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Accelerated Aggregation Studies of Monoclonal Antibodies: Considerations for Storage Stability

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Abstract

Aggregation of monoclonal antibodies is a crucial concern with respect to their safety and efficacy. Among the various properties of protein aggregates, it is emerging that their size can potentially impact their immunogenicity. Therefore, stability studies of antibody formulations should not only evaluate the rate of monomer loss but also determine the size distribution of the protein aggregates, which, in turn, depends on the aggregation mechanisms.

Here, we study the aggregation behavior of different formulations of two monoclonal immunoglobulins (IgGs) in the temperature range from 5°C to 50°C over 52 weeks of storage. We show that the aggregation kinetics of both antibodies follow non-Arrhenius behavior and that the aggregation mechanisms change between 40°C and 5°C, leading to different types of aggregates. Specifically, for a given monomer conversion, dimer formation dominates at low temperatures, while larger aggregates are formed at higher temperatures. We further show that the stability ranking of different molecules as well as of different formulations is drastically different at 40°C and 5°C while it correlates better between 30°C and 5°C.

Our findings have implications for the level of information provided by accelerated aggregation studies with respect to protein stability under storage conditions.

Introduction

In the last decades, therapeutic proteins like monoclonal antibodies (mAbs) have become an increasingly important class of pharmaceuticals [1,2]. With respect to small molecule drugs, the complexity of their macromolecular structure poses greater challenges in terms of chemical and physical stability, which must be carefully guaranteed throughout storage and delivery for drug safety and efficacy. Among the various possible instabilities, formation of protein aggregates can be a problem in the development of safe formulations, since proteinaceous particles have been associated with an increased risk of immunogenicity [3–6]. Such adverse immune responses can induce serious complications and even fatalities, and, in some cases, may lead to the retraction of biopharmaceuticals post-marketing [7,8].

The characterization and inhibition of protein aggregation is therefore a key topic in biopharmaceuticals development. John F. Carpenter and Theodore W. Randolph have been pioneers in recognizing the importance of this problem [9–11]. Starting from a first publication on the lyophilization of hemoglobin in 1996 [12], their work has tremendously contributed to the understanding of the sources and mechanisms of protein aggregation during production [13], formulation [14,15], shipment and storage [16,17], as well as handling by medical personnel and patients [5,18].

Together with advances in experimental characterization, this progress led to a fundamental understanding of the aggregation process at the molecular level [11,19,20]. It has now become clear that protein aggregation is a complex multi-step process, which involves a variety of possible microscopic reactions including protein conformational changes, nucleation and growth of the protein particles [21–26]. Different aggregation mechanisms lead to different types of aggregates and therefore therapeutic proteins may form a broad variety of soluble and insoluble particles, which can be reversible or irreversible [27–29].

The term “aggregation” should therefore not be used in a generic way, in particular in connection to possible consequences such as immunogenicity [4,30]. For instance, a recent study [31] suggests that mAb aggregates in the range of 100-1000 nm could be more problematic for immunogenicity with respect to smaller or larger species. In other reports, micrometer-sized aggregates were found to be more immunogenic [32,33]. Moreover, the

extent of chemical modifications of the primary structure within the aggregates can be another important factor [34–36]. The development of safe mAb formulations should therefore focus not only on the rate of monomer loss but also on the size distribution of the protein aggregates that are formed, which, in turn, requires understanding the aggregation mechanisms at the molecular level. This operation is very challenging, since the set of microscopic steps composing the aggregation reaction network is highly specific to molecule and solution conditions.

Development of new therapeutic mAbs commonly relies on forced degradation and accelerated stability studies performed at higher temperatures relative to storage conditions. These studies are aimed, respectively, at generating degradation products and at obtaining information about formulation stability within a shorter time frame [37–39]. However, the individual elementary reactions involved in the aggregation process typically exhibit different dependencies on temperature. Moreover, increasing temperature might not only raise the total fraction of partially unfolded monomers but also change their conformations, which probably exhibit different aggregation propensities. Consequently, extrapolating the aggregation mechanism to low temperature from forced degradation/accelerated stability studies is a challenging operation [40–43].

In addition, the identification of the relevant microscopic steps in the aggregation mechanism requires the acquisition of a large amount of high quality, time-resolved data. This operation is severely complicated at low temperatures due to the slow aggregation kinetics. Indeed, there are only few reports in literature presenting kinetic data for the aggregation of mAbs under refrigerated conditions [41,44–47]. This observation underlines the need for further research on the temperature-dependence of mAb aggregation in the temperature range that is relevant for standard stability testing and product storage.

Here, we investigated the aggregation behavior of two monoclonal antibodies at 5°C, 30°C, 40°C, and 50°C over 52 weeks of incubation. For both molecules, we studied formulations at 10 and 100 mg/mL mAb at five different pH values in the absence and presence of 150 mM sodium chloride. We show that the aggregation mechanism of those mAbs changes as a function of temperature. Consequently, the same degree of monomer loss at 5°C and 40°C corresponds to different protein aggregate populations. In particular, dimer formation dominates at low temperatures. This result demonstrates that a comprehensive picture of protein stability cannot rely on measurement of residual monomer only. In

addition, we show that the ranking of the stability of different molecules as well as the optimal formulations that minimize aggregation are different at 5°C compared to 40°C.

Materials and Methods

Materials

mAb-1 and mAb-2 were, respectively, an IgG1 with experimental pI of 8.2 and an IgG4 with experimental pI of 7.6. Sodium chloride, tris(hydroxymethyl)aminomethane (Tris) and sodium acetate anhydrous were purchased from Sigma-Aldrich, St. Louis, MO, USA. L-Histidine was purchased from Merck KGaA, Darmstadt, Germany. All chemicals were of analytical grade. Ultrapure water was prepared using a Milli-Q system (Merck MilliPore, Billerica, MA, USA).

Sample Preparation

Tris-HCl and L-Histidine-HCl buffers were prepared by dissolving the appropriate amount of buffer component in ultrapure water followed by pH adjustment through addition of 1 M HCl (Merck KGaA, Darmstadt, Germany). Acetate buffer was prepared by mixing appropriate volumes of 200 mM sodium acetate and 200 mM acetic acid solutions to obtain the desired pH. The identical buffers containing 3 M sodium chloride were prepared in parallel. All buffers were filtered using a Stericup® vacuum filtration system (Merck KGaA, Darmstadt, Germany) and stored in the refrigerator before use.

Buffer exchange of the mAb starting material was performed using Vivaflow 50 cross flow cassettes (Sartorius Stedim Biotech GmbH, Goettingen, Germany) equipped with 30 kDa cut-off PES membrane. The final permeate volume was approximately five times the volume of the mAb solution to ensure proper buffer exchange. The cassettes were cooled in a water bath during the entire procedure. The buffer-exchanged mAb solutions were concentrated to approximately 105 mg/mL after transfer into Amicon® Ultra 15 centrifugal filter tubes (Merck KGaA, Darmstadt, HE, Germany) equipped with a 30 kDa PES membrane. The concentrated solutions were sterile filtered using Steriflip-GP 0.22 µm sterile centrifuge tube top filter units (Merck KGaA, Darmstadt, HE, Germany) and stored under refrigerated conditions prior to further use. mAb concentration of the solutions was determined by UV absorption at 280 nm on a SoloVPE variable path length UV-Vis spectrophotometer (C technologies Inc., Bridgewater, NJ, USA). A specific extinction

coefficient $\hat{\varepsilon}$ of 1.58 mL mg^{-1} cm^{-1} and 1.34 mL mg^{-1} cm^{-1} was used for mAb-1 and mAb-2, respectively.

Stability Studies

The aggregation kinetics of the different mAb formulations was investigated during isothermal incubation at 5°C, 30°C, 40°C and 50°C using climate chambers. Different formulations were considered by varying pH and sodium chloride concentration. More specifically, pH was selected relative to each mAb's isoelectric point and set equal to 0, 1, 2, 3 and 4 units below the pI. For each pH value, one formulation without excipient and one with 150 mM sodium chloride was prepared. Next, 200 µL of formulation were placed in HPLC vials with 300 µL fixed inserts (Thermo Scientific, Langerwehe, Germany). The vials had been sterilized in a Laboklav 80-V (Detzel Schloss, Germany) autoclave prior to use. Those operations were performed inside a Herasafe HS 15 (Thermo Scientific™, Waltham, MA, USA) laminar flow cabinet. In regular time intervals, vials were withdrawn from the incubators and the samples were analyzed by SEC-MALS and DLS to determine the mass fraction of aggregates, as well as to determine average molecular weight and hydrodynamic size of the aggregate population.

Size-Exclusion Chromatography Coupled to Multi-Angle Light Scattering

Chromatographic analyses of the mAb formulations were run on an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) system. Prior to injection, samples were kept at 5°C within the autosampler of the unit. A Wyatt WTC-030S5 analytical size-exclusion chromatography (SEC) column (7.8x300 mm, 5 µm, 300 Å) was used in combination with a Wyatt WTC-030S5G guard column to separate the different species based on their hydrodynamic size. For analysis, 250 µg of protein were injected and eluted at 1 mL/min with 100 mM sodium phosphate, 200 mM sodium sulfate at pH 7.0 as mobile phase. Chromatograms were recorded in terms UV absorption at 280 nm. Further, the chromatography system was coupled to a DAWN HELEOS multi-angle light scattering (MALS) detector (Wyatt Technologies, Santa Barbara, CA, USA) to determine the molecular weight of the eluting species. The UV chromatograms were analyzed with the Empower 3 software (Waters, Milford, MA, USA) and the light scattering data with the Astra 6.1.7.17 software (Wyatt, Santa Barbara, CA, USA).

Results and Discussion

The Aggregation Rate Shows Non-Arrhenius Behavior

We focused our attention on the effect of temperature on the initial stages of the aggregation process, corresponding to less than 10% monomer conversion, since typically only few percent of monomeric mAb are converted into aggregates during the storage of commercial products [46]. The aggregation rate was determined from the mass fraction of the aggregates measured via size exclusion chromatography at the different time points. The total mass fraction of aggregates w_{Agg} at a generic incubation time t was computed using the following formula:

$$w_{\mathrm{Agg}}(t) = \frac{A_{\mathrm{Agg}}(t)}{A_{\mathrm{Tot}}(t)} + \frac{A_{\mathrm{Tot}}(0) - A_{\mathrm{Tot}}(t)}{A_{\mathrm{Tot}}(0)}.$$

Here, A_{Agg} denotes the combined peak area of all species eluting earlier than the monomer. A_{Tot} represents the total area under the curve and $A_{\mathrm{Tot}}(0)$ is the initial value. This formula also accounts for the potential formation of insoluble aggregates, which would lead to incomplete mass recovery in SEC analysis. Such mass loss, however, was observed only for a very few formulations at higher temperatures. The initial rate of aggregate formation $\left(\mathrm{d}w_{\mathrm{Agg}}/\mathrm{d}t\right)_{t\to 0}$ was determined by linear regression in the range $w_{\mathrm{Agg}}(t) \in \left[w_{\mathrm{Agg}}(0), w_{\mathrm{Agg}}(0) + 0.1\right]$ as shown in Figure 1. Finally, the initial aggregation rate r_0 was obtained by multiplying $\left(\mathrm{d}w_{\mathrm{Agg}}/\mathrm{d}t\right)_{t\to 0}$ by the total protein concentration c_0.

Figure 2 presents the influence of temperature on the initial aggregation rate for 100 mg/mL mAb concentration while the data at 10 mg/mL are reported in supplementary Figure S1. For all formulations, the initial aggregation rate showed non-linear dependence on temperature according to the Arrhenius framework. This result is in agreement with previous reports for other mAbs [41,44,46]. The deviation from Arrhenius-behavior was more pronounced for formulations at lower pH, which might be attributed to the lower melting temperature of the mAbs under those conditions.

Our results are not surprising, since for large proteins like mAbs Arrhenius-behavior is usually observed only over narrow temperature ranges [48]. Moreover, for mAbs this temperate range is typically above 45°C, which significantly exceeds the temperature

window relevant for formulation development and storage [49–51]. In agreement with our results, a bend in the Arrhenius plot is typically observed between 30°C and 40°C, which challenges the predictions of stability at low temperatures based on data generated under thermal stress.

The Apparent Reaction Order Increases with Decreasing Temperature

The possibility to measure the time evolution of the mass loss allows us to estimate the apparent reaction order ν, which is an important parameter that quantifies the dependence of the aggregation rate on the monomer concentration $[M]$ and is indicative of the dominant microscopic aggregation mechanism. We determined ν by analyzing the dependence of the initial aggregation rate r_0 on the initial monomer concentration $[M]_0$:

$$r_0 = \lim_{t \to 0} \left(\frac{\mathrm{d}[M]}{\mathrm{d}t} \right) = -k_{\mathrm{obs}} [M]_0^{\nu}. \qquad (1)$$

Here, k_{obs} denotes the observed rate constant of the aggregation rate. This expression can be linearized as

$$\ln(r_0) = \ln(k_{\mathrm{obs}}) + \nu \ln([M]_0), \qquad (2)$$

which can be applied to estimate ν from the slope of the linear plot $\ln(r_0)$ against $\ln([M]_0)$. In Figure 3, we show the dependence of ν on temperature. For both molecules and all formulation conditions, the reaction order changed as a function of temperature. Specifically, ν increased from values closer to one towards values close to two with decreasing temperature. This important result indicates that temperature affects both the overall aggregation rate (see Figure 2) and the aggregation mechanism (see Figure 3), thereby leading not only to different amounts but also to different types of protein aggregates.

We note that at 5°C data could only be acquired for mAb-1 formulations without excipient, while for all other formulations the aggregation rate at lower mAb concentration was too slow to robustly estimate the reaction order. Moreover, formation of insoluble aggregates was observed in a few formulations at 50°C (see supplementary Table S1), which could explain the increase of ν between 40°C and 50°C in those cases [23,52]. Finally, the kinetic data for $\mathrm{pI} - \mathrm{pH} = 4$ with sodium chloride was too noisy for proper analysis.

Lowering Temperature Arrests Growth and Coagulation of mAb Aggregates

The apparent reaction order conveys important information about the underlying microscopic aggregation mechanism, since the individual elementary steps exhibit different reaction orders with respect to the monomer concentration. For instance, dimer nucleation is second order, whereas addition of monomers to pre-existing aggregates is first order in terms of monomer concentration [24,53]. The apparent order will be a convolution of the two contributions and ν is expected to assume values closer to two, when dimer nucleation becomes the dominating aggregation route. Thus, the observed ν of two at lower temperatures suggests that aggregate growth by monomer addition is negligible for those conditions.

We verified this prediction by measuring the time evolution of the size of the aggregates at different temperatures, reported as the weight-average number of mAb monomers per aggregate (see Figure 4). Indeed, at 5°C and 30°C, the size of the aggregates remained constant over time and equal to $n_{\mathrm{w}}^{\mathrm{Agg}} \approx 2$ indicating that the aggregate population at low temperatures consisted of dimers that did not grow over time. In contrast, at higher temperatures (i.e. 40°C and 50°C), the size of the aggregates rapidly increased for both mAbs within few weeks, both in the absence and presence of sodium chloride as excipient.

An important consequence of this behavior is that the same extent of monomer loss can lead to different aggregate size distributions, which could potentially be associated with different risks of immunogenicity [31,54]. To illustrate this concept, in Figure 5 we plot the average aggregate size $n_{\mathrm{w}}^{\mathrm{Agg}}$ against the monomer conversion $1 - m$ for the different temperatures (with $m = [M]/[M]_0$ representing the fraction of unreacted monomer). At any given conversion, $n_{\mathrm{w}}^{\mathrm{Agg}}$ increased as function of temperature, clearly indicating that aggregate growth becomes more important relative to the nucleation of new dimers at higher temperatures.

Aggregation under Storage Conditions Correlates Poorly with Forced Degradation Studies at 40°C

Overall, the results discussed above indicate that higher temperature not only increases the overall aggregation rate but also changes the relative contribution of the different microscopic steps composing the aggregation reaction network, which has deep implications for formulation design. In general, different excipients will have different effects on the individual microscopic steps of the aggregation process. For instance, sugars strongly impact protein conformational changes, while salts typically affect nucleation and growth events by modulating electrostatic interactions between protein molecules. Consequently, a formulation chosen based on forced degradation studies at 40°C may not represent the optimal formulation for storage at refrigerated temperatures of 2-8°C.

To illustrate this point, we plotted the aggregation rate of both molecules at 5°C against the rate at 40°C for all investigated formulations (see Figure 6), which differ in terms of pH values and sodium chloride concentration. The two aggregation rates correlated poorly (Pearson correlation coefficient of $R = -0.01$), in particular for mAb-2 and for typical pH values of commercial formulation (orange symbols in Figure 6B).

Moreover, it is worth noting that most formulations of mAb-2 showed slower aggregation compared to formulations of mAb-1 at 5°C, while this trend was much less pronounced at 40°C, as highlighted in Figure 6B. In fact, the Spearman rank correlation coefficient ρ for the two aggregation rates is equal to 0.26. Thus, temperature cannot only change the ranking of different formulations of the same molecule but also the overall relative stability of formulations of several candidates. This observation underlines the inherent limitations of thermal stress as a mean to accelerate protein aggregation and obtain information about stability under conditions that are relevant for long-term storage [55,56].

These limitations are strongly associated with the interplay between protein conformational stability and aggregation propensity. The outcome of forced degradation studies at 40°C could be influenced by the thermal stability of the mAb conformation, while this biophysical parameter might have little relevance for stability at lower temperatures. Since antibodies generally possess differing melting temperatures, the same increase in temperature may induce different conformational changes in different molecules. In our study, the midpoint temperature of the first transition $T_{\mathrm{m},1}$ measured by differential scanning

calorimetry (DSC) was lower for the majority of the formulations of mAb-2 compared to those of mAb-1, i.e. an overall average of 62°C relative to one of 68°C (see supplementary Figure S2). Thus, at 40°C mAb-2 may undergo larger conformational changes compared to mAb-1. In contrast, those structural changes could be much less relevant at storage temperatures, which are far from the $T_{m,1}$ of both mAbs. This observation is confirmed by the additional analysis of the correlation between the aggregation rate at 5°C and 40°C for the two mAb separately, which is presented in Supplementary Figure S5. Individually, mAb-1 (which exhibits higher $T_{m,1}$) showed better correlation relative to mAb-2. For mAb-2, the correlation was very poor, even excluding the outlier in Figure 6A. Moreover, the correlation was better for formulations with pH close to or at the mAbs' isoelectric point, which is of limited practical relevance, since antibodies are normally formulated at pH values lower than the pI (see Supplementary Figure S5). In agreement with these considerations, we observed that the correlation between the kinetics of aggregation and the ranking of different formulations was much better between 5°C and 30°C (see Figure 7).

The interplay between thermal stability and aggregation is probably also responsible for the observed reversal in the ranking of the aggregation rates as a function of formulation pH between high and low temperatures: At 50°C, the aggregation rate increased with decreasing pH value (see Figure 2 (B)-(D) and 6). The exception were formulations of mAb-1 without salt, where the formulation at $\mathrm{pH} = \mathrm{pI}$ was the least stable at 50°C. In contrast, at 5°C, lower pH corresponded to slower aggregation in most cases. Decreasing pH simultaneously reduces the conformational stability (i.e. lower T_m) of the mAbs and increases their colloidal stability by increasing electrostatic repulsion between antibody molecules. At low temperature, the latter stabilizing effect appears to dominate, while at high temperature the abundance of unfolded species and attractive interactions among them become more important.

The role of conformational stability at different temperatures is also relevant in the context of the different microscopic aggregation mechanisms observed in forced degradation studies and under storage conditions, as summarized in Figure 8. Under thermal stress, mAb aggregates increase their size either by growth via monomer addition or through aggregate-aggregate coagulation. In contrast, at refrigerated temperatures at comparable monomer conversion only dimers are observed.

The growth of dimers into oligomers requires the establishment of a link between one of the monomers engaged in the dimer and a third mAb monomer. Probably, the most flexible domain of a mAb will be the one involved in the establishment of the initial bond within the dimer. Thus, formation of additional bonds with other mAb molecules will require flexibility also in other parts of the mAb. Increasing temperature may render other sections of the mAb structure also available for inter-molecular contacts, therefore increasing the likelihood of growth and coagulation of aggregates.

Overall, our results suggest that thermal stress also accelerates aggregation pathways that are not relevant for storage conditions, leading to the formation of an aggregate population that may not be representative of the aggregates formed over the shelf life of antibody drugs. Although these findings require further validation with a larger number of molecules and formulation conditions, these results suggest that under storage conditions dimer formation is an important process. Future directions should therefore focus on the characterization of mAb dimers, which can be very different in terms of both intermolecular linkages (i.e. covalent vs. non-covalent) and IgG domains involved in the connections [57–60]. In this context, it is important to combine modeling activities with advances in analytical methods. The characterization of dimers and other aggregate end products involves a variety of orthogonal techniques [61–63] and can benefit from emerging approaches based on microfluidic technology [64,65]. We refer to recent reviews and reports for discussion about the strengths and shortcomings of these techniques [66–68].

Finally, the design of accelerated stability studies would benefit from the establishment of rules (possibly based on T_m) to determine the temperature range in which the predominant aggregation mechanism corresponds to the pathway under storage conditions.

Conclusion

We investigated the aggregation kinetics of two monoclonal antibodies in the temperature range from 5°C to 50°C to access the window relevant for drug product storage as well as accelerated stability and forced degradation studies. Our results highlight several challenges for shelf life predictions based on thermal stress studies. First, the aggregation rate of mAb formulations shows non-Arrhenius behavior as a function of temperature. Secondly, changes in temperature also affect the underlying aggregation mechanism and the

corresponding reaction order. Specifically, aggregation at 5°C and 30°C is dominated by dimerization, while at the same monomer conversion larger aggregates are formed at higher temperatures. Consequently, the same extent of monomer loss leads to the formation of different aggregate populations, which may potentially be associated with different risks of immunogenicity.

Moreover, our results show that there is very poor correlation between the stability ranking of different molecules as well as of different formulations under storage conditions and under thermal stress at 40°C, in particular under typical pH values of commercial formulation.

Overall, our results demonstrate that, in addition to the rate of monomer loss, characterizing the microscopic aggregation mechanisms and the end aggregate products is crucial to design suitable accelerated stability studies.

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Figure Legend

Figure 1: Measurement of the initial aggregation rate. The plot shows the time evolution of the total mass fraction of aggregates for 100 mg/mL mAb-1 at $\mathrm{pH} = \mathrm{pI}$ and 30°C. The black line represents a linear regression analysis of the data to determine $\left(\mathrm{d}w_{\mathrm{Agg}}/\mathrm{d}t\right)_{t\to 0}$.

Figure 2: Arrhenius plot of the initial aggregation rate at 100 mg/mL mAb concentration and $\mathrm{pH} = \mathrm{pI}$ (blue squares), $\mathrm{pI} - \mathrm{pH} = 1$ (light blue circles), $\mathrm{pI} - \mathrm{pH} = 2$ (yellow upward triangles), $\mathrm{pI} - \mathrm{pH} = 3$ (orange downward triangles), and $\mathrm{pI} - \mathrm{pH} = 4$ (red diamonds). (A) mAb-1 without excipient, (B) mAb-2 without excipient, (C) mAb-1 with 150 mM sodium chloride, (D) mAb-2 with 150 mM sodium chloride.

Figure 3: Temperature dependence of the apparent reaction order ν of the initial aggregation rate with respect to the monomer concentration. $pI = pH$ (blue squares), $pI - pH = 1$ (light blue circles), $pI - pH = 2$ (yellow upward triangles), $pI - pH = 3$ (orange downward triangles), and $pI - pH = 4$ (red diamonds). (A) mAb-1 without excipient, (B) mAb-2 without excipient, (C) mAb-1 with 150 mM sodium chloride, (D) mAb-2 with 150 mM sodium chloride.

Figure 4: Time evolution of the weight-average number of mAb monomers per aggregate at 100 g/L mAb and $pI - pH = 2$ for 5°C (blue squares), 30°C (yellow circles), 40°C (orange upward triangles), and 50°C (red downward triangles). (A) mAb-1 without sodium chloride, (B) mAb-2 without sodium chloride, (C) mAb-1 with 150 mM sodium chloride, (D) mAb-2 with 150 mM sodium chloride.

Figure 5: Weight-average number of mAb monomers per aggregate as function of monomer conversion for $\mathrm{pI} - \mathrm{pH} = 2$ at 5°C (blue squares), 30°C (yellow circles), 40°C (orange upward triangles), and 50°C (red downward triangles). (A) mAb-1 without excipient, (B) mAb-2 without excipient, (C) mAb-1 with 150 mM sodium chloride, (D) mAb-2 with 150 mM sodium chloride. Lines are guidelines to the eye.

Figure 6: Correlation between stability under storage conditions and in accelerated studies. (A) Relationship between the aggregation rate at 5°C and at 40°C. (B) Plot of the stability rank of different formulations in terms of aggregation rate at 5°C versus 40°C. Data corresponds to 100 mg/mL formulations of mAb-1 (squares) and mAb-2 (circles) at $\mathrm{pH} = \mathrm{pI}$ (blue), $\mathrm{pI} - \mathrm{pH} = 1$ (light blue), $\mathrm{pI} - \mathrm{pH} = 2$ (yellow), $\mathrm{pI} - \mathrm{pH} = 3$ (orange), and $\mathrm{pI} - \mathrm{pH} = 4$ (red). Full symbols correspond to formulations without excipient and half-filled symbols to those with 150 mM sodium chloride.

Figure 7: Correlation between stability under storage conditions and in accelerated studies. (A) Relationship between the aggregation rate at 5°C and 30°C. (B) Plot of the stability rank of different formulations in terms of aggregation rate at 5°C versus 30°C. The symbols represent the same conditions as in Figure 6.

Figure 8: Schematic illustration highlighting the differences in mAb aggregation under thermal stress and under storage conditions. (A) At high temperature, the size of mAb aggregates increases due to growth by monomer addition and aggregate-aggregate coagulation. In contrast, the aggregation process arrests after dimer formation at lower temperatures. (B) Consequently, the same extent of monomer conversion leads to aggregate populations characterized by drastically different size distributions, with many, small oligomers formed at low temperature and fewer, large aggregates generated at high temperature.