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

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ABSTRACT

Controlling and predicting unwanted degradation, such as non-native aggregation, is a long-standing challenge for 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 rate method is used 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 versus citrate buffer. Parallel temperature initial rates are shown to agree with results from a traditional, isothermal method and permits direct comparison of the formulations across almost 3 orders of magnitude of aggregation rates. The apparent midpoint unfolding temperatures (through differential scanning calorimetry) and the effective activation energy values (E_a) are generally higher in acetate buffer compared with citrate buffer, which is consistent with preferential accumulation of citrate ions compared with 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 interactions in acetate compared with 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] mAbs are expected to be among the leading candidates for biologic drugs in the future, with >35 Food and Drug Administration–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] On heating or applying other stresses, proteins can lose higher order structure and biologic function. At temperatures significantly below the midpoint unfolding temperature(s), mAb unfolding/folding stages will be pre-equilibrated because they occur much more quickly than the rate-limiting steps for aggregation.[7] Although 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 are 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]

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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 or protein–protein interactions, whereas temperature changes can dramatically affect conformational stability.[10,12,14,15] Previous 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 (DSC), 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.[11,15]

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 semiquantitatively monitor aggregation.[10,19,20] 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 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.[6,21]

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.[21]

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 toward larger sized particles.[22] pH and ionic strength changes can alter aggregation mechanisms and produce large and heterogeneous aggregate populations that provide much larger scattering intensities compared with small-sized aggregates at an identical monomer loss rate. These challenges are compounded if fragmentation occurs, as is relatively common for mAbs[23-25] and other proteins.[21]

This report introduces a parallel temperature initial rate (PTIR) method to accurately and efficiently determine degradation rates as a function of temperature. PTIR is compared with 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.[14,26-29] Aggregation rates from accelerated (high temperature) to near-room temperature conditions are reported across multiple values of pH and NaCl concentration and different buffer species. The results not only 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 shown to be important 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.[14,30] 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

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

Differential Scanning Calorimetry

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°C to 90°C at a 1°C per minute 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 on a rescan. The absolute heat capacity was calculated from the buffer-subtracted DSC scans, as previously reported.[14,30]

Quantifying Aggregation Rates

IgG1 stock solutions were prepared at 1 mg/mL at a given pH, NaCl concentration, and buffer type, and aliquotted into hermetically sealed deactivated borosilicate glass high-performance liquid chromatography vials (Waters, Milford, MA). Isothermal incubations were performed by heating multiple samples in a water bath or custom-built PTIR device (see Supplementary Material) at a given temperature and removing samples at predetermined 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°C-23°C) before 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 earlier. Over approximately the first 10%-20% monomer loss, the rate of change of m remains nearly constant, and the observed rate law can be well described as zeroth order without the need to assume an underlying rate law.[31] The monomer fraction was regressed with Equation 1 to obtain the aggregation rate coefficient (units of