

High Throughput Thermostability Screening of Monoclonal Antibody Formulations

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ABSTRACT: Differential scanning fluorimetry (DSF) was employed to increase the throughput of the thermostability screening of monoclonal antibody (mAb) formulations. The method consists of measuring the fluorescence intensity of a polarity sensitive probe at gradually increasing temperatures, and obtaining the transition temperature of exposure of the hydrophobic regions of proteins (T_h). The change in fluorescence intensity was directly related to protein unfolding levels and temperatures. The results from thermostability measurements were compared with the data acquired using differential scanning calorimetry (DSC), and a good correlation between T_h and the temperature of protein unfolding or melting (T_m) was observed. The method was applied to screen four mAb molecules in 84 different buffers. The studies revealed a good correlation of T_h values with the known effects of pH and excipients on protein stability in solution. Specifically, the elevated aggregation levels induced by salt, low pH, and high protein concentrations could be successfully predicted by this thermal stability screening. This method is efficient, with high throughput capability, and could be widely applied in the biopharmaceutical industry for formulation and process development, and characterization. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 99:1707–1720, 2010

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INTRODUCTION

A successful biopharmaceutical is the product of process and formulation development focused on obtaining a protein that will be stable during manufacturing, storage, and delivery. An increasingly important class of biotherapeutics is the monoclonal antibodies (mAbs). A reliable formulation not only ensures clinical performance but also enhances drug manufacturability and

stability. During formulation development, many types of studies are typically performed in order to reach a final recommendation for the optimal formulation and storage conditions. One of the most important characteristics of the mAbs that enables their successful development as biotherapeutics is their conformational stability, which can be defined as the ability of a molecule to maintain the native secondary, tertiary, and quaternary structure under a broad range of conditions.^{1,2} Interactions with the surrounding solution can also play a critical role in the maintenance of proper protein structures and their biological activities. These protein–solution interactions are related to colloidal stability;

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both colloidal and conformational stability can be affected by a large number of variables including pH, ionic strength, excipients, and protein concentration.^{3–7} Factors that significantly reduce aggregation under certain conditions might enhance aggregation under others. This complexity increases with the number of excipients included in formulations. Typically, a number of compositions needs to be evaluated in order to identify several lead formulation conditions. An emerging view is that the stabilization of biophysical properties, such as homogeneity, solubility, and stability, are likely to be predictive of successful formulations.⁸ Even at ambient temperatures, slow unfolding rates can be observed for some proteins.⁹ Additional factors such as protein net charge, charge distribution, and the presence of ions and excipients in solution, may also significantly impact unfolding, and therefore the conformational and possibly colloidal stability of the protein.^{10,11} Very often the unfolded intermediates of a protein will have a greater propensity to aggregate than the native state, so stabilizing the native conformation results in a drug product that will have minimized aggregation.²

One way to evaluate thermal stability is to increase the temperature and measure the formation of unfolded proteins under this stress. Physical characteristics of mAbs at increased temperatures can be assessed by several different techniques, such as circular dichroism (CD),⁴ Fourier transform infrared (FTIR),¹² intrinsic fluorescence spectroscopy,⁴ differential scanning calorimetry (DSC),^{13–15} and extrinsic fluorescence spectroscopy using probes sensitive to the environment on the surface of the protein.¹⁶ mAbs have characteristic DSC profiles consisting of the melting transitions of the CH2, CH3, and Fab domains, and the thermal stabilities of IgGs are affected by the contribution of these different domains.¹⁷ DSC is a valuable tool to compare thermal stability of mAbs, but it takes a significant amount of time and material to obtain results and for this reason DSC has limited throughput capability. Furthermore, the range of concentrations that can be investigated in typical DSC experiments is usually limited. It is important to test proteins in formulation solutions at concentrations used for real-time storage and drug delivery, conditions which often exceed the capability of DSC.

Fluorescence-based assays are easy to set up and are often compatible with multi-well plate devices. A promising assay recently developed to

assess protein stability in a high throughput screening (HTS) format was introduced by Pantoliano et al.¹⁸ The method measures the fluorescence of an extrinsic probe that is sensitive to the hydrophobic environment on the protein surface. In aqueous solutions the fluorescence emission from free probes is negligible, but in the presence of unfolded proteins the fluorescence intensity increases significantly due to the increased exposure of the hydrophobic protein interior providing hydrophobic regions that the probe can bind to.^{18,19} Thermally induced unfolding is commonly a cooperative transition between folded and unfolded states with a characteristic T_m as the midpoint transition temperature. In this report we employed a thermostability assay to obtain the temperature values of hydrophobic exposure or T_h . The T_h data displayed a good correlation with T_m values determined using DSC. This thermostability assay has been previously applied to screen ligand libraries to study interactions between small molecules and target proteins.^{18,20} It has also been used for structural optimization of protein clones,²¹ crystallization propensity screening,²² screening of ligands promoting stability²⁰ and for structure determination.²³ This method is often referred to as differential scanning fluorimetry (DSF),²⁰ or thermal shift assay;²⁴ the term differential scanning fluorimetry or DSF will be used throughout this report. In this study, DSF was utilized to simultaneously screen various buffer conditions to optimize T_h for multiple IgGs. DSF was shown to significantly save time and material in the development of formulations for mAbs.

MATERIALS AND METHODS

Monoclonal Antibodies

Bulk drug lots of two IgG1 mAbs, mAb1 and mAb4, and two IgG2 mAbs, mAb2 and mAb3, were purified and stored in a mildly acidic buffer at a protein concentration of 70 mg/mL. The molecular weights of the four mAbs are between 144 and 147 kDa. The theoretical pI values based on the amino acid composition using the ExPASy server were 8.9 for mAb1, 7.8 for mAb2, 7.1 for mAb3, and 7.4 for mAb4.²⁵

Reagents

The SYPRO[®] Orange dye was purchased from Invitrogen, Inc. (Carlsbad, CA). It is supplied as a

dimethyl sulfoxide (DMSO) solution at 5000 times the concentration recommended for use. The concentration, structure, and molecular weight are considered proprietary information, and are not released to the public. Guanidine hydrochloride (GuHCl) solution (8 M in water), sodium acetate, sodium chloride, L-arginine, proline, histidine, sucrose, sorbitol, and glycerin were obtained from Fisher Scientific, Inc. (Pittsburgh, PA). Isopropanol was received from Sigma-Aldrich, Inc. (St. Louis, MO). All other chemicals used were of at the highest quality commercially available.

Buffers and mAb Sample Preparation

DSF experiments were employed using mAb molecules at the concentrations indicated. For measurements in GuHCl solutions, mAb molecules in their initial buffers at a concentration of 70 mg/mL were diluted to a final concentration of 0.1 mg/mL into the indicated concentration of GuHCl. The GuHCl solutions contained phosphate-buffered saline (PBS) and 0–3 M GuHCl. The four mAbs were also diluted to a 0.1 mg/mL concentration in the various buffers that were screened. The buffers were 40 mM acetate (pH 4, 4.5, 5, 5.5), 40 mM histidine (pH 5.5, 6, 6.5, 7), and 40 mM phosphate (pH 6.5, 7, 7.5, 8). Additional excipients including proline, sodium chloride, sucrose, sorbitol, arginine, and glycerol were also included in the buffer panel that was screened. The exact concentration of the excipients is proprietary information. All proteins were dialyzed into the same buffers, and the conclusions of the studies are based on relative stability of the proteins in these buffers. Each reaction plate contained a total number of 84 formulation buffers consisting of 12 blank buffers plus 6 solutions at each buffer pH condition containing one additional excipient.

DSF experiments to assess the effect of protein concentration of thermal stability as determined by this assay were performed using mAb1 and mAb2. In these studies, the mAb were dialyzed against the buffers to be tested (1:100,000 ratio) for 16 h at 4°C. The dialyzed protein samples were concentrated to a protein concentration of above 70 mg/mL using 10,000 MWCO Amicon Ultra-15[®] centrifugal filters (Millipore Corporation, Billerica, MA). The mAb samples at a 70 mg/mL concentration were diluted to 10, 1, and 0.1 mg/mL in their corre-

sponding buffers before performing the DSF experiments.

Differential Scanning Fluorimetry (DSF)

The SYPRO orange dye was supplied in DMSO at 5000 times the working concentration and diluted into the appropriate buffers prior to being added to the protein solutions, to achieve a final dilution of 1:5000 in assay samples, consistent with the recommended gel stain concentration. Fluorescence measurements were employed using a 7000 series real-time polymerase chain reaction (RT-PCR) instrument (Applied Biosystems, Foster City, CA). Optical reaction plates of 96 wells (Applied Biosystems) were used with 100 μ L of solution per well. The fluorescence emission was collected using a ROX filter (600–630 nm) with a fixed excitation wavelength at 488 nm. During the DSF experiment the temperature was increased from 25 to 95°C at a heating rate of 20°C/h and at 1°C increments with an equilibration time of 2 min at each temperature prior to measurement. The data were exported into Excel[®] (Microsoft Corporation, Redmond, WA) for further analysis. The temperature of hydrophobic exposure, T_h , is defined as the temperature corresponding to the maximum value of the first derivative of the first fluorescence transition. When multiple unfolding transitions were observed only the T_h value of the first transition was analyzed, due to the overlap of the transitions at higher temperatures. Prior to the application of DSF on IgG molecules, several mAb proteins were melted using a peltier-controlled spectrophotometer (Photon Technology International, Birmingham, NJ) in the presence and absence of SYPRO Orange. The dye (at 1:5000- to 1:1000-fold dilutions of the original reagent) did not change the melting transition of the proteins determined by both the intrinsic fluorescence and static light scattering data acquired during heating.

Differential Scanning Calorimetry (DSC)

The DSC measurements were obtained using a VP-Capillary DSC system (Microcal, Inc., Northampton, MA) equipped with tantalum 61 cells, each with an active volume of 125 μ L. Protein samples were diluted to 0.5 mg/mL while the corresponding buffer was used as a reference. The samples were scanned from 20 to 95°C at a rate of 20°C/h with an initial 15 min of equilibration at

20°C. A filtering period of 16 s was used and data were analyzed using Origin 7.0 software (OriginLab® Corporation, Northampton, MA). Thermograms were corrected by subtraction of buffer-only blank scans. The corrected thermograms were normalized for protein concentration. The lowest melting temperature, T_{m1} , was determined using the DSC functions built into the Origin 7.0 software.

RESULTS AND DISCUSSION

DSF Profile Is Determined by Hydrophobicity of the Environment

The typical DSF profile of an IgG molecule consists of a sharp sigmoid-like increase to the maximum level followed by a decrease in fluorescence intensity (Fig. 1A). This profile represents changes in the fluorescence resulting from changes in the environment of the dye. The initial increase in fluorescence most likely results from the exposure of the dye to hydrophobic areas of the protein during thermal unfolding, which is dependent on the transition from folded to unfolded states.¹⁸ Fluorescence quenching at increased solution temperatures, a nonspecific transition, also occurs and contributes to the overall sigmoidal shape. To clarify the nature of the fluorescence quenching, the fluorescence of the fluorophore alone was investigated using solutions with different levels of hydrophobicity achieved by the addition of variable concentrations of isopropanol. As shown in Figure 1B, the fluorescence gradually decreases with increasing temperature; the intensity of the initial fluorescence and the rate of decline are dependent on the hydrophobicity of the solution environment (protein and buffer). Increased hydrophobicity in the environment of the fluorescence probe can also be caused by the presence of partially or completely unfolded proteins. To simulate the presence of denatured proteins due to thermal unfolding mAb molecules were denatured in solutions containing GuHCl. The fluorescence results obtained with mAbs mAb1, mAb2, mAb3, mAb4 in the presence of different concentrations of GuHCl (shown in Fig. 2) as a function of temperature were determined, and are shown in Figure 2. As the concentration of GuHCl increased the temperature of hydrophobic exposure, T_h , decreased and the transitions became less pronounced. At the highest concentration of GuHCl tested the transi-

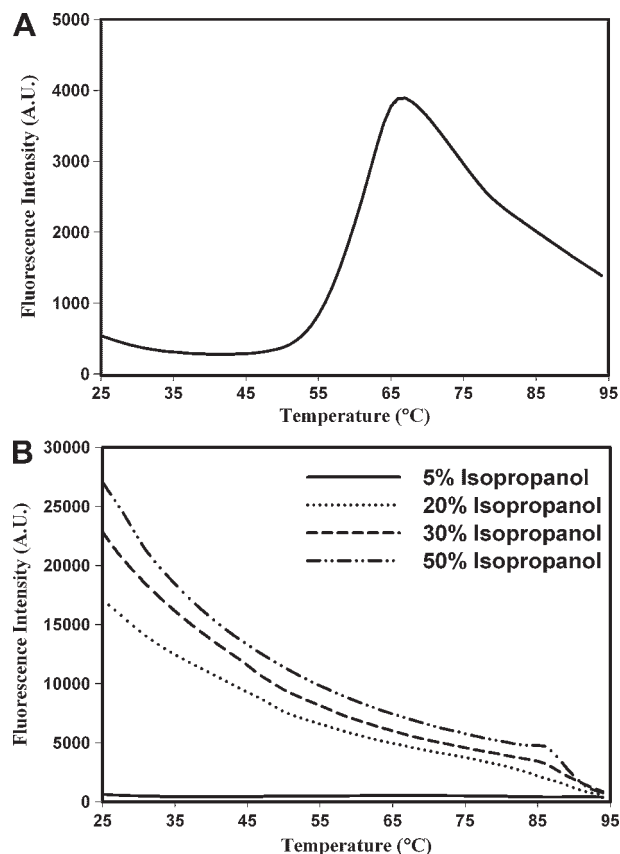


Figure 1. Fluorescence of SYPRO Orange obtained by DSF in the presence of a typical mAb molecule (A) and isopropanol (B) as a function of temperature. SYPRO Orange was diluted in 40 mM acetate, pH 5.5 to a final dilution of 1:5000. The mAb concentration was 0.1 mg/mL. The DSF procedure is described in Materials and Methods Section.

tion disappeared and the initial fluorescence was already at the maximum intensity, reflecting the large hydrophobic area exposed on the mAbs unfolded by the denaturant. The fluorescence quenching behavior in the presence of unfolded proteins is similar to that observed in the presence of isopropanol. This is a good indication that the data obtained by DSF is the result of a combination of an increase in fluorescence due to increased hydrophobicity of the environment of the dye due to the exposure of hydrophobic regions on the protein as it unfolds, and fluorescence quenching resulting from the gradual increase in the temperature of the entire solution. The fluorescence increase observed in DSF experiments with mAb molecules is a result of interaction or direct binding of the probe to hydrophobic regions of unfolded proteins. In addition, the multiple

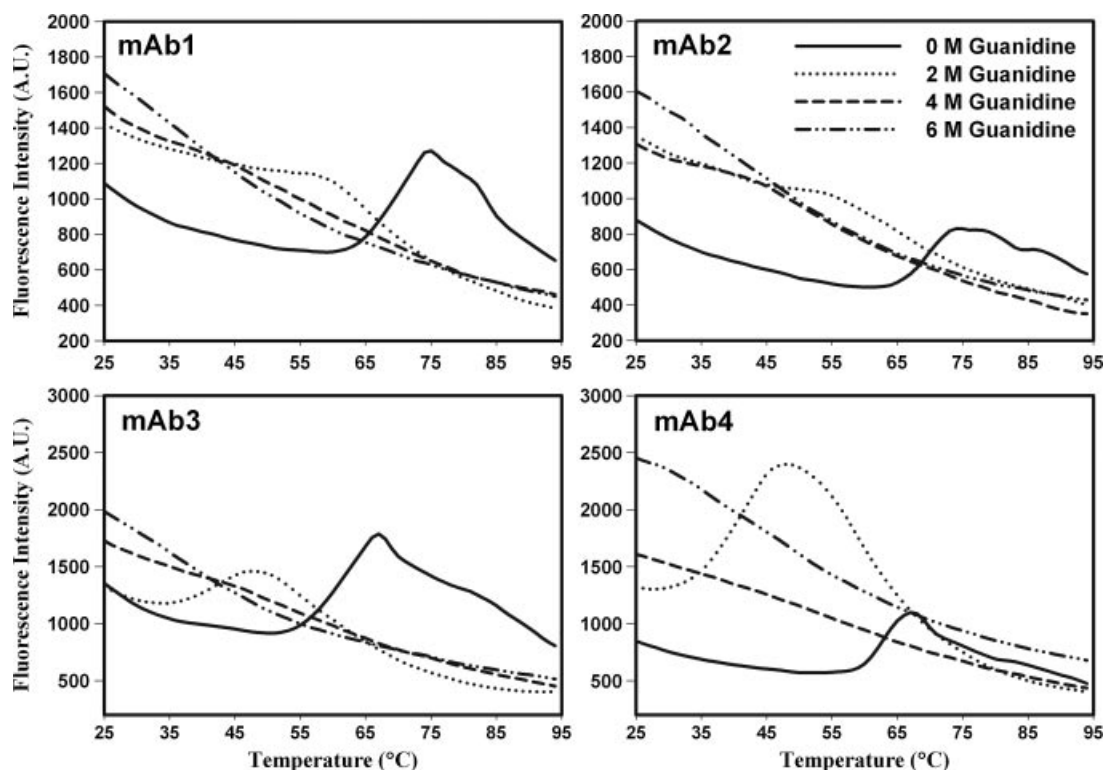


Figure 2. Representative DSF profiles obtained for mAb samples in PBS solutions containing GuHCl. Protein concentration was 0.1 mg/mL. DSF measurements were employed as described in Materials and Methods Section.

domains of the mAbs have different conformational stabilities, which resulted in multiple temperature-dependent transitions. The midpoint measured by DSF was determined simply as the local maximum value of the first derivative of the fluorescence curve for the transition occurring at the lowest temperature. Since this midpoint is not a true melting temperature the transition temperature observed in DSF was denoted as T_h or the temperature of hydrophobic exposure, and represents the transition of the least stable domain of the mAb.

GuHCl typically denatures IgG molecules at concentrations between 3 and 4 M.²⁶ The DSF results show that the increase in the initial fluorescence and the T_h shifts to lower values with increased concentrations of GuHCl. Under conditions where mAb molecules are mostly unfolded the DSF profile displays no transitions and high initial fluorescence emissions. The dependence of T_h on GuHCl concentration indicates that this technique is measuring the stability of the protein, with partially or fully unfolded protein having increased exposure of hydrophobic regions from the beginning, and also

being less stable to heat than the folded molecule. These results show that DSF can be a useful method for studying protein unfolding. The increase in the exposure of hydrophobic regions on the protein could be accompanied by, or even due to, protein aggregation.²⁷ The question whether aggregation precedes unfolding or vice versa requires more investigation and will not be addressed in this report. However, no matter what the underlying protein structure is, the thermal transitions detected by this method correlate with protein unfolding and stability.

The background fluorescence in the presence of properly folded IgG molecules is very low due to the hydrophobicity of the surface of these proteins when folded and the signal increased significantly upon protein unfolding. These features allowed us to measure protein thermostability with high sensitivity with minimal material. The resulting fluorescence changes can be simplified as a combination of a sigmoidal increase in the case of two-state unfolding and an exponential decrease described by temperature-dependent quenching.^{18,20} Despite the difficulty in deconvoluting the fluorescence profiles in the presence of

proteins, it was possible to estimate the temperature of melting and the saturation temperature when a domain was completely unfolded by an approximation model. In this study, the first derivative of the fluorescence intensity was used to denote T_h as a characteristic parameter to describe unfolding. Currently the interaction of SYPRO Orange with specific proteins is not thoroughly understood, due primarily to the fact that the supplier does not provide information on the chemical structure or molecular mass of the probe. It is possible that the interaction of SYPRO Orange can be driven not only by hydrophobicity of the protein but also by charges on the protein, as is sometimes the case for 8-anilino-1-naphthalene sulfonate (ANS), another fluorescent probe sensitive to hydrophobicity.^{16,28} Unfortunately ANS could not be assessed in the DSF format due to the absence of any appropriate filters for the collection of ANS fluorescence. As is the case even for ANS, as long as the interactions of the dye to different IgGs are similar the exact interactions of the probe to the unfolded protein do not need to be defined for the relative stabilities determined with it to be meaningful. As demonstrated above the SYPRO Orange is a suitable probe for comparing the stabilities of these molecules.

DSF and DSC Results Are Consistent

DSC is perhaps the most commonly used method to characterize conformational stability based on differences in thermal stability. Normalized DSC thermograms of IgG molecules and DSF data were compared under six different buffer conditions that were used in the formulation screening study described below. Two molecules, mAb1 and mAb2, were studied in acetate buffers at pH 4.0 and 5.5; histidine buffers at pH 5.5 and 7.0, and phosphate buffers at pH 6.5 and 8.0. The results are shown in Figure 3. The T_h obtained with DSF occurred at slightly lower temperatures than the first T_m determined by DSC under all experimental conditions. At pH 4.0 the DSF profile mimicked the transition corresponding to the CH2 domain which had the lowest melting temperature for these mAb molecules as determined by DSC (Fig. 3). The CH2 domain was more sensitive to pH changes than the other IgG domains, consistent with previously published results.^{29–31} The correlation between T_h and T_m was plotted for different buffer conditions and molecules (see Fig. 4). A good approximation to a linear

relationship between T_h and T_m was observed. The transition of the Fab domain did not vary much at this pH as analyzed by both DSC and DSF. At higher pH values the transition of the CH2 domain overlapped that of the Fab domain; the Fab domain transition did not change under the pH range tested. DSC measurements were repeated in the presence of the fluorescence probe in these same buffers and the thermograms were the same as those obtained in the absence of the dye, suggesting that the fluorescence probe did not alter the thermostability properties of the molecules under the analytical conditions used.

Direct comparison of DSF and DSC demonstrated that both methods detected the same trends in thermostability in different solutions, and the same relative stabilities of the domains. Results from mAb molecules indicated that T_h was consistently lower than T_m determined by DSC. The fluorescence probe could be more sensitive to changes induced by heat than the DSC, since anything that affects the surface hydrophobicity will be detected, including changes in protein–solvent interactions or local changes in surface hydrophobicity that so not include unfolding of the entire domain. In addition it is possible that aggregation could occur simultaneously during protein unfolding and complicate determination of T_h or T_m values. In this study, T_h , the temperature of hydrophobic exposure for the domain unfolding at the lowest temperature, was indicative of the unfolding of the CH2 domain. The Fab domain is less sensitive to pH than the CH2 domain;^{30,31} because of this, as the pH is increased, signals from the CH2 and Fab domains can overlap under these conditions, as was seen with both DSF and DSC in this study. In phosphate buffers at pH 6.5 the CH2 domain melted at essentially the same temperatures as the Fab domain (Fig. 3F). For some mAb molecules the CH2 domain can even melt at higher temperatures than the Fab domain. In these cases the T_h should be attributed to the appropriate transitions and interpretations should be derived accordingly. It is important, however, to determine the temperature of unfolding for the least stable domain of the protein. Results obtained in this study on mAb molecules suggest that T_h is in good correlation with the CH2 unfolding temperature T_m (Fig. 4). This linear correlation indicates that T_h determination is, indeed, a predictive method of protein thermostability. The comparison of the T_h of the mAbs in different buffer pH solutions demonstrated that the deglycosylated molecule, mAb4, had a lower

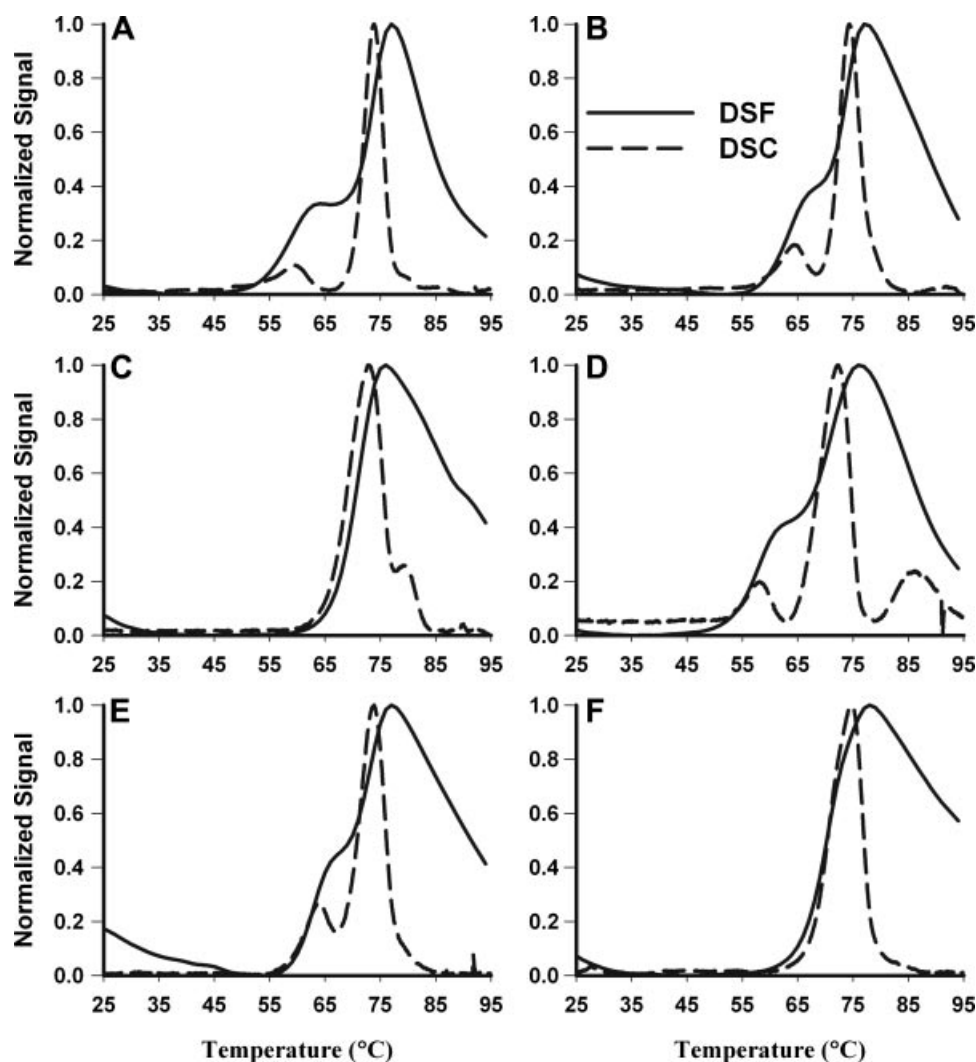


Figure 3. Comparison of representative DSF profiles and DSC thermograms. IgG molecules, mAb1 and mAb2, were at 0.5 mg/mL. C_p and fluorescence signals were normalized to the maximum values obtained. Solution compositions were the following: mAb1 in 40 mM acetate, pH 4.0 (A); mAb1 in 40 mM histidine, pH 5.5 (B); mAb1 in 40 mM phosphate, pH 8.0 (C); mAb2 in 40 mM acetate, pH 4.0 (D); mAb2 in 40 mM histidine, pH 5.5 (E); mAb2 in 40 mM phosphate, pH 8.0 (F). DSF and DSC experimental conditions are listed in Materials and Methods Section.

stability than the glycosylated proteins, and that the glycosylated mAbs had similar stability (Fig. 5). The stability of the entire molecule is likely to be directly related to the stability of its most unstable domain. Hence T_h indicates the temperature of exposure of protein hydrophobic areas and the temperature at which the probability of aggregation is significantly increased.^{31–}

³³ It follows that lower T_h values suggest the protein is thermally less stable, and this was verified by the correlation between DSF and DSC. Thus, DSF could be used as a tool for the

evaluation of thermal stability of mAbs and the method could be employed to compare the stabilities of different mAbs, and of the same mAb in different solutions.

DSF Reveals Effects of pH and Excipients on IgG Thermostability

The compatibility of DSF to a high throughput format allows screening of many conditions simultaneously. A total of 84 common formulations with

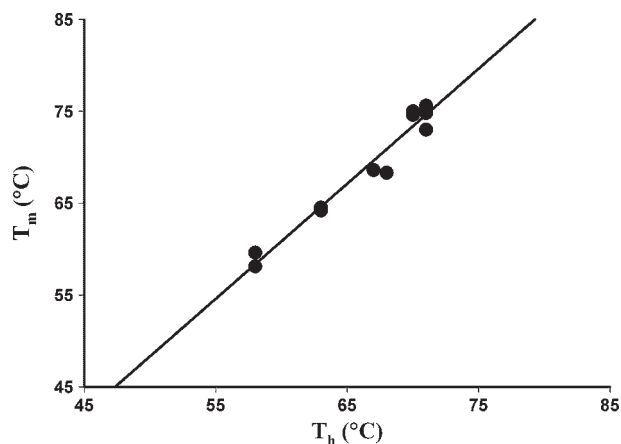


Figure 4. Correlation between T_h and T_m . Samples containing mAb1 or mAb2 (both at 0.5 mg/mL) were prepared in a variety of solutions including: 40 mM acetate, pH 4.0 and 5.5; 40 mM histidine, pH 5.5 and 7.0; 40 mM phosphate, pH 6.5 and 8.0. T_h values were determined as the midpoint of the first fluorescence transition in DSF profiles using the first derivative curves and T_m values were determined as the lowest melting temperature in DSC thermograms. Data acquisition was according to Materials and Methods Section. Linear regression yielded a linearity correlation coefficient $R^2 = 0.9605$.

previously known effects on mAbs were applied to show the predictive capability of DSF for the evaluation of physical stability of mAbs. As we have mentioned in the context above four mAb molecules were investigated in this study: mAb1 (IgG1), mAb2 (IgG2), mAb3 (IgG2), and mAb4 (IgG1). A deglycosylated molecule, mAb4, was included for the purpose of exploring the effects of pH, excipients and buffer composition on the stability of mAbs with different chemical properties. A variety of formulation conditions was employed to generate DSF profiles for each of the mAb's. Twelve buffer compositions were studied covering a wide pH range from 4.0 to 8.0. These pH conditions represented typical working ranges of the selected buffer components. Six excipients at concentrations widely used in pharmaceutical formulations were also studied (see Materials and Methods Section). A total of 84 combinations with or without one additional excipient were assessed in the 96-well plate format, to allow the simultaneous comparison of these formulations. When DSF was employed in the absence of proteins, all 84 buffer formulations displayed similar background fluorescence traces, with no detectable transitions, suggesting the excipients included in this study did not interfere with T_h

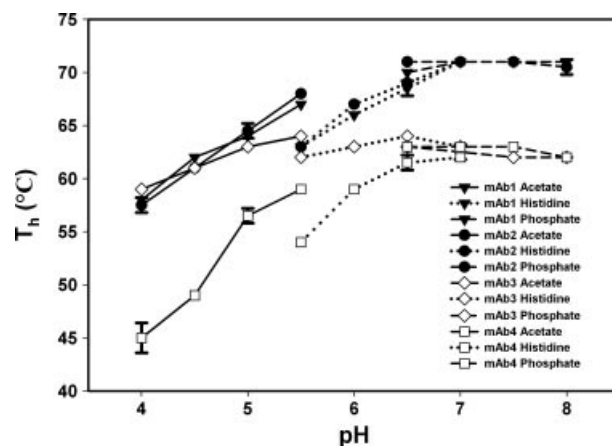


Figure 5. T_h values as a function of pH for mAb molecules (0.1 mg/mL) using SYPRO Orange fluorescence. Twelve solutions were included for each mAb: 40 mM acetate, pH 4.0, 4.5, 5.0, and 5.5; 40 mM histidine, pH 5.5, 6.0, 6.5, and 7.0; 40 mM phosphate, pH 6.5, 7.0, 7.5, and 8.0. Averaged T_h values and error bars obtained from duplicate measurements are shown. Symbols represent different mAb molecules and line patterns indicate buffer components. All measurements were carried out as described in Materials and Methods Section.

determination under the experimental conditions used.

The effect of pH on T_h for all four molecules in buffers without additional excipients is shown in Figure 5. The T_h increased with increasing pH up to 6.5, where it reached a plateau of maximum stability. The results also showed that at pH 5.5, the mAbs were more stable in acetate buffer than in the histidine buffer. The thermostability in the phosphate buffers appeared to be independent of pH over the range of 6.5–8.0. This is consistent with the results previously obtained with DSC.¹⁵ The effects of excipients on the thermostability of the mAbs are shown in Figure 6. In this case differences in T_h (ΔT_h) were calculated by subtracting the values obtained without excipients from those obtained with excipients. Six ΔT_h values are presented for each pH and buffer composition. The largest changes in stability were observed with sodium chloride and arginine, which both resulted in large negative ΔT_h values. This destabilizing effect was more pronounced at lower pH values than at higher ones. NaCl destabilized proteins more than arginine at low pH values while ΔT_h in the presence of these two excipients was comparable under more basic conditions.

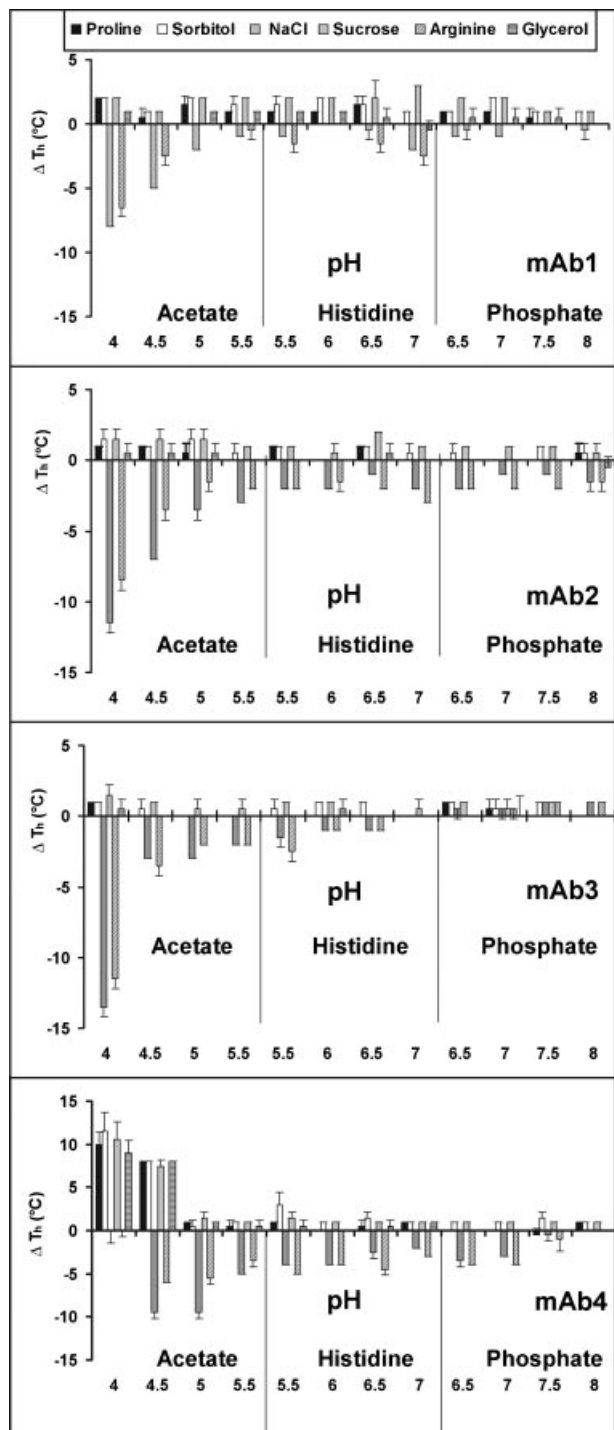


Figure 6. The effects of excipients and pH expressed in ΔT_h for mAb molecules. DSF was employed as described in Materials and Methods Section at a protein concentration of 0.1 mg/mL. Formulations included 84 conditions as indicated in the figure labels. ΔT_h was determined by subtracting T_h values obtained in blank buffers from those with excipients according. Vertical histograms and error bars represent results from duplicate experiments.

For mAb1, mAb2, and mAb3 the inclusion of proline and glycerol had a minimal effect on the stability of the proteins, resulting mostly in a slight increase in T_h values. The most significant stabilization was observed in the presence of sorbitol and sucrose, and was independent of pH. For mAb4 there was a dramatic positive shift in T_h at pH 4.0 and 4.5 in the presence of all excipients except for sodium chloride and arginine. In fact the transitions of the mAb4 samples with NaCl and arginine at pH 4.0 were poorly defined and nearly impossible to determine (see Fig. 7), indicating significant protein unfolding at 25°C at this pH. This is most likely due to the absence of carbohydrate on this protein.

The effects of pH on the T_h shift caused by salt or arginine were attenuated at higher pH's for all mAbs, and the magnitude of this effect depended on the specific protein. As shown in Figure 6 the most stable molecule was mAb1. mAb1 over the entire pH range was not as sensitive to the concentration of the NaCl or arginine as the other molecules. NaCl had no effect at pH 6.5 or higher on mAb1 stability. Sucrose and sorbitol had positive effects on all four of the proteins at all pH and buffer conditions tested. The least stable molecule was mAb4. At pH 4.0 in the presence of salt or arginine it was partially unfolded even at 25°C, demonstrated by the increased initial fluorescence and less pronounced transitions during melting (Fig. 7). Therefore, the

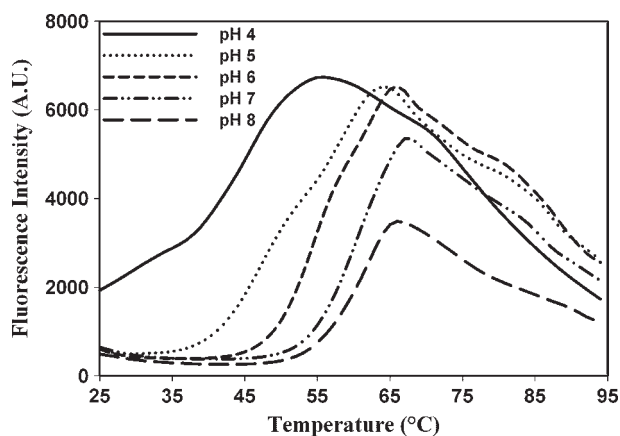


Figure 7. Representative SYPRO Orange fluorescence as a function of temperature in the presence of mAb4. 0.1 mg/mL mAb4 was prepared in buffers containing 140 mM sodium chloride and: 40 mM acetate, pH 4.0; 40 mM acetate, pH 5.0; 40 mM histidine, pH 6.0; 40 mM histidine, pH 7.0; and 40 mM phosphate, pH 8.0. Experimental procedures are stated in Materials and Methods Section.

ΔT_h determination for mAb4 under these conditions was not determined. Significant destabilization effects of salt and arginine on mAb4 were still visible at higher pH values while the dramatic positive T_h shifts caused by other excipients could only be seen at pH 4–4.5. For all other mAbs the positive shifts in T_h caused by sucrose and sorbitol were relatively independent of pH and did not seem to vary among buffer compositions.

The effects of the excipients studied were consistent with those previously observed.^{34–36} Effects of pH on mAb stability have previously been described in detail^{6,31} and the increased propensity to aggregate at low pH has been related to the low temperatures of unfolding that occur at these pHs.^{7,31,33,37} The NaCl and arginine may interact with proteins via a charge-dependent mechanism which is mediated by pH. In contrast, the stabilizing effects of sucrose and sorbitol previously shown for other proteins appear to be independent of pH, and can be the result of a different type of protein–solution interaction.^{36,38,39} Deglycosylation has been known to have a significant impact on the stability of IgGs.^{40,41} As described above DSF detects the unfolding of the CH2 domain in IgG. This is where the glycosylation sites are located and, as a result, the deglycosylated mAb4 molecule was the least stable among the four mAbs tested. In this study we have demonstrated that multiple conditions can be tested in the same experiment in a systematic manner, while consuming significantly less time and material than those required for other methods like DSC, yielding comparable results.

Higher Protein Concentration Shifts T_h to Lower Values

One of the advantages of DSF is the ability to carry out measurements of T_h in highly concentrated protein solutions. Whether investigating potential candidates for relative stability, or screening different formulation conditions for stabilization of proteins, it may be important to identify the properties of the protein solution at high concentrations where the number of characterization methods available is limited. A few pH's and excipients resulting in maximum T_h shifts were chosen to test DSF at high protein concentrations, based on the data previously obtained at 0.1 mg/mL. One IgG1 (mAb1) and one IgG2 (mAb2) were selected for this experi-

ment. Different SYPRO Orange concentrations (from a 5000- to 1000-fold dilution of the reagent) were evaluated in the presence of highly concentrated mAb molecules and no differences were detected in onset or midpoint of the fluorescence transition (data not shown). The dye was then used at a 5000-fold dilution for the remaining experiments in alignment with the literature.^{19,21} The antibodies were prepared in selected buffers at about 70 mg/mL and samples were prepared by dilution to 0.1, 1, and 10 mg/mL accordingly. In Figures 8A and 9A the dependence of T_h on pH is shown for the solutions without excipients, at different concentrations. The T_h decreased with increasing protein concentrations for all buffers tested. The changes in T_h (ΔT_h) are also plotted in Figures 8B and 9B for the molecules in the presence of excipients compared to the transitions obtained in their absence. Increased protein

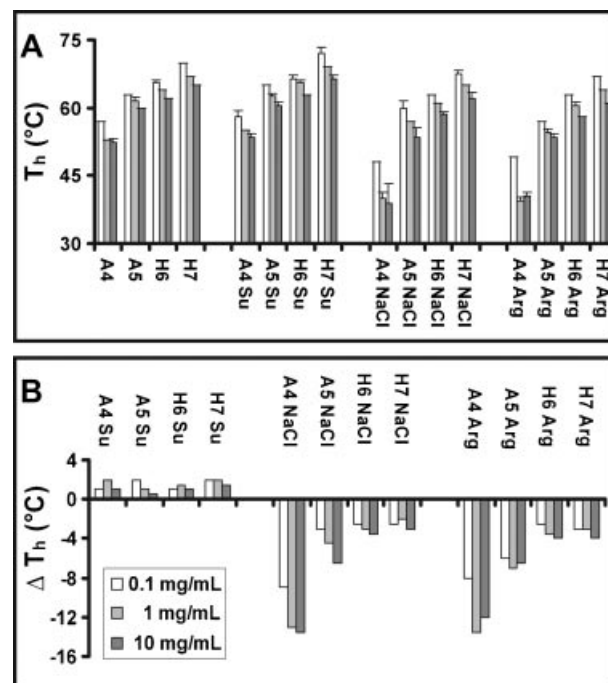


Figure 8. The effects of protein concentration on T_h value for mAb1. A: T_h values are shown for the samples in different buffers without excipients at the indicated protein concentrations. The error bars are based on duplicate measurements. B: ΔT_h expressed as the difference in T_h between conditions with excipient and those without excipients. Averaged T_h values from duplicate experiments were used in the calculation. Buffer conditions are labeled on the X-axis. A4 and A5: 40 mM acetate, pH 4 and 5; H6 and H7: 40 mM histidine, pH 6 and 7; Su: sucrose; NaCl: sodium chloride; Arg: arginine. All experimental parameters are as described in Materials and Methods Section.

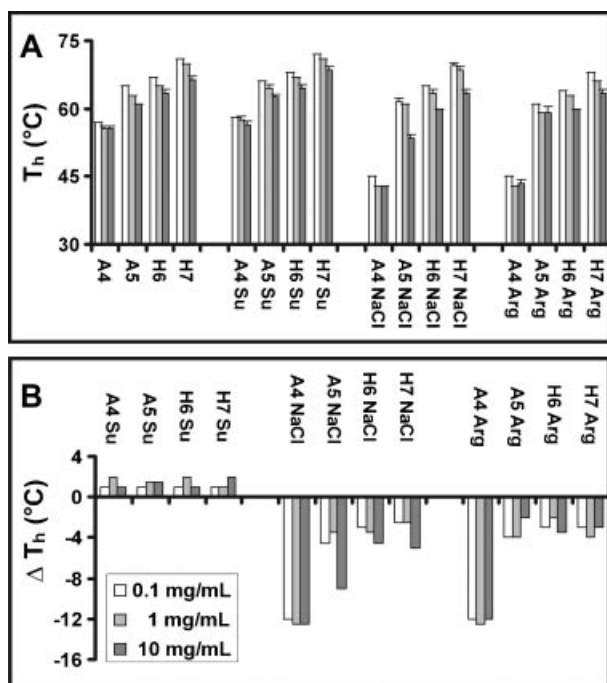


Figure 9. The effects of protein concentration on T_h value for mAb2 molecule. A: T_h values are shown for the samples in different buffers without excipients at the indicated protein concentrations. The error bars are based on duplicate measurements. B: ΔT_h expressed as the difference in T_h between conditions with excipient and those without excipients. Averaged T_h values from duplicate experiments were used in the calculation. Buffer conditions are labeled on the X-axis. A4 and A5: 40 mM acetate, pH 4 and 5; H6 and H7: 40 mM histidine, pH 6 and 7; Su: sucrose; NaCl: sodium chloride; Arg: arginine. All experimental parameters are as described in Materials and Methods Section.

concentrations at pH 4.0 enhanced the destabilizing effect of salt and arginine. At higher pH, protein concentration had less effect on ΔT_h .

In addition to pH and excipients, protein concentration is another important factor to consider during formulation development. Highly concentrated IgG proteins can be used to provide optimal dosage for delivery and storage. Analysis of highly concentrated protein solutions is challenging in general. The experiments described here demonstrate that DSF can be used to compare the thermostability of mAb molecules over a wide range of protein concentrations. The same experiments were also performed using mAb molecules at 70 mg/mL and the results will be included in a separate report. The T_h decreased with increasing protein concentration for all conditions and proteins tested. This trend has been previously observed by DSC and

was attributed to the increased probabilities of interactions between the IgG molecules, resulting in the increased reversible self-association, increased formation of hydrophobic patches on the protein, and increasing irreversible association as well.⁴ Higher concentrations can lead to greater aggregation levels under increased temperature stresses, which is in good correlation with the DSF observations.⁴² The shift in T_h caused by excipients was less dependent on protein concentration (Figs. 8B and 9B). The most apparent changes were observed for the samples at pH 4.0 and 5.0 with NaCl, presumably as a result of charge-mediated distributions by salt molecules.^{10,43} These are also the conditions under which the proteins are the least stable, indicating a greater proportion of unfolded intermediates with hydrophobic regions exposed and available for self-association as the protein concentration is raised.

DSF Is a Tool for High Throughput and Predictive Screening of IgG Formulations

We have shown that DSF can be employed successfully as an HTS assay during formulation development. Comparison of different conditions or different molecules can be achieved in a short time with small amounts of protein. DSF can be applied to the initial assessment of IgG properties in different solutions and the solution compositions applied for this product can be narrowed early in product development. DSF is also robust enough to be applied to highly concentrated protein solutions to simulate actual storage conditions. Concentrations could vary from 0.1 to 100 mg/mL (unpublished results). A bench size experiment (up to 96 formulation conditions) usually takes <4 h, which could be further reduced by automating the system. Additional high throughput capability could be achieved by increasing the number of samples tested to 384 and 1024 per experiment based on the multi-well plates chosen. Consistency with DSC results presents possibilities for DSF to replace DSC when fast screening is demanded, or when only a small amount of protein can be spared. T_h and T_m are distinctive measurements and the correlation between DSF and DSC presented here is solely based upon experimental observations. This analysis should always be established for particular types of proteins and conditions prior to substituting DSF for DSC. Though the experiments described here were performed on a single

instrument, it seems reasonable to expect that the technique could be adapted for use on other instruments capable of measuring fluorescence in multi-well plates while increasing the temperature in a controlled fashion.

DSF can predict the effects of excipients on aggregation levels when the mechanism of aggregation is related to protein unfolding. The thermostability of IgG molecules is decreased significantly at low pH and in the presence of NaCl and arginine. Decreased thermostability should be an indication of the increased propensity of protein to unfold and/or aggregate. It has been shown that unfolded proteins have a higher tendency to aggregate.^{44,45} Dependence of aggregation levels on the presence of salt has been observed previously in studies with IgGs where the samples were incubated at elevated temperatures.^{10,42} It is interesting that NaCl resulted in increased aggregation that was induced not only by high temperatures but also by agitation even though the mechanism of formation and the size of aggregates could be different in each case.^{10,42} Though aggregation could be caused by covalent interactions,¹¹ chemical modifications,¹⁰ air/liquid or liquid/surface interfaces among others,⁶ it is not clear that the same conditions can lead to protein unfolding. DSF (and indeed other techniques following thermostability) assess the stability of a protein based on conformational stability, and aggregation propensity. This assay screens for one aspect of formulation stability, selection of the final formulation will need to take into account other aspects as well.

The application of DSF to formulation studies has the potential to fit the design of experiment (DOE) methodology at both the screening and optimization stages of formulation development, which emphasize simultaneous and efficient testing of multiple factors. The HTS capability of DSF allows measurements to be employed with minimum variations in experimental/hardware settings. The vast amount of information obtained in thermostability screening will allow investigators to perform systematic studies over a large range of candidates and parameters. Ultimately it has the potential to revolutionarily decrease the time and amount of resources required during this type of biopharmaceutical development.

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