ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/237069040

# Nondenaturing Size-Exclusion Chromatography-Mass Spectrometry to Measure Stress-Induced Aggregation in a Complex Mixture of Monoclonal Antibodies

**ARTICLE** *in* ANALYTICAL CHEMISTRY · JUNE 2013

Impact Factor: 5.64 · DOI: 10.1021/ac401455f · Source: PubMed

CITATIONS
6

READS
43

**3 AUTHORS**, INCLUDING:

Ramil F Latypov
Amgen
**36** PUBLICATIONS **645** CITATIONS

SEE PROFILE

Available from: Ramil F Latypov
Retrieved on: 31 January 2016

analytical chemistry

Article

pubs.acs.org/ac

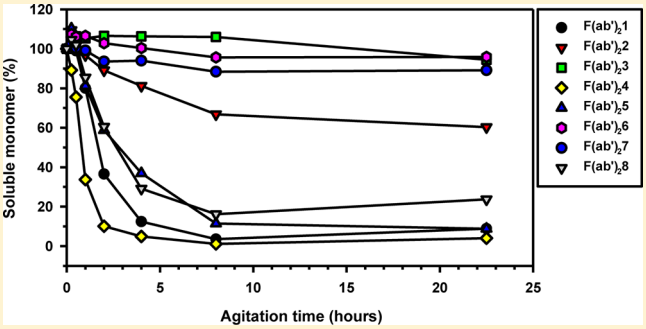
# Nondenaturing Size-Exclusion Chromatography-Mass Spectrometry to Measure Stress-Induced Aggregation in a Complex Mixture of Monoclonal Antibodies

Jonathan Woodard, Hollis Lau, and Ramil F. Latypov*

Drug Product Development, Amgen Inc., Seattle, Washington 98119, United States

Supporting Information

**ABSTRACT:** During therapeutic candidate selection, diverse panels of monoclonal antibodies (mAbs) are routinely subjected to various stress conditions, and assayed for biophysical and biochemical stability. A novel high throughput method has been developed to differentiate candidate molecules in a mixture based on their propensity for forming aggregates when subjected to agitation (vortexing) stress. Protein monomers are separated from soluble and insoluble aggregates using size exclusion chromatography, under nondenaturing conditions, and the individual components in the mixture are identified by mass spectrometry and quantitated relative to an unstressed control. An internal standard was added to the mixture after stress, and used to correct for differences in ionization between samples. Treatment of the samples with the enzyme IdeS (FabRICATOR) significantly reduces sample complexity, and allows for a large number of candidate molecules to be assessed in a single analysis. Simple and robust, the method is well suited for measuring relative aggregation propensity (RAP) in conjunction with molecule selection and coformulation development.




Aggregation is a major concern in the development of monoclonal antibodies (mAbs) as therapeutic agents.[1−3] Aggregation is known to occur via multiple pathways, and various stress conditions have been developed to induce different types of aggregation.[4−7] The susceptibility of various candidate molecules to aggregation under these stress conditions can be used to screen out molecules which may present challenges in development, and thus direct resources toward the most promising candidates at an early stage of development. Here, we describe a method which allows a large number of molecules to be screened simultaneously for relative aggregation propensity (RAP) by utilizing mass spectrometry to definitively identify individual molecules within a complex mixture. Differences in ionization efficiency between samples, caused by precipitation of less stable molecules, are corrected for by the addition of an internal standard. Inclusion of the internal standard prior to mass spectrometry analysis allows for meaningful quantitative comparison of different samples, with varying protein concentrations, before and after stress. Assessing molecules in a mixture ensures that all of the species are subjected to the same experimental conditions, facilitating comparisons and reducing the need for replicate analyses to gain confidence in the results.[5] This allows a large number of candidates to be screened quickly, with a limited amount of sample.

A method for assessing aggregation in mixtures of monoclonal antibodies has been established by Chen et al.[5] and is being used in our laboratory to rank different molecules according to their stability to various stresses. Mixtures of antibodies are subjected to stress (vigorous agitation, high temperature, low pH, etc.) sufficient to cause massive aggregation, and the remaining monomer is analyzed by cation exchange chromatography under native conditions.[8] As this method depends on chromatographic resolution of different species for identification, each protein must be characterized individually before being assessed in the mixture. Our extensive experience with this method has demonstrated good correlation between molecules stressed individually and in different mixtures. Here, we demonstrate a method based on size-exclusion chromatography with detection by mass spectrometry (SEC-MS), which offers significantly higher throughput than the existing method, by allowing for analysis of more complex mixtures and removing the need for chromatographic resolution. The method utilizes nondenaturing solvents and ambient column temperature and is not expected to unfold proteins or dissociate aggregates. In comparison with reversed-phase chromatography using high levels of organic solvents and strong acids,[9] our SEC-MS is specifically designed for analyzing noncovalent protein aggregation.

Additionally, we investigated the utility of the enzyme IdeS (FabRICATOR) to investigate the relative stabilities of Fab and Fc regions of two subclasses of immunoglobulin G (IgG). This

**Received:** April 2, 2013
**Accepted:** June 6, 2013
**Published:** June 6, 2013

 © 2013 American Chemical Society  dx.doi.org/10.1021/ac401455f | *Anal. Chem.* 2013, 85, 6429−6436

enzyme cleaves C-terminally of the hinge disulfide bonds at a very specific sequence which is present in all human IgG subclasses.[10,11] We show that this has the advantage of significantly reducing the complexity of the resulting MS spectra by decoupling heterogeneity due to the N-linked glycosylation in $C_H2$ domains of Fc from that due to the presence of diverse variable domains in Fab. Fc glycosylation is of particular concern due to the large number of potential glycan species present, even in highly purified mAbs. The ability of MS detection to resolve these species results in a reduction in the signal intensity for each individual species. Glycan heterogeneity also increases the number of species which must be considered when determining whether a given mAb can be included in a mixture. IdeS treatment helps to circumvent these issues and allows for a larger number of candidates to be tested simultaneously.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** High-purity recombinant human monoclonal IgG1 and IgG2 antibodies were produced at Amgen Inc. (see Table 1). FabRICATOR digestion enzyme (IdeS) was manufactured by Genovis (Lund, Sweden) and obtained from QED Biosciences (San Diego, CA). All other reagents and chemicals were of analytical grade or better. All buffer solutions were filtered through a 0.22 $\mu$m filter prior to use.

**Table 1. Molecules, Alone or in Combination, Used**

| molecule | IgG subclass | expected MW *(G0/G0 glycoform)* |
|---|---|---|
| mAb 1 | IgG1 aglycosylated | 145 289.03 (aglycosylated) |
| mAb IS (anti-streptavidin, aSA) | IgG1 | 144 779.68 |
| mAb 2 | IgG1 | 148 312.29 |
| mAb 3 | IgG2 | 147 623.01 |
| mAb 4 | IgG2 | 148 246.52 |
| mAb 5 | IgG2 | 148 344.12 |
| mAb 6 | IgG2 | 148 628.10 |
| mAb 7 | IgG2 | 149 220.77 |
| mAb 8 | IgG2 | 149 495.21 |

**Agitation-Induced Aggregation.** Non-native aggregation of mAbs was induced by vigorous vortexing at 4 °C, as described previously.[5] Vortexing was performed in 100 mM sodium acetate, 50 mM sodium chloride at pH 5.2 (100A52N) at the maximal speed from a G-560 vortexer (Scientific Industries, Bohemia, NY) for a total duration up to 24 h. The high ionic strength and buffering capacity of 100A52N facilitated aggregation (by reducing interprotein repulsion) while maintaining constant pH. Various time points were analyzed throughout the study by collecting small sample aliquots for SEC-MS and cation-exchange (CEX) analyses (see below for method details). Agitated protein solutions quickly became turbid; therefore, all experiments were conducted in 0.5−1.5 mL eppendorf tubes so that centrifugation can be performed prior to high-pressure liquid chromatography (HPLC) analysis.

**Native SEC-MS Analysis.** In-line SEC-MS analysis has been previously reported, utilizing relatively high levels of organic solvents and strong acids in the mobile phase.[12] For our experiments, it was desirable to match chromatographic conditions as closely as possible to the mAb aggregation conditions (i.e., 100A52N), in order to minimize on-column formation or resolubilization of aggregates. For this reason, the volatile salt ammonium acetate was used to generate an MS compatible mobile phase matching the formulation pH. All experiments were performed on a Waters Acquity UPLC (Waters Corporation, Milford, MA) coupled to a 6200 series time-of-flight (TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA). Separation was performed using an Acquity UPLC BEH200 SEC 1.7 $\mu$m 4.6 × 150 mm column (Waters Corporation, Milford, MA) at ambient temperature under isocratic conditions at 220 $\mu$L/min. The mobile phase was 25 mM ammonium acetate and 5% acetonitrile in water, adjusted to pH 5.2 with acetic acid. The mass spectrometer was tuned and calibrated using the integrated calibrant delivery system, and MS spectra were acquired from 100 to 6000 $m/z$. Deconvoluted spectra were generated by maximum entropy, using Agilent MassHunter qualitative analysis software.

**Cation-Exchange HPLC.** Aggregation of IgGs within mixtures was monitored by cation-exchange chromatography at pH 5.2.[5,8] The method was run on an Agilent 1100 series HPLC system using a ProPac WCX-10 analytical column (weak cation exchange, 4 mm × 250 mm; Dionex, Sunnyvale, CA) preceded by a ProPac WCX-10G guard column (weak cation exchange, 4 mm × 50 mm; Dionex) at ambient temperature. Various amounts of protein were 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, 0.0025% sodium azide, pH 5.2), and protein was eluted with a linear gradient of buffer B (20 mM sodium acetate, 300 mM sodium chloride, 0.0025% sodium azide, pH 5.2) from 0 to 100% over 35 min. Following elution, the column was washed with buffer C (20 mM sodium acetate, 1 M sodium chloride, 0.0025% sodium azide, pH 5.2) for 5 min and re-equilibrated with buffer A for 16 min. Data were analyzed with Dionex Chromeleon software, and the 280 nm signal was integrated to estimate protein peak area.

**Enzymatic Digestion with IdeS (FabRICATOR).** Different mAbs were premixed in 100A52N and subsequently used to reconstitute a fresh aliquot of FabRICATOR to achieve the enzyme/substrate ratio of 2000 units/mg IgG. Incubation was performed at 37 °C for up to 2 h. Even though 100A52N was not recommended by the manufacturer and provided suboptimal conditions, digestion was found to be specific and complete (data not shown). After digestion, mixtures were subjected to agitation stress without any additional treatment and analyzed as described above.

## RESULTS

**Aggregation Rank Order of Intact mAbs and Fragments by SEC-MS.** Our main aggregation studies were performed on a mixture of 8 antibody molecules, mAb 1−mAb 8 (Table 1), where each protein was at 0.5 mg/mL. Lower protein concentrations, such as 0.1 mg/mL, were also evaluated and provided comparable results (data not shown). The mixture was prepared by diluting 70 mg/mL protein stock solutions into 100A52N (100 mM sodium acetate, 50 mM sodium chloride, pH 5.2). Eppendorf tubes filled with this solution were subjected to rigorous agitation (vortexing) which primarily caused formation of insoluble aggregates.[5] Multiple time points were analyzed throughout the course of agitation by removing small aliquots for analysis. A method has previously been developed to analyze mixtures of mAbs using CEX.[5] In the present work, we demonstrate a complementary technique,


dx.doi.org/10.1021/ac401455f | Anal. Chem. 2013, 85, 6429−6436

using SEC to desalt and separate monomers from aggregates, and the specificity of MS detection to eliminate the need for chromatographic separation of the various mAbs. This allowed us to assess the amount of monomer remaining in solution after application of stress conditions and to determine the relative stabilities of the molecules tested. Figure 1 illustrates this

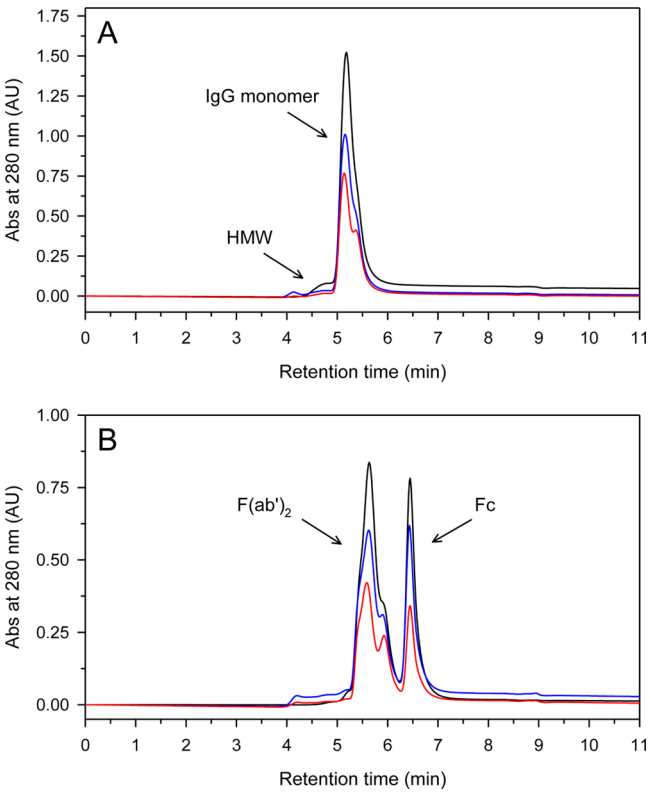



**Figure 1.** 280 nm UV chromatograms from SEC-MS analysis of a mixture of (A) 8 intact mAbs at $t$ = 0 h (black), 1 h (blue), and 4 h (red) of agitation, and (B) the fragments generated by digestion with FabRICATOR, at the same time points. The growth of HMW did not account for the overall monomer loss because of the formation of insoluble aggregates.[5]

separation for intact mAbs as well as for the protein fragments ($F(ab')_2$ and Fc) generated by IdeS. As expected, SEC provided an incomplete resolution between IgG1 and IgG2 species of similar MW. The SEC monomer peak comprised of different monomeric mAb species was deconvoluted via MS to allow a stability comparison between different candidates. The relative aggregation propensity (RAP) rank order observed by SEC-MS is given in Table 2. As each mAb in the mixture was itself a mixture of several major glycoforms, the resulting MS data was very complex, limiting the number of mAbs which can be mixed (see Figure 2A). As a consequence, quantitative determination of aggregation for species with a mass difference of less than 32 Da became difficult (see Supplemental Figure 1 in the Supporting Information). For this reason, FabRICATOR was used to generate $F(ab')_2$ and Fc species of mixed mAbs prior to agitation. Separation of $F(ab')_2$ from Fc significantly reduced the complexity of the resulting MS spectra by decoupling heterogeneity due to the N-linked glycosylation in the Fc from that due to the presence of diverse variable domains (Figure 2B). Indeed, $F(ab')_2$ fragments gave rise to much simpler spectra and facilitated the interpretation of data from mixtures of a large number of different candidates. In these experiments,

**Table 2. Relative Aggregation Propensity (RAP) Rank Order for Intact and Fragmented mAbs Listed from the Highest Aggregation Propensity to the Lowest[a]**

| SEC-MS | | CEX | |
|---|---|---|---|
| intact | $F(ab')_2$ | intact | $F(ab')_2$ |
| mAb 4 | $F(ab')_2$ 4 | N/A[b] | N/A[b] |
| mAb 1 | $F(ab')_2$ 1 | mAb 1 | $F(ab')_2$ 1 |
| mAb 5 | $F(ab')_2$ 5 | N/A[b] | N/A[b] |
| mAb 8 | $F(ab')_2$ 8 | mAb 8 | $F(ab')_2$ 8 |
| mAb 2 | $F(ab')_2$ 2 | mAb 2 | $F(ab')_2$ 2 |
| mAb 7 | $F(ab')_2$ 7 | N/A[b] | N/A[b] |
| mAb 3 | $F(ab')_2$ 3 | N/A[b] | N/A[b] |
| mAb 6 | $F(ab')_2$ 6 | mAb 6 | $F(ab')_2$ 6 |

[a]Major differences in RAP lie between unstable (mAb 1, mAb 4, mAb 5, mAb 8) and stable (mAb 2, mAb 3, mAb 6, mAb 7) mAbs. The exact rank order within each of these categories is relatively less certain. [b]This protein was not resolved by CEX because of an overlap with other proteins in the mixture.

a mass difference of as little as 27 Da in the $F(ab')_2$ was sufficient for resolving the species. Comparisons of RAP rank orders for intact mAbs and $F(ab')_2$ fragments revealed that they aggregated similarly, which suggested that IgG stability during vortexing was primarily controlled by the Fab regions (see the Discussion). An alternative approach where FabRICATOR is used not prior but after vortexing was not pursued to avoid artifacts associated with partial digestion of aggregates and aggregate to monomer reversal.

**Ionization Suppression and Quantitative Analysis Using an Internal Standard.** A common concern when performing quantitative analysis with MS data is differential ionization efficiencies and ion suppression effects which vary with protein concentration.[13−15] For our experiments, this consideration is particularly important, as the concentration of soluble monomers is expected to change as less stable molecules convert into aggregates. Supplemental Figure 2 in the Supporting Information shows the changes in signal intensity observed for a $F(ab')_2$ species held at a constant concentration, as the concentration of other mixture components is varied. The inherent variability of the MS signal intensity is typically overcome by the addition of isotopically labeled internal standards, which are chemically identical to the analytes of interest, but are readily resolved by their mass differences. Here, we demonstrate that a chemically matched, isotopically labeled internal standard is not necessary and that any mAb which is resolved from the analytes of interest can serve as an appropriate internal standard for all mixture components, within the concentration range tested. Supplemental Figure 3 in the Supporting Information shows the effect of normalizing target analyte responses to internal standard (IS) responses ($F(ab')_2$ from mAb 3 in this experiment), using a dilution series where all nominal concentrations are known. Normalizing all analyte responses to a single internal standard yields linear curves with $r^2$ values ranging from 0.979 to 0.999. Figure 3 shows the responses for all $F(ab')_2$ species from the agitation time course, normalized to an internal standard ($F(ab')_2$ aSA). In this representation the data clearly demonstrates the rank order of RAP for these molecules when exposed to this stress condition as well as the magnitude of the stability differences.

**Aggregation Rank Order of Intact mAbs and Fragments by CEX.** Figure 4 shows an overlay of CEX


dx.doi.org/10.1021/ac401455f | Anal. Chem. 2013, 85, 6429−6436

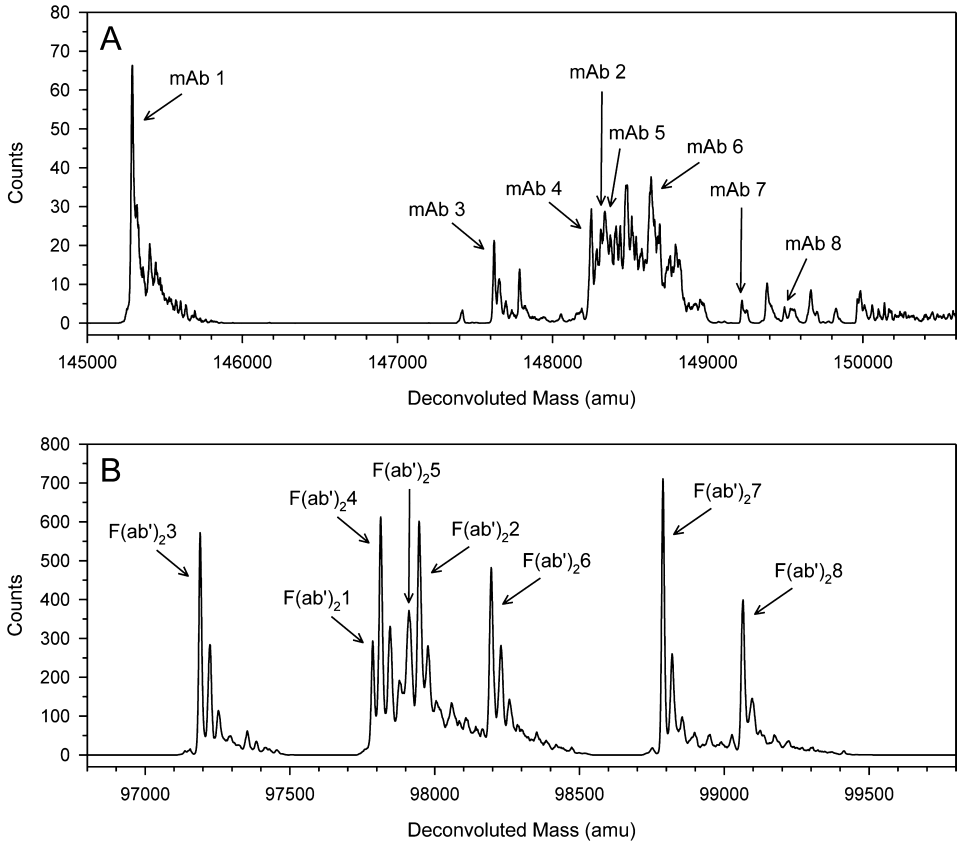



**Figure 2.** Comparison of deconvoluted spectra for an unstressed mixture of (A) 8 intact mAbs, and (B) the $F(ab')_2$ fragments generated by digestion with FabRICATOR. Complexity in panel B is much reduced, facilitating data analysis.

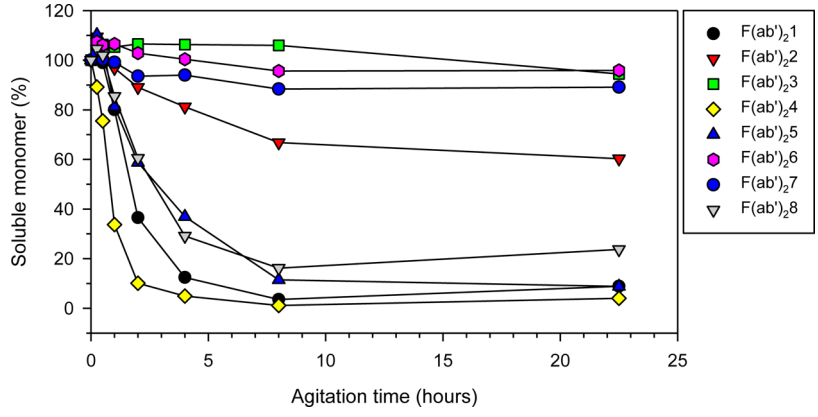



**Figure 3.** Protein monomers remaining after agitation, normalized to internal standard response. Shown is the amount of each $F(ab')_2$ species remaining in solution, based on integration of the deconvoluted spectra, after normalization to an internal standard ($F(ab')_2$ aSA). The internal standard is used to control for changing matrix conditions as the protein concentration changes. Each data series is presented as a ratio to the response at $t = 0$ to facilitate comparison between molecules with different ionization efficiencies and RAP.

chromatograms for the same eight individual and mixed mAbs that were analyzed by SEC-MS (see above). Note that there was an insufficient separation between mAb 3, mAb 4, mAb 5, and mAb 7 antibodies. Arrows in Figure 4A highlight the disappearance of different monomeric mAb species as a result of aggregation; only selected time points are shown for clarity. A dramatic change in the peak areas for mAb 1 and mAb 8 due to the loss of soluble monomer is apparent. Changes are evident also in the case of the large unresolved peak comprised of mAb 3, mAb 4, mAb 5, and mAb 7. Unfortunately, the lack of chromatographic resolution precludes rank ordering of these molecules. In contrast, no significant changes are seen in the case of mAb 2 and mAb 6 indicating their superior stability. The CEX aggregation results for mAbs 1, 2, 6, and 8 are summarized in Figure 5A. The data are presented in terms of percent monomer recovery calculated based on chromatographic peak area. Reproducibility of these results was verified in several repeated experiments (data not shown). An overall correspondence was found between the aggregation data by CEX and SEC-MS for these four intact mAbs (Table 2).

Our last experiment was performed on the same 8-mAb mixture after FabRICATOR digestion. Figure 6 shows an


dx.doi.org/10.1021/ac401455f | *Anal. Chem.* 2013, 85, 6429−6436

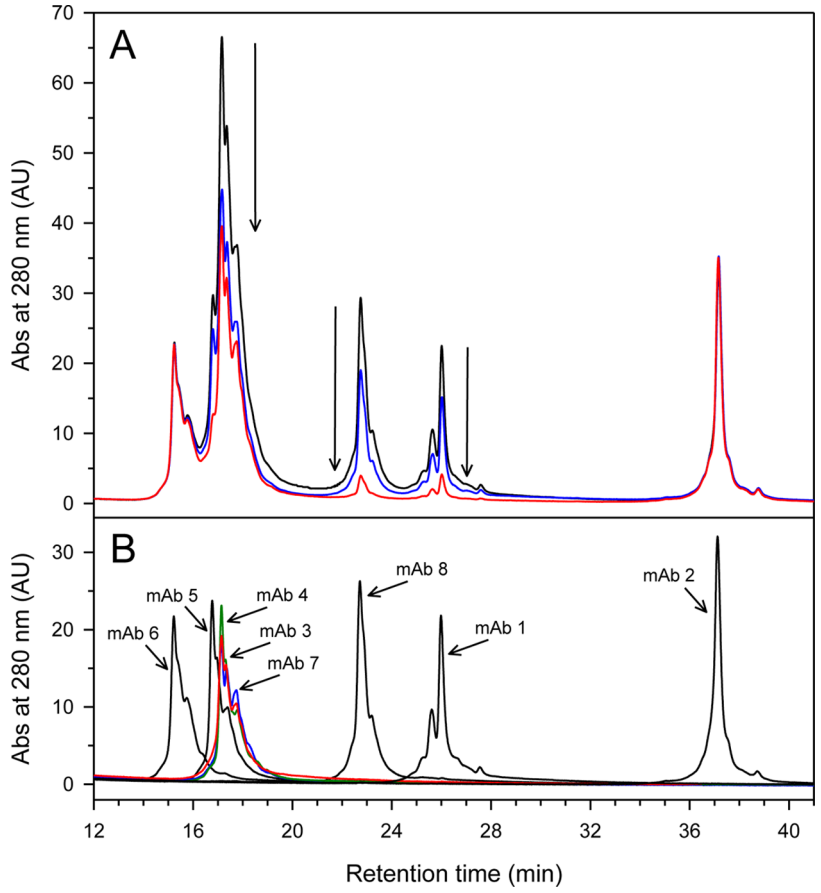



**Figure 4.** 280 nm UV chromatograms from CEX analysis of a mixture of (A) 8 intact mAbs at $t$ = 0 h (black), 1 h (blue), and 4 h (red) of agitation and (B) individual mAbs comprising the mixture. Chromatographic changes resulting from the loss of soluble monomer due to aggregation are shown by the arrows. Overlapping mAbs 3, 4, and 7 are shown in color for clarity.

overlay of CEX chromatograms for the individual and mixed $F(ab')_2$ and Fc species. As with the intact mAbs (Figure 4), there was an insufficient separation between the $F(ab')_2$ fragments originating from mAb 3, mAb 4, mAb 5, and mAb 7. Figure 6A illustrates CEX changes reflecting the progression of aggregation upon vortexing at three different time points. Peak areas for $F(ab')_2$ 1 and $F(ab')_2$ 8 change very early compared to other molecules; changes are also evident in the case of the unresolved peak comprised of $F(ab')_2$ 3, $F(ab')_2$ 4, $F(ab')_2$ 5 and $F(ab')_2$ 7 species. In contrast, no changes are seen in the case of the $F(ab')_2$ 2 and $F(ab')_2$ 6 species, indicating that they have a higher stability toward aggregation. In summary, the following rank order for the $F(ab')_2$ species was revealed (listed from the highest RAP to the lowest): mAb 1 ≈ mAb 8 > mAb 2 ≈ mAb 6. An overall correlation was found between (1) aggregation of the intact mAbs and their $F(ab')_2$ fragments (cf. parts A and B of Figure 5); (2) the RAP data generated by CEX and SEC-MS analyses (cf. Figures 3 and 5B; Table 2). In addition, some insights were gained regarding agitation-induced aggregation of the various Fc. The Fc species for all six IgG2 molecules eluted at the same retention time (~18.5 min) because they were identical in sequence and fully glycosylated in $C_H2$ domains. In contrast, the Fc of the two IgG1 were different as they were either fully glycosylated (mAb 2) or aglycosylated (mAb 1). These two forms of IgG1 Fc were separated from each other by CEX and eluted at ~22 and 29.5 min, respectively. Therefore, available data supported an assessment for the relative aggregation propensity of three different Fc. We found that agitation-induced aggregation of Fc depended on both the IgG subclass (IgG1 vs IgG2) and $C_H2$ glycosylation, in agreement with our previous findings from low pH experiments.[6,16] The following rank order for the Fc species was revealed (listed from the highest RAP to the lowest): aglyco-Fc IgG1 > Fc IgG2 > Fc IgG1 (see Figure 5C). A similar trend was observed by SEC-MS despite the complexity of MS data resulting from the presence of multiple glycoforms (data not shown). Such inherent differences in Fc aggregation propensity, however, were practically insignificant in the case of intact mAbs. As stated above, aggregation of the intact mAbs and their $F(ab')_2$ fragments matched closely indicating the leading role of the Fab regions in IgG aggregation on agitation (see the Discussion).

## DISCUSSION

Agitation or shaking experiments are now widely implemented in the biopharmaceutical industry for selection of stable therapeutic candidates and preformulation screening.[1,3] The detailed mechanism of agitation-induced protein aggregation is not fully understood, but it likely involves a combination of factors.[4,5,17] Available literature suggests that besides the air−liquid interface, protein surface characteristics such as charge and hydrophobicity must be considered.[18] Results of agitation experiments are influenced by protein concentration,[19] presence of excipients (especially, surfactants), as well as primary container configuration, fill volume, and shaking speed.[20] All of the above make agitation results inherently


dx.doi.org/10.1021/ac401455f | Anal. Chem. 2013, 85, 6429−6436

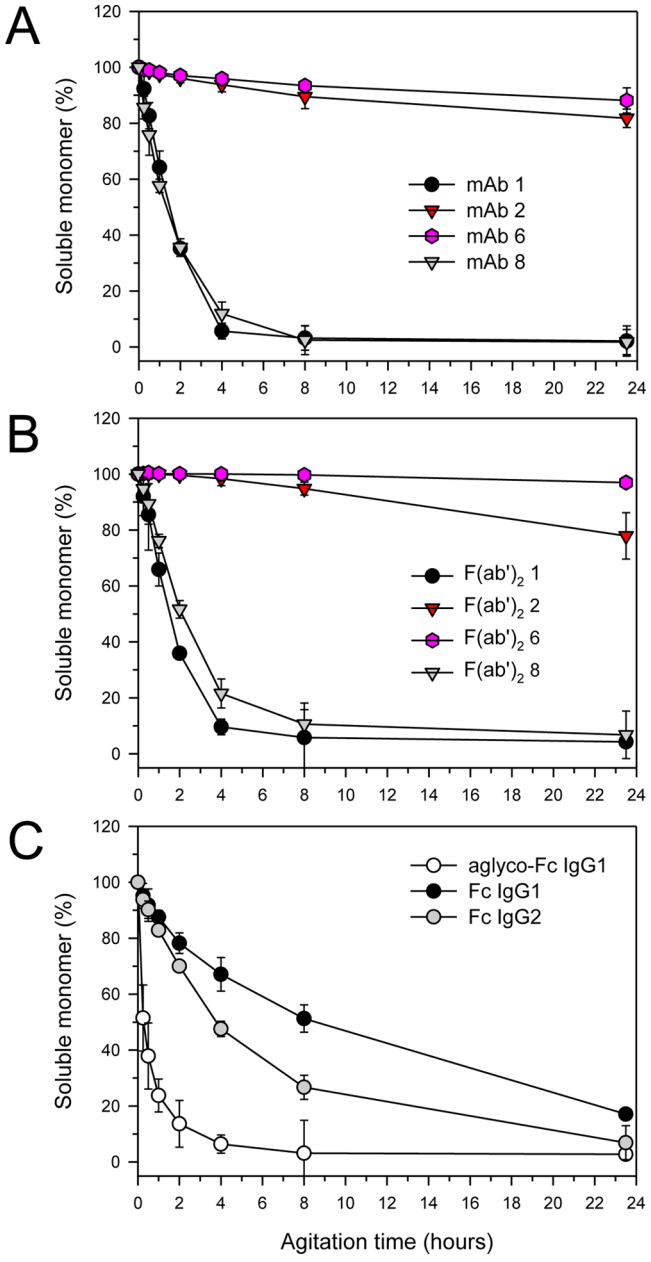



**Figure 5.** Plot of percent soluble monomer vs agitation time for (A) intact mAbs, (B) $F(ab')_2$ fragments, and (C) Fc fragments resolved by CEX.

difficult to reproduce. Despite the best experimental efforts, sample to sample variations may preclude meaningful selection among candidates and fine-tuning of excipient concentrations. However, every major biopharmaceutical company has adopted an agitation assay in one format or the other. What makes agitation experiments so valuable? There are at least three factors. First, agitation stressing is believed to offer an accelerated way of assessing stability of protein therapeutics during transportation. Second, agitation testing does not require changes in sample composition and can be performed using virtually any type of container without even opening it, thus protecting sterility. The third reason is that agitation stressing can sometimes reveal instability that is not associated with reduced thermal stability of a protein.[5] Some mAb candidates that appear to be thermally stable by DSC may aggregate rapidly on vortexing at 4 °C. Such candidates are usually disqualified from further development based on agitation data.

The issue with reproducibility of agitation data may be solved via mixing protein candidates in a single container. RAP data extracted from a single container can provide information for as many candidates as can be analytically resolved. There are powerful techniques capable of resolving complex protein mixtures, such as high-resolution chromatography and MS. These techniques can be adopted for analyzing protein mixtures for the purpose of selecting stable candidates and preformulation screening. Our previous work provided a foundation for the use of protein mixtures in studies of IgG aggregation via CEX.[5] The success of this earlier approach provided a framework for the exploration of other strategies for RAP analysis, such as SEC-MS.

In the work described herein, we investigated the aggregation of antibodies with and without FabRICATOR enzymatic treatment. The goal of our experiments was 2-fold: (1) developing a universal analytical method capable of investigating aggregation in complex antibody mixtures and (2) understanding how different protein regions ($F(ab')_2$ vs Fc) contributed to the aggregation process caused by shaking. The results presented demonstrate our success at developing such a method and highlight the importance of Fab domains in antibody aggregation upon agitation. The latter follows from the fact that the $F(ab')_2$ fragments created by IdeS mirrored the aggregation behavior of intact antibodies (Table 2 and Figure 5). Such a correlation reveals the possible role of variable domains and, potentially, CDRs. The dominant role of Fab in agitation-induced aggregation is in striking contrast with the low pH process which is primarily driven by the Fc.[5,6,16] Addressing the reasons behind such disparity in the role of IgG regions in aggregation remains a matter of further investigation. For the purpose of this study, it will suffice to note that such differences should have practical implications. Perhaps, agitation and low pH aggregation experiments can be adopted as orthogonal tools for selectively assessing instability in the Fab and Fc regions of mAbs. Molecule assessment aimed at stable candidate selection is an area that may benefit from such a targeted approach, as indicated by the following examples. On the basis of historical evidence, mAb 2 and mAb 6 exhibited no aggregation issues during manufacturing or formulation, in agreement with our new results. In contrast, stability issues with the least stable mAb 1 and mAb 8 ranged from irreversible denaturation during low pH viral inactivation to particle formation during ultrafiltration-diafiltration (data not shown).

The results of our novel SEC-MS analytical approach are in agreement with those that were obtained in parallel by using CEX (Table 2). However, SEC-MS allows for the rank ordering of all species in a complex mixture, without the need for chromatographic separation, provided that there is a sufficient mass difference between the species. In the current work, we demonstrate a quantitative determination of aggregation propensity for species with a mass difference of greater than 32 Da within a mixture of 8 different intact antibodies and greater than 27 Da for antibody fragments. By appropriate selection of components, it should be possible to apply our method to increasingly complex protein mixtures. The amount of individual protein required for such analysis can be as little as 10 $\mu$g, a quantity that is often available even at an early stage of candidate engineering. As instrumentation and software continues to improve, we expect the sensitivity and resolving power of this method to further improve and it's utility to


dx.doi.org/10.1021/ac401455f | Anal. Chem. 2013, 85, 6429−6436

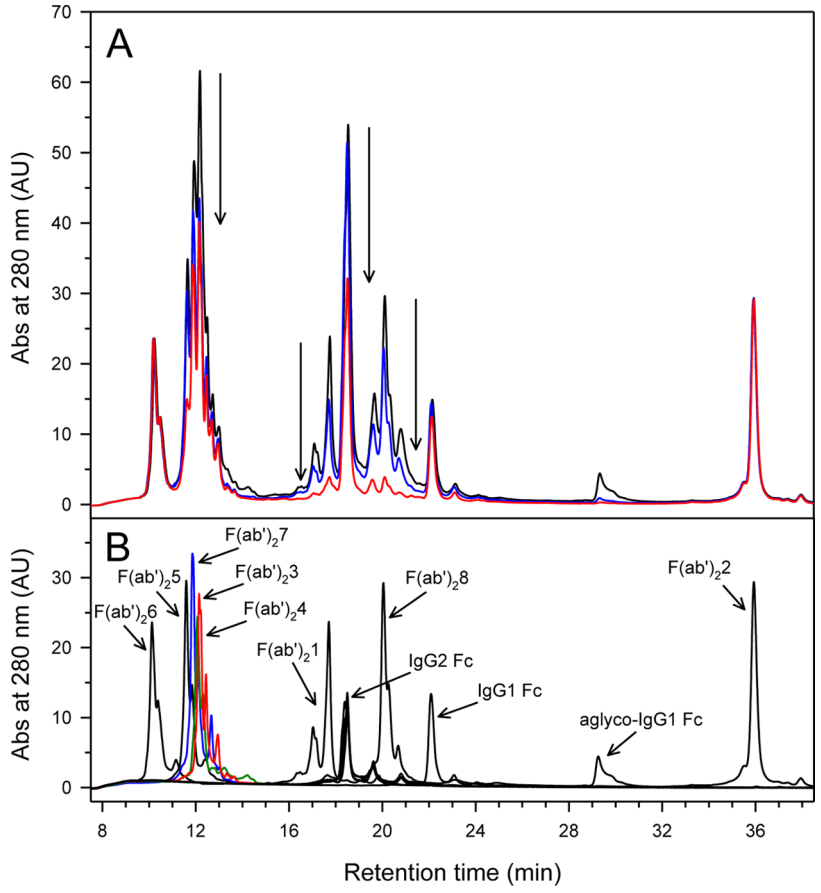



**Figure 6.** 280 nm UV chromatograms from CEX analysis of (A) a FabRICATOR treated mixture of 8 mAbs at $t$ = 0 h (black), 1 h (blue), and 4 h (red) of agitation and (B) FabRICATOR treated individual mAbs comprising the mixture. Major chromatographic changes resulting from the loss of soluble monomer due to aggregation are shown by the arrows. Overlapping $F(ab')_2$ fragments of mAb 3, 4, 5, and 7 are shown in color for clarity. The IgG2 Fc peak in panel A is contributed by Fc fragments from 6 different IgG2s.

broaden. An interesting and important application of the method also lies in the emerging field of coformulation development where different antibodies are combined in a drug product to achieve greater therapeutic efficacy. Indeed, our SEC-MS method is uniquely suited for analyzing such mixtures with respect to both physical (aggregation) and chemical (covalent modifications and fragmentation) degradation processes.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Address: Drug Product Development, Amgen Inc., 1201 Amgen Court West, Seattle, WA 98119. Phone: (206) 265-8851. Fax: (206) 217-0346. E-mail: rlatypov@amgen.com.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors thank Rutilio Clark, Jane Carter, and Randal R. Ketchem for providing proteins, Lowell J. Brady for technical assistance, and Gerald W. Becker, Gerd R. Kleemann, and Michael J. Treuheit for discussions and support.

## ■ REFERENCES

(1) Wang, W. *Int. J. Pharm.* **2005**, *289*, 1−30.
(2) Rosenberg, A. S. *AAPS J.* **2006**, *8*, E501−507.
(3) Manning, M. C.; Chou, D. K.; Murphy, B. M.; Payne, R. W.; Katayama, D. S. *Pharm. Res.* **2010**, *27*, 544−575.
(4) Kiese, S.; Papppenberger, A.; Friess, W.; Mahler, H.-C. *J. Pharm. Sci.* **2008**, *97*, 4347−4366.
(5) Chen, S.; Lau, H.; Brodsky, Y.; Kleemann, G. R.; Latypov, R. F. *Protein Sci.* **2010**, *19*, 1191−1204.
(6) Hari, S. B.; Lau, H.; Razinkov, V. I.; Chen, S.; Latypov, R. F. *Biochemistry* **2010**, *49*, 9328−9338.
(7) Joubert, M. K.; Luo, Q.; Nashed-Samuel, Y.; Wypych, J.; Narhi, L. O. *J. Biol. Chem.* **2011**, *286*, 25118−25133.
(8) Lau, H.; Pace, D.; Yan, B.; McGrath, T.; Smallwood, S.; Patel, K.; Park, J.; Park, S. S.; Latypov, R. F. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2010**, *878*, 868−876.
(9) Hearn, M. T. W.; Grego, B. *J. Chromatogr.* **1984**, *296*, 61−82.
(10) von Pawel-Rammingen, U.; Johansson, B. P.; Björck, L. *EMBO J.* **2002**, *21*, 1607−1615.
(11) Vincents, B.; von Pawel-Rammingen, U.; Björck, L.; Abrahamson, M. *Biochemistry* **2004**, *43*, 15540−15549.
(12) Brady, L. J.; Valliere-Douglass, J.; Martinez, T.; Balland, A. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 502−509.
(13) Annesley, T. M. *Clin. Chem.* **2003**, *49*, 1041−1044.
(14) Mallet, C. R.; Lu, Z.; Mazzeo, J. R. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 49−58.
(15) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **1998**, *70*, 882−889.
(16) Latypov, R. F.; Hogan, S.; Lau, H.; Gadgil, H.; Liu, D. *J. Biol. Chem.* **2012**, *287*, 1381−1396.

(17) Maa, Y.-F.; Hsu, C. C. *Biotechnol. Bioeng.* **1997**, *54*, 503−512.
(18) Baszkin, A.; Boissonnade, M. M.; Kamyshny, A.; Magdassi, S. *J. Colloid Interface Sci.* **2001**, *239*, 1−9.
(19) Treuheit, M. J.; Kosky, A. A.; Brems, D. N. *Pharm. Res.* **2002**, *19*, 511−516.
(20) Eppler, A.; Weigandt, M.; Hanefeld, A.; Bunjes, H. *Eur. J. Pharm. Biopharm.* **2010**, *74*, 139−147.