The use of native cation-exchange chromatography to study aggregation and phase separation of monoclonal antibodies

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Abstract: This study introduces a novel analytical approach for studying aggregation and phase separation of monoclonal antibodies (mAbs). The approach is based on using analytical scale cation-exchange chromatography (CEX) for measuring the loss of soluble monomer in the case of individual and mixed protein solutions. Native CEX outperforms traditional size-exclusion chromatography in separating complex protein mixtures, offering an easy way to assess mAb aggregation propensity. Different IgG1 and IgG2 molecules were tested individually and in mixtures consisting of up to four protein molecules. Antibody aggregation was induced by four different stress factors: high temperature, low pH, addition of fatty acids, and rigorous agitation. The extent of aggregation was determined from the amount of monomeric protein remaining in solution after stress. Consequently, it was possible to address the role of specific mAb regions in antibody aggregation by co-incubating Fab and Fc fragments with their respective full-length molecules. Our results revealed that the relative contribution of Fab and Fc regions in mAb aggregation is strongly dependent on pH and the stress factor applied. In addition, the CEX-based approach was used to study reversible protein precipitation due to phase separation, which demonstrated its use for a broader range of protein–protein association phenomena. In all cases, the role of Fab and Fc was clearly dissected, providing important information for engineering more stable mAb-based therapeutics.

Keywords: antibody aggregation; antibody phase separation; monoclonal antibody; Fab and Fc fragments; limited proteolysis; cation-exchange chromatography

Abbreviations: AUC, analytical ultracentrifugation; CD, circular dichroism; CEX, cation-exchange chromatography; CEX52, cation-exchange chromatography at pH 5.2; DSC, differential scanning calorimetry; Fab, fragment antigen-binding; Fc, fragment crystallizable; FFF, field-flow fractionation; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; mAb, monoclonal antibody; pI, isoelectric point; SEC, size-exclusion chromatography; UV Abs, absorbance in the UV region.

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Introduction

Aggregation of monoclonal antibodies (mAbs) is an unwanted but frequently observed degradation process that poses challenges for biopharmaceutical development.^{1–4} Protein aggregation is generally described as a complex, multi-stage process involving unfolding or misfolding of free monomers followed by one or more assembly steps to form soluble or insoluble oligomers.^{5,6} Throughout the manufacturing process, protein therapeutics may be exposed to various stress conditions, and understanding the

effects of these factors on protein aggregation is important.

Gaining a detailed, molecular-level understanding of IgG1 and IgG2 aggregation is analytically challenging and time consuming due to the large size of antibodies (~ 150 kDa). Therapeutic mAbs are multi-domain and multi-chain glycosylated proteins, which exhibit significant heterogeneity in structure and stability.^{7–10} Specifically, the Fab and Fc regions of mAbs bear different functionality or surface properties and are expected to respond differently to various stress conditions. Therefore, these regions may contribute differently to protein stability, aggregation, and phase separation. Dissecting the role of these regions in aggregation and other degradation processes is critical for engineering more stable mAb-based therapeutics.

A large number of analytical techniques are currently available for measuring protein aggregation. These techniques can be categorized as direct or indirect assays depending on whether the aggregated protein is physically separated from the native (monomeric) protein.⁴ Spectroscopy-based (indirect) techniques (CD, fluorescence, derivative UV, IR spectroscopy, etc.) probe changes in protein structure and correlate them with aggregation. In these cases, protein aggregation is inferred from the relative spectral changes, rendering these techniques less adequate for quantifying aggregation. In contrast, separation-based methods (SEC, FFF, AUC, etc.) offer a more direct way to quantify and characterize aggregates. Many are potentially high throughput (e.g., SEC and FFF) and can provide information on the rates of aggregation and the size of protein aggregates. However, even these techniques may be insufficient for understanding the underlying mechanisms of antibody aggregation.

In this work, we have attempted to assess the impact of different stress conditions (high temperature, low pH, presence of fatty acids, and agitation) on the aggregation propensity of several IgG1 and IgG2 molecules. When applied with sufficient intensity, all of these stress factors promote massive antibody aggregation, which may or may not result in protein precipitation. Given that the stress conditions are well optimized, the extent of protein aggregation should differentiate less stable mAbs from their more stable counterparts. This idea may also be applicable to protein formulation development involving the optimization of pH and excipients to minimize protein aggregation. In this report, we introduce a chromatography-based approach for investigating mAb aggregation by measuring the loss of native soluble protein. This approach is justified based on the fact that denaturation of IgG1 and IgG2 molecules is highly irreversible.¹¹ It has also been established that denatured or misfolded mAbs aggregate quickly,¹¹ often

with no detectable accumulation of soluble (oligomeric) species.^{12–14}

Previously, we demonstrated the use of native cation-exchange chromatography (CEX) in solving analytical problems associated with charge heterogeneity, covalent modifications, and dimerization in mAbs.^{15,16} Here, we continue to exploit the separation capability of CEX to analyze mixtures of full-length mAbs and their respective Fab and Fc fragments. To the best of our knowledge, this is the first account illustrating the use of CEX as a primary tool for investigating mAb aggregation. As a result, we were able to reveal previously unknown aspects of antibody aggregation by assessing the relative contributions of Fab and Fc to mAb solution stability. In addition, a highly reversible mAb phase separation (precipitation) process has been investigated, demonstrating the potential of CEX for studying a wider range of protein–protein association phenomena.

Results and Discussion

Measurement of protein aggregation by CEX52

Proteins may exhibit a high propensity to aggregate when subjected to elevated temperatures, rigorous agitation, extreme pH variations, or fatty acid precipitation.^{17–23} The aggregation reactions may proceed to a considerable extent and result in massive protein aggregation and precipitation. In the present work, we demonstrate that this leads to a measurable loss of soluble monomer (or native protein population), which can be monitored by a non-denaturing chromatographic method such as CEX52.¹⁵ Among the stress factors tested, only acid-induced aggregation resulted in the accumulation of soluble aggregates which eluted from the column at high salt concentrations (see below). Other stress factors promoted complete and irreversible precipitation of denatured protein molecules. The resulting aggregates were removed by centrifugation before CEX52 analysis. Aggregates that did not precipitate upon centrifugation were effectively eliminated by the use of a CEX guard column (see Materials and Methods). This allowed straightforward quantification of the remaining monomeric protein in the supernatant. A reduction in peak area after stress treatment served as a surrogate measure of total protein aggregation/precipitation. Relative changes in the chromatographic peak area for different antibodies served as a ranking tool to identify proteins with stability issues against a particular stress factor.

Analytical CEX is a quick and robust chromatographic method capable of resolving complex protein mixtures. Even minor *pI* differences, charge distribution variations, or structural changes may be sufficient to separate proteins.^{15,16} A typical CEX52 chromatogram of an antibody mixture consisting of

Figure 1. Comparative analysis of a four-mAb mixture by (A) cation-exchange chromatography at pH 5.2 and (B) size-exclusion chromatography at pH 6.8. The antibody mixture contained IgG1-A, IgG1-B, IgG2-B, and IgG2-C.

IgG1-A, IgG1-B, IgG2-B, and IgG2-C is shown in Figure 1(A). It is evident that CEX52 provides complete baseline separation of the different mAbs. In contrast, analytical SEC is unable to resolve the same mixture because of insufficient size differences between the proteins [see Fig. 1(B)]. Therefore, the resolving power of CEX52 cannot be matched by any conventional SEC method that is routinely used for monitoring protein aggregation. Furthermore, the composition and pH of the mobile phases in CEX52 are mild and do not induce protein denaturation during analysis, as previously demonstrated for a noncovalent IgG1-B dimer.¹⁵

Temperature-induced mAb aggregation

Samples containing individual or mixed antibodies (IgG1-A, IgG2-A, and IgG2-B) were incubated at 70°C for 10 min without agitation. This treatment was sufficient to induce protein precipitation in 20 mM sodium phosphate (pH 6.8). The samples were then briefly centrifuged, and their supernatants analyzed by CEX52 (see Materials and Methods for details). The data revealed dramatic differences in the protein resistance against aggregation. IgG1-A appeared to be the most stable as its soluble monomeric fraction (represented by the chromatographic

peak area) remained constant before and after heat treatment [Fig. 2(A)]. IgG2-A, on the other hand, was found to be the least stable because its

Figure 2. Temperature-induced mAb aggregation in 20 mM sodium phosphate (pH 6.8). Individual and mixed mAb solutions were incubated at 70°C for 10 min without agitation. Control (25°C) and stressed (70°C) samples are shown in black and red, respectively. CEX52 data for individual mAbs are shown for IgG1-A (A), IgG2-A (B), and IgG2-B (C). CEX52 data for a mixture containing IgG1-A, IgG2-A, and IgG2-B are shown in (D).

Figure 3. DSC thermograms in 20 mM sodium phosphate (pH 6.8). A: DSC results for IgG1-A (solid line), IgG2-A (dashed line), and IgG2-B (dotted line). B: DSC results for intact IgG1-B (solid line) and its Fab (dashed line) and Fc (dashed and dotted line) fragments. The thermograms correspond to equimolar protein concentrations.

concentration in solution dropped by $\sim 90\%$ [Fig. 2(B)]. In contrast, $\sim 50\%$ of IgG2-B remained in solution, indicating that this molecule had an intermediate stability among the three mAbs tested [Fig. 2(C)]. Incubation at 80 °C or at higher temperatures resulted in massive precipitation of all three mAbs and was, therefore, less informative for differentiating their stability (data not shown). Temperatures up to 60 °C, however, induced no protein precipitation within the short, 10-min, incubation window presumably due to the low level of protein denaturation. Indeed, DSC results generated under the same experimental conditions showed no evidence of protein denaturation up to 65 °C [see Fig. 3(A)]. These data were in agreement with the fact that below the unfolding temperature, unfolding (denaturation) is the rate-determining step for protein aggregation.¹¹ Importantly, no new CEX peaks were observed after the temperature incubations, indicating that denatured antibodies quickly assembled into large aggregates, and were removed from the soluble (native) protein. Regarding the formation of small soluble oligomers, these species were expected to have distinct elution times compared to monomeric pro-

teins.¹⁵ It was evident from the chromatographic data that such species did not populate to any significant degree.

Our CEX52 results for the IgG1-A, IgG2-A, and IgG2-B mixture were surprisingly similar to the individual mAb data, suggesting little interference between the molecules [cf. Fig. 2(A–C) with 2(D)]. The relative peak areas in various mixtures are compared with the individual protein data in Figure 4, which demonstrates that co-incubation of different

Figure 4. Relative percentage (%) of soluble (A) IgG1-A, (B) IgG2-A, and (C) IgG2-B remaining in the supernatant after a 70 °C heat treatment. The bar graphs correspond to temperature-induced aggregation experiments in 20 mM sodium phosphate (pH 6.8) supplemented with 0M, 0.1M, or 1.0M NaCl. The various mAb combinations are shaded differently; the experimental errors have not been determined.

Table I. DSC-Based and CEX52-Based Data on mAb Denaturation and Precipitation

Protein	Melting temperature (°C)	Precipitation temperature (°C)
IgG1-A	80.0 (Fab)	~ 80.0
IgG1-B	76.7 (Fab)	~ 75.0
IgG1-B Fab	78.8	~ 75.0
IgG1-B Fc C _{H2}	71.7	n/a
IgG1-B Fc C _{H3}	82.9	n/a
IgG2-A	72.3 (Fab)	~ 70.0
IgG2-B	73.7 (Fab)	~ 75.0

mAbs only leads to minor changes. The aggregation tendency of IgG2-A was similar in the presence of more stable mAbs (IgG1-A and IgG2-B), and IgG2-A did not significantly alter the stability of the other two antibodies. A similar trend was observed for IgG1-A and IgG2-B. The relative propensity of these three mAbs to aggregate (IgG2-A > IgG2-B > IgG1-A) remained the same in the case of both individual and mixed protein solutions.

DSC results were generally consistent with this ranking order of protein aggregation, especially with respect to Fab denaturation [Fig. 3(A) and Table I]. The DSC profiles shown in Figure 3(A) are characterized by the presence of a single major endothermic peak and one or two minor peaks. As follows from comparative analysis of the full-length IgG1-B and its Fab and Fc fragments [see Fig. 3(B) and Table I], the major peak originates from Fab denaturation. In contrast, the Fc fragment exhibits two minor cooperative transitions that partly overlap with the Fab melting transition and are due to the independent denaturation of C_{H2} and C_{H3} domains.²⁴ The extent of this overlap is known to be protein specific and highly dependent on the pH of the solution.¹¹ Generally, melting of the C_{H2} domain occurs at lower temperatures compared to that of the C_{H3} domain.²⁴ IgG2-A provides an example where the Fab is thermodynamically less stable and denatures within the same temperature range as the C_{H2} domain. Unlike IgG2-A, the Fab portion of IgG1-A is more stable than the C_{H2} and melts along with the C_{H3} region. Overall, IgG2-A Fab melts at a lower temperature than that of IgG2-B (cf. 72.3°C and 73.7°C in Table I, respectively), whereas denaturation of IgG1-A Fab (80.0°C, Table I) is shifted to much higher temperatures compared to both IgG2-A and IgG2-B. This correlates with the mAb aggregation propensities at 70°C as measured by CEX52 and serves as unequivocal evidence that heat-induced antibody aggregation requires protein denaturation. The leading role of Fab instability in this process is also corroborated by the fact that the lowest temperature of mAb precipitation (the lowest temperature at which the protein solution becomes turbid) falls within the range of Fab denaturation by DSC (Table I).

Another practically important but poorly understood aspect is the effect of solution ionic strength on the extent and specificity of mAb aggregation. It is frequently observed that mAbs form more aggregates and visible particulates (insoluble aggregates) in salt-based formulations.¹² It has been recognized that ion–protein binding can modulate the charge properties of proteins and promote aggregation and self-association.^{25–28} Assuming that salt has a significant effect on protein stability, an increase or decrease in the extent of mAb aggregation as a function of salt concentration can be expected. To investigate the effect of salt on antibody aggregation, 70°C heat treatment was applied to protein solutions supplemented with 0.1M and 1.0M NaCl. Interestingly, we found that the aggregation propensities of the mAbs did not change appreciably with increasing salt concentrations (Fig. 4). This result demonstrated that heat-induced mAb aggregation was not controlled by the ionic strength of the solvent. Therefore, we conclude that compared to the role of protein denaturation, the effect of protein–protein charge interactions (repulsion or attraction) is small.

Identification of aggregation-prone regions

Identification of aggregation-prone regions is important for determining antibody aggregation mechanisms. This may be achieved by experimenting with native Fab and Fc fragments under the same stress conditions used for intact mAbs. Fab and Fc fragments are easily produced from IgG1 molecules via Lys-C or papain digestion.^{15,29} However, limited proteolysis of IgG2 molecules is much more difficult and requires optimization. The protocol shown in the Materials and Methods section was derived from systematic experimentation on IgG2-A aimed at maximizing the yield of native Fab and Fc fragments. Yet, complete digestion was not achieved even after 20 h of papain treatment, which necessitated a subsequent semi-preparative CEX fractionation [see Fig. 5(A) and Materials and Methods]. Purified Fab and Fc fragments were verified by mass spectrometry (data not shown). Their stability and aggregation were then studied as a function of 10-min incubations at 60, 65, 70, and 75°C [see Fig. 5(B,C)] and compared to the results for the full-length molecule [in Fig. 5(D)].

Intact IgG2-A was shown to be unstable at 70°C [Fig. 2(B)], and a similar observation was made for its Fab fragment [Fig. 5(B)]. In contrast, the Fc portion was more resistant to high temperature and aggregated only at 75°C [Fig. 5(C)]. Data for % soluble protein, summarized in Figure 5(D), shows that temperature-induced aggregation of the full-length mAb correlated well with Fab precipitation. Therefore, Fab instability was proven to be the main driver behind IgG2-A aggregation under the experimental conditions.

Figure 5. Identification of aggregation-prone regions in temperature-induced mAb aggregation. A: CEX52 separation of papain-digested IgG2-A, illustrating the incomplete digestion of the molecule into constituent Fab and Fc fragments. B: Temperature dependence of IgG2-A Fab precipitation. C: Temperature dependence of IgG2-A Fc precipitation. D: Temperature-dependent aggregation of intact IgG2-A and its Fab and Fc fragments.

Table II. Relative % Soluble IgG1-B Fab and Fc After a 5-min Co-Incubation at Indicated Temperatures

IgG1-B fragments	37°C	70°C	80°C	85°C
Fab	~ 100%	100.0%	12.2%	0.5%
Fc	~ 100%	100.0%	43.8%	6.7%

The antibody was quantitatively digested by Lys-C to produce native Fab and Fc fragments.

Similar observations were made in an independent experiment on IgG1-B subjected to limited Lys-C digestion. In this case, the protein sample was fully digested into Fab and Fc fragments and required no further purification. Also, co-incubation of both fragments ensured that they experienced identical conditions. The fragments were incubated at temperatures spanning the entire DSC profile of the molecule [Fig. 3(B)]. Only the temperature increase from 70 to 80°C induced rapid loss of soluble fragments, and the Fc portion was clearly more resistant to aggregation (see Table II). Once again, the stability of Fab resembled that of the full-length mAb and was, therefore, primarily responsible for its temperature-induced aggregation.

The examples above provided justification for the use of Fab and Fc fragments in the identification of aggregation-prone regions in mAbs. Indeed, it has been shown previously that the Fab and Fc regions denature independently even within the context of the full-length molecule.^{11,24} Although the IgG1-B Fab appeared to be thermodynamically more stable when isolated [by ~ 2°C, see Table I and Fig. 3(B)], the magnitude of the difference did not invalidate our conclusions. Once again, comparison of the DSC and CEX52 data showed the expected correlation between protein denaturation and heat-induced aggregation. Incubation at 70°C was optimal for elucidating the relative role of different protein domains, whereas heating at 80°C and above caused severe structural damage to the protein, resulting in massive precipitation.

Another significant observation made from these experiments is related to the role of domain unfolding in Fab and Fc aggregation. Assuming that denaturation of the C_H2 domain is responsible for IgG1-B aggregation, the aggregation propensity of Fc should be higher than that of Fab but similar to the intact mAb [Table I and Fig. 3(B)]. However, this is not the case. The Fab fragment actually shows higher aggregation propensity despite being more structurally stable by DSC. Thus, while it may be useful to know the order of melting transitions in antibodies, this information alone is not sufficient for understanding and predicting mAb aggregation propensity.

Agitation-induced mAb aggregation

Agitation is one of the most frequently used stress factors in mAb candidate selection and formulation

Figure 6. Agitation-induced mAb aggregation in 20 mM sodium phosphate (pH 6.8). A mixture of IgG1-A, IgG2-A, and IgG2-B was rigorously agitated at ambient temperature for up to 48 h. A: CEX52 chromatograms corresponding to different time points during agitation. The species eluting at ~ 28 min is contained in the IgG2-A drug substance material. The minor peaks eluting at ~ 36 min are components of the IgG1-A drug substance material. B: Real-time agitation-induced aggregation of the IgG1-A (triangles), IgG2-A (squares), and IgG2-B (circles) mixture.

development.^{12–14,30,31} Agitation of protein solutions can cause formation of insoluble aggregates and particles which contribute to increased turbidity.¹⁴ A mAb mixture (IgG1-A, IgG2-A, and IgG2-B) was used to establish whether mechanisms of agitation-induced aggregation differ from those of temperature-induced aggregation. The protein mixture was subjected to rigorous agitation at ambient temperature in 20 mM sodium phosphate, pH 6.8. To monitor the aggregation process, small sample aliquots were taken for CEX52 analysis at different time points throughout a 48-h study [Fig. 6(A)]. The corresponding time-dependent loss of native peak area is shown in Figure 6(B). The most unstable protein was found to be IgG2-A, which almost completely precipitated within the first day of agitation. In contrast, IgG1-A and IgG2-B were more stable and showed very similar aggregation kinetics. Their respective final concentration in solution was $\sim 60\%$ even after 2 days of continuous agitation. These

results provided the following rank order for mAb aggregation propensity: IgG2-A > IgG2-B = IgG1-A. This order was different from that obtained in the high temperature aggregation experiments (see above) and demonstrated that agitation-induced aggregation invoked different mechanisms. Unexpectedly, the most thermodynamically stable mAb, IgG1-A, appeared to be as susceptible to agitation-induced aggregation as the moderately stable IgG2-B. This result was inconsistent with the DSC data [Fig. 3(A)] and not dependent on the antibody subclass (IgG1 or IgG2). In fact, our ongoing investigation revealed a discrepancy between the DSC and agitation-induced aggregation data for a number of antibody molecules (a more detailed analysis of the data will be presented elsewhere). Similar observations were recently made by Thirumangalathu *et al.*³⁰

The importance of protein colloidal stability and pI was also probed, but we did not see a good correlation between these two parameters. Our agitation experiments were performed at a pH matching the overall pI of IgG2-B (6.8, see Materials and Methods), yet the molecule proved to be as resistant to aggregation as IgG1-A ($pI = 8.9$). The role of pI in IgG2-A aggregation was likely insignificant too since its overall pI (8.8) was similar to that of IgG1-A. Although it has been shown that colloidal stability is an important factor in modulating mAb aggregation,^{23,30} our data suggested that denaturation may be more important than protein net charge.

In another study, the effect of milder agitation conditions was investigated in 20 mM sodium phosphate (pH 6.8) as a function of temperature (4°C vs. 25°C) and salt concentration (0–1M NaCl). A complete set of individual and mixed protein solutions was prepared, shielded from light, and slowly inverted for up to 6 days. In none of the cases was protein precipitation observed indicating that the massive aggregation seen in the previous experiment resulted from excessive agitation. In both experiments, the air–liquid interface was present, yet this interfacial area alone was insufficient to drive aggregation upon milder agitation. We do not dispute the importance of the air–liquid interface in protein instability^{32,33} but argue that the main factor determining protein aggregation in our experiments was the concentration of non-native species in solution. The observed disparity between the mild and rigorous agitation experiments can be explained by reversible changes in protein structure. Solutions with low concentrations of non-native molecules will not exhibit aggregation if diffusion-limited association of these species is fully negated by refolding. This helps to re-evaluate a hypothesis that agitation-induced protein aggregation predominantly occurs at the air–water interface. Assuming that protein denaturation and aggregation are limited to

the interfacial area, even mild agitation should result in a slow but steady loss of native monomer. However, this is not the case, and our results are more consistent with a diffusion-limited aggregation mechanism irrespective of whether protein denaturation occurs in solution or at the air–liquid interface. Thus, in contradiction with previous contentions, the diffusion-limited character of the data identifies the bulk solution as the main aggregation medium. This also agrees with the fact that stirring can induce more aggregation than shaking, even in the complete absence of headspace.³⁴ It is worth mentioning, however, that aggregation in the presence of the interfacial area must be influenced by protein adsorption, which determines the surface coverage. It was shown previously that mAbs differ significantly in their surface affinity depending on overall hydrophobicity and *pI*.³⁵ Some of these factors may be responsible for the lack of correlation between the DSC data and the rank order of aggregation from agitation experiments (see above). In addition, consistent aggregation levels in the presence and absence of NaCl provide evidence that at neutral pH mAb stability is not significantly modulated by the ionic strength of the solvent (we reached a similar conclusion with respect to temperature-induced aggregation). With respect to IgG2-B, this finding effectively means that even a combination of pH = *pI* and high salt concentrations is insufficient for promoting rapid aggregation in the absence of protein denaturation.

Milder agitation conditions were also used to examine the possibility of seeding or accelerating antibody aggregation by addition of preformed aggregates. Insoluble antibody aggregates of IgG1-A and IgG1-B (see details of aggregate preparation in the Materials and Methods) were spiked into a mAb mixture containing the same molecules (in 20 mM sodium phosphate, pH 6.8), and the mixture was gently inverted over a period of 2 days at 25°C. CEX52 analysis of the resulting supernatant did not show any depletion of soluble protein and, therefore, any recruitment of mAbs into the aggregates (data not shown). The absence of any effect from preformed aggregates was consistent with the low concentration of soluble non-native species (due to insufficient denaturation) in such experiments.

The fact that protein aggregation requires more rigorous agitation raises a question as to the level of agitation needed. Whereas rigorous agitation should help in eliminating the least stable mAbs, it appears to be overly disruptive for differentiating more stable molecules. A similar concern was raised previously by Kiese *et al.*³⁴ with respect to selecting the optimal type and duration of a mechanical stress. Results from a more recent systematic investigation by Eppler *et al.*¹⁴ demonstrate the possibility of fine tuning the agitation stress to produce practically relevant data.

Acid-induced mAb aggregation

Exposure of therapeutic mAbs to low pH is often unavoidable for purification and viral inactivation purposes.^{36,37} Antibodies may be exposed to pH as low as 3.5 for a few hours at ambient temperature, which can affect stability of the protein structure and induce aggregation.^{36,38} In particular, the C_H2 domains of Fc are likely to be sufficiently destabilized or unfolded to initiate the aggregation process.^{11,24}

Aggregation of IgG1-A, IgG2-A, and IgG2-B was measured in 100 mM sodium acetate, pH 3.5. Unlike the high temperature and rigorous agitation experiments, all protein solutions remained free of visible protein precipitate for up to 48 h of static storage. Nevertheless, there was a significant decrease or even a complete loss in native CEX peak area for IgG2-A and IgG2-B at 4°C and 25°C (Fig. 7). At the same time, a new peak emerged at ~ 41 min, corresponding to a fraction of soluble aggregates which elute at higher salt concentrations. In contrast, IgG1-A showed greater resistance to aggregation, revealing higher stability of IgG1s compared to IgG2s under acidic conditions.

The low pH samples experienced a sudden increase in pH upon injection onto the CEX52 column, possibly triggering protein refolding. However, no evidence of on-column refolding was seen, suggesting that any refolding that may have occurred was insignificant. The experiments provided the following rank order for acid-induced mAb aggregation: IgG2-A = IgG2-B > IgG1-A. This order was different from that observed in our heat and rigorous agitation experiments at pH 6.8 (see above) and reflected pH-dependent changes in mAb aggregation mechanisms. Taking into account that the Fc domains of IgG2-A and IgG2-B are identical, the fact that IgG2-B showed similar stability compared to IgG2-A demonstrated the importance of Fc instability in antibody aggregation at pH 3.5 (a more detailed analysis of the low pH antibody aggregation will be presented elsewhere).

Minimizing low pH aggregation in mAb candidates is highly desirable and requires selection processes that are based on aggregation resistance of both native and acid-denatured states.³⁸ Both of these aspects can be investigated by applying CEX52 to measure the recovery of native antibodies after low pH treatment and neutralization.

Fatty acid-induced mAb aggregation

Precipitation of proteins by short-chain fatty acids is a recognized method for protein recovery, viral inactivation, and plasma protein precipitation.^{17–19,39} However, the effect of fatty acids in promoting protein aggregation and precipitation is not fully understood. At low concentrations (40–60 mM), these acids induce the formation of insoluble complexes composed of protein, water, and a fatty acid.^{17,40}

Figure 7. Acid-induced mAb aggregation in 100 mM sodium acetate (pH 3.5). Individual and mixed mAb solutions were incubated at 4°C and 25°C for up to 48 h without agitation. Control samples (black) were prepared in 20 mM sodium phosphate (pH 6.8) and incubated at 4°C. Acid-treated samples are shown in blue and red for 4°C and 25°C, respectively. CEX52 results after a 48-h incubation of individual IgG1-A (A), IgG2-A (B), and IgG2-B (C) are shown. Corresponding data for a mixture containing IgG1-A, IgG2-A, and IgG2-B are shown in (D).

Figure 8. Fatty acid-induced mAb aggregation. A mixture of IgG1-A, IgG2-A, and IgG2-A Fab and Fc fragments was subjected to precipitation with caprylic acid (red) and heptanoic acid (black) and analyzed by CEX52. The control without fatty acids is shown in blue. The species eluting at ~ 28 min is contained in the IgG2-A drug substance material. The minor peaks eluting at ~ 36 min are components of the IgG1-A drug substance material. Note the close correspondence between the loss of intact IgG2-A and its Fc fragment.

Depending on the protein, the complexes may contain irreversibly denatured and aggregated protein molecules⁴¹ or reversibly precipitated proteins that retain biological activity.⁴⁰

An insight into fatty acid mAb precipitation was gained by combining IgG1-A with intact IgG2-A and IgG2-A Fab and Fc fragments. First, we found that heptanoic acid was more effective in precipitating mAbs than caprylic acid when both were at roughly similar molar concentrations (see Materials and Methods and black and red traces in Fig. 8). Second, IgG1-A was resistant to caprylic acid, whereas IgG2-A was readily precipitated by both. This was consistent with our observations that IgG1-A is generally more resistant to aggregation than IgG2-A (see above). With respect to the antibody fragments, Fc precipitation closely mirrored the behavior of intact IgG2-A (Fig. 8), suggesting that the precipitation was driven primarily by the Fc region. This result demonstrated the importance of specific protein regions (i.e., Fc) and the antibody subclass (IgG1 *vs.* IgG2) in the fatty acid precipitation process.

Denaturation and aggregation of mAbs

The underlying mechanisms of protein aggregation ultimately dictate the extent, reversibility, specificity, and kinetics of the aggregation process. IgG1 and IgG2 antibodies show complex aggregation behavior because they are composed of domains with distinct surface properties and unequal stabilities. As mAb aggregation can be induced by a variety of stresses, the resulting aggregates may form via different mechanisms and have unique structural properties. Indeed, Hawe *et al.*⁴² showed that heat- and

freeze-thaw-induced mAb aggregates differed significantly in their physicochemical characteristics. Such structural differences likely arise from a combination of stress-dependent (domain-specific) protein denaturation processes and details of intermolecular association. However, only a few published cases have highlighted the role of instability in specific antibody domains.^{12,43,44}

As follows from our results, forced mAb aggregation is a largely irreversible process irrespective of the stress factor applied. Often, but not always, the least stable domain controls the aggregation process, and some of the data generated here implicate Fc. Although this may hold true for aggregation experiments where conditions are moderately to strongly acidic (i.e., pH ~ 5 and below), the situation changes as pH approaches neutral values. In such cases, the role of Fab denaturation becomes as important as, if not more prominent than, Fc instability. This highlights one issue with deriving mAb aggregation propensities from simplified thermodynamic predictions based on DSC or other biophysical data. It mainly stems from the fact that typical IgG1 and IgG2 mAbs are composed of one Fc and two Fab arms. Moderate instability in Fab regions may have great impact due to their higher effective (local) concentration. Consequently, the presence of a less stable Fc may not necessarily mean that changes in this region will drive protein aggregation. As it was pointed out previously, the rate and extent of the aggregation process are ultimately determined by the concentration of non-native protein conformations.^{45–48} Therefore, regions that are structurally perturbed and present at higher concentrations will likely have greater contributions toward antibody aggregation. Another practical complication stems from the fact that agitation-induced aggregation shows little correlation with thermodynamic stability and may even show an inverse dependence on protein concentration (see our results above, as well as Fesinmeyer *et al.*,¹² Thirumangalathu *et al.*,³⁰ and Treuheit *et al.*⁴⁹). However, agitation stress is used frequently in mAb candidate selection and formulation development.¹⁴ Therefore, it is necessary to learn more about the capabilities of agitation-based approaches for predicting real-time storage stability of protein therapeutics. Fortunately, forced aggregation can be modulated to an extent which allows the ranking of mAbs based on aggregation propensity rather than thermodynamic predictions. By using protein mixtures and the CEX52 method, this can be done with relative ease and without consuming much material (~ 100–200 µg).

Phase separation of mAbs

Concentrated protein formulations have become a desirable outcome of formulation development to reduce injection volumes and increase therapeutic

dosing. This adds to the number of examples where protein–protein interactions influence macroscopic properties of the system such as viscosity, opalescence, gelation, and so forth.^{25,27,50} Many cases exist that illustrate the tendency of mAbs to participate in these phenomena.^{3,51}

In contrast to the largely irreversible non-native aggregation discussed above, interactions involved in phase separation tend to be weak, highly reversible, and transient in nature. Their investigation requires different approaches, one of which is based on studying the protein partitioning between different phases. As mAbs are large proteins consisting of independently folded regions, their native Fab and Fc fragments can provide useful information on the phase separation process.

Acid-treated and neutralized Protein A-purified material of IgG2-A (see Materials and Methods) was found to particulate heavily upon refrigerated storage. Short-term incubation of the resulting suspension at room temperature was sufficient to dissolve the particles, but subsequent refrigeration induced new particle formation characteristic of a reversible liquid–solid phase separation (precipitation) process. Its temperature dependence and highly reproducible nature were recognized and duly used in our phase separation partitioning experiments. Initially, we established that the molecules which formed the precipitate represented a more homogeneous fraction of the protein [Fig. 9(A)]. This reflected the high selectivity of the phase separation process, apparently resembling that of protein crystallization.⁵² Native Fab and Fc fragments of IgG2-A were then introduced at low concentrations to the room temperature IgG2-A material, and the resulting mixture was allowed to cool. Following 2 days of storage on ice, the suspension was centrifuged at 0°C to collect the precipitate. The supernatant was quickly removed and transferred into another tube, while the pellet (~ 1/20 of the total sample volume) was allowed to dissolve at room temperature. Figure 9(B) compares CEX52 data for the resulting supernatant and pellet from two different samples supplemented with either Fab or Fc. The distribution of Fc was found to be identical between the supernatant and corresponding precipitate, indicating that this region of IgG2-A did not participate in particle formation (cf. red and black traces). In contrast, Fab was enriched in the precipitate fraction compared to the supernatant (cf. green and blue traces), revealing its preferential partitioning into the solid state. These results were sufficient to conclude that reversible precipitation of IgG2-A was driven by weak interactions involving the Fab portion of the molecule. Although the exact nature of these interactions is currently unknown, this finding illustrated the importance of Fab regions in mAb phase separation, corroborating a number of recent reports.^{25,27,28}

Figure 9. Phase separation of mAbs. A: CEX52 analysis of acid-treated and neutralized Protein A-purified IgG2-A from the precipitate (solid line) and supernatant (dashed line). As protein concentration in the precipitate was ~ 6 times higher than in the supernatant (94.1 ± 1.6 mg/mL vs. 16.2 ± 0.8 mg/mL), the data for the supernatant was normalized with respect to the pellet. Note the differences in homogeneity of precipitated IgG2-A compared to its soluble fraction. B: Protein partitioning experiment on acid-treated and neutralized Protein A-purified IgG2-A. Native IgG2-A Fab and Fc fragments were added to IgG2-A at low quantities before sample refrigeration (see text). Illustrated are four CEX52 traces corresponding to supernatant (blue) and pellet (green) formed in the presence of Fab, and supernatant (black) and pellet (red) formed in the presence of Fc. Note the equal recovery of the Fc fragment from the supernatant and the pellet (the black trace is obscured by the red trace), indicating a lack of preferential partitioning into the solid phase.

Materials and Methods

Materials

High-purity recombinant human monoclonal IgG1 [IgG1-A ($pI = 8.9$), IgG1-B ($pI = 9.0$)] and IgG2 [IgG2-A ($pI = 8.8$), IgG2-B ($pI = 6.8$), IgG2-C ($pI = 8.0$)] antibodies were produced at Amgen. The antibody samples were stored at -80°C and thawed immediately before use. Endoproteinase Lys-C and papain were purchased from Roche (Catalog #11047825001 and 10108014001, Basel, Switzerland).

All other reagents and chemicals were of analytical grade or better.

Methods

Cation-exchange chromatography at pH 5.2

The CEX52 method¹⁵ was run on an UltiMate 3000 Series (Dionex, Sunnyvale, CA) HPLC system. Chromatography was performed on a ProPac WCX-10 analytical column (weak cation exchange, 4×250 mm; Dionex) preceded by a ProPac WCX-10G guard column (weak cation exchange, 4×50 mm; Dionex) at 25°C . Fifty micrograms of protein sample was loaded onto the column and analyzed at a flow rate of 0.7 mL/min. The column was equilibrated with Buffer A (20 mM sodium acetate, pH 5.2), and protein was eluted with a linear gradient of Buffer B from 0 to 100% (20 mM sodium acetate, 300 mM sodium chloride, pH 5.2) over 35 min. Following elution, the column was washed with Buffer C (20 mM sodium acetate, 1 M NaCl, pH 5.2) for 5 min and re-equilibrated with Buffer A for 16 min. Absorbance was measured at 215 and 280 nm.

Size-exclusion chromatography

Size-exclusion chromatography was performed on a TSKgel G3000SW_{XL} analytical column (7.8×300 mm; Tosoh Bioscience LLC, Montgomeryville, PA) at 25°C . Twenty micrograms of protein sample was loaded onto the column and analyzed at a flow rate of 0.5 mL/min. Protein was eluted isocratically with 100 mM sodium phosphate, 250 mM sodium chloride, pH 6.8 buffer over 35 min. Separations were carried out on an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA), and absorbance was measured at 215 and 280 nm.

Limited proteolysis of an IgG1 mAb using endoproteinase Lys-C

Fab and Fc fragments from an IgG1 antibody (IgG1-B) were obtained by limited proteolysis using endoproteinase Lys-C.^{15,29} The antibody sample was diluted to a concentration of 1 mg/mL in 100 mM Tris-HCl buffer (pH 7.5) and incubated with Lys-C at an enzyme-to-substrate ratio of $1:200$ (w/w) at 37°C for 10 min. The digestion reaction was quenched by adding $1/4$ reaction volume of 150 mM ammonium acetate buffer (pH 4.7).

Limited proteolysis of an IgG2 mAb using papain

Limited proteolysis of an IgG2 antibody (IgG2-A) was performed at an antibody concentration of 1 mg/mL in a buffer consisting of 100 mM Tris-HCl (pH 7.6), 4 mM EDTA, 10 mM cysteine, and 10 mM dithiothreitol (DTT). Papain was added to achieve an enzyme-to-substrate ratio of $1:100$ (w/w). The digestion mixture was incubated at 37°C for 20 h.

Purification of Fab and Fc fragments from IgG-2A limited proteolysis

The semi-preparative purification of Fab and Fc fragments was performed on an ÄKTA Explorer 10 system (GE Healthcare, Uppsala, Sweden) using a ProPac WCX-10 semi-preparative column (weak cation exchange, 10 × 250 mm; Dionex). The mobile phases used were the same as those from the analytical CEX52 method (see above). The elution was scaled proportionally from the analytical method based on the column size and the loading volume. After sample loading, a linear gradient (v/v) from 100% of Buffer A to 100% of Buffer B over 35 min was applied followed by a 5-min isocratic column regeneration step with 100% of Buffer C. The column was re-equilibrated with 100% of Buffer A for 15 min before next injection. The flow rate was 0.7 mL/min, and the UV absorption was measured at 215, 254, and 280 nm. The effluent was fractionated consecutively with equal time intervals. The fractions containing Fab and Fc fragments were collected for subsequent experiments.

Differential scanning calorimetry

Proteins were dialyzed overnight at 4°C against 20 mM sodium phosphate (pH 6.8). DSC measurements were taken using a VP-Capillary DSC system (MicroCal, Northampton, MA) equipped with tantalum 61 cells, each with an active volume of 125 µL. Protein samples were diluted to 0.5 mg/mL in the dialysis buffer, and the same buffer was used as a reference. The samples were scanned from 20°C to 90°C at a rate of 20°C/h following an initial 15-min equilibration at 20°C. A filtering period of 16 s was used, and the data were analyzed using Origin 7.0 software (OriginLab® Corporation, Northampton, MA). Resulting thermograms were corrected by subtraction of buffer-only blank scans. The corrected thermograms were normalized for protein concentration.

mAb Aggregation induced by high temperature, agitation, fatty acids, and low pH

The aggregation propensity of mAbs under various stress conditions was investigated. Solutions containing individual or mixed proteins were subjected to the same treatment. After the stress treatment, samples were centrifuged at 16,100 rcf (Eppendorf 5415D, Hamburg, Germany) for 10 min to remove insoluble protein precipitate and analyzed by CEX52.

Heat-induced aggregation was studied at various temperatures as a function of salt concentration. Protein samples were at 1 mg/mL concentration for each mAb (i.e., total protein concentration of a mixture consisting of 3 mAbs was 3 mg/mL). Buffers contained 20 mM sodium phosphate (pH 6.8) and

0M, 0.1M, or 1.0M NaCl. Protein samples were heated for 10 min and centrifuged before CEX52 analysis. The unstressed material was used as a control.

Rigorous agitation at ambient temperature was performed in 20 mM sodium phosphate (pH 6.8) buffer supplemented with 0M, 0.1M, or 1.0M NaCl at the maximal speed from a VX-2500 Multi-Tube Vortexer (VWR, West Chester, PA) for a total duration of 48 h. Various time points were analyzed throughout the study by removing small sample aliquots for CEX52 analysis. Protein samples were at 1 mg/mL concentration for each mAb. Agitated samples quickly became turbid and required centrifugation before chromatography.

Mild agitation experiments were performed in 20 mM sodium phosphate (pH 6.8) buffer supplemented with 0M, 0.1M, or 1.0M NaCl at 4°C and 25°C for a total duration of 6 days using a RKVSD Vortexer (Appropriate Technical Resources, Laurel, MD). Agitated protein samples remained free of visible precipitation throughout incubation. Samples used in aggregation seeding experiments were supplemented by IgG1-A or IgG1-B precipitate (see below) at a 1:20 volume ratio with respect to the soluble fraction. Agitation was continued for 2 days at 25°C. Protein samples were at 1 mg/mL concentration for each mAb.

Low pH aggregation was studied in 100 mM sodium acetate, pH 3.5 (adjusted by glacial acetic acid/HCl). Protein solutions were protected from light and incubated without agitation at 4°C and 25°C for up to 48 h. The samples remained free of visible protein precipitate throughout the study. Protein samples were at 1 mg/mL concentration for each mAb.

Caprylic (Catalog # 129390025, Acros Organics) and heptanoic acids (Catalog # 54905123, Fluka) were used to study fatty acid precipitation of mAbs. To achieve massive precipitation of IgG1 mAbs, 1.5% (115 mM) heptanoic acid was used. Samples of pure IgG1-A (11.5 mg/mL) and IgG1-B (25.0 mg/mL) were dialyzed into 50 mM acetate. Heptanoic acid was added to dialyzed samples to initiate the precipitation process. This reaction mixture was incubated overnight at room temperature at pH 5.2. White amorphous protein precipitate was collected by centrifugation at 4°C at 2600 rcf (Allegra™ 25R centrifuge, Beckman Coulter, Fullerton) for 5 min. The pellet was resuspended in 20 mM sodium phosphate (pH 6.8) and centrifuged to eliminate soluble protein. The process was repeated multiple times (>10) to ensure complete removal of weakly associated protein monomers.

Precipitation of IgG1-A, IgG2-A, and IgG2-A Fab and Fc fragments was induced by either 1% (63 mM) caprylic acid or 1% (77 mM) heptanoic acid in 10 mM sodium acetate (pH 5). Upon addition of the fatty acid, protein solutions were rigorously vortexed

at room temperature for 1 min to accelerate the aggregation process. The reaction mixtures were subsequently centrifuged to produce particle-free solutions for CEX52 analysis.

Phase separation experiments with IgG2-A

Phase separation experiments were performed with acid-treated and neutralized Protein A-purified material of IgG2-A containing 18.6 mg/mL protein. Buffer composition of the protein sample was 100 mM acetate, 25 mM phosphate, and 100 mM Tris (pH 7.1). This material was susceptible to reversible temperature-dependent protein precipitation during static refrigerated storage.

Conclusions

CEX52 is a highly versatile method, which can be adopted to study protein mixtures for the purpose of elucidating mAb aggregation and phase separation. Stress conditions such as high temperature, agitation, low pH, and fatty acid precipitation induce mAb aggregation by promoting protein denaturation. These stresses are useful for eliminating thermodynamically unstable candidates but are not interchangeable as they promote different aggregation mechanisms. Therefore, consistent use of the various stress conditions in candidate selection is advised. Although none of these stress factors may be adequate for assessing the colloidal stability of concentrated protein solutions, these phenomena can be addressed by analyzing protein phase partitioning with CEX52.

Potential drawbacks of the method include protein co-precipitation and the intensity of stress required for producing massive aggregation. Protein co-precipitation can be assessed by performing control experiments with individual protein solutions. The second problem relates to a more general issue of selecting representative stress conditions for aggregation. Our experiments illustrated the use of CEX52 in unraveling the complexity of mAb aggregation under accelerated conditions. However, its utility in predicting real-time storage stability of protein formulations requires further investigation.

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