

# Ion mobility-mass spectrometry and collision induced unfolding of designed bispecific antibody therapeutics

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**ABSTRACT:** Bispecific antibodies (bsAbs) represent a critically important class of emerging therapeutics capable of simultaneously two different antigens simultaneously. As such, bsAbs have been developed as effective treatment agents for diseases that remain challenging for conventional monoclonal antibody (mAb) therapeutics to access. Despite this, bsAbs are intricate molecules, requiring both the appropriate engineering and pairing of linked heavy and light chains derived from separate parent mAbs. Current analytical tools for tracking the bsAb construction process have demonstrated a limited ability to robustly probe the higher order structure (HOS) of bsAbs. Native ion mobility-mass spectrometry (IM-MS) and collision induced unfolding (CIU) have proven to be useful tools in probing the HOS of mAb therapeutics. In this report, we describe a series of comprehensive IM-MS and CIU datasets that reveal HOS details associated with knob-into-hole (KiH) bsAb model system. We find that quantitative analysis of CIU data indicates that global bsAb stability occupies and intermediate space between the stabilities recorded for its parent mAbs. Furthermore, our CIU data identifies the hole-containing half of the bsAb construct to be least stable, thus driving much of the overall stability of the bsAb. An analysis of both intact bsAb and enzymatic fragments allows us to link the first and second CIU transitions observed for intact bsAbs to the Fab and Fc domains, respectively. This result is likely general for CIU data collected from low charge state mAb ions and is supported by data acquired for deglycosylated bsAb and mAb constructs, each of which indicate greater destabilization of the second CIU transition observed in our data. When integrated, our CIU analysis allows us to link changes in the first CIU transition primarily to the Fab region of the hole-containing halfmer, while the second CIU transition is likely strongly connected to the Fc region of the knob-containing half of the bsAb construct. Taken together, our results provide an unprecedented roadmap for evaluating the domain-level stabilities and HOS of both bsAbs and mAb constructs using CIU.

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## Introduction

Bispecific antibodies (bsAbs) have become promising therapeutic modalities since they merge the specificities of two different monoclonal antibodies (mAbs). This unique quality of bsAbs not only permits the targeting of two distinct epitopes or antigens simultaneously, but it also paves the way for innovative functionalities that are unattainable with conventional mAb-based therapeutics. Examples of such treatment strategies include the redirection and activation of immune effector cell cytotoxic activity to specifically eradicate tumor cells,<sup>1-3</sup> the selective inhibition of enzymes across the blood brain barrier responsible for amyloid- $\beta$  (A $\beta$ ) peptide production,<sup>4,5</sup> and the dual targeting and neutralization of two independent growth factors associated with neovascular eye diseases.<sup>6,7</sup> These therapeutic capabilities have led to the market approval of two bsAbs by the US Food and Drug Administrator, while well over 100 bsAbs are currently in clinical development.<sup>8</sup>

Advancements in antibody engineering and development have cultivated many different commercialized technology platforms within pharmaceutical and biotechnology companies for novel bsAb construction.<sup>9</sup> These technologies have led to the generation of primarily two different architectural classes of bsAbs: 1) fragment-based formats that join various antigen-binding moieties into one entity without a fragment crystallizable (Fc) region and 2) immunoglobulin G (IgG)-like molecules containing two different

fragment antigen-binding (Fab) regions connected by an Fc domain. In most cases, the later 'IgG format' for bsAbs exhibits greater solubility, stability, and plasma half-life when compared to other constructs due to its large size and resistance to catabolism by the neonatal Fc receptor, FcRn.<sup>10</sup> However, the production of bsAbs using this format is challenging since dual specificity is dependent on the co-expression of variable domains from two separate light chains and two separate heavy chains. Therefore, one of the major challenges is the chain association issue, where different chain combinations can theoretically result in 10 different antibodies of which only one matches the target functional bsAb.<sup>9,11</sup>

Protein engineering approaches, such as the "knobs-into-holes" (KiH) concept, have been developed to address the bsAb chain association problem and enforce the proper heterodimerization of the engineered heavy chains. This design strategy involves the mutation of amino acid residues at the interface between C<sub>H</sub>3 domains of each heavy chain, where targeted residues are replaced bulkier amino acids in the "knob" variant and smaller amino acids in the "hole" variant.<sup>12</sup> However, the characterization of bsAb therapeutics extends beyond their proper assembly. Compared to conventional small-molecule drugs, antibody therapeutics are large (~150 kDa), exhibiting significantly more structural complexity and heterogeneity than small molecule drugs. Additionally, changes in higher order structure (HOS)

caused by KiH engineering, post-translational modifications, or degradation can result in the inhibition of molecular binding, an increased potential for immunogenicity, and a higher rate of aggregation.<sup>13-16</sup> Therefore, a thorough characterization of antibody HOS is crucial not only to define bsAb structure-function relationships but also to ensure stability, efficacy, and safety of the eventual therapeutic.<sup>17,18</sup>

A comprehensive range of biophysical techniques have been well-established for the characterization of mAb therapeutic HOS and stability. Tools such as circular dichroism (CD),<sup>19</sup> differential scanning calorimetry (DSC),<sup>20</sup> and size exclusion chromatography (SEC)<sup>21</sup> offer a global perspective on mAb stability and conformation, but lack the resolution needed to distinguish subtle conformational changes. By contrast, techniques such as nuclear magnetic resonance (NMR) spectroscopy,<sup>22</sup> X-ray crystallography,<sup>23</sup> and cryogenic electron microscopy (cryo-EM)<sup>24</sup> provide atomically resolved structural information, but typically require extensive sample preparation, large quantities of homogenous sample, lengthy data acquisition times, and complex data interpretation.<sup>25</sup> Currently, no full-length IgG-like bsAb crystal structures are publicly available, and only bsAb fragment structures have been reported.<sup>26,27</sup>

Recently, native ion mobility-mass spectrometry (IM-MS) has emerged as a useful structural biology tool capable of probing the HOS of mAb therapeutics from a few micrograms of sample in the presence of structural heterogeneity and impurities. IM separates gas-phase protein ions based on their charge and rotationally averaged collision cross sections (CCSs) on the millisecond timescale.<sup>28</sup> When coupled with MS, two ions of the same mass-to-charge ratio but different CCSs can be readily distinguished. IM-MS measurements have been shown to monitor the dynamics of bsAb formation resulting from Fab-arm exchange (FAE),<sup>29</sup> resolve disulfide structural isoforms of IgG2 mAbs,<sup>30</sup> and assess antibody-drug conjugate (ADC) structural heterogeneity.<sup>31</sup> Moreover, collision induced unfolding (CIU) has enabled IM-MS to synchronously provide both protein structure and conformational stability information.<sup>32</sup> In CIU experiments, protein ions are collisionally-heated prior to IM separation to elicit protein unfolding in the gas-phase. CIU has discriminated differences based on disulfide patterns,<sup>33</sup> glycosylation levels,<sup>34</sup> and drug conjugation.<sup>35,36</sup> CIU has also probed a bsAb formed via FAE of wt-IgG4s, highlighting that such bsAb can retain structural information from both its parent mAbs.<sup>37</sup> Despite past progress, quantitative CIU has yet to be deployed in order to evaluate extensively engineered bsAb modalities, such as KiH constructs, and the individual contributions of parent mAbs to bsAb HOS remain unclear.

In this report, we extend the capabilities of native IM-MS and CIU to robustly characterize the HOS of a model KiH bsAb and its parental 'knob' and 'hole' homodimer (HD) mAbs. We define biophysical connections between these constructs by quantifying the global differences observed between CIU fingerprints and comparing their stabilities. We further convert the bsAb and its parent mAbs into fragments of various types to evaluate their domain-level stabilities. By comparing the stabilities of isolated domains, we can localize contributions from each parent mAb in our bsAb CIU transitions. After enzymatically removing N-

linked glycans from the Fc regions of our constructs, we find evidence of destabilization occur primarily in higher-energy CIU transitions, permitting us to pinpoint the unfolding of the Fc region in our CIU fingerprints. We conclude by discussing the potential impact of native IM-MS and CIU workflows on the HOS characterization of bsAbs assembled using other technology platforms.

### Experimental Section

**Sample Preparation.** BsAb heterodimer (10 mg/mL) and "Knob" and "Hole" homodimer mAbs (2 mg/mL) were produced, purified, and formulated in their respective formulation buffer at Bristol Myers Squibb (New Brunswick, NJ). Glycerol-free PNGase F (500,000 units/mL) was purchased from New England Biolabs (Ipswich, MA). Papain from papaya latex was supplied as a buffered aqueous suspension and acquired from Sigma Aldrich (St. Louis, MO).

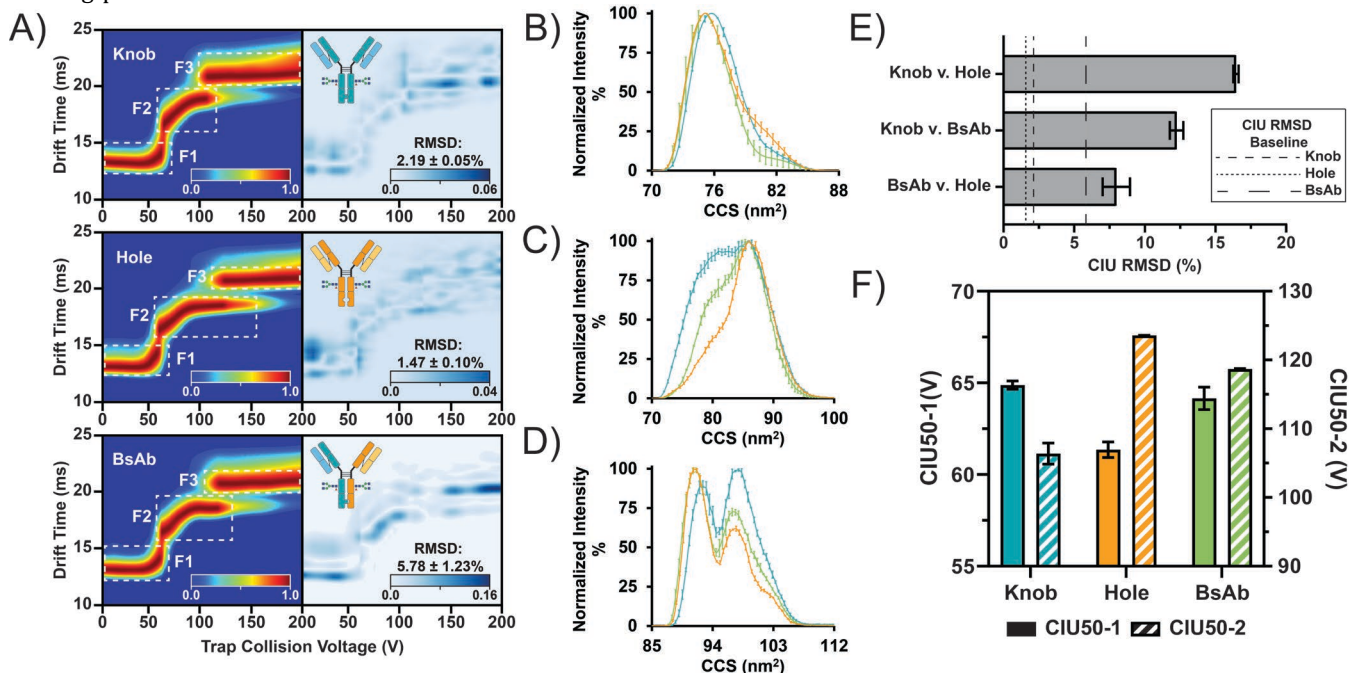
All native, unmodified samples were buffer exchanged into 200 mM ammonium acetate (pH 6.8 – 7.0) using Micro Bio-Spin P-6 columns (Bio-Rad, Hercules, CA) and diluted to working concentrations of 1 mg/mL (~6.7  $\mu$ M). Removal of N-glycans from antibody constructs was achieved using PNGase F under non-denaturing conditions overnight per the vendor's recommended protocol. A control without PNGase F was incubated concurrently. For the papain digestion of BsAb, papain was first activated with 5 mM cysteine for 30 min at 37°C. Excess cysteine was then removed by buffer exchanging the activated papain into digestion buffer (200 mM ammonium acetate, 5 mM EDTA, pH 6.8 – 7.0) using Micro Bio-Spin P-6 columns. BsAb (1 mg/mL) sample was buffer exchanged into digestion buffer followed by the addition of activated papain to achieve a 1:100 (papain:BsAb) ratio. The digest was then incubated for 2 hr at 37°C to generate intact Fab and Fc fragments. A control without papain was also incubated to track potential disulfide bond reduction caused by any residual cysteine in the digestion buffer. Little to no disulfide bond reduction was observed. All deglycosylated and papain-digested samples were quenched on ice and immediately buffer exchanged into 200 mM ammonium acetate (pH 6.8 – 7.0) using Bio-Spin P-6 columns.

**Native MS Analysis.** High resolution native MS experiments were performed on a standard commercial Q Exactive Orbitrap MS with Ultra High Mass Range (UHMR) platform (Thermo Scientific, San Jose, CA). Samples were further diluted to ~2  $\mu$ M before analysis. Sample (~3  $\mu$ L) was transferred to a gold-coated borosilicate capillary needle (prepared in house), and ions were generated via direct infusion using a commercial Thermo Fisher Nanospray Flex Ion Source (ES071) with a static nanospray ionization (NSI) probe operated in positive ion mode. Capillary voltages were held at 1.1 – 1.2 kV, and the inlet capillary was heated to 275°C. Nitrogen was used as the collision gas, and the trapping pressure was set to 3. Low  $m/z$  detector optimization and high  $m/z$  transfer optics were used. In-source trapping was enabled with the desolvation voltage fixed at -50 V for improved ion transmission and efficient salt adduct removal. Transient times were set at 128 ms (resolution of 25,000 at  $m/z$  400). Mass spectra were processed and deconvoluted using UniDec software.<sup>38</sup>

**Native IM-MS and CIU.** IM-MS and CIU experiments were performed using a quadrupole-ion mobility-time-of-flight mass spectrometer (Q-IM-ToF-MS) instrument

(Synapt G2 HDMS, Waters, Milford, MA). Sample (~3  $\mu$ L) was transferred to a gold-coated borosilicate capillary needle, and ions were generated by direct infusion utilizing a nano-electrospray ionization (nESI) source set to the positive mode. The nESI capillary was operated at voltages of 1.1 – 1.5 kV. For intact and halfmer species, the sampling cone was operated at 40 V, while for Fab and Fc fragments, it was operated at 20 V to prevent in-source activation. The backing pressure was set to ~7.3 to 7.5 mbar. The helium

cell flow rate was operated at 200 mL/min and pressurized to  $1.4 \times 10^{-3}$  mbar. The trap travelling wave ion guide was pressurized to  $4.9 \times 10^{-2}$  mbar of argon gas. The traveling-wave IM separator was operated at a pressure of ~3.4 mbar, and IM separation was achieved with a travelling wave height and velocity of 40 V and 600 m/s, respectively. The ToF-MS was operated over an  $m/z$  range of 1000 – 12,000 at a pressure of  $2.3 \times 10^{-6}$ . Ions were subjected



**Figure 1.** CIU experiments of native, unmodified knob and hole homodimers and bsAb heterodimer. (A) Averaged CIU fingerprints ( $n = 3$ ) for the 24+ charge state (left) with corresponding replicate RMSD baselines (right). All fingerprints show three main features indicated by the dashed white boxes. Normalized  $^{TW}CCS_{N2}$  distributions at (B) 5 V, (C) 65 V, (D) 110 V. At lower activation potentials, all antibodies adopt similar CCS distributions, which significantly diverge at higher acceleration potentials. (E) Pairwise RMSD analysis reveals global HOS differences among antibodies compared to replicate RMSD baselines (dashed lines). (F) CIU50 analysis illustrates how bsAbs adopt a stability between that of the knob and hole parent homodimers.

to collisions in the traveling-wave ion trap prior to IM separation to perform CIU. For intact BsAb, Knob, and Hole constructs, tandem-MS was utilized to select charge state 24+. For ions corresponding to halfmer, charge state 16+ was selected. The collisional voltages were then ramped from 5 V to 200V in 5 V intervals to construct each CIU fingerprint. For Fab and Fc fragment measurements, charge state 13+ was selected, and collision voltages were ramped from 5 V to 140 V in 5 V increments since collisional activation above 140 V resulted in fragmentation. Polyalanine ions, bovine serum albumin, and glutamate dehydrogenase were used as CCS calibrants. All data collection was performed in triplicate.

IM and MS data were viewed using DriftScope and Masslynx V4.1 software, respectively (Waters, Milford, MA). Mass spectra were deconvoluted using UniDec software.<sup>38</sup> Drift times were extracted at each collision voltage using TWIMExtract (v1.5).<sup>39</sup>  $^{TW}CCS_{N2}$  calculations were performed using IMSCal software.<sup>40</sup> Extracted drift time data were then analyzed using a home-built software package, CIUSuite 2 (v2.2).<sup>41</sup> CIU fingerprints were 2-D smoothed with a Savitzky-Golay function with a smoothing window of 5 and 2 smoothing iterations. The collision voltage axis was interpolated with an axis scaling factor of 2 for intact constructs

and 4 for halfmers and Fab and Fc fragments. Standard feature detection was performed using a minimum feature length of 3 steps, an allowed width of 0.75 drift time axis units, and a maximum CV gap length of 0. CIU<sub>50</sub> values were then assigned using max centroiding mode with a transition region padding of 15 CV and a maximum CV gap length of 0. CIU<sub>50</sub> represents the collision voltage at which 50% of a more compact state of the antibody transitions to a more unfolded state, and it defines the midpoint between adjacent features. Root-mean-square-deviation analysis (RMSD) analysis was performed using the compare function in CIUSuite 2. All CIU fingerprints shown are the average of three replicates.

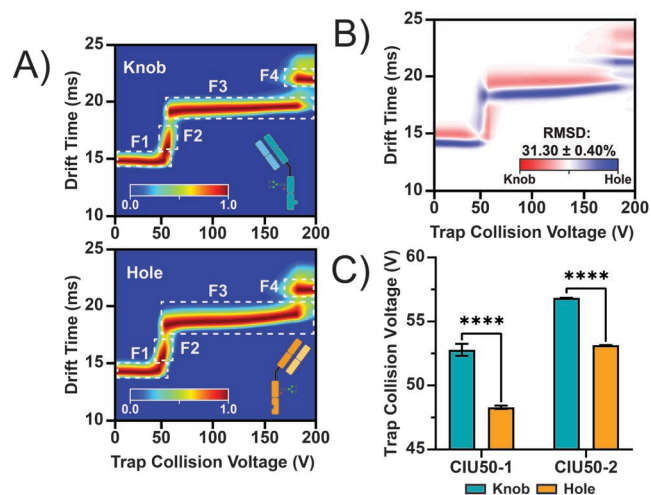
## Results and Discussion

**Native IM-MS and CIU of Knob, Hole, and BsAb.** The native IM-MS spectra of Knob, Hole, and BsAb reveal various ion populations corresponding to intact homodimer and heterodimer, as well as low and high molecular weight (MW) species (Figure S1A). Knob and Hole spectra show high abundances of halfmers, highlighting that KiH (Knob: T366W, Hole: T366S, L368A, Y407V) mutations impede efficient halfmer dimerization into homodimer. IM-MS data for BsAb, on the other hand, showed very minimal halfmer populations, revealing the effective association between

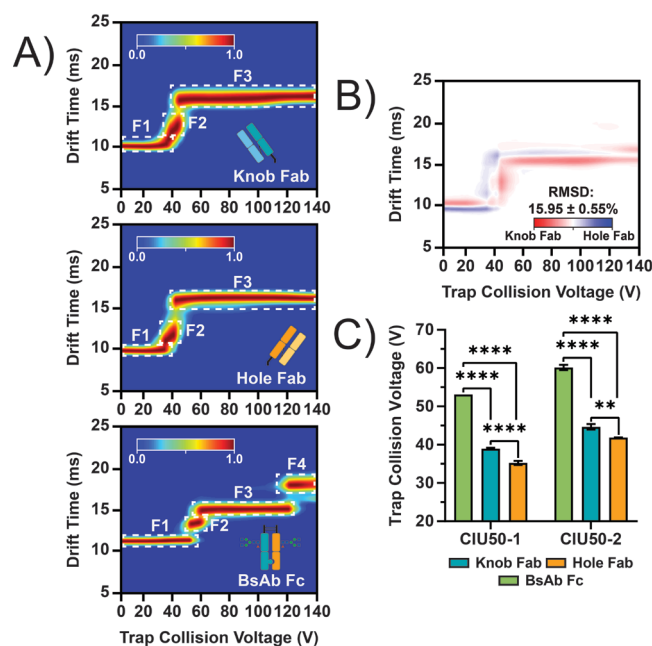
Knob and Hole halfmers. All intact antibodies present narrow charge state distributions ( $21^+ - 26^+$ ), indicating the preservation of native-like structural information in the gas-phase compared to denatured species.<sup>42-44</sup> Moreover, all glycosylation sites are conserved for each antibody, and averaged deconvoluted masses include all glycoforms such as those commonly found in standard mAbs (Figure S2B).

To further probe the HOS of Knob, Hole, and BsAb, we performed CIU on a range of charge states ( $22^+ - 24^+$ ). Previous studies have indicated that ion charge state influences the number of transitions in its CIU fingerprint.<sup>45,46</sup> As expected, the lowest charge state,  $22^+$ , requires higher acceleration voltages to unfold, giving rise to fewer CIU intermediates when compared to more highly-charged ions (Figure S2). We observe three main CIU features for  $23^+$  and  $24^+$  bsAb ions, where the intensity of the most unfolded feature detected at higher collision voltages is most prominent in the later charge state. For our analyses described below, we chose to focus on  $24^+$  ions due to their large relative intensities and more prominent third CIU features. Globally, our analysis reveals both similarities and differences across the parent mAbs and bsAb studied here (Figure 1). Notably, all antibodies undergo similar number of CIU transitions. Technical replicates produce baseline RMSDs of  $< 6\%$  indicate a consistent, reproducible CIU data for all samples (Figure 1A). To better quantify differences among our intact mAb and bsAb constructs, we first utilized the classification workflow within CIUSuite2 to select collision voltages that exhibit the greatest differences in arrival time distributions (ATDs) between CIU fingerprints.<sup>41,47</sup> Conversion of IM drift times to  $^{TW}CCS_{N2}$  values indicates occupy broad CCS ensembles that are essentially indistinguishable by IM-MS alone (Figure 1B). However, CCS data captured at collision voltages of 65 V (Figure 1C) and 110 V (Figure 1D), reveal significantly different distributions of  $^{TW}CCS_{N2}$ . Interestingly,  $^{TW}CCS_{N2}$  values recorded for the bsAb construct seem similar to those produced by knob mAb CIU at 65V, but switch to produce a CCS distribution similar to hole mAb CIU data at 110V, providing early evidence suggesting that bsAb stability is discreetly connected to both parent mAbs.

To build upon these observations, we performed a pairwise RMSD analyses between CIU data recorded for all antibodies in order to probe the global differences in mAb structures (Figure 1E). Comparing knob and hole homodimers yields an RMSD of  $16.45 \pm 0.19\%$ , indicating significant differences in global mAb HOS. When CIU data collected for knob and hole homodimers are compared to bsAb data, however, we observe decreased RMSD values. Specifically, a comparison between knob homodimer and the bsAb CIU fingerprints produces an RMSD of  $12.25 \pm 0.48\%$ , while a comparison between hole homodimer and the bsAb produces an RMSD of  $7.98 \pm 0.96\%$  (Figure S3). These comparisons reveal that the bsAb CIU data, and by extension bsAb global stability, most closely resembles that of the hole homodimer. Next, we shifted our focus to evaluating differences in CIU<sub>50</sub> based stability values between knob, hole, and bsAb. CIU<sub>50</sub> analyses of knob, hole, and bsAb constructs indicate that bsAb stability can indeed be characterized as intermediate between



**Figure 2.** CIU data recorded for knob and hole halfmers. (A) Averaged CIU fingerprints ( $n = 3$ ) for  $16^+$  charge states. (B) Pairwise RMSD analyses reveal significant differences between halfmer HOSs. (C) CIU<sub>50</sub> analysis shows significant differences (\*\*\*\*  $p < 0.0001$ ) in protein stabilities.



**Figure 3.** CIU data recorded for Fab and Fc fragments. (A) Averaged CIU fingerprints ( $n = 3$ ) for  $13^+$  charge states. (B) Pairwise RMSD analysis reveals significant differences between knob and hole Fab fragments. (C) CIU<sub>50</sub> analysis indicates significant differences (\*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ ) in fragment stabilities.

its parent homodimers if initial protein unfolding is used as the primary method of evaluating protein stability. Specifically, CIU<sub>50</sub>-1 values, which correspond to the first CIU transition recorded in our fingerprints, indicate that the hole homodimer ( $61.36 \pm 0.42$  V) is less stable than both the knob homodimer ( $64.88 \pm 0.23$  V) and bsAb ( $64.16 \pm 0.62$  V) (Figure 1F). Conversely, CIU<sub>50</sub>-2 values, which are linked to the second, higher-energy CIU transition detected in our experiments, reveal that the knob homodimer ( $106.38 \pm 1.52$  V) unfolds at collision voltages lower than those of both the hole homodimer ( $123.57 \pm 0.02$  V) and bsAb ( $118.67 \pm 0.07$

V). Taken together, these results suggest that the CIU<sub>50-1</sub> and CIU<sub>50-2</sub> values recorded in our fingerprints are likely reporting on the local stabilities of different domains within the bsAb and mAb constructs studied here.

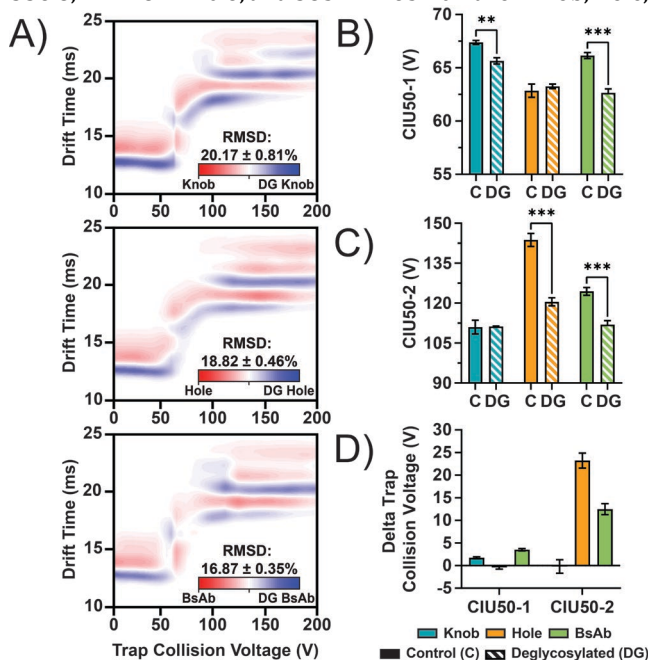
**Middle-level CIU reveals Knob and Hole contributions to BsAb HOS.** To better assign regions of the bsAb to specific CIU features, we performed CIU experiments targeting mAb and bsAb fragments generated under native conditions. First, we recorded CIU data for 16+ knob and hole halfmers (the 75kDa light/heavy chain construct corresponding to half of the homodimers probed above, Figure 2). In these fingerprints, we observe four main CIU features across both constructs, but RMSD analysis reveals significant differences their native-like structures ( $31.30 \pm 0.40\%$ ) when compared baseline RMSD values recorded for technical replicates ( $3.02 \pm 0.54\%$  for knob and  $1.48 \pm 0.23\%$ , hole halfmers). CIU<sub>50</sub> analysis further reveals that knob halfmers are significantly more stable than their hole counterparts across the two unfolding transitions observed. These differences in stability correlate strongly with our prior measurements of intact hole and knob homodimer CIU<sub>50-1</sub> values.

To continue our efforts to assign CIU features observed in the intact bsAb to specific domains or regions within its structure, we performed CIU on the Fab and Fc fragments generated via papain digestion, which cleaved above the bsAb hinge to produce a hole Fab, a knob Fab, and a chimeric Fc (Figure S4). CIU fingerprints for Fab fragments reveal three CIU features for both knob and hole Fab fragments (Figure 3). CIU of the chimeric Fc domain, on the other hand, contains four CIU features. Technical replicates recorded for knob Fab, hole Fab, and chimeric Fc fragments yield RMSD baselines of  $2.08 \pm 0.28\%$ ,  $1.41 \pm 0.21\%$ , and  $4.85 \pm 0.70\%$ , respectively. Although knob and hole Fabs share qualitatively similar CIU fingerprints, difference analysis reveals an RMSD of  $15.59 \pm 0.55\%$ , indicating that these fragments differ greatly in terms of their HOS. Furthermore, a stability assessment of these fragments quantitatively assigns the knob Fab is significantly more stable than the Hole Fab, across both CIU<sub>50-1</sub> and CIU<sub>50-2</sub> values recorded (Figure 3C). Interestingly, our data also indicate that the Fc domain is significantly more stable than both of the bsAb Fab domains. It is likely that this difference in stability is related to the strong non-covalent interactions that exist between the C<sub>H</sub>3 domains within each heavy chain, as well as the knob and hole mutations promoting heavy chain dimerization.<sup>48-50</sup>

**Removal of N-glycans impact Knob, Hole, and BsAb conformation and stability.** Previous work from our group has demonstrated that CIU can detect the influence of different levels of glycosylation on both intact mAb and mAb fragment stabilities.<sup>33,34</sup> Specifically, the removal of glycans has been shown to decrease the thermal stability of C<sub>H</sub>2 domains within the mAb Fc region.<sup>51</sup> Therefore, to better assign CIU transitions corresponding to the unfolding of the Fc domain of bsAbs, we removed N-linked glycans from the C<sub>H</sub>2 domains of knob, hole, and bsAb constructs, and probed their resulting stabilities using CIU.

To better track the progress of our deglycosylation reactions, we performed high-resolution native orbitrap MS experiments in order to resolve individual mAb and bsAb glycoforms both prior to and after the addition of PNGase F.

Our results provide evidence for a wide range of glycoforms for knob, hole, and bsAb constructs. Notably, bsAb exhibits greater heterogeneity than the mAbs studied here, containing at least 12 different glycoforms (Figure S5). Specifically, we not only observe mass shifts of 162 Da, corresponding to hexose, but also shifts of 291 Da, corresponding to either a N-acetyl neuraminic acid (Neu5Ac) or a sialic acid. In contrast, knob and hole homodimers glycoforms differ primarily by 162 Da, with the knob homodimer native MS containing a single 291 Da shift in the mAb glycoforms detected. Glycan removal results in average mass shifts of  $4362.4 \pm 336.8$ ,  $2121.8 \pm 210.6$ , and  $3854.7 \pm 654.0$  Da for Knob, Hole,



**Figure 4.** CIU of deglycosylated knob, hole, and bsAb 24+ ions ( $n = 3$ ). (A) RMSD analysis comparing control and deglycosylated antibodies reveals significant differences in HOS. (B) CIU<sub>50-1</sub> and (C) CIU<sub>50-2</sub> analysis reveals significant destabilization of antibodies after deglycosylation (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). (D) Plot of differences in CIU<sub>50</sub> values between control and deglycosylated knob, hole, and bsAb.

and BsAb constructs, respectively, all in line with expectations for PNGase F declysylation.<sup>34</sup> The variability observed in the mAb or bsAb mass lost following PNGase F treatment are a reflection of the unique glycosylation patterns observed for each antibody studied here.

CIU experiments for both control and deglycosylated antibodies reveals the same number of features as those observed in our initial CIU data, alongside clear shifts in protein stability. RMSD analysis reveals significant differences between control and deglycosylated forms ( $\sim 17 - 20\%$ ) compared to baseline RMSDs of  $< 4\%$  (Figure 4A). Pre-CIU IM drift times for deglycosylated mAbs and bsAbs are universally shorter than those produced by control (glycosylated) antibodies, and thus produce CIU intermediates that are similarly shifted. Interestingly, an RMSD analysis comparing deglycosylated knob, hole, and bsAb constructs reveals that the deglycosylated bsAb most closely resembles the deglycosylated hole homodimer (Figure S6), a result that agrees well with our initial CIU experiments on the

glycosylated forms of these antibodies discussed above (Figure 1).

A quantitative analysis of CIU<sub>50</sub> data reveals an asymmetric effect on protein stability upon the removal of glycans from each antibody (Figure 4 B,C). CIU<sub>50-1</sub> values point to a significant decrease in gas-phase stability for deglycosylated knob homodimers ( $65.65 \pm 0.28$  V) and bsAb ( $62.65 \pm 0.37$  V) when compared to controls ( $73.35 \pm 0.36$  V and  $66.15 \pm 0.28$  for knob homodimer and bsAb, respectively). Deglycosylated hole homodimers, on the other hand, show no detectable change in CIU<sub>50-1</sub>. In comparison, our CIU<sub>50-2</sub> results point to a significant decrease in stability for deglycosylated hole homodimers ( $120.50 \pm 1.53$  V) and bsAb ( $111.94 \pm 1.46$  V) compared to controls ( $143.73 \pm 2.44$  V and  $124.41 \pm 1.49$  V for hole homodimer and bsAb, respectively), with no change observed in knob CIU<sub>50-2</sub>. By plotting the differences in CIU<sub>50</sub> recorded between our control and deglycosylated antibodies, we can delineate which CIU transition is most affected by deglycosylation (Figure 4D). Overall, we observe greater differences in CIU<sub>50-2</sub> when compared to CIU<sub>50-1</sub>, with the later transition shifting <5V (maximally ~6%) across all constructs and the former generating shifts of 10-25V (maximally ~17%) for bsAb and hole antibodies. In contrast to this general trend, our CIU data for the knob homodimer does not exhibit strong shifts in CIU<sub>50</sub> for either transition upon deglycosylation. We note that the intact knob construct exhibits a destabilized CIU<sub>50-2</sub> when compared with both bsAb and hole constructs, as discussed above. Furthermore, x-ray data collected for knob-knob Fc fragments have indicated destabilization of this domain upon glycosylation due to a change in the relative orientation of C<sub>H</sub>3 domains provoked by the Knob point mutation.<sup>52</sup>

### Conclusions

Here, we demonstrate the capabilities of IM-MS in combination with CIU to establish HOS connections between a model KiH bsAb and its parent homodimer mAbs. We found CCS alone to be insufficient to resolve the subtle HOS differences between the antibodies studied here. In contrast, CIU fingerprints permitted us to resolve and differentiate each iso-cross-sectional antibody. Overall, our findings indicate that KiH bsAbs adopt an intermediate stability profile when compared to parent knob and hole homodimers. Quantitative CIU identifies the hole homodimer as most closely resembling the fingerprint recorded for the bsAb, an observation that is driven primarily on the similarity of the early stages of CIU for both antibodies. Conversely, CIU data collected for the knob homodimer adopts stronger similarity to the bsAb at higher acceleration potentials.

Importantly, our analysis of bsAb and its parent mAbs has enabled us to assign many details surrounding mAb and KiH bsAb CIU for the first time. By studying mAb and bsAb fragments, alongside deglycosylated constructs, our data strongly point to the first and second CIU transitions of KiH bsAbs, and mAbs in general, to be related to Fab and Fc domain unfolding, respectively. In particular, the CIU profiles recorded for Fab fragments in comparison to liberated chimeric Fc domains indicated lesser stabilities for the Fab domains in a manner that reflected their relative stabilities in larger constructs (e.g. halfmers and homodimers). The larger stability of the Fc, in combination with the larger influence of deglycosylation CIU<sub>50-2</sub>, supports the notion that

higher-energy CIU transitions in bsAbs and mAbs are related to Fc unfolding, while lower energy transitions are connected to Fab CIU. A greater degree of granularity can be assigned to bsAb CIU than the generalities detailed above, which we argue are likely applicable to all mAb CIU collected for ions having sufficiently for low charge states. For KiH bsAbs specifically, our results indicate that the lower energy, Fab dominated, unfolding event is likely driven by the stability of the hole Fab domain, which all of our CIU data indicates is the least stable element of the bsAb construct, and subsequent higher-energy CIU transitions are related to CH<sub>2</sub> and CH<sub>3</sub> domains within the Fc domain of the knob. This latter assignment is supported by X-ray data that suggests deglycosylation greatly destabilizes its modified CH<sub>2</sub> domains.<sup>52</sup>

Our ability to interpret the details of mAb CIU, coupled with our enhanced understanding of the biophysical underpinnings of KiH bsAb stability, has the potential to provide critical information in support of bsAb discovery and development. Future work in our group aims to further develop the IM-MS and CIU workflows in order to rapidly probe the suitability and HOS details of bsAb construction. For example, we envision combined native MS, IM-MS and CIU, available in a high-throughput mode, to reveal changes in bsAb conformational dynamics and stability provoked changes in protein sequence, enabling us to relate HOS changes to overall bsAb fitness for subsequent development efforts.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

IM-MS spectra and theoretical and experimental masses of Knob, Hole, and BsAb; CIU fingerprints for 22<sup>+</sup> - 24<sup>+</sup> ions of intact Knob, Hole, and BsAb; RMSD plots of 24<sup>+</sup> ions; papain digestion cleavage site, papain digest IM-MS spectra, and theoretical and experimental masses of papain generated fragments; high-resolution spectra of Knob, Hole, and BsAb; CIU fingerprints and RMSD analysis of deglycosylated 24<sup>+</sup> ions.

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B.T.R., T.S., G. C., and L.T. designed and conceived of the experiments described. R.V.V. collected Synapt G2 and UHMR data and drafted the manuscript. B.T.R and R.V.V. analyzed all data. H.L. assisted in collecting and analyzing Synapt G2 data. T.S., N.C., G.G., and L.T. provided all antibodies used in this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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