein concentration by this method. DLS showed predominantly monomers (approximately 5 nm radius), with no changes in hydrodynamic size of protein in these samples after shake-stress. For NTA analysis, submicron sized particles in the range of 100-300 nm were observed in the samples with no changes noted in particle concentration and size distribution as a function of shake-stress (data not shown).

In contrast, differences in solution turbidity and micron size particle concentrations (detected by MFI) were observed between the liquid and lyophilized mAb samples after shaking as shown in Figure 1A and 1B, respectively. The turbidity of the liquid sample does

not appear to change even after 15 h of shaking. In contrast, for the lyophilized sample, the turbidity of the solution (upon reconstitution) increases as a function of shaking time (Figure 1A). After stressing the liquid and lyophilized samples for 5 min up to 15 h, the liquid and lyophilized samples both showed a small increase in the total number of micron sized particles as measured by MFI (Figure 1B). Given the variability in the particle concentration determinations, it was concluded that both the liquid and lyophilized stressed samples generated similar levels of micron particles, with the 15 h time point showing a trend such that the lyophilized stressed sample may actually produce a greater number of micron sized particles than the liquid stressed samples.

The relative instability of the lyophilized protein was not anticipated. In fact, it was assumed that the protein in liquid state would be more susceptible to physical degradation by shaking, compared to the lyophilized state, even though the formulation contained polysorbate 80, a non-ionic surfactant which is known to stabilize against shaking-induced degradation in liquid formulations. To better understand these observations, a series of experiments were performed as described below to examine the effect of shaking the lyophilized formulation on the physical stability of the IgG1 mAb (measured upon reconstitution) as determined by solution turbidity and formation of micron sized particles (MFI).

Characterization of particle formation in shake stressed lyophilized mAb samples

In an initial set of experiments, the effect of moisture content on the physical stability of the lyophilized mAb after shaking was assessed. Both “low” moisture (0.6%) and “high” moisture (6.8%) freeze-dried samples were prepared and then shaken for 24 h, turning both lyophilized samples into broken apart, finer powders, prior to reconstitution. No increases in size or in concentration of soluble aggregates or submicron particles were observed as a function of stress or moisture content by SE-HPLC and NTA, respectively (data not shown). In addition, DLS analysis did not show the presence of species other than the monomer across the four samples (data not shown). Twenty-four hours of shaking increased the solution turbidity and micron sized particle counts for both samples (Figure 2). The shake-stressed 6.8% moisture lyophilized mAb sample showed somewhat increased levels of turbidity and particle counts when compared to the 0.6% moisture samples.

The MFI total particle concentration data displayed in Figure 2 were further analyzed and displayed as a radar chart to better visualize the particle size distributions in the four samples as shown in Figure 3. Two different scales, shown on the right side of the figure, are used to analyze these MFI data. The “particle number” scale, used to display the actual MFI particle size and concentration data for samples at time 0 and after 24 hours of shaking, has the innermost circle representing the lowest particle concentration of ∼0 particles/mL and the outermost corresponding to the highest concentration of $\sim 2.5 \times 10^6$ particles /mL. The 2-5 μm size bin starts at the top and increases clockwise up to a 25-40 μm size bin. The “normalized” scale shows particle concentrations of the stressed sample relative to its unstressed control for each size bin. Thus the scale increases from 0× (innermost circle) to 50× (outermost circle), where the latter indicates the number of particles in a size bin after shake-stressing are 50× greater than the number of particles in the same size bin at time 0 (no shaking).

For both the low and high moisture samples, the lyophilized mAb shows a low number of micron-sized particles upon reconstitution. After 24 h of shaking, the number of 2-5 μm particles increased (along with a small increase in 5-10 μm particles) in both moisture level samples. However, the 6.8% moisture samples formed more 2-5 μm particles than the 0.6% samples after shaking. This effect is more clearly reflected in the normalized radar plots where, it can be seen that the shake-stressed 6.8% moisture sample shows a larger relative increase in particles compared to its control than the shake-stressed 0.6% moisture sample. For example, there is an ∼40 fold increase in the formation of 2-5 μm particles compared to the unstressed control for the 6.8% moisture sample, but only about a 10 fold increase of the 2-5 μm for the shaken 0.6% moisture sample. Similarly, a larger relative increase is observed for the formation of 5-10 μm sized particles, with an almost 35 fold increase for the higher moisture sample after shaking.

In addition to sizing and counting particles, the nature of the protein within the particles formed was examined by SDS-PAGE and FTIR analysis. In both the control and shake-stressed lyophilized samples at the two different moisture levels, non-reduced and reduced SDS-PAGE gels were compared. In the non-reduced gel, mostly IgG1 monomers with some fragments and dimers were observed. Upon reduction with dithiothreitol, however, dimers were reduced and only heavy and light chains of the IgG1 can be seen (see Supplemental Figure S1). However, it appeared that these dimers were not forming as a function of the shake-stress and were present in all of the samples. Additionally, upon centrifugation of samples into supernatant and pellet components, no notable differences were seen in both the non-reduced and reduced SDS-PAGE gels for the supernatant, pellet, or the non-centrifuged samples.

FTIR and FTIR Microscopy were used for the evaluation of overall secondary structure of the mAb in solution and of the mAb within the particles formed as a function of shaking stress in the lyophilized samples, respectively. Representative FTIR spectra, and corresponding wavenumber positions from triplicate measurements, are shown for two control samples and for the 0.6% and 6.8% moisture lyophilized mAb samples in Figures 4A and 4B, respectively. The solid black line corresponds to the FTIR second derivative spectrum of the Amide I band of the unstressed, control mAb in solution and shows spectra with minima around 1636 and 1690 cm^{-1}, which correspond to the intramolecular beta sheets that are in the main secondary structure of antibodies. To determine the extent of secondary structure loss that is possible with this mAb, the mAb solution was extensively heated and the resulting isolated particles were analyzed by FTIR Microscopy. The second derivative spectra, depicted as blue dotted graphs, possess minima at 1622 cm^{-1} and 1619 cm^{-1} and 1692 cm^{-1} and 1693 cm^{-1} indicating loss of intra-molecular beta sheets and formation of inter-molecular beta sheets (i.e., aggregation). The red dotted lines are the second derivative spectra of the isolated protein particles obtained from each of the two shake-stressed samples by FTIR Microscopy (after reconstitution of the low and high moisture lyophilized mAb samples followed by filtration and capture of particles on a gold filter). Compared to the two control samples (unstressed sample in solution and heat control), the IgG1 in the protein particle from the T=24 h shake-stressed sample has similar overall secondary structure to the unstressed control. However, these isolated protein particles may have a slightly altered overall secondary structure content compared to the

protein in the unstressed sample since their spectra show a small shift in the average minima around 1633 cm^{-1} and 1691 cm^{-1}. Importantly, the particles obtained from the 0.6 and 6.8% moisture samples showed similar levels of change in protein secondary structure indicating that for this IgG1 mAb, moisture content in conjunction with shake stress do not seem to largely impact the secondary structure of the protein within the particles.

Characterization of particle formation in shake stressed lyophilized mAb samples as function of reconstitution medium type and addition time

For both the low and high moisture containing lyophilized mAb samples, the control (unstressed) vials fully reconstituted in about 30 s, while the shake-stressed vials took ~ 1.5 min. This was similar regardless of the medium type or medium addition rate. There was no difference in reconstitution time as a result of moisture content, so the effect of medium type and addition rate were further examined with the low moisture lyophilized sample. The 0.6% moisture lyophilized sample was shaken for 24 h and reconstituted with 5 mL of different mediums at two different rates and then monitored for their effects on physical stability of the mAb by measuring solution turbidity and the concentration of micron-sized particles. As shown in Figures 5A and 5B, the shake stressed lyophilized samples were more turbid and contained higher concentrations of micron-sized particles than the non-shaken samples upon reconstitution. Reconstitution with water consistently led to higher solution turbidity and higher micron-sized particle concentrations than reconstitution with the other diluents. In addition, the role of medium addition rate was examined and no effects were observed for most of the conditions with two exceptions: (1) for the 24 h shake-stressed lyophilized samples reconstituted at a slow rate with 150 mM NaCl + 0.05% polysorbate 80, higher turbidity and increased particle counts were observed compared to the fast addition of this medium, and (2) while the turbidity did not change, reconstituting the shake-stressed lyophilized sample slowly with 150 mM NaCl solution produced more micron-sized particles than the same sample reconstituted at the fast rate.

Radar plot analysis of the MFI particle data showed some distinct relative changes in particle size distribution depending on the medium selection and the rate of addition (Figures 6 for water as the medium and Figure 7 for the other diluents; note the scale differences in the two figures). Regardless of medium type, predominantly 2-5 μm particles are formed upon shake-stress lyophilized samples upon both the slow and fast medium addition. When water was used as the reconstitution medium (Figure 6), there was almost a 15 fold increase in the formation of 5-10 μm particles upon slow reconstitution compared to the unstressed sample similarly reconstituted. In contrast, rapid addition of water resulted in a smaller relative increase in particles formed in this same size range. For the other medium types, the addition rate was found to have some effect on the micron particle concentration as shown in Figure 7. Slow reconstitution of the shake-stressed lyophilized samples with 150 mM NaCl and 150 mM NaCl+0.05% polysorbate 80 produced a larger relative increase (as seen by the normalized plot) in the formation of micron particles across the size bins. Such a relative increase in particle formation was not observed upon more rapid addition of these reconstitution media.

Effect of shake stressing lyophilized mAb samples on subsequent storage stability

A three month stability study was performed using the lyophilized mAb samples (0.6% moisture) after exposure to different amounts of shake stress. Intact lyophilized cakes (unstressed controls), slightly broken cakes (shaken for 2.5 min) and completely broken lyophilized cakes (shaken for 24 h) were stored at 4, 40, and 55°C for 3 months (at ambient humidity) in stoppered glass vials. Representative pictures of the physical integrity of these lyophilized cakes corresponding to the varying levels of shake stress are shown in Figure 8A. Upon fast addition with 5 mL deionized water, samples were analyzed by a combination of NTA, MFI, and turbidity. NTA showed no changes in aggregation due to stress (data not shown). In contrast, solution turbidity (Figure 8A) and micron-sized particles measured by MFI (Figure 8B) did reveal some stability differences as a function of shake stress and storage temperature (in addition, at the higher temperatures and longer time periods, slight yellow color changes were noted in all of the samples upon reconstitution; data not shown). As shown in Figure 8A, all samples showed an increase in turbidity with increasing storage temperature (4°C < 40°C < 55°C). If samples stored for identical time periods are considered, turbidity did not significantly increase in the intact and 2.5 min shaken samples. The samples stored at 55°C were the most turbid, followed by the samples stored at 40°C (4°C < 40°C < 55°C). Figures 8C and 8D show normalized turbidity and normalized micron particle concentrations obtained by taking a ratio of turbidity (or micron particle concentration) of the stressed sample to its control (T=0) for a given shaking duration. This shows the change in turbidity or number of micron particles in a sample relative to its unstressed control. At 40°C, after three months of storage, the relative change in turbidity and total subvisible particle concentration for all shaken samples is approximately 1.5× and 2-3×, respectively. At 55°C after three months, the relative change for the shaken samples is about 3.5-5× and 7-15× for turbidity and total micron particle concentration, respectively.

In summary, Figures 8A and 8B show increasing turbidity and particle counts with increasing shaking duration, storage time, and temperature. However, the T=0 samples themselves show an increase in turbidity and micron sized particle counts with increasing shaking duration (T=0 Intact cake < T=0 2.5 min shaking < T=0 24 h shaking). Higher temperatures and longer storage show an increase in turbidity and particle counts relative to the control (4°C < 40°C < 55°C and T=0 ∼ T=1 month < T=3 months) within each shaking time. When different shaking times are compared, in terms of relative changes versus time zero (Figures 8C and 8D), however, both relative turbidity and relative micron particle concentration changes stay constant or even decrease, especially for the samples stored at 55°C for three months as the cake structure is increasingly collapsed.

Discussion

The focus of this work was to better characterize how shaking of a freeze-dried IgG1 mAb formulation can affect protein stability upon reconstitution. We first examined the lyophilized IgG1 mAb control (no shake-stress), which showed minimal levels of physical instability after reconstitution. These initial results indicated the formulation composition and lyophilization cycle resulted in a stable protein preparation. While there is a plethora of literature on how to effectively formulate and stabilize protein drugs during the

lyophilization process and subsequent long term storage,[1,5,17,24,35] the effect of subsequent mechanical stresses applied to the lyophilized dosage form on physical stability and aggregation of mAb, which could potentially occur during shipping and handling, has not been examined to the same extent.

Physical Stability of Shake-stressed mAb in Lyophilized State

In this work, mechanical shaking-stress was applied to the liquid and lyophilized state of an IgG1 mAb formulation, and a variety of analytical techniques were used to assess the physical stability of the mAb. These results highlight the need to examine protein aggregation across a wide size range since no one analytical approach covers the different size ranges of protein aggregates and particles that may form[26,29,36]. SEC and DLS, two very commonly used techniques to monitor soluble, nanometer sized aggregates, along with NTA, to detect submicron sized particles, showed no differences in particle levels between control (unstressed) and shake-stressed samples across the various experiments. Interestingly, although the shaking stress had minimal effects on the formation of soluble aggregates and smaller submicron sized particles, physical instability was detected by turbidity measurements and by formation of larger micron size particles (as shown by MFI). Thus, shake-stressing the lyophilized mAb, followed by reconstitution, led to increased levels of protein particles in the subvisible size range (~2-100 microns) but not in the smaller, submicron size ranges. These results highlight the need to examine protein aggregation across a wide size range since no one analytical approach covers the different size ranges of protein aggregates and particles that may form[26,29,36]

The shake-stress had minor effects on the conformation and composition of the protein contained within these particles as evidenced by SDS-PAGE and FTIR analysis. Upon shake stressing freeze-dried cakes, the resulting protein particles, formed in two different moisture level samples, showed similar levels of non-native disulfide crosslinks (SDS-PAGE) and the presence of slightly altered overall secondary structure content compared to the native protein (FTIR analysis). In contrast, the role of non-native disulfide bond formation in the generation of aggregates appears less influential than has been observed previously with other lyophilized mAbs[17]. A recent study performed in our laboratory studied the aggregation behavior of a mAb undergoing shaking stress. While this was a different IgG1 mAb, in a liquid solution and in a different formulation, shake stress also led to formation of particles with only minor changes in overall conformational integrity of the protein within the particles as seen by SDS-PAGE, FTIR and by ANS fluorescence spectroscopy[26].

The effect of the residual moisture content of the lyophilized cake on the physical stability of the mAb after shake-stress was also evaluated. There is an abundance of literature describing the effects of moisture content on the stability of lyophilized protein therapeutics[19-22] Improved protein stability is often observed at lower moisture contents of a lyophilized cake, with values not exceeding 2.0% [6], however, exceptions have been noted. For example, when the stability of a lyophilized humanized mAb produced with varying moisture contents and stored at elevated temperatures up to a year was analyzed,[22] no cake collapse or changes in protein secondary structure were observed[22] but the intermediate moisture level samples were more resistant to aggregation. The relationship between

moisture content and protein aggregation potential is complex and may be very dependent on the protein itself, formulation composition and freeze-drying conditions[1,5-7,22]. In our study with an IgG1 mAb, the physical stability of the 6.8% moisture sample was not drastically different than the 0.6% moisture sample during lyophilization or upon exposure to subsequent mechanical stress.

Effect of Reconstitution on Shake-Stressed Degradation of Lyophilized mAb

The choice of medium used to reconstitute the lyophilized IgG mAb was an important factor in this study[6,37]. Using water as the diluent for both the unstressed and shake-stressed lyophilized mAb samples resulted in higher solution turbidity, and in an increased concentration of micron sized particles, compared to the use of other diluents. The addition of sodium chloride solutions partially inhibited the formation of micron particles in our studies probably because the sodium chloride reduces protein-protein colloidal interactions.[638] Previous studies have shown the importance of ionic strength in reconstitution solutions where either increased or decreased ionic strength of the reconstitution medium inhibited protein aggregation, highlighting the protein and formulation specific nature of these observations. [33,37-40] However, sodium chloride containing diluents result in reconstituted mAb solutions with higher solution osmolality, which might be a concern depending on the route of administration (e.g., subcutaneous injection into the patient). The presence of polysorbate 80 in the reconstitution buffer perhaps had a small stabilizing effect on the formation of subvisible particles upon reconstitution. Numerous studies have shown that polysorbate can decrease aggregation when used in the reconstitution medium[38,39,41-43]. It has been suggested that surfactants may increase wettability of powders leading to increased dissolution rate of lyophilized powders, inhibit surface induced denaturation during reconstitution, or stabilize the native state of the protein by increasing the free energy of unfolding. [39,41,43] Stabilizing compounds, such as polysorbate 80 or NaCl, in the diluent may prevent or reduce protein-protein interactions, and/or the physical dilution imparted by their addition can lead to separation of protein molecules and hinder aggregation[7].

Upon reconstitution, the type of diluent and the rate of diluent addition can affect not only protein stability, [6,40] but also reconstitution times. The rate of diluent addition on the physical stability of the reconstituted, lyophilized IgG1 mAb in this study was, in general, not an important factor. However, the time required for reconstitution was longer for the shake-stressed lyophilized samples than the unstressed vials (approx. 0.5 vs. 1.5 minutes), regardless of the rate of addition or the type of diluent used. Since the physical state of the lyophilized mAb impacts dissolution time upon reconstitution, it is possible this results in differences in local protein or excipient concentrations and subsequently to the observed differences in subvisible particle formation. The higher surface area and porosity present in the lyophilized cake, compared to the finer powder formed after shaking, may allow it to dissolve faster than the powder[44]. However, disruption of lyophilized cake structure may not be the only destabilizing mechanism. Vibrational forces, or local heating effects generated from shaking itself, can potentially increase the contact area of powder particles allowing increased interactions between them[45] leading to a cohesive powder that is “sticky.”[46]

These differences in reconstitution time did not change, however, when the samples were reconstituted at different rates and with different diluents. While this lyophilized protein formulation had a relatively short reconstitution time, for other lyophilized protein samples in which the reconstitution time is longer and may be problematic during clinical administration, techniques mentioned by Cao et al[15] could be beneficial. For example, reconstituting under vacuum, adding wetting agents, and/or using low diluent volumes were observed to be methods that can decrease reconstitution times in a high concentration lyophilized formulation of a Fc-fusion protein[15]. Additionally, three reconstitution procedures were described, in which the reconstitution medium is added and the vial is gently swirled for different periods of time, resulting in different reconstitution times[15]. In addition to reconstitution time, such reconstitution procedures could be evaluated in the future to assess their ability to minimize subvisible particle formation during reconstitution of shake stressed lyophilized protein powders.

Storage of Shake-stressed Lyophilized mAb Samples

The effects of the extent of shaking stress on subsequent storage stability of the freeze dried IgG1 formulation were also analyzed in this work. Lyophilized mAb samples were prepared as follows: (1) unstressed, physically intact cakes, (2) brief shaking (2.5 min) resulting in some cake breakage, and (3) extensive shaking (24 h) resulting in the cake being broken down to a powder. These samples were stored at different temperatures over a three month period, and at each time point, were reconstituted rapidly with 5 mL of water. Increasing shaking stress on the freeze dried cake showed some small differences with increased levels in turbidity and subvisible particles. Solution turbidity and subvisible particle concentration increased with increasing storage (T=0 < T=1 month <T=3months), especially for the samples stored at 40°C and 55°C. Similar to Schersch et al[19-21], we also noticed a color change during storage of the lyophilized protein at higher temperatures in this formulation, which they reasonably attributed to the well-known non-enzymatic browning (Malliard-type) reaction between reducing end sugars (potentially due to degraded sucrose) and lysine residues in the protein. The turbidity levels and subvisible particle concentrations are highest for the 24h shaken samples compared to the intact and 2.5 min shaken samples (Intact ∼ 2.5 min shaking < 24 h shaking). Even though Figures 8A and 8B show increasing turbidity and particle counts with increasing shaking duration, storage time, and temperature, comparing samples to appropriate controls provides fuller understanding of the importance of shaking stress in inducing turbidity and micron particle formation. Figure 8C and Figure 8D compare a particular sample with its relevant control (T=0). Within each shaking time, higher temperatures and longer storage show increase in turbidity and particle counts relative to the control (4°C < 40°C < 55°C and T=0 ∼ T=1 month < T=3 months). When different shake times are compared, however, both turbidity and micron particle counts stay relatively constant or decrease, especially for the samples stored at 55°C for three months. This is largely because the controls themselves (T=0) increase in both turbidity and particle counts with increasing shaking duration so the instability associated with storage temperature and storage duration appear less profound.

In comparison, Schersch et al. have examined the effect of cake collapse (due to a variety of causes) during freeze-drying and subsequent storage at elevated temperatures on the stability

of a different IgG1 mAb.[19-21] For example, when lyophilized cakes were collapsed by using different amounts of excipients or by using different freeze-drying protocols, the stability of protein in the collapsed and non-collapsed cakes were not different from one another. In addition, conformational integrity of the IgG1, as measured by FTIR analysis of the overall secondary structure, was also not affected by cake collapse.[19] Additionally, when the effect of cake collapse on long-term storage at various temperatures ranging from 2-50°C up to 6 months was analyzed, protein stability was retained in the collapsed and non-collapsed cakes after storage.[20] In addition, the stability of the freeze-dried samples that were collapsed during freeze-drying vs. samples collapsed only during subsequent storage at 40 and 50°C for 3 months were compared to determine if the method of collapse affected protein stability. In this case, the stability of the protein in the collapsed lyophilized cakes (collapse due to freeze-drying) was better than the stability of protein in the storage-collapsed samples. Overall, the authors concluded that the collapse (freeze-dried) samples appeared to be more stable than the collapsed (storage) samples.[21]

The method of cake collapse could be another important parameter to consider as well in terms of effects on storage stability. In this work, we mechanically stressed the samples to alter the cake integrity to different extents and then stored the cakes (with varying levels of physical collapse) at different temperatures. In comparison, Schersch et al. have examined in detail the effect of different methods of cake collapse on the stability of a different IgG mAb.[19-21] The stability of the samples collapsed during freeze-drying compared to samples during subsequent storage at elevated temperatures showed that the stability of the protein in the freeze-drying collapsed cake was better than the protein in the storage-collapsed samples. Overall, the authors concluded that the collapse (freeze-dried) samples appeared to be more stable than the collapsed (storage) samples[21].

Conclusions

This case study highlights that post-lyophilization mechanical stresses, potentially encountered during shipping and transportation excursions, can result in physical instability of a lyophilized protein upon reconstitution. For this particular IgG1 mAb formulation, a liquid dosage form (5 mL of a 30 mg/mL protein solution in a 20 mL stoppered glass vial) showed instability due to shaking stress, despite the presence of stabilizers including polysorbate 80. The common sense approach of lyophilization did not successfully address the issue since the lyophilized dosage form of the same formulation was also shake-sensitive. The increase in subvisible particle formation seen with the shake-stressed lyophilized IgG1 mAb upon reconstitution correlated with the formation of a finer powder and increased dissolution times, while no major differences in the structural integrity of the protein within the particles was noted. Thus, local differences in protein and excipient concentrations, upon the wetting and dissolution of the shake-stressed vs. control lyophilized cakes, likely contribute to these observations.

It is acknowledged that the level of shake stress that the cakes were exposed to in this study was relatively high, but this was a useful means of rapidly characterizing various conditions with respect to product quality impact (e.g., cake moisture, reconstitution rate, medium type, etc). The level of mechanical stress that a product will be exposed to during typical shipping

and handling can be variable, and it is easier to interpret their effects by comparing to stressed, worst-case study designs. In addition, within the general ASTM shipping guidance documents[25], which are not specific for pharmaceutical products, the level of stress these documents suggest may be relatively high based on the schedule rating a user chooses to analyze.

The observations highlight the importance of considering shake sensitivity of lyophilized cakes in terms of protein stability as part of formulation development activities including the formulation composition, lyophilization process and reconstitution medium selection. Potential degradation is expected to be manageable by implementing an appropriate packaging and shipping configuration that will minimize or prevent extensive cake breakage. The use of orthogonal subvisible particle counting and sizing techniques such as light obscuration (HIAC) and/or coulter counters are suggested for future work for comparison to the MFI analytical results. Additional work is also required to further elucidate the nature of the physical degradation pathway(s) leading to protein particle formation during reconstitution of shake-stressed lyophilized mAb preparations, as well as to evaluate how this physical instability may vary with different IgG mAbs, other proteins and in the presence of different excipients and stabilizers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank and acknowledge Human Genome Sciences (currently GlaxoSmithKline) for financial support and for providing the IgG1 mAb for this study. Additional financial support was provided by the Kansas Bioscience Authority, and ST financial support was provided by NIH biotechnology training grant 5-T32-GM008359. The authors would also like to acknowledge Kevin O'Brien of GlaxoSmithKline for his help in preparing the mAb samples at various moisture contents. The authors would also like to thank the reviewers for their helpful suggestions.

References

1. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. Rational design of stable lyophilized protein formulations: theory and practice. Pharm Biotechnol. 2002; 13:109–133. [PubMed: 11987749]
2. Matejtschuk P. Lyophilization of proteins. Methods Mol Biol. 2007; 368:59–72. [PubMed: 18080462]
3. Tang X, Pikal MJ. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 2004; 21(2):191–200. [PubMed: 15032301]
4. Chang LL, Pikal MJ. Mechanisms of protein stabilization in the solid state. J Pharm Sci. 2009; 98(9):2886–2908. [PubMed: 19569054]
5. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res. 1997; 14(8):969–975. [PubMed: 9279875]
6. Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm. 2000; 203(1-2):1–60. [PubMed: 10967427]
7. Wang, W.; R, C., editors. Aggregation of therapeutic proteins. Hoboken, New Jersey: Wiley; 2010.
8. Kasper J, Winter G, Friess W. Recent advances and further challenges in lyophilization. Eur J Pharm Biopharm. 2013; 85:162–169. [PubMed: 23751601]

9. Awotwe-Otoo D, Agarabi C, Wu GK, Casey E, Read E, Lute S, Brorson KA, Khan MA, Shah RB. Quality by design: impact of formulation variables and their interactions on quality attributes of a lyophilized monoclonal antibody. Int J Pharm. 2012; 438(1-2):167–175. [PubMed: 22944306]
10. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009; 98(9):2909–2934. [PubMed: 18823031]
11. Heljo VP, Harju H, Hatanpaa T, Yohannes G, Juppo AM. The effect of freeze-drying parameters and formulation composition on IgG stability during drying. Eur J Pharm Biopharm. 2013; 85(3): 752–755. [PubMed: 23623795]
12. Abdul-Fattah AM, Dellerman KM, Bogner RH, Pikal MJ. The effect of annealing on the stability of amorphous solids: chemical stability of freeze-dried moxalactam. J Pharm Sci. 2007; 96(5): 1237–1250. [PubMed: 17455341]
13. Awotwe-Otoo D, Agarabi C, Read EK, Lute S, Brorson KA, Khan MA, Shah RB. Impact of controlled ice nucleation on process performance and quality attributes of a lyophilized monoclonal antibody. Int J Pharm. 2013; 450(1-2):70–78. [PubMed: 23618961]
14. Bhatnagar BS, Bogner RH, Pikal MJ. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol. 2007; 12(5):505–523. [PubMed: 17963151]
15. Cao W, Krishnan S, Ricci MS, Shih LY, Liu D, Gu JH, Jameel F. Rational design of lyophilized high concentration protein formulations-mitigating the challenge of slow reconstitution with multidisciplinary strategies. Eur J Pharm Biopharm. 2013; 85(2):287–293. [PubMed: 23702275]
16. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Critical reviews in therapeutic drug carrier systems. 1993; 10(4):307–377. [PubMed: 8124728]
17. Andya JD, Hsu CC, Shire SJ. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS PharmSci. 2003; 5(2):E10. [PubMed: 12866937]
18. Davis JM, Zhang N, Payne RW, Murphy BM, Abdul-Fattah AM, Matsuura JE, Herman AC, Manning MC. Stability of lyophilized sucrose formulations of an IgG1: subvisible particle formation. Pharm Dev Technol. 2013; 18(4):883–896. [PubMed: 22813478]
19. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: stability after freeze-drying. J Pharm Sci. 2010; 99(5):2256–2278. [PubMed: 20039389]
20. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: stability during storage at elevated temperatures. J Pharm Sci. 2012; 101(7):2288–2306. [PubMed: 22517663]
21. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins III: collapse during storage at elevated temperatures. Eur J Pharm Biopharm. 2013; 85(2):240–252. [PubMed: 23727369]
22. Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. Pharm Res. 2001; 18(9):1345–1353. [PubMed: 11683251]
23. Towns JK. Moisture content in proteins: its effects and measurement. J Chromatogr A. 1995; 705(1):115–127. [PubMed: 7620565]
24. Costantino HR, Langer R, Klibanov AM. Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin: effect of moisture and stabilization by excipients. Biotechnology (N Y). 1995; 13(5):493–496. [PubMed: 9634790]
25. International A. Standard Test Methods for Vibration Testing of Shipping Containers D 999-08. West Conshohocken, PA: ASTM International; 2008. ASTM Standards; p. 1-5.
26. Telikepalli SN, Kumru OS, Kalonia C, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Structural Characterization of IgG1 mAb Aggregates and Particles Generated Under Various Stress Conditions. J Pharm Sci. 2014; 103(3):796–809. [PubMed: 24452866]
27. Kalonia C, Kumru OS, Kim JH, Middaugh CR, Volkin DB. Radar Chart Array Analysis to Visualize Effects of Formulation Variables on IgG1 Particle Formation as Measured by Multiple Analytical Techniques. J Pharm Sci. 2013; 102(12):4256–4267. [PubMed: 24122556]

28. Kim JH, Iyer V, Joshi SB, Volkin DB, Middaugh CR. Improved data visualization techniques for analyzing macromolecule structural changes. Protein science : a publication of the Protein Society. 2012; 21(10):1540–1553. [PubMed: 22898970]
29. Wang, T.; Joshi, SB.; Kumru, OS.; Telikepalli, S.; Middaugh, CR.; Volkin, DB. Case studies applying biophysical techniques to better characterize protein aggregates and particulates of varying size. In: Narhi, LO., editor. Biophysics for therapeutic protein development. Springer; New York: 2013. p. 205-243.
30. Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. Advanced drug delivery reviews. 2011; 63(13):1118–1159. [PubMed: 21855584]
31. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang XC, Pikal MJ. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J Pharm Sci. 2005; 94(7):1427–1444. [PubMed: 15920775]
32. Carpenter JF, Prestrelski SJ, Dong A. Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur J Pharm Biopharm. 1998; 45(3):231–238. [PubMed: 9653627]
33. Prestrelski SJ, Pikal KA, Arakawa T. Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy. Pharm Res. 1995; 12(9):1250–1259. [PubMed: 8570516]
34. Murphy BM, Zhang N, Payne RW, Davis JM, Abdul-Fattah AM, Matsuura JE, Herman AC, Manning MC. Structure, stability, and mobility of a lyophilized IgG1 monoclonal antibody as determined using second-derivative infrared spectroscopy. J Pharm Sci. 2012; 101(1):81–91. [PubMed: 21918984]
35. Costantino HR, Carrasquillo KG, Cordero RA, Mumenthaler M, Hsu CC, Griebenow K. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. J Pharm Sci. 1998; 87(11):1412–1420. [PubMed: 9811499]
36. Kumru OS, Liu J, Ji JA, Cheng W, Wang YJ, Wang T, Joshi SB, Middaugh CR, Volkin DB. Compatibility, physical stability, and characterization of an IgG4 monoclonal antibody after dilution into different intravenous administration bags. Journal of pharmaceutical sciences. 2012; 101(10):3636–3650. [PubMed: 22733600]
37. Zhang MZ, Wen J, Arakawa T, Prestrelski SJ. A New Strategy for Enhancing the Stability of Lyophilized Protein: The Effect of the Reconstitution Medium on Keratinocyte Growth Factor. Pharm Res. 1995; 12(10):1447–1452. [PubMed: 8584478]
38. Roy S, Jung R, Kerwin BA, Randolph TW, Carpenter JF. Effects of benzyl alcohol on aggregation of recombinant human interleukin-1-receptor antagonist in reconstituted lyophilized formulations. J Pharm Sci. 2005; 94(2):382–396. [PubMed: 15614819]
39. Sarciaux JM, Mansour S, Hageman MJ, Nail SL. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. J Pharm Sci. 1999; 88(12):1354–1361. [PubMed: 10585234]
40. Zhang MZ, Pikal K, Nguyen T, Arakawa T, Prestrelski SJ. The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukin-2 and ribonuclease A. Pharm Res. 1996; 13(4):643–646. [PubMed: 8710761]
41. Webb SD, Cleland JL, Carpenter JF, Randolph TW. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. J Pharm Sci. 2002; 91(2):543–558. [PubMed: 11835212]
42. Jones LS, Randolph TW, Kohnert U, Papadimitriou A, Winter G, Hagmann ML, Manning MC, Carpenter JF. The effects of Tween 20 and sucrose on the stability of anti-L-selectin during lyophilization and reconstitution. J Pharm Sci. 2001; 90(10):1466–1477. [PubMed: 11745706]
43. Chang BS, Kendrick BS, Carpenter JF. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J Pharm Sci. 1996; 85(12):1325–1330. [PubMed: 8961147]
44. Sinko, PJ.; Singh, Y., editors. Martin's Physical Pharmacy and Pharmaceutical Sciences. 6. Baltimore, MD: Lippincott Williams & Wilkins; 2011. p. 659

45. Duran, J. Sands, Powders, and Grains: An Introduction to the Physics of Granular Materials. New York: Springer-Verlag; 2000. p. 214

46. Chaudhuri B, Mehrotra A, Muzzio FJ, Tomassone MS. Cohesive effects in powder mixing in a tumbling blender. Powder Technology. 2006; 165(2):105–114.

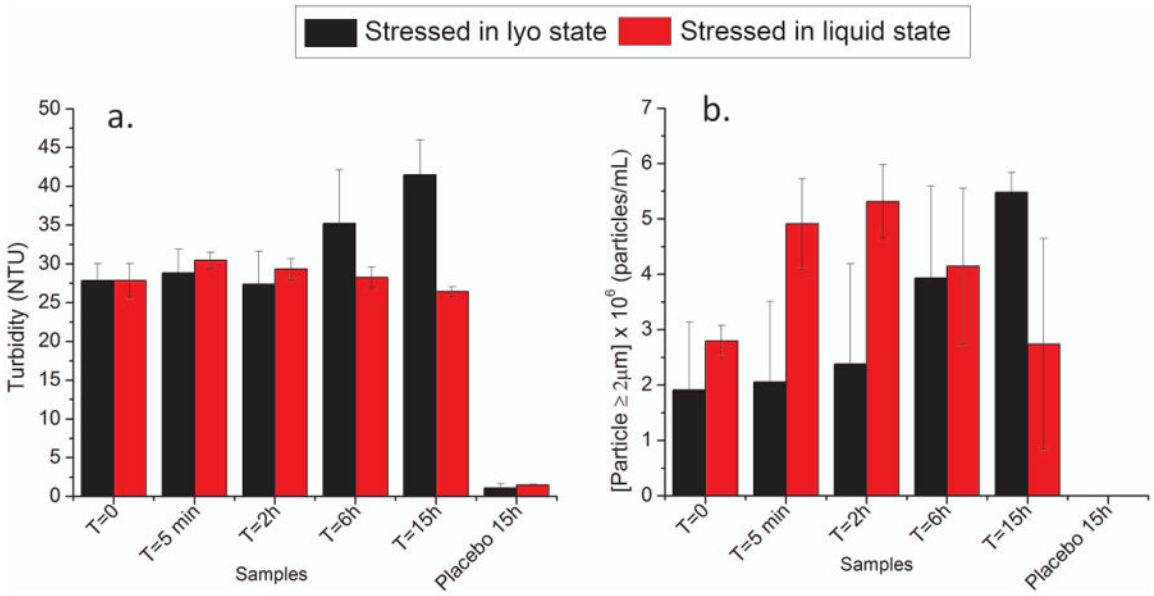


Figure 1.

Physical instability of an IgG1 mAb formulation after shaking in the solid (freeze-dried cake) or liquid state for 5 min, 2h, 6h, and 15h. Samples were monitored by (A) solution turbidity and (B) total concentration of subvisible particles (≥2 μm as measured by MFI). Lyophilized samples were reconstituted prior to analysis. Each graph represents the average of three separate experiments (n=3) and error bars represent one standard deviation. Placebo samples were measured after 15 h of shaking showed negligible turbidity and concentration of micron sized particles compared to the protein containing samples.

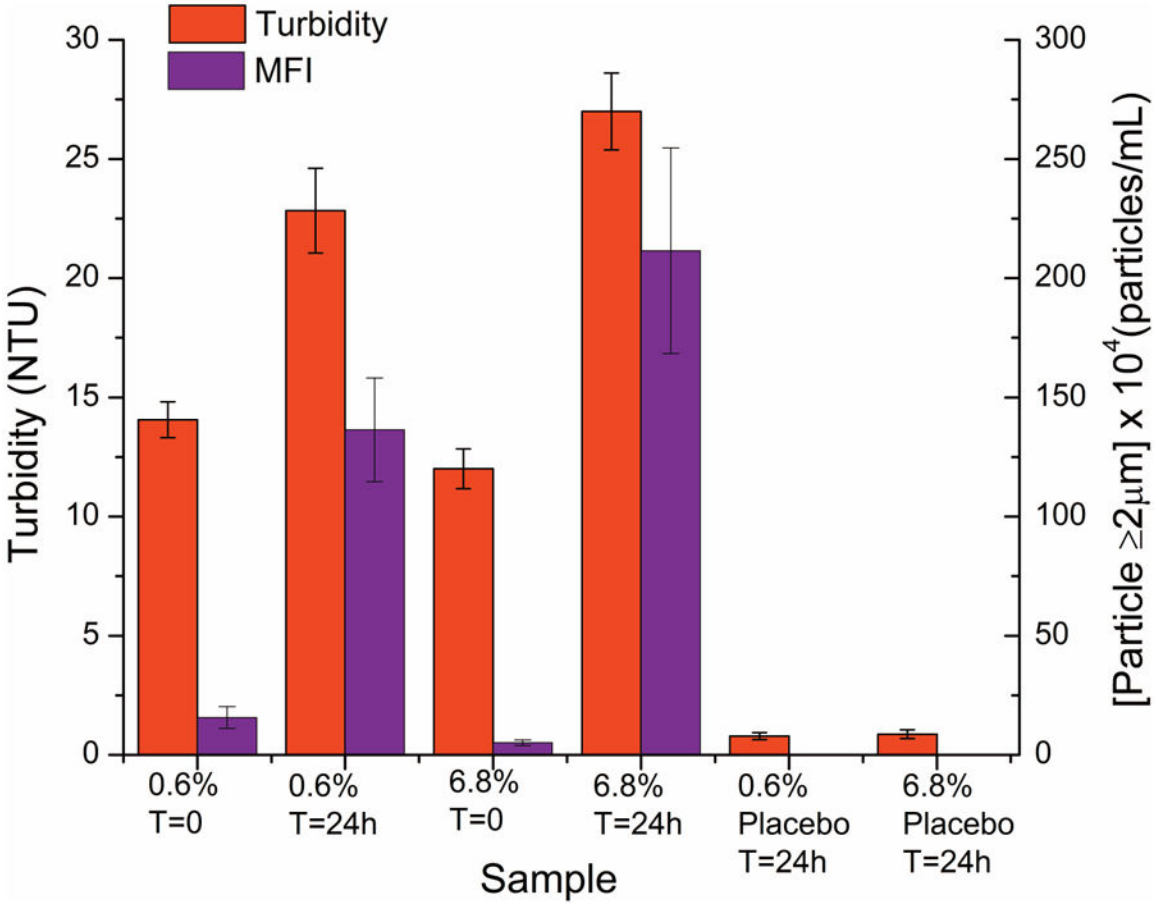


Figure 2.

Physical instability of a lyophilized IgG1 mAb samples prepared with 0.6% and 6.8% moisture content and shake-stressed for 24 h in the solid state. Samples were reconstituted with water and monitored for solution turbidity (left Y- axis) and subvisible particles by MFI (right Y-axis). Placebo samples were measured after 24 h of shaking and showed negligible turbidity and concentration of micron sized particles compared to the protein containing samples. The graph represents the average of three separate experiments (n=3) and error bars represent one standard deviation.

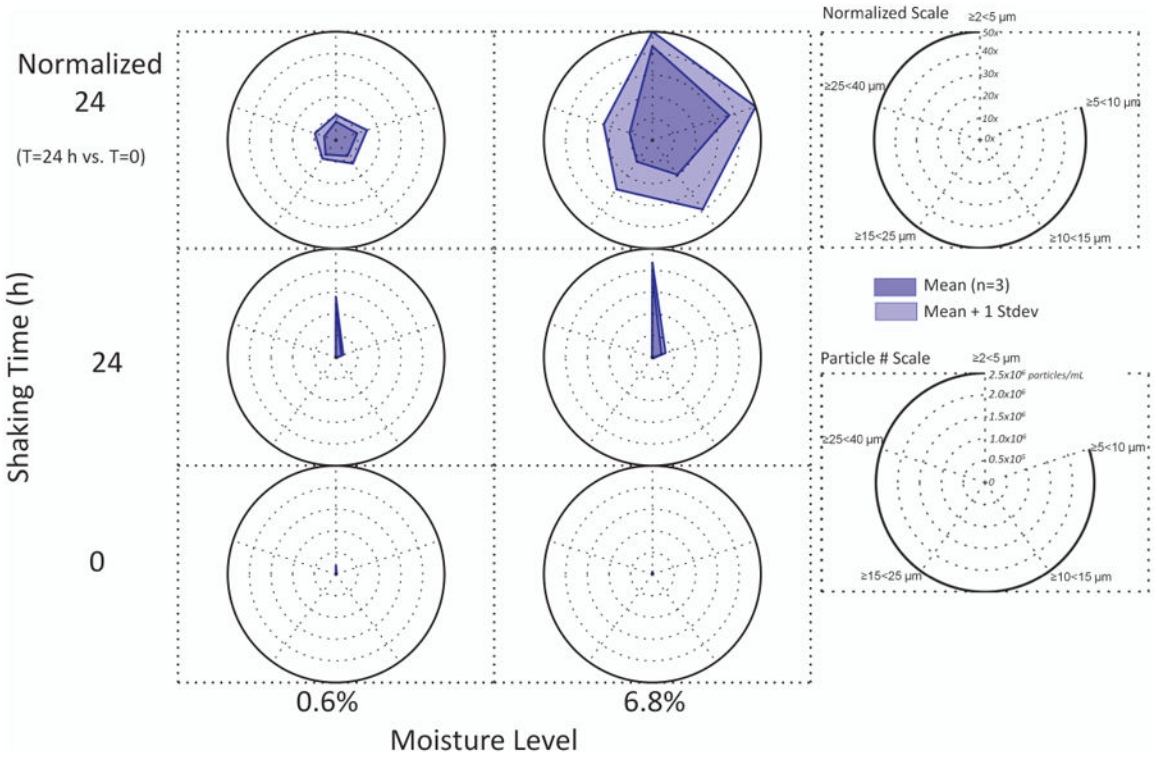
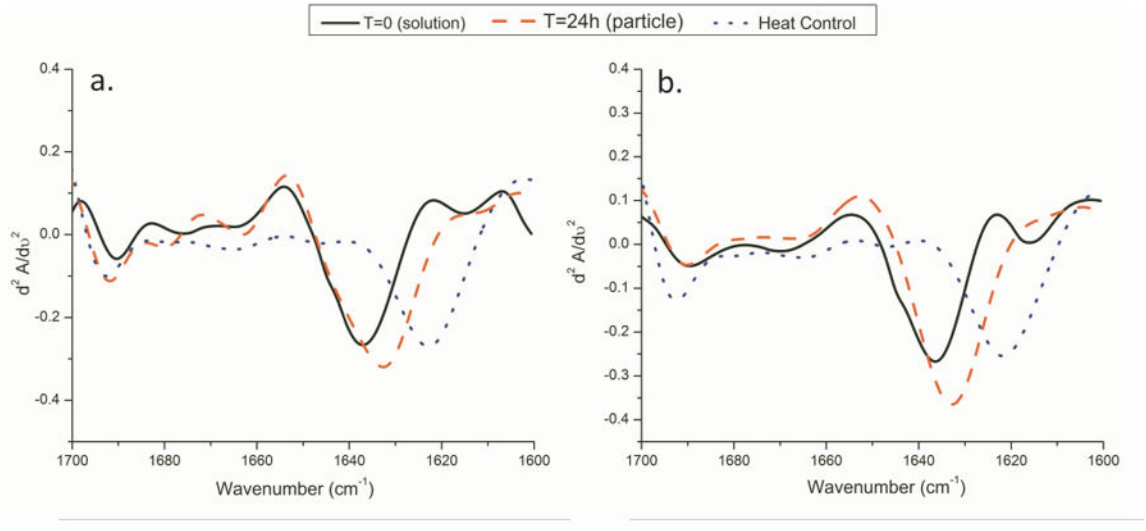


Figure 3.

Radar plot analysis of MFI particle concentration and size distribution results before and after shake-stressing lyophilized mAb samples. IgG1 mAb lyophilized samples were prepared with 0.6% and 6.8% moisture content, shake-stressed for 24 h, reconstituted with water, and subvisible particle levels were measured by MFI (See Figure 2). The “particle # scale” radar plot shows actual particle concentration values in different size ranges and the “normalized scale” radar plot shows relative change in subvisible particle concentration and size distribution of shake stressed samples compared to the corresponding T=0 sample (unstressed control).



	Sample	Wavenumber 1 (cm^{-1})	Wavenumber 2 (cm^{-1})
	T=0 (solution)	1636.8 ± 0.4	1690.1 ± 0.3
0.6%	Heat Control	1622.1 ± 1.1	1692.4 ± 0.3
	T=24h (particle)	1633.2 ± 0.8	1691.7 ± 1.8

	Sample	Wavenumber 1 (cm^{-1})	Wavenumber 2 (cm^{-1})
	T=0 (solution)	1636.5 ± 0.7	1690.1 ± 0.1
6.8%	Heat Control	1619.3 ± 3.9	1692.9 ± 0.5
	T=24h (particle)	1633.4 ± 0.9	1691.1 ± 1.2

Figure 4.

Representative second-derivative FTIR spectra of protein particles isolated from lyophilized IgG1 mAb samples containing 0.6% and 6.8% moisture levels after shake-stress. Samples include: native, unstressed protein in solution (T=0); protein particles isolated from shake stressed lyophilized mAb (a) 0.6% and b) 6.8% moisture) after reconstitution (T=24h); and particles isolated from mAb solution heated at 80°C for 20 min (heat control). The characteristic peaks in the Amide I region indicative of intra and inter molecular beta sheet secondary structure are shown in the table. Values in table represent the average of three separate experiments (n=3) along with one standard deviation.

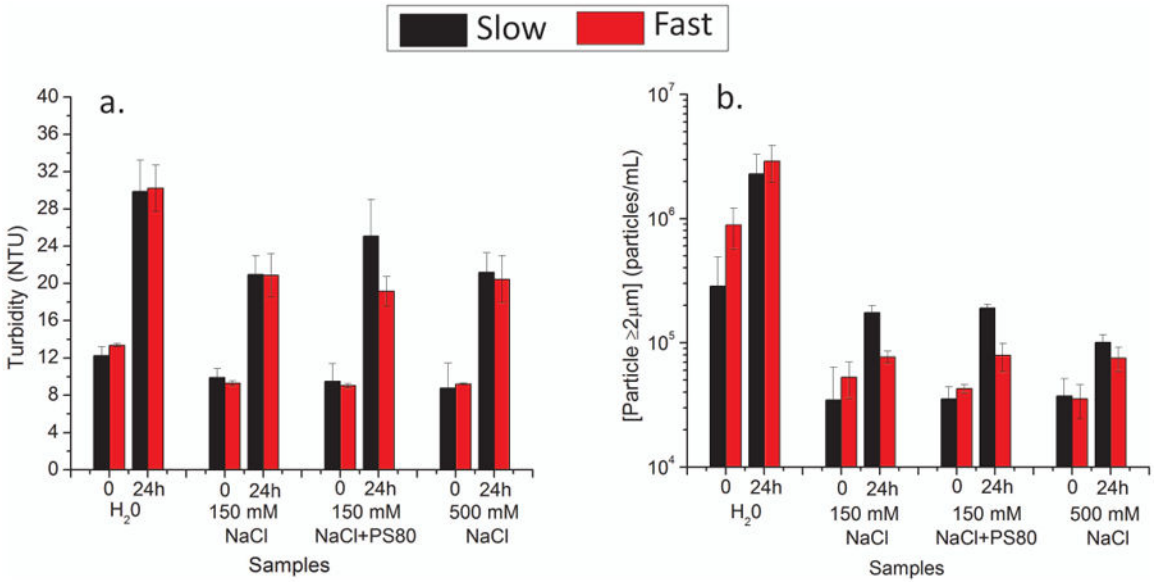


Figure 5.

Effect of reconstitution medium and addition rate on the physical instability of a lyophilized IgG1 mAb samples reconstituted after shaking. Lyophilized IgG1 mAb samples were shake-stressed for 24 h and reconstituted slow (5 mL over 2.5 minutes) or fast (5 mL over 10 seconds) conditions with the following mediums: (1) deionized H_20, (2) 150 mM NaCl, (3) 150 mM NaCl containing 0.05% polysorbate 80, and (4) 500 mM NaCl. The resulting (A) solution turbidity and (B) total concentration of subvisible particles (≥2 μm as measured by MFI) values are shown. Each graph represents the average of three separate experiments (n=3) and error bars represent one standard deviation.

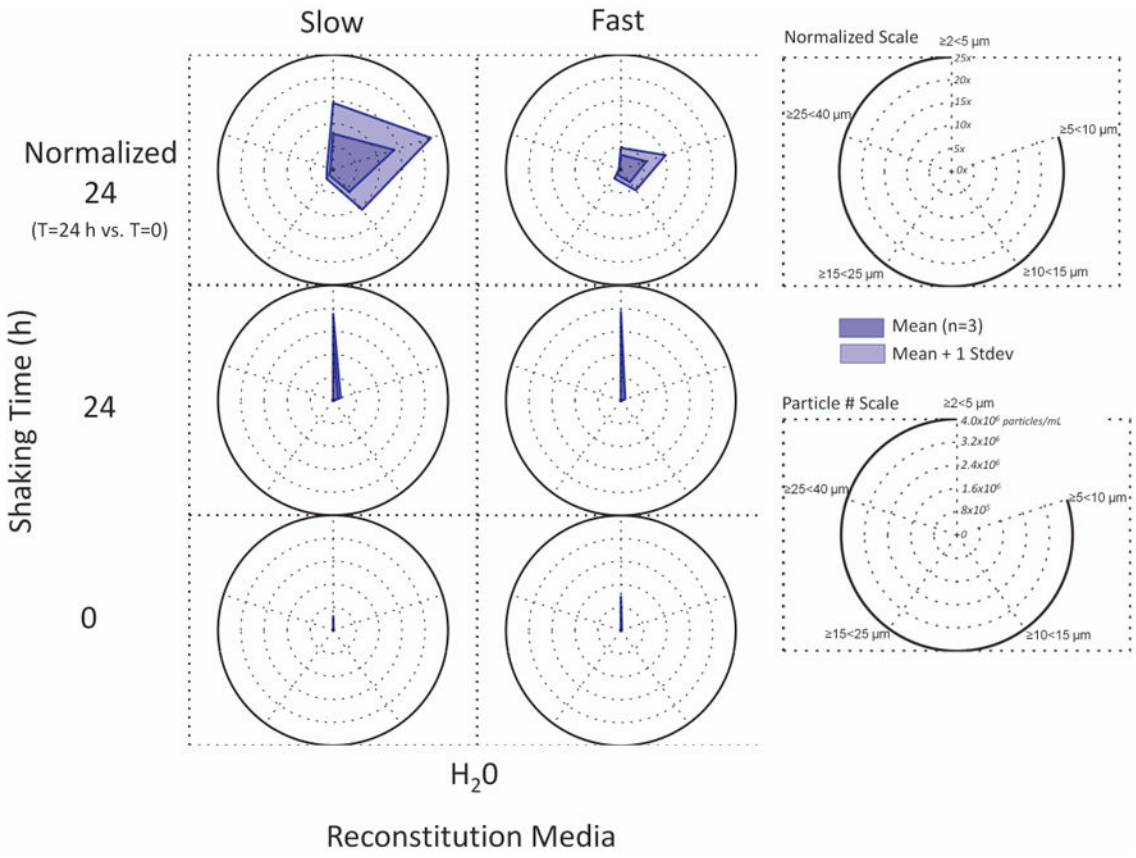


Figure 6.

Radar plot analysis of MFI particle concentration and size distribution results as a function of the rate of reconstitution of shaking-stressed lyophilized mAb samples. Lyophilized IgG1 mAb samples (without shaking, T=0) and 24 h of shaking (T=24) were reconstituted with deionized H_20 slowly (5 mL over 2.5 min) or rapidly (5 mL over 10 s) are shown. The "particle # scale" radar plot shows actual particle concentration values in different size ranges and the "normalized scale" radar plot shows relative change in subvisible particle concentration and size distribution of shake stressed samples compared to the corresponding T=0 sample (unstressed control).

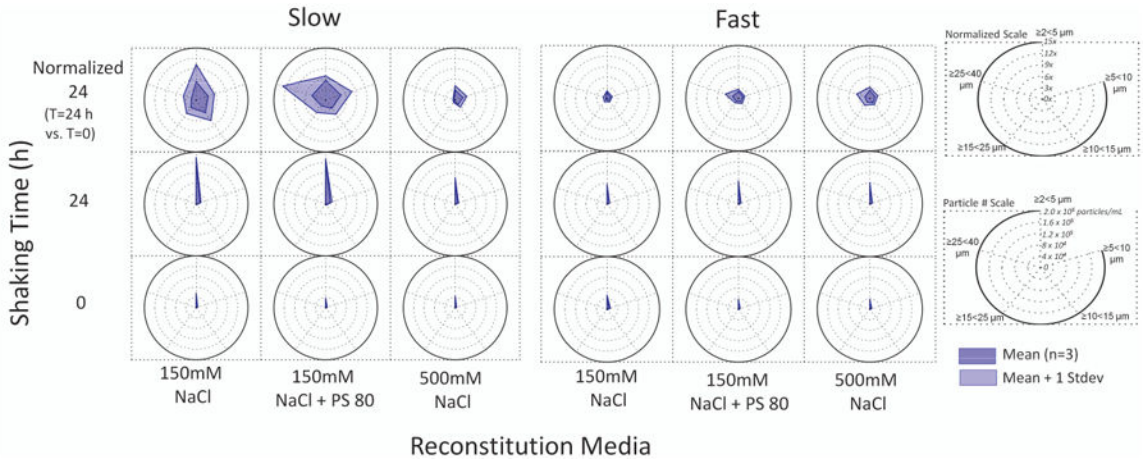


Figure 7.

Radar plot analysis of MFI particle concentration and size distribution results as a function of diluent type used to reconstitute shake-stressed lyophilized mAb samples. Lyophilized IgG1 mAb samples (without shaking, T=0) and 24 hours of shaking (T=24) were reconstituted with various mediums under slow (5 mL over 2.5 min) or fast (5 mL over 10 s) conditions as indicated in the figure. Reconstitution diluents included: (1) 5 mL of 150 mM NaCl, (2) 5 mL of 150mM NaCl + 0.05% Polysorbate 80, and (3) 5 mL 500 mM NaCl. The "particle # scale" radar plot shows actual particle concentration values in different size ranges and the "normalized scale" radar plot shows relative change in subvisible particle concentration and size distribution of shake stressed samples compared to the corresponding T=0 sample (unstressed control).

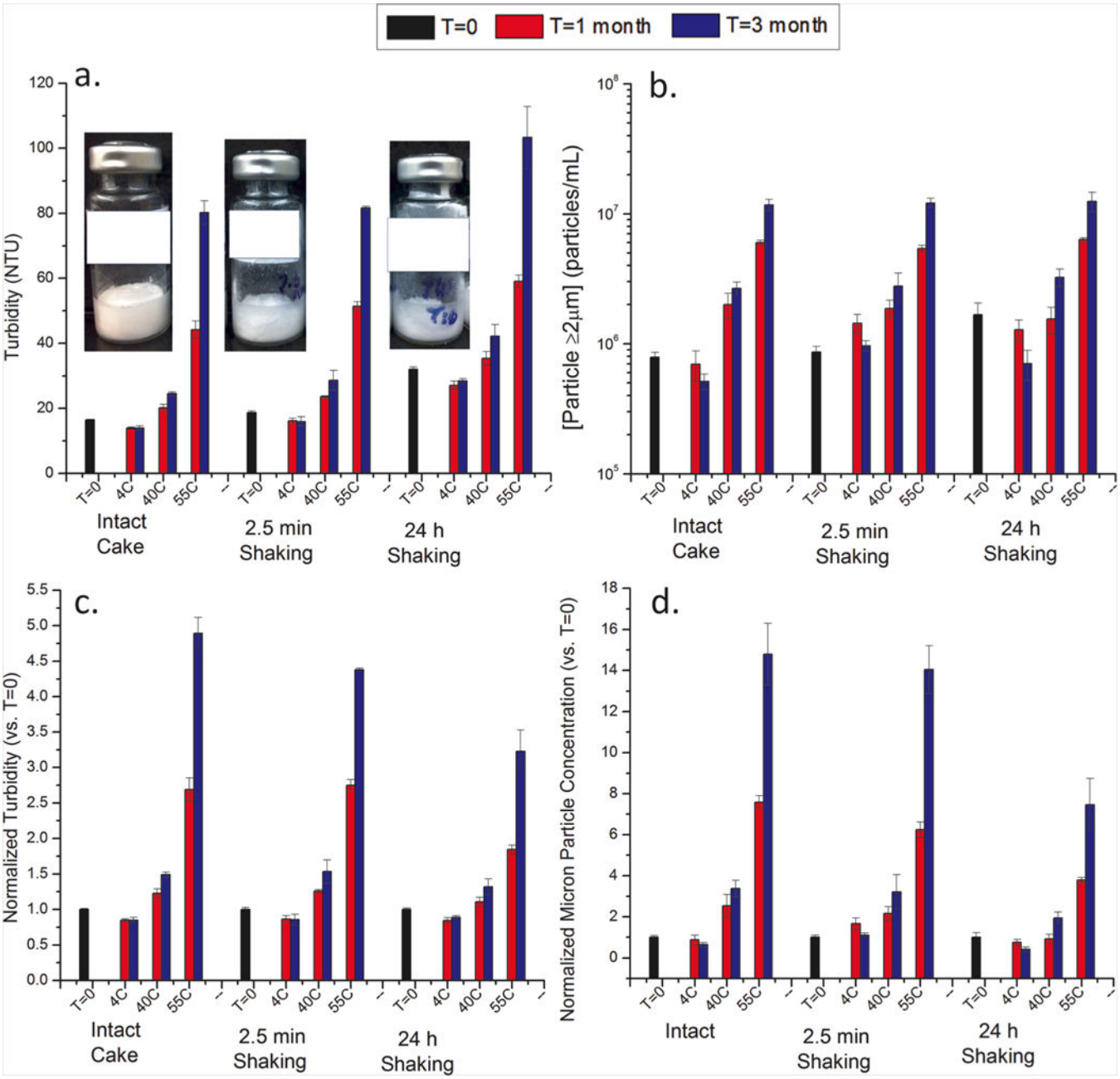


Figure 8.

Storage stability of lyophilized IgG1 mAb samples as a function of cake integrity. Lyophilized samples included unstressed control (Intact cake), shake stressed for 2.5 min, and shake stressed for 24 hrs. Representative pictures of intact cake, 2.5 min shaking, and 24h shake stressed cakes at time zero are shown. Samples were then stored for up to 3 months at three different temperatures (4, 40, 55°C), reconstituted and monitored for (A) solution turbidity and (B) total concentration of subvisible particles (≥2 μm as measured by MFI). Stressed samples, which have been shaken for a certain amount of time and stored at different temperatures, were compared to controls (T=0, but with shaking) and resulting C) normalized turbidity or D) normalized subvisible particle concentrations are shown. The y-

axis values for both (C) and (D) were obtained by dividing the turbidity values (or micron particle concentration) of a stored sample by its relevant control at time zero experiencing similar duration of shaking. Each graph represents the average of three separate experiments (n=3) and error bars represent one standard deviation. Placebo samples were measured after 3 months storage at 55°C h and showed low turbidity (∼1 NTU) and a relatively low number of micron sized particles (∼12,000 particles/mL).