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Freezing-Induced Perturbation of Tertiary Structure of a Monoclonal Antibody

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Abstract

We studied the effects of pH and solution additives on freezing-induced perturbations in the tertiary structure of a monoclonal antibody (mAb) by intrinsic tryptophan fluorescence spectroscopy. In general, freezing caused perturbations in the tertiary structure of the mAb, which were reversible or irreversible depending on the pH or excipients present in the formulation. Protein aggregation occurred in freeze–thawed samples in which perturbations of the tertiary structure were observed, but the levels of protein aggregates formed were not proportional to the degree of structural perturbation. Protein aggregation also occurred in freeze–thawed samples without obvious structural perturbations, most likely because of freeze concentration of protein and salts, and thus reduced protein colloidal stability. Therefore, freezing-induced protein aggregation may or may not first involve the perturbation of its native structure, followed by the assembly processes to form aggregates. Depending on the solution conditions, either step can be rate limiting. Finally, this study demonstrates the potential of fluorescence spectroscopy as a valuable tool for screening therapeutic protein formulations subjected to freeze–thaw stress.

Keywords

excipients; formulation; liquid chromatography; monoclonal antibody; protein aggregation; proteins; stability; surfactants

INTRODUCTION

Most proteins are marginally stable and degrade by numerous pathways in aqueous solution.^{1,2} The degradation processes can often be inhibited by keeping properly

formulated proteins in a frozen state (i.e., cryopreservation).^{3–5} At a laboratory scale, cryopreservation provides an effective way for researchers to preserve precious protein samples and standards, minimize interexperiment variability and maximize flexibility for experimental design and scheduling. Likewise, large-scale cryopreservation is an important step in the manufacturing of therapeutic proteins.^{6–8} Frozen storage of process intermediates and bulk drug substance may be necessary to control product quality and to make efficient use of production facilities. In addition, liquid formulations of therapeutic proteins may be accidentally frozen and thawed during shipping and long-term storage because of mishandling or temperature excursions.^{9,10}

Freeze–thawing can cause aggregation of proteins, presumably because of protein structural perturbation caused by freezing.^{9,11–13} In turn, aggregates of therapeutic proteins may elicit undesirable immune responses, such as the generation of antidrug antibodies.¹⁴ The mechanism(s) of freezing–thawing-induced protein aggregation are not well understood, in part, because of the limitations of many commonly used spectroscopic techniques for the analysis of protein structure within frozen samples.¹⁵ Freezing-induced structural changes in proteins have been addressed previously in studies using confocal Raman microspectroscopy,¹⁶ fluorescence spectroscopy,^{15,17,18} and infrared microscopy¹⁹ to examine the structures of lysozyme, azurin, and lactate dehydrogenase. More recently, confocal Raman microspectroscopy and Fourier transform infrared spectroscopy were also used to study the interaction between bovine serum albumin molecules and ice crystals.²⁰ These studies of nontherapeutic proteins have documented that freezing can cause protein structural perturbations. But the earlier work did not address the potential connections between freezing-induced structural alterations and aggregation after thawing.

Furthermore, the majority of therapeutic proteins currently under development and clinical trials are monoclonal antibodies (mAbs).²¹ But very limited data are available on potential freezing-induced structural perturbations of mAbs.¹² The objectives of the current study were: (1) to investigate the application of fluorescence spectroscopic analysis of freezing-induced protein structural perturbation to a mAb in various formulations; and (2) to investigate potential correlations between the extent of freezing-induced structural perturbations of the mAb and the quantities of aggregates detected after freeze–thawing.

Fluorescence spectroscopy has been used to study perturbations of protein tertiary structure induced by chemical denaturants, pH changes, and temperature.^{15,22} The wavelength of maximal fluorescence emission (hereinafter referred as λ_{max}) is often used as an indicator of the solvent exposure of tryptophan (Trp) residues in a protein molecule.^{22,23} For example, azurin contains only a single Trp residue which, in the protein's native conformation, is buried in the protein's hydrophobic core.²⁴ The λ_{max} value for native azurin is 308 nm.²⁴ In contrast, glucagon, a peptide with a single Trp residue that is highly solvent exposed, exhibits a λ_{max} value of 355 nm.²³ For proteins whose Trp residues are solvent exposed to intermediate extents, λ_{max} usually falls somewhere between the above two wavelengths.^{22,23} When the microenvironment of Trp residues changes, λ_{max} may shift to lower (i.e., blue shift) or higher wavelength (i.e., red shift), a phenomenon that is commonly correlated to perturbation of protein tertiary structure.²²

There are a limited number of publications investigating protein structures in frozen solutions by intrinsic Trp fluorescence.¹⁵ In an early publication, Visser et al.²⁵ observed a blue shift in the Trp fluorescence of the holoenzyme of lipoamide dehydrogenase as solutions were cooled from 20°C to 193°C, suggesting corresponding changes in protein tertiary structure. Strambini and coworkers^{17,18} measured intrinsic Trp fluorescence using a custom-built fluorometer to investigate the effects of anions, sugars, and polyols on the stability of azurin in ice and found that freezing-induced structural perturbations could be reduced by known protein stabilizers such as sucrose and glycerol.

In our study, to monitor freezing-induced perturbations of the tertiary structure of a mAb during cooling, freezing, and rewarming a commercially available fluorescence spectrometer was used to measure intrinsic Trp fluorescence. Size-exclusion high-performance liquid chromatography (SE-HPLC) was used to quantify the levels of monomer and soluble aggregates in freeze-thawed samples. We investigated the impacts of pH and solution additives such as KCl, sucrose, guanidine hydrochloride (Gdn HCl), and polysorbate 80 (PS80) on freezing-induced perturbation of protein tertiary structure and aggregate levels in freeze-thawed samples.

MATERIALS AND METHODS

Materials

The mAb used in this study was an IgG2 (hereafter referred to as “mAb”) and was generously provided by Pfizer (Chesterfield, Missouri). Isoelectric point (pI) values for this mAb, determined by isoelectric focusing, ranged from 8.65 to 9.30.⁹ Monobasic and dibasic potassium phosphate, potassium chloride, phosphoric acid, sodium azide, sodium acetate, acetic acid, guanidinium HCl (Gdn HCl), N-acetyl-L-tryptophanamide (NATA), and ribonuclease A (RNase A) were purchased from Sigma-Aldrich (St. Louis, Missouri). PS80, (containing low carbonyl and peroxide) was purchased from Thermo Scientific (Rockford, Illinois). Sucrose was obtained from Pfanstiehl Laboratories (Waukegan, Illinois). Distilled, deionized water was used throughout.

Sample Preparation

The mAb in a stock solution was purified to remove excipients by a Pharmacia FPLC system (GE Healthcare, Piscataway, New Jersey) as previously described.⁹ Protein solutions were prepared by diluting the purified mAb to 0.5 mg/mL (unless otherwise noted) with 10 mM potassium phosphate buffer (pH 3 or 8) or 10 mM sodium acetate buffer (pH 4). The choice of pH was based on a previous study with this protein from our laboratory wherein substantial levels of protein aggregates were detected at pH 3 and 4 after freeze-thawing, but only minimal aggregation occurred after freeze-thawing at pH 5–8.⁹

To study freezing-induced protein structural perturbations and resulting aggregation, we examined solutions with three different pH (3, 4, and 8) and the effects of solution additives. Stock solutions of 1 M KCl, 2 M sucrose, 6 M Gdn HCl, and 0.5% (w/v) PS80 were prepared in the buffers at each pH. Samples were prepared by mixing the purified mAb

solution and stock additive solutions, in the respective buffers, to obtain 0.5 mg/mL protein and the target excipient concentrations.

Fluorescence Spectroscopy

Fluorescence spectra were recorded by a QuantaMaster spectrofluorometer equipped with a Peltier-thermostatted cuvette holder (Photon Technologies International, Lawrenceville, New Jersey). Two milliliter of sample solution was loaded into a 4.5 mL disposable cuvette (VWR Cat. No. 58017-875). Samples were not degassed because preliminary experiments demonstrated that there was no significant difference in results between nondegassed and degassed samples (data not shown). A thermocouple with a long rod-like probe (diameter $\times$ length = 1.7 mm $\times$ 49 mm) was used to measure sample temperatures. The thermocouple was put through a hole in a square Teflon cuvette cap where it did not interfere with the light path. On both left and front wall of the fluorometer chamber, there was a hole accessible to a digital camera. A black plastic cap was used to seal the hole during fluorescence measurements. When necessary, to record video images of the sample, after closing the shutter to the emission detector, one of the caps could be removed. The video recordings of the freezing process demonstrated that the nucleation of ice crystals started from the air–liquid interface in the vicinity of the thermocouple (data not shown). Then, ice crystals progressed from the top of the sample to the bottom, and simultaneously from the cuvette wall toward the center.

Intrinsic Trp fluorescent measurements were made using an excitation wavelength of 295 nm and emission scanning range between 310 and 400 nm. The slit widths were 3 and 2 nm for excitation and emission, respectively. The dimension of the light beam was 750 μ m (horizontal) $\times$ 5 mm (vertical).

The effects of pH and solution additives were studied during cooling and heating between 20°C and –30°C at 2°C intervals by preset program using software FeliX 32 (Photon Technologies International). Between measurements, the cuvette holder temperature was changed at a rate of 2°C/min. During cooling and heating in the subzero temperature range, the temperature of cuvette holder was set at 1°C lower than the target sample temperature to promote heat transfer and reduce the time needed to reach the target sample temperature. The tolerance to take the fluorescence measurements between the set and the actual temperatures was 1°C. The fluorescence measurement was triggered by the thermocouple probe inserted in the sample, which took the actual sample temperature. Therefore, the sample temperature was still at target temperature during the fluorescence measurement even though the set temperature was 1°C lower.

During temperature ramping, temperature gradients were created in the sample, with maximum temperature differences expected to occur between the center and inner wall of the cuvette. Thermocouples were placed at these two locations. The temperature differences were measured at 10-s intervals using same freeze–thawing protocol for the sample. The temperature differences ranged from 0°C to 0.4°C except at the moment of ice formation (data not shown). Between two fluorescence measurements, when the cuvette holder temperature changed at 2°C/min, the temperature difference was around 0.4°C. The

temperature difference was reduced to near zero at the target temperature where the fluorescence measurement was made.

To minimize the impact of the background spectroscopic signal because of effects such as light scattering after the sample was frozen, the signal for a blank solution was collected and subtracted from those for the mAb samples. To ensure that the remaining spectral features were mainly contributed by the Trp residues, for comparison, the signal of 0.5 mg/mL RNase A, a Trp-free protein, prepared in 10 mM potassium phosphate buffer (pH 3 and 8) was also collected in liquid and frozen states. There was no distinct difference of the fluorescence signal between the blank and the RNase solution before and after freezing (Fig. 1). Therefore, the subtracted spectrum for frozen mAb solutions was ascribed to mAb Trp residues. The corrected Trp fluorescence spectrum was then fitted using the extreme peak function in Origin software (Origin 7; OriginLab, Northampton, Massachusetts) to obtain the $\lambda_{\max}$ value.

To ascribe shifts in $\lambda_{\max}$ to changes in protein structure, we needed to rule out the possibility that any $\lambda_{\max}$ shifts might be because of cryoconcentration of the mAb and potential inner filter effects. The total number of Trp residues in this mAb is 24. For 0.5 mg/mL of mAb used in our studies, the equivalent concentration of Trp residues is 79 μ M for NATA. We measured Trp fluorescence of 79, 790, and 7900 μ M NATA prepared in buffer at pH 3 and 8. The 100-fold difference between the low and high concentrations of NATA should cover the expected range of Trp concentrations in freeze-concentrated liquid phases.²⁶ In addition, we also measured the fluorescence spectra of 0.5 and 86 mg/mL mAb (pH 5.5, the ratio of concentration is 172-fold).

Size-Exclusion High-Performance Liquid Chromatography

Following intrinsic Trp fluorescence measurements, the freeze-thawed samples were removed from the cuvette, pipetted into Eppendorf tubes and centrifuged. The supernatants were analyzed on a HP 1090 HPLC (Agilent, Palo Alto, California) using a Tosoh Super SW3000 column (Tosoh Bioscience, Tokyo, Japan). The volume of injection was 30 μ L, with a running time of 20 min. The flow rate of the mobile phase [200 mM potassium phosphate, 0.05% (w/v) sodium azide, pH 7.0] was 0.35 mL/min. The average total peak area for protein monomer of three replicate samples—without additives and that were not freeze-thawed—served as a control value. The area under the curve (AUC) for the monomer peak divided by the average total AUC of the control samples ($\times 100$) was taken as the percent-age of monomer, and AUC for the peak representing aggregates divided by the average total AUC of the control samples ($\times 100$) was taken as percentage of aggregates.

Statistical Analysis

Two-tailed unpaired Student's *t*-tests with 95% confidence interval were performed to determine the significance of differences for the means between two samples.

RESULTS

Effects of Freezing and Thawing on the Intrinsic Trp Fluorescence of the mAb in Buffers Without Any Additives

Representative intrinsic Trp fluorescence emission spectra are shown in Figure 1. Compared with the signal of liquid buffer blank, the fluorescence intensity substantially increased because of ice-induced light scattering when the buffer blank was completely frozen, with greater increases in intensity at shorter wavelengths. However, there was no distinct difference between the spectra of the blank and RNase A (a protein without Trp residues) solutions before and after freezing. Thus, spectrum obtained by subtracting the blank spectrum from that of the frozen mAb solution was mainly because of the Trp residues in the mAb protein.

No significant peak shift was observed at 20°C for NATA with concentration ranged from 79 to 7900 μM (data not shown). Likewise, the fluorescence spectra for the mAb did also not show peak shifts when measured at mAb concentrations of 0.5 and 86 mg/mL (data not shown). Therefore, the shift in λ_{max} for the mAb during freezing was not because of freezing-induced concentration of the protein and potential resulting inner filter effects.

For the clarity of presentation, only data for prefrozen (20°C), frozen (−30°C), and post-thawed (20°C) steps are shown for the λ_{max} and peak shifts of protein because of freezing and freeze–thawing (Figs. 2–4). Plots of λ_{max} as a function of temperature for all formulations are found in the supplemental Supporting Materials (Figs. S1–S3). In the absence of additives, at both pH 3 and 4, λ_{max} showed approximately 6 nm blue shift ($p < 0.001$) at −30°C compared with λ_{max} at 20°C. At pH 8, freezing and thawing caused minimal, insignificant change (about 0.3 nm, $p = 0.15$) in λ_{max} .

Representative SE-HPLC chromatograms for all samples at pH 4 are shown in Figure 5. SE-HPLC results in Figure 6 showed that mAb aggregates formed during freeze–thawing at all tested pH, with the lowest level observed in samples at pH 8. Also, aggregation level was lower after freeze–thawing at pH 3 than at pH 4.

Effects of Additives on the Intrinsic Trp Fluorescence of the mAb During Freezing and Thawing

Representative intrinsic Trp fluorescence emission spectra for the mAb in the absence and presence of additives are shown in Figure 7.

KCl

At pH 8 in the presence of 150 mM KCl, similar shifts in λ_{max} were observed as in its absence (Fig. 4). In contrast, samples with added KCl at pH 3 and 4 showed smaller blue shifts after freezing than observed in these buffers alone (Figs. 2 and 3).

mAb aggregates were detected by SE-HPLC analysis after freeze–thawing in the presence of KCl at all pH, although soluble aggregates were not observed in samples freeze–thawed at pH 3 (Fig. 6). The monomer percentage of the samples with 150 mM KCl at pH 3 was also

relatively low, reflecting a substantial loss of monomer because of the formation of insoluble aggregates.

Sucrose

The presence of 1 M sucrose in mAb samples at pH 8 caused a 3.4-nm blue shift during freezing (Fig. 4). At pH 3 and 4, the presence of 1 M sucrose reduces the extent of the $\lambda_{\max}$ blue shift (Figs. 2 and 3).

Size-exclusion high-performance liquid chromatography showed that the quantities of aggregates were substantially reduced at pH 3 and 4 with the presence of 1 M sucrose (Fig. 6). In contrast, the quantities of insoluble aggregates were increased for pH 8 when 1 M sucrose was included in the mAb solution.

Guanidine HCl

The $\lambda_{\max}$ values for the mAb in solutions at each of the three pH tested showed minor red shifts after addition of 45 mM of Gdn HCl (Figs. 2–4). However, freezing in the presence of this denaturant caused a significant red shift at all pH. In contrast, an addition of 4 M Gdn HCl to samples at pH 8 caused a significant 9-nm red shift of $\lambda_{\max}$ prior to freeze–thawing (Fig. 4). Freezing made the emission peak red shift even further. At pH 3 and 4, 4 M Gdn HCl caused the mAb to denature prior to freezing (Figs. 2 and 3). Freezing further red-shifted the emission peak (Fig. S3). However, $\lambda_{\max}$ returned to around 350 nm when samples were cooled to -30°C (Fig. S3), presumably because of the crystallization of Gdn HCl and resultant removal of the denaturant molecules from protein's surface. The eutectic temperature for Gdn HCl²⁷ is approximately -24°C , which was confirmed by the analysis of mAb samples by differential scanning calorimetry (data not shown).

Size-exclusion high-performance liquid chromatography analysis of samples containing 45 mM Gdn HCl at pH 3 detected only small residual peaks for monomeric or aggregated mAb, because the protein formed insoluble aggregates during freeze–thawing (Fig. 6). Samples of mAb at all study pH prepared with 4 M Gdn HCl were not tested by SE-HPLC after freeze–thawing because SE-HPLC measurement of the initial solutions did not show detectable monomer or soluble aggregates. This observation was likely because of the denatured mAb-forming aggregates upon dilution with SE-HPLC mobile phase, which were then filtered out by the inline filter and the frit of the SE-HPLC column.

After freeze–thawing of mAb samples containing 45 mM Gdn HCl, large amounts of insoluble aggregates were observed, likely because of the combination of inherent stresses of freezing and increased concentration of Gdn HCl, followed by dilution as the samples thawed. Thus, the Gdn HCl concentrations to which the mAb was exposed during freezing and thawing could have been similar to those experienced when mAb samples prepared in the presence of 4 M Gdn HCl were injected into and diluted with the mobile phase of the SE-HPLC system.

Polysorbate 80

Compared with samples without additive, samples formulated at pH 8 containing 0.05% (w/v) PS80 (a concentration greater than critical micelle concentration, ~0.002%, w/v^{28,29}) showed a blue shift of about 2.5 nm upon freezing (Fig. 4). At pH 3 and 4, samples showed the same blue shift as samples without PS80 (Figs. 2 and 3).

Size-exclusion high-performance liquid chromatography showed that the quantities of soluble aggregates were increased for samples at pH 3 and 8 after freeze–thawing in the presence of 0.05% (w/v) PS80 (Fig. 6). In contrast, the quantities of monomers increased in the presence of 0.05% (w/v) PS80 during freeze–thawing at pH 4 (Fig. 6).

DISCUSSION

The fluorescence spectra in this study were because of the average emissions of all 24 Trp residues in the mAb, and thus polarity changes in the microenvironments for individual Trp residues in the protein could not be resolved. However, any change in the average $\lambda_{\max}$ should be attributable to a change in the protein's tertiary structure. Furthermore, when multiple Trp residues are present, the lack of a detectable shift cannot be unequivocally interpreted as a lack of perturbation of the tertiary structure, because structural perturbations might induce blue shifts in $\lambda_{\max}$ for some Trp residues and red shifts in $\lambda_{\max}$ for others.

For mAb solutions without additives during freezing, $\lambda_{\max}$ showed approximately 6 nm blue shift at pH 3 and 4 but a minimal shift at pH 8. The percentage of monomer recovered after freeze–thawing was highest at pH 8, most likely because of the minimal freezing-induced structural perturbation of the protein. The percentage of monomer detected was significantly higher at pH 3 than that at pH 4. Therefore, the level of protein aggregation was not directly proportional to the extent of the $\lambda_{\max}$ shift observed for frozen mAb samples.

Aggregation during freeze–thawing is likely a result of various influencing factors including the conformational and colloidal stabilities of the protein. Chi et al.³⁰ studied the aggregation of recombinant human granulocyte stimulating factor (rhGCSF) during isothermal incubation in aqueous solution. They found that the aggregation pathway of rhGCSF first involves the perturbation of the protein's native conformation, followed by the assembly processes to form aggregates. The first step can be modulated by changes in the conformational stability of the protein, whereas the second step is dominated by the energetics of protein–protein interactions, so-called “colloidal stability.” Depending on the solution conditions, either step can be rate limiting. For example, by adding sucrose, rhGCSF aggregation decreased because of increased conformational stability. Under low pH or ionic strength, rhGCSF aggregation was reduced because of increased colloidal stability. This aggregation mechanism was further supported by another study, which explored mechanisms for benzyl alcohol-induced aggregation of rhGCSF.³¹ In that study, Thirumangalathu et al.,³¹ found 0.9% benzyl alcohol-altered rhGCSF tertiary structure at pH 3.5 and 7. However, benzyl alcohol-induced structural perturbation accelerated rhGCSF aggregation at pH 7, but not at pH 3.5. At the acid pH, aggregation could not proceed from the structurally perturbed protein molecules because of the dominant colloidal stability of the protein.

In our case, aggregation of the mAb during freeze–thawing was less at pH 3 than that at pH 4, perhaps because the mAb's colloidal stability was increased as a result of charge–charge repulsion at pH 3 despite the increased perturbation in the tertiary structure during freezing. To test this hypothesis, an effort was made to measure the second virial coefficients (B_{22}) for the protein using a light scattering method. Light scattering data were obtained for the protein at all three pH in the absence and presence of KCl. Unfortunately, efforts to obtain reliable dn/dc values for the protein in these solutions were not successful. Thus, the B_{22} values were not included in the results. However, we can assume $dn/dc = 0.185$ mL/g (a typical dn/dc value used for proteins in the literature^{32,33*****}) for all solution conditions. The dn/dc value will only impact the absolute value of the calculated B_{22} and not the sign of the parameter, as shown in these equations: where:

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2B_{22}c$$

where

$$K = \frac{4\pi^2 n_0^2 \left(\frac{dn}{dc}\right)^2}{N_A \lambda^4}$$

On the basis of the assumed dn/dc value and the measured light scattering values, B_{22} for samples at pH 3 and 4 were positive. Therefore, intermolecular interactions were repulsive at both pH, and it appears—based on the limited dataset available from our studies—that greater colloidal stability at pH 3 cannot explain the lower level of aggregation noted during freeze–thawing at pH 3 than at pH 4. The different aggregation behavior observed at pH 3 and 4 might be because, at least partly, of buffer species effects because different buffers were used at pH 3 (potassium phosphate) and pH 4 (sodium acetate).

At pH 8, the B_{22} value was negative, most likely because this pH is close to the pI of the protein. Thus, although significant perturbation of the tertiary structure was not observed for the frozen mAb, perhaps the freeze concentration of buffer salts sufficiently reduced protein colloidal stability to foster protein aggregation.

In the presence of KCl, B_{22} values at pH 3, 4, and 8 were negative, indicating that in the presence of the salt intermolecular interactions between protein molecules were attractive. In the presence of 150 mM KCl, peak shifts for the mAb at pH 8 upon freezing and aggregate levels after freeze–thawing were similar to those noted in buffer alone. With KCl, smaller blue shifts were observed during freezing at pH 3 and 4 than in buffer alone. With KCl, the percentage of monomer loss was less at pH 4 than that at pH 3, which is opposite to that observed in buffer alone at the two pH values. The addition of KCl reduced the structural perturbation of the mAb at pH 4 (i.e., increased conformational stability) during freezing, resulting in less aggregation during freeze–thawing. In contrast, although the addition of KCl reduced the structural perturbation at pH 3 during freezing, the reduced colloidal stability because of charge shielding could have dominated and led the increase in mAb

aggregation observed after thawing. Similarly, charge shielding was observed to increase rhGCSF aggregation in solution at pH 3.5 in the presence of 150 mM NaCl.³⁴

The presence of 1 M sucrose reduced the λ_{max} blue shift at pH 3 and 4 (Figs. 2 and 3), consistent with the well-known stabilization of protein native conformation by sucrose. In contrast, the presence of 1 M sucrose at pH 8 caused a 3.4-nm blue shift during freezing (Fig. 4), which reflects a surprising perturbation of protein tertiary structure by sucrose. These phenomena can be explained by preferential exclusion mechanism.³⁵ Sucrose is preferentially excluded from the surface of protein, and there is a concomitant increase in protein chemical potential. The increase of chemical potential is greater for unfolded than for native protein molecules, which leads to higher free energy of unfolding in the presence of sucrose. Therefore, the native protein is stabilized, and a compact protein conformation and the least exposure of protein surface to solvent are more favorable. This mechanism is straightforward to apply to the mAb during freeze–thawing at pH 3 and 4 because sucrose inhibited protein partial unfolding and consequently reduced protein aggregation during freeze–thawing. However, because the mAb in buffer alone remained native at pH 8 during freezing but had slightly perturbed tertiary structure when frozen with sucrose, one potential way to decrease surface area is through protein–protein association, that is, aggregation.³⁶ Furthermore, pH 8 was close to the *pI* value (8.65–9.3) of the mAb, which could make intermolecular interactions during freezing relatively favorable because of minimum charge–charge repulsion, especially in the freeze-concentrated liquid phase.

In the presence of 45 mM Gdn HCl, freezing caused a significant red shift of the protein's spectrum at all tested pH, documenting that the tertiary structure of the mAb was altered by freezing and the concomitant freezing concentration of the Gdn HCl. The λ_{max} values for the mAb in the presence of 45 mM Gdn HCl at -30°C were close to λ_{max} for mAb in the presence of 4 M Gdn HCl at 20°C . Similarly, Strambini and Gonnelli measured the Trp fluorescence spectra of an azurin mutant with various Gdn HCl concentrations at -14°C .¹⁵ The λ_{max} shifted from around 308 to 357 nm when the fraction of Gdn HCl was increased from 0% to 100% in the 19-mM Gdn HCl–NaCl salt mixture + 1 mM Tris (pH 7.5). Interestingly, in our study, the λ_{max} returned to around 350 nm when samples were cooled to -30°C , presumably because of the crystallization of Gdn HCl (eutectic temperature for Gdn HCl²⁷ is approximately -24°C) and the removal of Gdn HCl molecules from protein's surface.

At pH 3 and 4, mAb samples containing 0.05% (w/v) of PS80 showed similar peak shifts upon freezing as observed for samples without the surfactant, but at pH 8, the surfactant led to structural perturbation of the protein that was not observed without surfactant. However, at pH 3 and 8, there were higher levels of soluble aggregates formed during freeze–thawing in the presence of PS80 than in the absence of the surfactant. A similar result was observed by Kreilgaard et al.³⁷ when they studied the effect of PS20 on freeze–thawing-induced aggregation of recombinant human factor XIII (rFXIII).³⁷ They found that PS20 did not prevent rFXIII aggregation during freeze–thawing but reduced the apparent formation of insoluble aggregates.

In contrast, in this study, at pH 4, the quantities of mAb monomer lost to aggregates during freeze–thawing was reduced in the presence of 0.05% (w/v) PS80 during freeze–thawing, which is consistent with the stabilization of several proteins observed during freeze–thawing. This observation is consistent with the finding of Kuelto et al.⁹—with the same mAb used in the current study—that protein unfolding at the ice–water interface may play a critical role during freeze–thawing at pH 4. They found that increasing the initial protein concentration resulted in a smaller fraction of protein aggregating during freeze–thawing, because presumably there was finite ice–water surface area for protein damage. Thus, in a given sample volume, there is a certain mass of protein that could be damaged at the ice–water interface. Similar results were observed by Kreilgaard et al. for Factor XIII.³⁷ Given this mechanism for protein damage during freezing, the inclusion of a surfactant such as PS80 could reduce protein aggregation during freeze–thawing by inhibiting protein adsorption to the ice–water interface.^{9,35–37}

CONCLUSIONS

Freezing of a mAb solution induced tertiary structural perturbation in the mAb, which depended on the composition of the formulations. Protein aggregation occurred in freeze–thawed samples in which perturbations of the tertiary structure were observed in the frozen state, but the amount of protein aggregates formed was not proportional to the extent of the λ_{max} shift. Protein aggregation also occurred in some freeze–thawed samples without obvious structural perturbations of the frozen protein, most likely because of freeze concentration of protein and salts, and thus reduced protein colloidal stability. Therefore, freezing-induced protein aggregation may or may not first involve the perturbation of its native structure, followed by the assembly processes to form aggregates. Depending on the solution conditions, either conformational or colloidal stability could be dominant. Finally, the fluorescence spectroscopic methods employed in this study are simple and effective, and should be useful in formulation screening for therapeutic proteins subjected to freezing stress and for gaining insights into the mechanism(s) for freeze–thawing-induced protein aggregation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative intrinsic (Trp) fluorescence spectra of 10 mM potassium phosphate buffer, 0.5 mg/mL RNase A, and 0.5 mg/mL mAb at pH 3 before and after freezing. The excitation wavelength is 295 nm. All spectra are original signals without correction.

Figure 2.

The wavelength of Trp fluorescence emission maxima ($\lambda_{\max}$) for all samples at pH 3. Data represent mean $\pm$ standard deviation of triplicate samples. Prior to the determination of $\lambda_{\max}$, each spectrum was corrected by subtracting the signal collected from its blank solution at the same temperature.

Figure 3.

The wavelength of Trp fluorescence emission maxima ($\lambda_{\max}$) for all samples at pH 4. Data represent mean $\pm$ standard deviation of triplicate samples. Prior to the determination of $\lambda_{\max}$, each spectrum was corrected by subtracting the signal collected from its blank solution at the same temperature.

Figure 4.

The wavelength of Trp fluorescence emission maxima ($\lambda_{\max}$) for all samples at pH 8. Data represent mean $\pm$ standard deviation of triplicate samples. Prior to the determination of $\lambda_{\max}$, each spectrum was corrected by subtracting the signal collected from its blank solution at the same temperature.

Figure 5.

Representative size-exclusion chromatographs of mAb with or without additives at pH 4 after freeze-thawing, except control sample was the sample without additive and not subjected to freeze-thawing stress.

Figure 6.

The effects of additives on freeze-thawing-induced aggregation of mAb by SE-HPLC. Data represent mean $\pm$ standard deviation of triplicate samples. HMW%: percentage of dimer and high molecular weight species. The average total peak area for protein monomer of three replicate samples—without additives and that were not freeze-thawed—served as a control value. The AUC for the monomer peak divided by the average total AUC of the control samples ($\times 100$) was taken as the percentage of monomer, and AUC for the peak representing aggregates divided by the average total AUC of the control samples ($\times 100$) was taken as percentage of aggregates. mAb +4 M Gdn HCl samples were not tested as per the reason explained in the text.

Figure 7.

Representative intrinsic (Trp) fluorescence spectra of 0.5 mg/mL mAb (pH 3) with no additive, 150 mM KCl, 1 M sucrose, 45 M Gdn HCl, 4 M Gdn HCl, and 0.05% PS80 at -30°C . The excitation wavelength is 295 nm. Each spectrum was corrected by subtracting the signal collected from its relative blank solution at the same temperature.