# Ion Mobility-Mass Spectrometry Reveals the Structures and Stabilities of Biotherapeutic Antibody Aggregates

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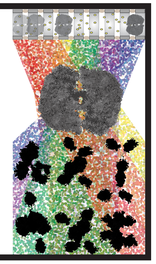
## Introduction

Biotherapeutic monoclonal antibody (mAb) development typically requires screening tens-of-thousands of candidate molecules for changes in higher order structure (HOS) caused by stress or environment that alters aggregation, immunogenicity, and efficacy[1,2]. However, the structures of the resulting aggregates are largely unknown[3]. Current assays to monitor aggregation and stability shifts are well validated, but are often slow, require large amounts of mAb, and lack access to HOS information. Ion mobility-mass spectrometry (IM-MS) and collision induced unfolding (CIU), are capable of measuring protein size and stability rapidly from small amounts of sample[4,5]. Here, IM-MS and CIU are used to characterize mAb dimers. Our data indicate compact, overlapped dimer structures, the formation of which depend upon sequence differences within mAb complementarity determining regions (CDRs).

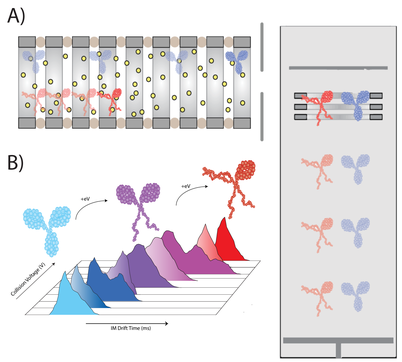
Figure 1 (Right). A cartoon representation of ion mobility being used to elucidate the ensemble of aggregate structures antibodies form as a result of their inherant structural flexibility.



## Methods

Two model IgG1 proteins were analyzed under native and stressed conditions. Aggregation time course experiments were performed under heat (50°C) and pH3 stress conditions. IM-MS and CIU data for IgG1 samples were acquired using both a Synapt G2 q-IM-ToF platform and an Agilent 6560 IM-q-ToF instrument. Collision cross sections (CCSs) were calibrated using standard proteins. Analysis software includes: MassLynx, IM-MS browser, and CIUSuite 2. Computational CCS calculations were performed using 120 IgG1 structures modeled to mimic the solution HOS ensemble. Six archetypical dimer structures were generated using the median solution-phase IgG structure. Monomer and archetypical dimer solution-phase IgG structures were charged (25+ and 40+, respetively) and relaxed in the gas-phase using GROMACS and CCS values were calculated using the trajectory method within Collidoscope. Compact dimers were calculated using IMPACT and then corrected to trajectory method $CCS_{N_2}$ measurements.

Figure 2 (Right). A general ion mobility-mass spectrometry (IM-MS) workflow for the analysis of mAbs. (A) An ion packet is introduced into the IM separator where larger, more extended ions (red) will take longer to traverse the IM region then smaller, more compact ions (blue). We then measure the m/z using a ToF mass analyzer. Collision induced unfolding (CIU) has emerged as a valuable technique for distinguishing subtly different protein structures though their distinct unfolding pathways in the gas phase. (B) We perform CIU by increasing the energy of collisions prior to the IM-MS experiment to measure protein unfolding which is measured by the increase in arrival time distrubtion as a function of said collision voltage (V). Fingerprints can then be contoured into CIU "fingerprints".



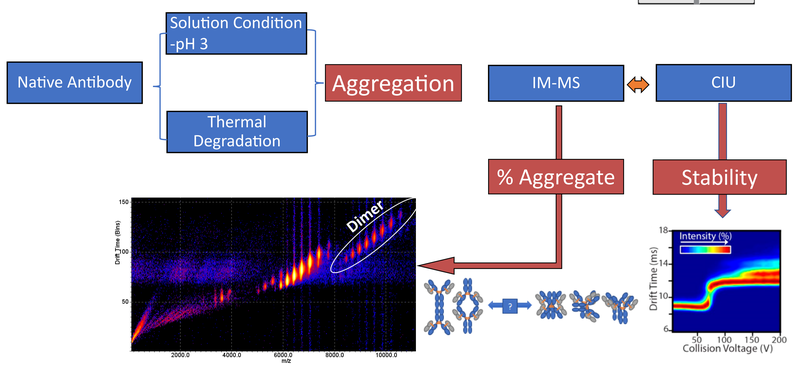




Figure 3. Workflow for the stress experiments to induce aggregation. Native antibodies were subjected to thermal (50°C) and chemical (pH 3) stress conditions. The resulting changes in aggregation and stability was qualitatively and quantiatively evaluated using IM-MS and CIU analysis, respectively. We similiarly calculated CCS values for all aggregates formed under native, heat, and pH stressed conditions.

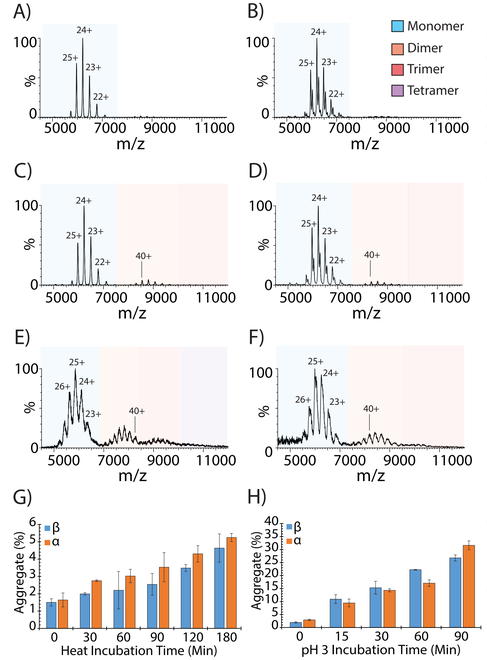



Figure 4 (left). Mass spectrum of native (A) IgG1 α and (B) IgG1 β presenting native charge states and dimer aggregate levels. Mass spectrum of 90-minute heat stressed (C) IgG1 α and (D) IgG1 β presenting native-like charge states and higher dimer aggregate levels. Mass Spectrum of 90-minute pH 3 stressed (E) IgG1 α and (F) IgG1 β with shifted charge states indicating denaturation and significant increases in oligomeric levels. Bar plots of both model antibodies showing increase of aggregate over their time course under (G) heat stress and (H) pH3 conditions.

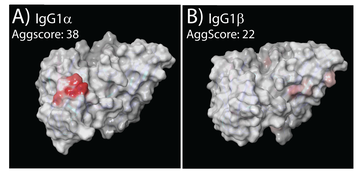
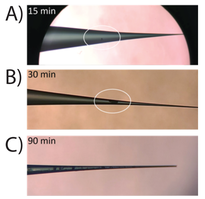



Figure 5 (top). Spatial aggregation propensity (SAP) scores for (A) IgG1α and (B) IgG1β. The higher indicates IgG1β to possess a greater propensity for aggregation via a hydrophobic patch of amino acid residues within its Fab region

Figure 6 (right). ESI emitters showing the increasing levels of insoluble aggregate formation at time-points (A) 15, (B) 30, (C) and 90 minutes for pH 3 conditions. These aggregates led to extensive needle clogging preventing acquisition past the 90-minute time point



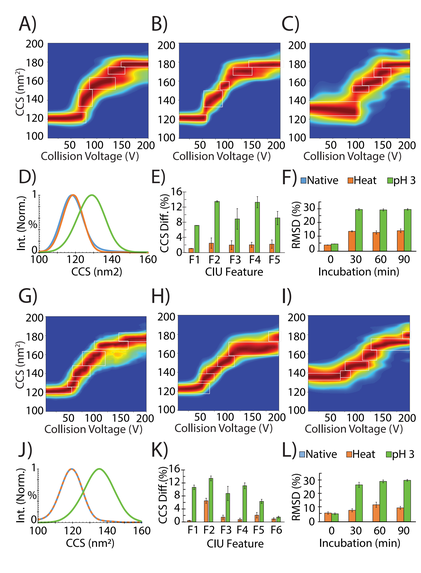
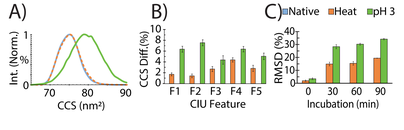




Figure 7 (left). CIU fingerprints of the 40+ charge state of IgG1 α dimer under (A) native, (B) heat stressed, and (C) pH 3 conditions. All fingerprints present five features, but the 2nd and 3rd feature for pH 3 will be combined for purposes of data analysis. (D) IM ATD show a 1% increase in ground state CCS for heat stress and 8% under pH 3 conditions. (E) CIU feature comparison show pH 3 conditions have substantial impacts on CCS for all features, and heat stress show little impact on features. (F) RMSD analysis show global changes in fingerprints throughout the stress time course. CIU fingerprints of the 40+ charge state of IgG1 β dimer under (G) native, (H) heat stressed, and (I) pH 3 conditions. All fingerprints present six features, (J) IM ATD show a <1%% increase in ground state CCS for heat stress and 10% under pH 3 conditions. (K) CIU feature comparison show pH 3 conditions have large impacts on CCS throughout in the early features, and heat stress impact features 2. (L) RMSD analysis show global changes in fingerprints throughout the stress time course.

Figure 8 (bottom). (A) Similiar trends observed in IgG1 β monomer IM ATD show a 1.8% increase in ground state CCS for heat stress and 6% under pH 3 conditions. (E) CIU feature comparison show pH 3 conditions have large impacts on CCS throughout all features, and heat stress have large impacts on later features. (F) RMSD analysis show global changes in fingerprints throughout the stress time course.



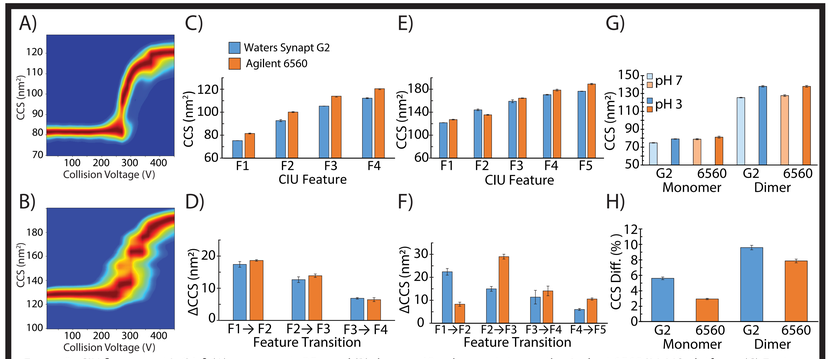
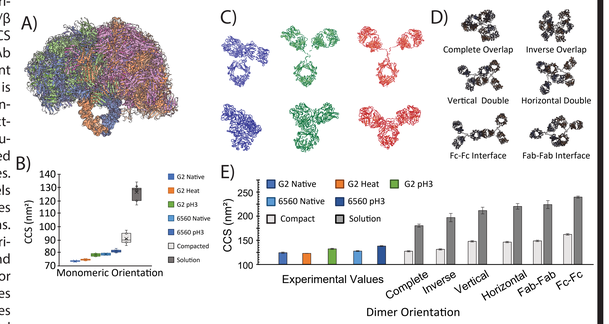




Figure 9 . CIU fingerprint IgG1 β (A) monomeric 25+ and (B) dimeric 40+ charge states on the Agilent 6560 IM-MS platform. (C) Feature analysis of 25+ monomeric IgG1 β between Synapt G2 (blue) and 6560 (orange). The 6560 presents higher CCS for each feature throughout the CIU fingerprint. (D) ΔCCS reveals the change in feature CCS is near systematic between features. (E) Feature analysis of 40+ dimer fingerprints between two IM-MS platforms. Feature 2 for 6560 shows compaction, but otherwise each other feature has a higher CCS compared to the G2. (F) ΔCCS reveals that large differences in feature CCS are related to the shift in feature 2, and that other feature transitions are the same. (G) Comparison of ground state CCS between two platforms for two pH conditions, pH 7 & 3. (H) CCS percent difference between pH 7 & 3 conditions reveal the G2 results in larger ground state unfolding compared to 6560.

Figure 10. (A) Overlay of 120 IgG1 structures demonstrating the flexibility of mAb Fab regions. (B) Box-and-whisker plot with experimental CCS values from IgG1 α/β and Collidoscope calculated CCS values of 120 monomeric mAb structures. These values represent solution phase structures, but it is clear that there is a large discrepancy with experimental and compacted CCS measurements. (C) Solution-phase and compacted gas-phase monomeric structures. (D) Several docked dimeric models built using canonical IgG structures and covering several orientations. (E) CCS measurements of experimental values from IgG1 α/β and Collidoscope CCS calculations for hand-docked solution phases structures. Compacted structures are calculated using IMPACT and further corrected to TM CCS.




## Conclusion and Future Directions

- Antibodies respond differently to different stress conditions
  - α has a higher magnitude response to heat stress
  - β has a higher magnitude response to pH stress
- IM-MS and CIU capture qualitative and quantitative differences between native and stressed antibodies
- Inter-instrument comparisons reveal broad similiarities in data trends
- Computational modelling provides first hypothesize structures for aggragates

## Acknowledgements

IM-MS and CIU method development in the Ruotolo group for antibody aggregation is supported through an AbbVie. Additional support for this project was provided by the Agilent Technologies Thought Leader Award and University Relations grant programs.

## References

(1) Roberts, C. J., et al., Trends Biotechnol. 2014, 32 (7), 372–380.
(2) Moussa, E. M., et al., J. Pharm. Sci. 2016, 105 (2), 417–430.
(3) Paul, R., et al., Pharm. Res. 2012, 29 (8), 2047–2059.
(4) Polasky, D. A., et al., Anal. Chem. 2019, 91, 3147–3155
(5) Vallejo, D. D., et al., Anal. Chem. 2019, 91 (13), 8137–8146.