“Acetate and Citrate Specific-Ion-Effects on Unfolding and Temperature-Dependent Aggregation Rates of Anti-Streptavidin IgG1”

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Keywords: Calorimetry (DSC), Protein aggregation, HPLC (high performance/pressure liquid chromatography), Formulation, Stability

Abbreviations: [PTIR, Parallel-Temperature-Initial-Rates; AS-IgG1, Anti-Streptavidin Immunoglobulin Gamma-1; mAb, monoclonal antibodies; SEC, size exclusion chromatography; DSC, differential scanning calorimetry; Fc, Fragment crystallizable; Fab, Fragment antigen-binding; C_H2 (C_H3), constant-heavy-domain 2 (3)]

Abstract

Controlling and predicting unwanted degradation, such as non-native aggregation, is a long-standing challenge for monoclonal antibodies (mAbs) and other protein-based products. mAb aggregation rates are typically sensitive to temperature, pH, and the addition of excipients. Quantitatively comparing temperature-dependent aggregation rates across multiple possible formulations is a challenge in product development. A Parallel Temperature Initial Rates (PTIR) method is employed to efficiently and accurately determine initial rates for anti-streptavidin (AS) IgG1 aggregation as a function of pH, [NaCl], and in the presence of acetate vs. citrate buffer. PTIR is shown to be in good agreement with results from a traditional, isothermal method, and permits direct comparison of the formulations across almost three orders of magnitude of aggregation rates. The apparent mid-point unfolding temperatures (via differential scanning calorimetry) and the effective activation energy values (E_a) are generally higher in acetate buffer compared to citrate buffer, which is consistent with preferential accumulation of citrate ions compared to acetate ions that was speculated in previous work (Barnett et al., *J Phys Chem B*, 2015). Static light scattering and Kirkwood-Buff analysis show that AS-IgG1 has stronger net repulsive protein-protein interaction in acetate compared to citrate buffer, also consistent with increased values of E_a . In an extreme case, aggregation of AS-IgG1 is effectively eliminated across all practical temperatures at pH 4 in 10 mM sodium acetate, but proceeds readily in citrate buffer.

Introduction

Protein-based pharmaceuticals are one of the fastest growing sectors of the pharmaceutical pipeline.¹ Monoclonal antibodies (mAbs) are expected to be among the leading candidates for biologic drugs in the future, with over 35 FDA approved therapeutic products on the market.² This class of proteins has the potential to treat many diseases, including various forms of cancer, autoimmune diseases, and life-threatening infections.¹ However, mAbs and other protein-based therapeutics have inherent stability problems that can be problematic during manufacturing and storage. During processing, proteins may experience chemical, thermal, or mechanical stresses that lead to unwanted chemical or physical degradation.³ In particular, aggregation has the potential to jeopardize patient safety and drug efficacy if product administration leads to unwanted patient immune responses.^{4,5}

pH, salt type and concentration, and the identity and concentration of other excipients may alter the chemical potential of the folded and unfold states.⁶ Upon heating or applying other stresses, proteins can lose higher-order structure and biological function. At temperatures significantly below the midpoint unfolding temperature(s), mAb unfolding/folding stage(s) will be pre-equilibrated because they occur much more quickly than the rate-limiting step(s) for aggregation.⁷ While thermodynamics may favor aggregates being the lowest free energy state, kinetics typically dictate the timescales and concentrations of the final aggregated populations.⁸ As such, measurement and prediction of aggregation rates is a major focus of effort during drug product development, as surrogate quantities such as virial coefficients, unfolding temperatures, and aggregation onset temperatures do not provide quantitative information about aggregation rates.⁹⁻¹²

Predicting aggregation rates *a priori* for a given protein formulation remains an outstanding challenge for a variety of fundamental and practical reasons.^{10,11,13} The solution pH, choice of buffer species, and addition of salt and other excipients may affect conformational stability and/or protein-protein interactions, while temperature changes can dramatically effect conformational stability.^{10,14,12,15} Prior work has indicated that conformational stability is a key factor affecting aggregation rates in solution, as the midpoint temperature of thermal unfolding from differential scanning calorimetry, or the onset temperature of aggregation from scanning techniques, can be at least qualitatively predictive of aggregation rates across different formulations.^{12,16–18} However, there can also be a competing effect between changes in conformational stability and protein-protein interactions as one changes solution conditions such as pH.^{11,19}

Accurately and efficiently determining protein aggregation rates across a range of conditions has been a long-standing challenge. A number of temperature-scanning techniques have been developed to at least qualitatively or semi-quantitatively monitor aggregation.^{10,20,21} An inherent issue with temperature-scanning techniques is thermal history. For example, in the process of scanning through lower temperatures, one creates aggregates that can act as “seeds” to accelerate aggregation at subsequent (higher) temperatures, and thus overestimate aggregation rates.¹⁰ It is difficult to predict when this will or will not be the case, as simple changes in the formulation pH and ionic strength can alter aggregation mechanisms and “seeding” effects.^{6,22}

A large majority of biophysical techniques that are currently used to rapidly monitor aggregation use an indirect measure of monomer loss and are only surrogate measures of aggregation rates. A direct measurement of monomer concentration necessitates a separation of monomer from aggregate species or the ability to measure a monomer-specific marker. For

example, in spectroscopic techniques such as circular dichroism, ThT dye-binding or intrinsic fluorescence, the spectra are ensemble averages. Therefore, they have contributions from monomer and aggregate species, and the spectral changes may or may not correlate with monomer consumption.²²

An indirect measure of monomer loss rates may also have a bias based on the measurement technique. For example, aggregation rates monitored using scattering techniques have a bias towards larger sized particles.²³ pH and ionic strength changes can alter aggregation mechanisms, and produce large and heterogeneous aggregate populations that provide much larger scattering intensities compared to smaller-sized aggregates at an identical monomer loss rate. These challenges are compounded if fragmentation occurs, as is relatively common for mAbs^{24–26} and other proteins.²²

This report introduces a Parallel-Temperature-Initial-Rates (PTIR) method to accurately and efficiently determine degradation rates as a function of temperature. PTIR is compared to rates determined using traditional isothermal incubations, and the method shows good quantitative agreement for aggregation rates for an anti-streptavidin (AS) immunoglobulin gamma 1 (IgG1) that has been investigated previously.^{14,27–30} Aggregation rates from accelerated (high temperature) to near-room temperature conditions are reported across multiple values of pH and NaCl concentration, as well as different buffer species. The results highlight conformational stability as a key factor in determining accelerated aggregation rates, but also illustrate strong contributions from electrostatic protein-protein interactions. Ion-specific effects are also highlighted, as the choice of buffer (acetate vs. citrate) significantly alters thermal unfolding transitions, protein-protein interactions, and aggregation rates.

Materials and Methods

Sample Preparation

AS-IgG1 (> 98% monomer) was provided by Amgen as a stock solution at a concentration of 30 mg/mL. Additionally, purified fragment crystallizable region (Fc-IgG1) was provided by Amgen as a stock solution at a concentration of 20 mg/ml. The protein was dialyzed as previously reported.^{14,31} The protein concentration was confirmed using UV-Vis absorbance at 280 nm (Agilent 8453 UV-Vis, Agilent Technologies, Santa Clara, CA) using an IgG1 extinction coefficient of 1.586 mL/mg cm and an Fc-IgG1 extinction coefficient of 1.36 mL/mg cm. All solutions were diluted gravimetrically to working concentrations.

Size exclusion chromatography (SEC)

The monomer concentration of a given sample was quantified using size exclusion chromatography (SEC). An Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) was connected in-line to a Tosoh (Montgomeryville, PA) TSK-Gel 3000SWxL column. Samples were injected with an autosampler (100 microliter injections), with samples held at room temperature prior to injection. Concentration was determined by peak area, using a variable wavelength detector (Agilent technologies, Santa Clara, CA) and absorbance at 280 nm, with external standards. Additional details are the same as previously reported.¹⁴

Differential scanning calorimetry (DSC)

DSC was performed using a VP-DSC (Malvern Instruments, Malvern, UK) for solutions at a given pH and salt concentration (1 mg/mL IgG1 or 0.33 mg/mL Fc-IgG1). Scans were

performed from 20 to 90 °C at a 1 °C /min scan rate. If precipitation did not occur after the scan, as indicated by the lack of a large exotherm, a rescan was performed to check for reversibility. None of the conditions that were tested exhibited reversibility upon a rescan. The absolute heat capacity was calculated from the buffer-subtracted DSC scans, as previously reported.^{14,31}

Quantifying Aggregation Rates

IgG1 stock solutions were prepared at 1 mg/mL at a given pH, NaCl concentration, and buffer type, and aliquot into hermetically sealed deactivated borosilicate glass HPLC vials (Waters, Milford, MA). Isothermal incubations were performed by heating multiple samples in a water bath or custom-built PTIR device (see Supporting Information) at a given temperature and removing samples at pre-determined incubation times. Incubation temperatures were chosen such that multiple time points could be taken during the early periods of monomer loss ($m=1-0.8$, m is defined as the concentration of monomer divided by the initial monomer concentration, as measured by SEC peak area). At each time point, a given vial was immediately quenched by immersion in an ice-water bath to arrest aggregation, and was subsequently held at room temperature (20-23 °C) prior to analysis with SEC.

Aggregation rates were determined by monitoring the monomer fraction remaining as a function of incubation time. The monomer fraction was quantified using SEC, described above. Over approximately the first ten to twenty percent monomer loss, the rate of change of m remains nearly constant and the observed rate law can therefore be well described as zeroth order without the need to assume an underlying rate law.³² Therefore, the monomer fraction was regressed with Equation [1] to obtain the aggregation rate coefficient (units of inverse time), k_{obs} , from the regime where m was between approximately 1 and 0.8.

$$m = 1 - k_{obs}t \quad [1]$$

Parallel Temperatures Initial Rates (PTIR)

Incubations were performed by heating a single vial at a given temperature using a water bath or the parallel temperature device (see Figure S1 in Supporting Information). The key difference between the PTIR approach and conventional approaches is that for conventional approaches one selects a small number of temperatures (sometimes only one, e.g., 40 °C) and measures multiple samples over a predetermined time course (e.g., as proscribed by ICH guidelines¹³); in the PTIR approach, one instead measures a small number of samples (e.g., one sample in the extreme example below) at multiple temperatures for the same incubation time. That is, rather than choose multiple time points at a given temperature, one chooses multiple temperatures with a given incubation time.

In the present examples, incubation temperatures were chosen so that samples quenched after 2 or 24 hours would have monomer loss values that fell in the initial-rate regime. The incubation time was selected to be not less than two hours so as to allow for sufficient temperature equilibration and elimination of artificial lag times at shorter incubation time scales when samples were heating to the set-point temperature. Sample temperatures were confirmed independently with a separately calibrated thermocouple. The longer incubation timescale of 24 hours was chosen to achieve initial rates approximately one order of magnitude slower than two hours. For some examples, 10-day incubations were also performed. Once a sample was removed from incubation it was quenched on ice as described above, prior to analysis with SEC.

Aggregation rates using the PTIR approach are based on Equation [2], which is derived by rearranging Eq. 1 and solving for k_{obs} .

$$k_{obs}(T) = \frac{1 - m(T)}{t} \quad [2]$$

In Eq. 2, it has been shown explicitly that temperature, (T) is the variable of interest, as t is held constant for a given experiment. The PTIR analysis method is valid for initial-rate conditions, where the rate of degradation remains approximately constant. This is expected to hold for other degradation processes (e.g., chemical degradation) not tested here, as the principle of initial rates in reaction kinetics is more general than just the example shown here.³³

Results and Discussion

Differential Scanning Calorimetry (DSC)

DSC was performed as a qualitative and semi-quantitative measure of IgG1 thermal stability. It is only a surrogate for the true conformational stability (unfolding free energy), as unfolding in DSC was found to be irreversible and the absolute heat capacity (C_p) was convoluted by a combination of unfolding and aggregation. Figures 1 illustrate thermograms for AS-IgG1 and the corresponding Fc fragment at pH 4 (black), pH 5 (blue), and pH 6 (red) in 10 mM acetate. Previous work reported DSC thermograms for AS-IgG1 at the same pH and NaCl concentrations, but in 5 mM citrate buffer.¹⁴ In Fig. 1, profiles for conditions with 100 mM added NaCl are offset vertically to distinguish them from those with 0 mM added NaCl. The peaks of the Fc-IgG1 thermograms overlay with the smaller peaks or shoulders of the full IgG1 thermograms in each case. As expected based on previous reports,^{14,34,35} there are only two, relatively small, transitions for the Fc-IgG1 when compared with the full IgG1; the peak at lower (higher) temperature is assigned to the C_H2 (C_H3) domain of the Fc. For the full IgG1, the peak for the Fab domains overlaps with one or both peaks from the Fc domains, depending on the

solution pH. The DSC profiles are consistent with the pH dependent thermograms reported previously for a range of other IgG1 molecules.^{14,34,35}

Visual inspection of Figure 1 shows that increasing the value of the pH from 4 to 6 increases the temperatures for the calorimetric maxima (T_m^{app} values) for all of the peaks for the full IgG1 and for the Fc fragment. The addition of 100 mM NaCl decreases the T_m^{app} values in each case. At pH 4, the calorimetric transition for the C_H2 peak occurs at a significantly lower temperature than the Fab, and the IgG1 thermogram shows three distinguishable peaks. At low pH, previous reports concluded that unfolding of the C_H2 was a primary step in exposing aggregation-prone sequences for monoclonal antibodies.^{14,28,34} Recent results indicate that both the C_H2 domain and the Fab domain(s) within the same protein can contain highly-aggregation prone sequences that become exposed upon unfolding.³⁴

The results in Figure 1 at pH 6 (0 mM and 100 mM NaCl) and pH 5 with 100 mM NaCl display large exotherms (i.e., decreases in C_p , and ultimately exotherms) at higher temperatures. Previous work has shown that this is indicative of irreversible processes such as protein aggregation and precipitation when using this particular instrument configuration⁶. From these results in isolation, it is unclear for pH 5 and 6 whether unfolding of the C_H2 or the Fab region is most important with regards to promoting aggregation. However, prior work¹⁴ showed that Fab unfolding was primarily responsible for aggregation of AS-IgG1 at elevated temperatures and a similar range of pH values. Supporting Information provides the T_m^{app} values associated with Fig. 1.

Temperature-Dependent Aggregation Rates

The DSC thermograms guided the initial choices for incubation temperatures for accelerated aggregation rates. All incubation temperatures were selected to be below the DSC Fab peak temperatures for a given solution condition, based on the discussion above. Aggregation rates were determined using the PTIR method and quantitatively compared to those determined by canonical isothermal-rate experiments. Briefly, for the PTIR approach one sample was incubated for a set incubation time at a given temperature, and many temperatures were used in parallel. For the standard isothermal approach, multiple samples were held for a series of incubation times at a single temperature. After quenching to cold temperature to arrest aggregation, the aggregation rate or initial-rate coefficient (k_{obs}) value was calculated based on Eq. 1 or 2 (see Methods). Using a single sample at each temperature for the PTIR approach provides a “worst case” example, as one could easily supplement this with more than one time point for each temperature. The results below indicate that may not be necessary if one has sufficiently high-precision results with the assay of choice (e.g., SEC in the present case).

Figure 2 illustrates the results one obtains from the two different approaches. The standard isothermal monomer loss method (panel A) and the PTIR method (panels B and C) are explicitly illustrated using 1 mg/mL IgG1 in pH 5 buffer (5 mM citrate) with 100 mM added NaCl. Figure 2A shows isothermal monomer loss as a function of incubation time (t) for 325.5 K, 330 K, and 332 K. Visual inspection of Figure 2A illustrates that monomer loss is linear versus t over the experimental range tested ($m=1-0.8$). Previous work also showed linear kinetics during initial periods of aggregation¹⁰, which is expected based on general mass action kinetic arguments when the extent of reactant consumption is small.³² This an advantage, in that the initial-rate regime does not require one to know or assume the mechanism. However if one considers much larger extents of monomer loss ($m \ll 1$), the monomer loss profile is expected to

become non-linear, and accurately quantifying the net or observed rate coefficient for monomer loss (k_{obs}) requires one to determine or assume the underlying aggregation mechanism.^{10,11,36,37}

Figure 2B illustrates how aggregation rates are determined with the PTIR approach. The closed symbols show m as a function of incubation temperature for 2-hour (circles, diamonds, triangles and red squares,) and 24-hour (blue squares) incubation times. For the 2-hour experiments, three separate protein stocks were prepared and the experiment was repeated on separate days to provide a simple assessment of variability. Scatter in the data in Figure 2B-C illustrates typical error expected from PTIR approach. Additional details are included in Supporting Information. The value of m for each symbol in Figure 2B was converted to k_{obs} using Eq. 2, with the corresponding values of $\ln(k_{obs})$ given in Figure 2C. The PTIR approach used a single value of incubation time in the initial rate regime ($m = 1$ to approx. 0.8), therefore the choice of incubation time at lower temperatures were necessarily larger values to allow for slower aggregation rates at those conditions. The 24-hour time-scale experiments were chosen to extend the range of accessible k_{obs} values by at least an order of magnitude. 10-day incubations were also performed (data not shown in Figure 2), but in many cases significant fragmentation occurred, and this convoluted the interpretation and analysis to properly determine monomer loss rates for monoclonal antibodies (see below).^{6,38} No data are included in the Figures for temperature/solution conditions where appreciable fragmentation occurred.

The results in Figure 3 show a comparison of the values of k_{obs} versus inverse temperature (i.e., an Arrhenius diagram) for the different methods, and illustrate that the PTIR approach allows one to efficiently and reasonably accurately measure temperature-dependent aggregation rates across a range of temperatures. Each data set corresponds to a different solution condition with 5 mM sodium citrate buffer: pH 5, no added NaCl (diamonds); pH 5,

100 mM added NaCl (triangles); pH 6, no added NaCl (squares); and pH 6, 100 mM added NaCl (closed circles). Open symbols correspond to aggregation rates determined from traditional isothermal incubation such as in Figure 2A, while closed symbols are for the PTIR method such as illustrated in Figure 2B.

The aggregation rates from the PTIR approach are comparable in accuracy to those from the standard isothermal aggregation method that uses many samples at the same temperature, but the PTIR method provides rates for many more temperatures, with comparable consumption of protein material and user time. As noted above, the current results are a “worst case” example, in that only a single time point was used for a given temperature in the PTIR method. The method could easily be extended to use a small number of time points at a given temperature to provide even more robust values of k_{obs} vs. T .

An important use for the behavior of k_{obs} vs. T is to determine an accurate value of the effective activation energy (E_a) of aggregation, so that accelerated aggregation rates may be more effectively extrapolated to lower temperatures – e.g., for predicting room temperature shelf life.^{9–11} Intuitively, having k_{obs} values at more T values will allow one to regress E_a values with much better statistical confidence intervals and will provide greater utility for extrapolation of k_{obs} to lower temperatures. Supporting Information reports the fitted parameters and 95% confidence intervals from non-linear regression of the $k_{obs}(T)$ data sets from the traditional approach and PTIR approach. The data were regressed using the Arrhenius equation (Eq. 3), where k_{obs} is the experimentally determined value for reaction rate coefficient (units of inverse time), E_a is defined above, k_0 is value of k_{obs} at an arbitrarily chosen temperature, T_0 . In each case below, k_0 was a fitting parameter and T_0 was selected as 333.15 K because that is near the

median of all incubations temperatures. As expected, choosing different values for T_0 shifted the fitted value and confidence interval for k_0 , but not for E_a .

$$k_{obs} = k_0 \exp\left(\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right) \quad [3]$$

As anticipated based on the discussion above, the 95% confidence intervals for the E_a values from the PTIR data are much smaller than those from a traditional approach. Notably, current guidelines from regulatory agencies for accelerated stability tests for pharmaceutical products require even fewer than three incubation temperatures.^{9,10} Even with three temperature values, the fitted E_a values from the canonical isothermal approach are statistically insignificant (i.e., the error bars are comparable to E_a values), and therefore those E_a values are effectively worthless for extrapolating rates to lower temperatures (see Supporting Information). In contrast, the E_a values from the PTIR approach provide much improved precision and confidence intervals (see also, below). For the examples shown here, both the PTIR methods and the traditional method utilize comparable amounts of protein material and user time.

Although not shown in this report, in principle this PTIR approach can be extended to longer incubation times (multiple days to months) to yield results that may be predictive of rates at even lower temperatures. In the present case, aggregation rates were measured over reasonably small temperature windows (i.e., net change in rates on the order of 10^2) and therefore an Arrhenius equation is expected to be valid.^{9,11} However, when extrapolating rates over a broader range of time scales, non-Arrhenius behavior may become significant for non-native aggregation, as discussed elsewhere.¹¹ Therefore, the present approach is expected to provide quantitative rate data for a range of temperatures but should be adjusted to lower temperatures if one seeks to minimize such non-Arrhenius behavior in the case of protein aggregation.⁹

Effects of pH, buffer type, and [NaCl] on Temperature-Dependent Rates

$k_{obs}(T)$ was determined as a function of pH (4, 5, 6), added NaCl concentration (0 mM or 100 mM), and buffer species (citrate or acetate). Figure 4A illustrates an Arrhenius diagram based on the PTIR method for all solution conditions that were tested. Symbols shown in Figure 4A-4B correspond to pH 4 (black), pH 5 (red), pH 6 (blue), 0 mM NaCl (circles), 100 mM NaCl (triangles). The two different buffer conditions were chosen to assess specific-ion effects for commonly employed buffers for this pH range: 10 mM acetate buffer (open symbols); 5 mM citrate buffer (closed symbols). Previous work³⁹ showed qualitatively that changing buffer species could significantly alter aggregation rates, even at low buffer concentrations. Additionally, IgG1 formulated at pH 4, 10 mM acetate buffer with no added salt resulted in no aggregation (data not shown) even after heating at 85 °C for one hour.

From a practical perspective, visual inspection of Figure 4A shows that no single incubation temperature would be practical to achieve aggregation rates on a comparable timescale (hours to weeks) for all solution conditions. For example, if one selected a temperature to achieve a rate corresponding to $\ln(k_{obs}) = -7$ for solution conditions indicated with the closed black circles, then the rates for solution conditions depicted with closed black triangles would be so large as to be impractical to measure (and vertically far off-scale in Figure 4A). The PTIR approach enables one to more easily obtain $k_{obs}(T)$ profiles for head-to-head comparison between solution conditions or different proteins that would otherwise be untenable to quantitatively compare if one had to select a common temperature for measuring aggregation rates.

Starting with a qualitative analysis of the results in Figure 4, the shifts in $k_{obs}(T)$ with changing solution conditions correlate well with the trends for T_m^{app} values of the Fab domain from DSC in Table 1. Figure 4B rescales the Arrhenius diagram based on the Fab peak temperatures, and this collapses the k_{obs} values onto more of a common profile. This illustrates the importance of conformational stability in determining aggregation rates, in that k_{obs} is strongly influenced by how close the incubation temperature is to the T_m^{app} for unfolding of the domain involved in exposing aggregation-prone sequences of the protein, at least for temperatures corresponding to accelerated aggregation conditions.^{11,14}

However, differences in conformational stability cannot explain all of the trends in aggregation rates, as there are significant differences in the slopes (i.e., E_a values) over the different solution conditions. Figure 5 shows E_a values and 95% confidence intervals determined from fitting 2-hr PTIR data to Eq. 3 for formulation conditions prepared in 10 mM acetate buffer (panel A) and 5 mM citrate buffer (panel B). Formulations prepared without added NaCl are shown in red; those with 100 mM NaCl concentration are shown in blue. Notably, pH 4 with no added NaCl and 10 mM acetate had an unmeasureably large E_a value, as IgG1 heated in this formulation did not aggregate (as mentioned above). This is indicated with a question mark in Figure 5 for those solution conditions.

For solutions with 100 mM added NaCl, E_a increases with increasing pH, which is expected as conformational stability (i.e. T_m^{app}) increases with pH. Based on thermodynamic arguments and qualitative mechanistic arguments for non-native aggregation,^{11,40,41} larger T_m^{app} values imply increased unfolding enthalpy values, and therefore higher E_a values. However, E_a values at low ionic strength conditions (no added NaCl) for acetate buffer show the opposite behavior. That is, T_m^{app} values decrease as one decreases pH, but E_a values increase

substantially; in the extreme, at the lowest pH value tested there is no aggregation over multiple hours at temperatures close to boiling (i.e., effectively infinite E_a in the present context). This is despite the fact that the Fab T_m^{app} value (and all T_m^{app} values in Figure 2 for pH 4) are much lower than the selected incubation temperatures.

Previous work has highlighted increased conformational stability and decreased electrostatic protein-protein interactions for mAbs as the pH increases towards the pI of a protein¹². Prior work with AS-IgG1 indicated there are large electrostatic repulsions between proteins under these conditions, which presumably help to prevent monomers from coming into contact.³⁹ Figure 6A (reproduced from Barnett et al.³⁹) shows normalized Kirkwood-Buff integrals for protein-protein interactions (G_{22}^*) determined via static light scattering from pH 4, 5, and 6 in 10 mM acetate (red bars) and 5 mM citrate (blue bars). Kirkwood-Buff integral values are normalized to the steric contribution for a hard sphere with same hydrodynamic radius of as AS-IgG1 (5.1 nm)³⁹. Therefore, G_{22}^* values higher (lower) than one correspond to net repulsive (attractive) protein-protein interactions. In addition, Figure 6B plots T_m^{app} for AS-IgG1 thermal unfolding as a function of pH with no added salt. T_m^{app} values for acetate (red bars) are determined from Fig. 1, and values for citrate (blue bars) are reproduced from Kim et al.¹⁴

Across the pH range measured in the present case, one observes higher G_{22}^* values in 10 mM acetate compared to 5 mM citrate buffer. This result indicates AS-IgG1 net protein-protein interactions are more repulsive at a given pH in 10 mM acetate compared to 5 mM citrate. Similarly, T_m^{app} values are higher in acetate compared to citrate buffer, which suggests increased unfolding free energies for AS-IgG1 in acetate compared to citrate.^{42,43} If the hypothesis is correct that citrate ions are preferentially accumulated at the protein surface,³⁹ then this effect might result in reduced unfolding free energies if this caused the unfolded state chemical

potential be reduced, relative to that for the folded state. However, such effects are usually observed at much higher salt concentrations.⁴⁴ An alternative hypothesis is that preferential accumulation of citrate ions (compared to acetate) results in reduced repulsions between proteins, and this increases aggregation rates at a given temperature. Increased aggregation rates will also shift T_m^{app} values to lower temperatures.⁴³ It is not possible to distinguish conclusively between these two hypotheses based on data here.

The present results are only determined at one concentration of acetate or citrate buffer. Supporting Information shows preliminary static light scattering data and corresponding G_{22}^* values at pH 5 as a function of acetate and citrate concentrations. Those results show stronger net electrostatic protein-protein interactions for AS-IgG1 in acetate compared to citrate over a range of ionic strengths (~ 0-0.1 M). Prior work has also shown specific-ion-effects in protein-protein interactions and aggregation behavior for mAbs.^{39,45}

Interestingly, prior work showed that solutions with weaker protein-protein repulsions resulted in slower aggregation rates at a given temperature, because reduced electrostatic repulsions between proteins were more than offset by changes in the conformational stability of the protein molecules.^{8,12} Alternatively, in some cases it has been argued that electrostatic repulsions are the dominant factor for changing aggregation rates.^{25,46-48} However, the resulting activation barriers that are inferred from regressing colloidal models are often unphysically large.^{25,46} They also do not account for the importance of protein conformational changes that are needed to explain the stability of the resulting aggregates and the long time scales involved in nucleating such aggregates. Those questions notwithstanding, the results in Figure 3, Figure 6 and Supporting Information highlight that statistically precise E_a values are needed to be able to better assess different contributions to aggregation rates

In that context, Figures 5 and 6 highlight both conformational stability and protein-protein interactions play a discernable role in AS-IgG1 monomer loss aggregation rates. The effects of conformational stability are evident under essentially all conditions, while those for electrostatic repulsions are most prevalent under conditions of low ionic strength and high net charge on the protein. While these conditions are not typical of *in vivo* conditions for most proteins, they are relevant for proteins under manufacturing conditions for biotechnology products⁴⁹. Finally, high-precision density measurements have the potential to detect preferential interactions of solutes with proteins or other macromolecules.^{50,51} Preliminary results (not shown) indicate that such instrumentation might be effective to detect such interactions at high solute concentrations but is not sensitive enough to detect differences at the low concentrations of buffer ions here. The specific-ion effects observed here and in previous work³⁹ occur at ion concentrations much lower than those typically reported for so-called Hofmeister effects.^{44,52} Future work will focus on determining whether this behavior holds for a wider range of proteins and salts in the Hofmeister series at low salt concentrations.

Summary and Conclusions

A Parallel Temperatures Initial Rates (PTIR) device and method were introduced and validated against more traditional approaches. The PTIR approach was applied to determine IgG1 aggregation rates across a range of solution conditions that covered more than three orders of magnitude for the initial-rate coefficient (k_{obs}) of monomer loss. The results for AS-IgG1 aggregation highlight the importance of conformational stability, as well as electrostatic protein-protein interactions, for mediating k_{obs} and its effective activation energy. AS-IgG1 generally shows higher E_a , G_{22}^* , and T_m^{app} values in 10 mM acetate compared to 5 mM citrate. The results

are consistent with citrate being preferentially accumulated near the surface of AS-IgG1, compared to acetate. The net effects are decreases in apparent conformational stability, weaker electrostatic repulsions between proteins, and increased aggregation rates.

Acknowledgements

GVB and CJR gratefully acknowledge support from Amgen, the National Institute of Standards and Technology (NIST 70NANB12H239), and the National Science Foundation (CBET 0931173).

References

1. Walsh, G. Biopharmaceutical Benchmarks 2014. *Nat Biotechnol* 2014, 32 (10), 992–1000.
2. Aggarwal, S. R. What's Fueling the Biotech engine—2012 to 2013. *Nature* 2014, 201, 4.
3. Vázquez-Rey, M.; Lang, D. A. Aggregates in Monoclonal Antibody Manufacturing Processes. *Biotechnol Bioeng* 2011, 108 (7), 1494–1508.
4. Wang, W.; Singh, S. K.; Li, N.; Toler, M. R.; King, K. R.; Nema, S. Immunogenicity of Protein aggregates—Concerns and Realities. *Int J Pharm* 2012, 431 (1-2), 1–11.
5. Rosenberg, A. S. Effects of Protein Aggregates: An Immunologic Perspective. *AAPS J* 2006, 8 (3), E501–E507.
6. Roberts, C. J.; Nesta, D. P.; Kim, N. Effects of Temperature and Osmolytes on Competing Degradation Routes for an IgG1 Antibody: Competing Degradation Routes for an IgG1 Antibody. *J Pharm Sci* 2013, 102 (10), 3556–3566.
7. Hartl, F. U.; Hayer-Hartl, M. Converging Concepts of Protein Folding in Vitro and in Vivo. *Nat. Struct. Mol Biol* 2009, 16 (6), 574–581.
8. Roberts, C. J.; Das, T. K.; Sahin, E. Predicting Solution Aggregation Rates for Therapeutic Proteins: Approaches and Challenges. *Int J Pharm* 2011, 418 (2), 318–333.
9. Wang, W.; Roberts, C. J. Non-Arrhenius Protein Aggregation. *AAPS J* 2013, 15 (3), 840–851.
10. Brummitt, R. K.; Nesta, D. P.; Roberts, C. J. Predicting Accelerated Aggregation Rates for Monoclonal Antibody Formulations, and Challenges for Low-Temperature Predictions. *J Pharm Sci* 2011, 100 (10), 4234–4243.
11. Weiss, W. F.; Young, T. M.; Roberts, C. J. Principles, Approaches, and Challenges for Predicting Protein Aggregation Rates and Shelf Life. *J Pharm Sci* 2009, 98 (4), 1246–1277.
12. Sahin, E.; Weiss, W. F.; Kroetsch, A. M.; King, K. R.; Kessler, R. K.; Das, T. K.; Roberts, C. J. Aggregation and pH-Temperature Phase Behavior for Aggregates of an IgG2 Antibody. *J Pharm Sci* 2012, 101 (5), 1678–1687.
13. Hawe, A.; Wiggenhorn, M.; van de Weert, M.; Garbe, J. H. O.; Mahler, H.; Jiskoot, W. Forced Degradation of Therapeutic Proteins. *J Pharm Sci* 2012, 101 (3), 895–913.
14. Kim, N.; Remmele, R. L.; Liu, D.; Razinkov, V. I.; Fernandez, E. J.; Roberts, C. J. Aggregation of Anti-Streptavidin Immunoglobulin gamma- 1 Involves Fab Unfolding and

- Competing Growth Pathways Mediated by pH and Salt Concentration. *Biophys Chem* 2013, *172*, 26–36.
15. He, F.; Woods, C. E.; Becker, G. W.; Narhi, L. O.; Razinkov, V. I. High-Throughput Assessment of Thermal and Colloidal Stability Parameters for Monoclonal Antibody Formulations. *J Pharm Sci* 2011, *100* (12), 5126–5141.
 16. Goldberg, D. S.; Bishop, S. M.; Shah, A. U.; Sathish, H. A. Formulation Development of Therapeutic Monoclonal Antibodies Using High-Throughput Fluorescence and Static Light Scattering Techniques: Role of Conformational and Colloidal Stability. *J Pharm Sci* 2011, *100* (4), 1306–1315.
 17. Cheng, W.; Joshi, S. B.; He, F.; Brems, D. N.; He, B.; Kerwin, B. A.; Volkin, D. B.; Middaugh, C. R. Comparison of High-Throughput Biophysical Methods to Identify Stabilizing Excipients for a Model IgG2 Monoclonal Antibody: Conformational Stability and Kinetic Aggregation Measurements. *J Pharm Sci* 2012, *101* (5), 1701–1720.
 18. Bhambhani, A.; Kissmann, J. M.; Joshi, S. B.; Volkin, D. B.; Kashi, R. S.; Middaugh, C. R. Formulation Design and High-Throughput Excipient Selection Based on Structural Integrity and Conformational Stability of Dilute and Highly Concentrated IgG1 Monoclonal Antibody Solutions. *J Pharm Sci* 2012, *101* (3), 1120–1135.
 19. He, F.; Woods, C. E.; Becker, G. W.; Narhi, L. O.; Razinkov, V. I. High-Throughput Assessment of Thermal and Colloidal Stability Parameters for Monoclonal Antibody Formulations. *J Pharm Sci* 2011, *100* (12), 5126–5141.
 20. Ablinger, E.; Leitgeb, S.; Zimmer, A. Differential Scanning Fluorescence Approach Using a Fluorescent Molecular Rotor to Detect Thermostability of Proteins in Surfactant-Containing Formulations. *Int J Pharm* 2013, *441* (1-2), 255–260.
 21. Menzen, T.; Friess, W. High-Throughput Melting-Temperature Analysis of a Monoclonal Antibody by Differential Scanning Fluorimetry in the Presence of Surfactants. *J Pharm Sci* 2013, *102* (2), 415–428.
 22. Li, Y.; Ogunnaike, B. A.; Roberts, C. J. Multi-Variate Approach to Global Protein Aggregation Behavior and Kinetics: Effects of pH, NaCl, and Temperature for α -Chymotrypsinogen A. *J Pharm Sci* 2009, 645–662.

23. Drenski, M. F.; Brader, M. L.; Alston, R. W.; Reed, W. F. Monitoring Protein Aggregation Kinetics with Simultaneous Multiple Sample Light Scattering. *Analytical Biochem* 2013, *437* (2), 185–197.
24. Vlasak, J.; Ionescu, R. Fragmentation of Monoclonal Antibodies. *mAbs* 2011, *3* (3), 253–263.
25. Arosio, P.; Rima, S.; Lattuada, M.; Morbidelli, M. Population Balance Modeling of Antibodies Aggregation Kinetics. *J Phys Chem B* 2012, *116* (24), 7066–7075.
26. Yi, L.; Beckley, N.; Gikanga, B.; Zhang, J.; Wang, Y. J.; Chih, H.-W.; Sharma, V. K. Isomerization of Asp-Asp Motif in Model Peptides and a Monoclonal Antibody Fab Fragment. *J Pharm Sci* 2013, *102* (3), 947–959.
27. Banks, D. D.; Latypov, R. F.; Ketchem, R. R.; Woodard, J.; Scavezze, J. L.; Siska, C. C.; Razinkov, V. I. Native-State Solubility and Transfer Free Energy as Predictive Tools for Selecting Excipients to Include in Protein Formulation Development Studies. *J Pharm Sci* 2012, *101* (8), 2720–2732.
28. Hari, S. B.; Lau, H.; Razinkov, V. I.; Chen, S.; Latypov, R. F. Acid-Induced Aggregation of Human Monoclonal IgG1 and IgG2: Molecular Mechanism and the Effect of Solution Composition. *Biochemistry* 2010, *49* (43), 9328–9338.
29. Latypov, R. F.; Hogan, S.; Lau, H.; Gadgil, H.; Liu, D. Elucidation of Acid-Induced Unfolding and Aggregation of Human Immunoglobulin IgG1 and IgG2 Fc. *J Biol Chem* 2012, *287* (2), 1381–1396.
30. Mason, B. D.; Schöneich, C.; Kerwin, B. A. Effect of pH and Light on Aggregation and Conformation of an IgG1 mAb. *Mol Pharm* 2012, *9* (4), 774–790.
31. Brummitt, R. K.; Nesta, D. P.; Chang, L.; Chase, S. F.; Laue, T. M.; Roberts, C. J. Nonnative Aggregation of an IgG1 Antibody in Acidic Conditions: Part 1. Unfolding, Colloidal Interactions, and Formation of High-Molecular-Weight Aggregates. *J Pharm Sci* 2011, *100* (6), 2087–2103.
32. Laidler, K. J. *Chemical Kinetics*, 3 edition.; Prentice Hall: New York, 1987.
33. Rawlings, J. B.; Ekerdt, J. G. *Chemical Reactor Analysis and Design Fundamentals*, 2 edition.; Nob Hill Pub: Madison, Wisconsin, 2013.
34. Wu, H.; Kroe-Barrett, R.; Singh, S.; Robinson, A. S.; Roberts, C. J. Competing Aggregation Pathways for Monoclonal Antibodies. *FEBS Lett.* 2014, *588* (6), 936–941.

35. Ionescu, R. M.; Vlasak, J.; Price, C.; Kirchmeier, M. Contribution of Variable Domains to the Stability of Humanized IgG1 Monoclonal Antibodies. *J Pharm Sci* 2008, 97 (4), 1414–1426.
36. Li, Y.; Roberts, C. J. Lumry–Eyring Nucleated-Polymerization Model of Protein Aggregation Kinetics. 2. Competing Growth via Condensation and Chain Polymerization. *J Phys Chem B* 2009, 113 (19), 7020–7032.
37. Andrews, J. M.; Roberts, C. J. A Lumry–Eyring Nucleated Polymerization Model of Protein Aggregation Kinetics: 1. Aggregation with Pre-Equilibrated Unfolding. *J Phys Chem B* 2007, 111 (27), 7897–7913.
38. Wang, T.; Kumru, O. S.; Yi, L.; Wang, Y. J.; Zhang, J.; Kim, J. H.; Joshi, S. B.; Middaugh, C. R.; Volkin, D. B. Effect of Ionic Strength and pH on the Physical and Chemical Stability of a Monoclonal Antibody Antigen-Binding Fragment. *J Pharm Sci* 2013, 102 (8), 2520–2537.
39. Barnett, G. V.; Razinkov, V. I.; Kerwin, B. A.; Laue, T. M.; Woodka, A. H.; Butler, P. D.; Perevozchikova, T.; Roberts, C. J. Specific-Ion Effects on the Aggregation Mechanisms and Protein–Protein Interactions for Anti-Streptavidin Immunoglobulin Gamma-1. *J Phys Chem B* 2015, 119 (18), 5793–5804.
40. Roberts, C. J. Kinetics of Irreversible Protein Aggregation: Analysis of Extended Lumry–Eyring Models and Implications for Predicting Protein Shelf Life. *J Phys Chem B* 2003, 107 (5), 1194–1207.
41. Roberts, C. J.; Darrington, R. T.; Whitley, M. B. Irreversible Aggregation of Recombinant Bovine Granulocyte-Colony Stimulating Factor (bG-CSF) and Implications for Predicting Protein Shelf Life. *J Pharm Sci* 2003, 92 (5), 1095–1111.
42. Beckett, W. J.; Schellman, J. A. Protein Stability Curves. *Biopolymers* 1987, 26 (11), 1859–1877.
43. Sanchez-Ruiz, J. M. Theoretical Analysis of Lumry–Eyring Models in Differential Scanning Calorimetry. *Biophys J* 1992, 61 (4), 921.
44. Zhang-van Enk, J.; Mason, B. D.; Yu, L.; Zhang, L.; Hamouda, W.; Huang, G.; Liu, D.; Remmele, R. L.; Zhang, J. Perturbation of Thermal Unfolding and Aggregation of Human IgG1 Fc Fragment by Hofmeister Anions. *Mol Pharm* 2013, 10 (2), 619–630.

45. Roberts, D.; Keeling, R.; Tracka, M.; van der Walle, C. F.; Uddin, S.; Warwicker, J.; Curtis, R. Specific Ion and Buffer Effects on Protein–Protein Interactions of a Monoclonal Antibody. *Mol Pharm* 2015, *12* (1), 179–193.
46. Nicoud, L.; Arosio, P.; Sozo, M.; Yates, A.; Norrant, E.; Morbidelli, M. Kinetic Analysis of the Multistep Aggregation Mechanism of Monoclonal Antibodies. *J Phys Chem B* 2014, *118* (36), 10595–10606.
47. Saluja, A.; Fesinmeyer, R. M.; Hogan, S.; Brems, D. N.; Gokarn, Y. R. Diffusion and Sedimentation Interaction Parameters for Measuring the Second Virial Coefficient and Their Utility as Predictors of Protein Aggregation. *Biophys J* 2010, *99* (8), 2657–2665.
48. Chi, E. Y.; Krishnan, S.; Kendrick, B. S.; Chang, B. S.; Carpenter, J. F.; Randolph, T. W. Roles of Conformational Stability and Colloidal Stability in the Aggregation of Recombinant Human Granulocyte Colony-Stimulating Factor. *Protein Sci* 2003, *12* (5), 903–913.
49. Wang, W.; Roberts, C. J. *Aggregation of Therapeutic Proteins*; John Wiley & Sons, 2010.
50. Ben-Naim, A. *Statistical Thermodynamics for Chemists and Biochemists*, Softcover reprint of the original 1st ed. 1992 edition.; Springer: S.I., 2013.
51. Arakawa, T.; Timasheff, S. N. Preferential Interactions of Proteins with Salts in Concentrated Solutions. *Biochemistry (Mosc.)* 1982, *21* (25), 6545–6552.
52. Baldwin, R. L. How Hofmeister Ion Interactions Affect Protein Stability. *Biophys J* 1996, *71* (4), 2056–2063.

Figure Captions

Figure 1. DSC for AS-IgG1(solid) and Fc-IgG1 (dashed) formulated in 10 mM acetate buffer at pH 4 (black), pH 5 (blue), and pH 6 (red) with 0 mM or 100 mM NaCl added salt. Profile for AS-IgG1 with 100 mM NaCl are offset vertically for easier visualization. The double sided arrow provides a scale bar, with the length of the arrow equaling 100 kcal/mol.

Figure 2. Illustrative isothermal and PTIR results for determining IgG1 aggregation rates at pH 5, (5 mM citrate buffer), and with the addition of 100 mM NaCl. (A) Isothermal monomer loss versus time at 325.5 K (triangles), 330 K (circles), and 332 K (squares shown in the inset). (B) PTIR monomer loss at two hours (triangles, circles, diamonds, and red squares) or 24 hours (blue squares) as a function of incubation temperature. Multiple two hourr PTIR data sets were repeated to illustrate experiment-to-experiment variability. (C) $\ln(k_{obs})$ from PTIR data and Equation [2]. Symbols correspond to the closed symbol points at the same temperature in panel B. Error bars are smaller than the size of the symbols unless visible in either panel.

Figure 3. Arrhenius plot: natural logarithm of the aggregation rate plotted as a function of inverse temperature. Accelerated aggregation rates were determined using PTIR method for IgG1 at pH 5 and 0 mM NaCl concentration (closed diamonds), pH 5 and 100mM NaCl concentration (closed triangles), pH 6 and 0 mM NaCl concentration (closed squares), and pH 6 and 100 mM NaCl concentration (closed circles). Open symbols correspond to aggregation rates determined using traditional isothermal incubations.

Figure 5. (A) Arrhenius plot using PTIR for two hour and 24 hour incubations. (B) Arrhenius plot rescaling incubation temperature by the DSC peak temperatures for a given formulation.

Figure 5. IgG1 effective aggregation activation energy determined from PTIR data in Figure 4 for (A) 10 mM acetate (B) 5 mM citrate. Red bars are shown for no added NaCl and blue bars are shown for 100 mM NaCl concentration. The question mark indicates that E_a is unknown for that condition because aggregation was too slow to measure. Error bars are shown for 95% confidence intervals.

Figure 6. (A) AS-IgG1 G_{22}^* values in 10 mM acetate (red bars) and 5 mM citrate (blue bars). Values are reproduced from Barnett et al.³⁹ T_m^{app} values for AS-IgG1 unfolding in 10 mM acetate (red bars) and 5 mM citrate (blue bars). Values for citrate are reproduced from Kim et al.¹⁴

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6