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Towards automation of Collision Induced Unfolding experiments through online Size Exclusion Chromatography coupled to native Mass Spectrometry.

Evolène Deslignière¹, Anthony Ehkirch¹, Thomas Botzanowski¹, Alain Beck², Oscar Hernandez-Alba¹, Sarah Cianférani^{1*}

¹ Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC UMR 7178, 67000 Strasbourg, France.

² IRPF - Centre d'Immunologie Pierre-Fabre (CIPF), 74160 Saint-Julien-en-Genevois, France.

*Corresponding author: Sarah Cianférani. Email: sarah.cianferani@unistra.fr

ABSTRACT: Ion mobility-based collision induced unfolding (CIU) has gained increasing attention to probe gas-phase unfolding of proteins and their noncovalent complexes, notably for biotherapeutics. CIU detects subtle conformational changes of proteins and emerges as an attractive alternative to circumvent poor IM resolution. However, CIU still lacks in automation for buffer exchange and data acquisition, precluding its wide adoption. We present here an automated workflow for CIU experiments, from sample preparation to data interpretation using online size exclusion chromatography coupled to native ion mobility mass spectrometry (SEC-CIU). Online automated SEC-CIU experiments offer several benefits over nanoESI-CIU, among which i) improved and fast desalting compared to manual buffer exchange used for classical CIU experiments; ii) drastic reduction of the overall data collection time process along with iii) maintaining the number of unfolding transitions. We then evaluate the potential of SEC-CIU to distinguish monoclonal antibodies (mAbs) subclasses, illustrating the efficiency of our method for rapid mAb subclass identification at both intact and middle levels. Finally, we demonstrate that CIU data acquisition time can be further reduced either by setting up a scheduled CIU method relying on diagnostic trap collision voltages or by implementing mAbs-multiplexed SEC-CIU analyses to maximize information content in a single experiment. Altogether, our results confirm the suitability of SEC-CIU to automate CIU experiments, particularly for the fast characterization of next generation mAb-based products.

INTRODUCTION

Native mass spectrometry (MS) is now broadly used in structural biology to characterize proteins, protein-protein complexes and protein-ligands interactions¹. In the last decade, ion mobility (IM) combined with mass spectrometry has grown into a valuable asset for the study of proteins and noncovalent complexes^{2, 3} mainly due to the implementation of IM, notably travelling wave ion mobility spectrometry⁴ (TWIMS), in commercially available mass spectrometers. TWIMS separates ions in the gas-phase based on their size, shape and charge under the influence of a low electric field. This new dimension provides structural information through arrival time distribution (ATD) which can be converted into rotationally averaged collision cross sections, the latter being related to the global conformation of the ion⁵. However, IM fails to differentiate co-drifting species with closely related conformations due to its low resolution. Collision induced unfolding (CIU) approaches help to assess ion gas-phase unfolding, and appear as a promising alternative to circumvent poor IM separation⁶⁻⁸. In TWIMS instruments, CIU experiments are performed by raising

collision voltages (CVs) in the trap cell before IM separation, which induces further ion activation by energetic collisions with a trapping gas. As the accelerating voltage is increased, collisions become more energetic, leading to a build-up of internal energy in the ions⁶. Hence, ions may cross energy barriers and transition through a series of conformational intermediates in which the size of the ion can increase or decrease compared to the initial folded state, due to the unfolding or compaction of the protein⁹. These conformational changes are reflected through ATDs variations. Thereby, CIU experiments generate multi-dimensional datasets, with ATDs being acquired at each CV.

Early works on collisional activation were carried out by Shelimov *et al.* in 1997 and include the observation of apomyoglobin conformations¹⁰, and the different gas-phase unfolding of cytochrome *c* vs bovine pancreatic trypsin inhibitor which differ in their number of disulfide bridges (none and three, respectively)¹¹. Following the commercialization of quadrupole/TWIMS/time-of-flight mass spectrometers⁴, modern technological developments have further extended the range of CIU examples. Current

applications of CIU encompass probing protein-protein complexes^{12, 13}, protein-ligand interactions^{9, 14-18}, and binding of lipids to membrane proteins^{19, 20}. CIU also plays a key role to investigate therapeutically-relevant proteins, allowing more conformational insights into monoclonal antibodies (mAbs)²¹⁻²⁴, biosimilars²⁵ and antibody-drug-conjugates^{26, 27}. In particular, Tian *et al.*⁷ demonstrated that subtle differences between intact mAbs subclasses, with different numbers and patterns of disulfide bridges, could be distinguished based on their gas-phase unfolding. Recently, Botzanowski *et al.* expanded this strategy to the middle-level classification of mAbs subclasses, which provides a more clear-cut categorization than the intact level²⁸.

Even if native MS approaches have entered R&D laboratories of biopharma companies, the use of CIU approaches is still scarce, mostly due to a lack of expertise required for this type of experiments but also because of a lack of automation of the CIU pipeline. Most efforts have been focused on the development of open-source software packages for CIU data treatment. CIU data are better visualized as unfolding plots, also named fingerprints, offering a unique representation of each protein's characteristics. ATDs are extracted at a selected mass-to-charge (*m/z*) ratio as a function of the applied CV. After normalization and smoothing at each CV, ATDs are stacked into a two-dimensional plot that can be generated using different programs^{9, 29-33}. CIUSuite, CIUSuite 2, PULSAR and ORIGAMI^{ANALYSE} also provide direct comparison of fingerprints through root-mean-square deviation (RMSD) plots.

Although significant software developments have helped to ease and automate data interpretation, the main bottleneck of the CIU workflow remains its lack of automation for sample preparation (buffer exchange) and on-line data acquisition. Increase of CV is usually done either manually or in a semi automatic way using sequential data acquisition through a pre-programmed sample list. Altogether, the classical CIU data acquisition pipeline results in a tedious and time-consuming process which hampers its routine use. Classical "semi-automated" experiments (manual desalting followed by automated sequential acquisition) require around three hours ranging from sample preparation to data acquisition. Vallejo *et al.*³⁴ suggested focusing only on median voltages of CIU features and transitions for faster mAbs differentiation, once full fingerprints have been established. Only one open-source software (ORIGAMI^{MS}) allows automated acquisition of CIU data, but prior manual desalting is still necessary³¹.

In this context, the use of size exclusion chromatography (SEC) is of main interest for fast online desalting. Indeed, SEC has been successfully hyphenated to native MS for therapeutic protein analyses, allowing improved desalting efficiencies compared to manual desalting and offering the possibility for routine implementation of native MS approaches in the industry³⁵. We thus explored the feasibility of SEC hyphenated to native IM-MS for CIU analyses.

We present here a fully automated CIU data acquisition setup, from buffer exchange to data interpretation, using SEC coupled to native IM-MS (SEC-CIU). We first developed and optimized the SEC-CIU method for different mAbs. Comparisons with classical nanoESI-CIU experiments pinpointed very similar fingerprints, proving the suitability of the SEC coupling for CIU automation. Overall, our SEC-CIU workflow allowed the high-throughput classification of mAbs subclasses at both intact and middle levels, while overall diminishing threefold the acquisition time necessary to record CIU experiments. Lastly, we demonstrated the potential of targeted scheduled SEC-CIU and multiplexed SEC-CIU to enhance information content in an even shorter amount of time.

MATERIALS AND METHODS

Sample preparation.

Adalimumab (Humira, Abbvie), ofatumumab (Arzerra, GSK), trastuzumab (Herceptin, Roche), panitumumab (Vectibix, Amgen), reslizumab (Cinqair, Teva) and nivolumab (Opdivo, BMS) were obtained from their respective manufacturers. Each intact mAb was N-deglycosylated by incubating one unit of IgGZERO (Genovis) per microgram of mAb, for 30 min at 37 °C. For middle-level enzymatic digestion, one unit of IdeS enzyme (FabRICATOR, Genovis) was added per microgram of mAb prior deglycosylation. The mixture was incubated at 37 °C for 60 min.

Manual buffer exchange.

For ESI- and nanoESI- CIU experiments, samples were desalted against 100 mM ammonium acetate at pH 7.0, using eight cycles of centrifugal concentrator (Vivaspin, 30 kDa cutoff, Sartorius, Göttingen, Germany). Protein concentration was then determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, France). Each solution was diluted in 100 mM ammonium acetate at pH 7.0 to 5 μ M prior to native ESI- and nanoESI- CIU acquisitions.

Native SEC-CIU experiments.

An ACQUITY UPLC H-Class system (Waters, Manchester, UK) comprising a quaternary solvent manager, a sample manager set at 10 °C, a column oven, and a TUV detector operating at 280 nm and 214 nm was hyphenated to a Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK). The mobile phase used for online buffer exchange was composed of 100 mM ammonium acetate at pH 7.0. Two SEC columns with different nominal lengths and pore sizes were evaluated: an Acquity BEH SEC 200Å, 1.7 μ m, 4.6 x 300 mm and an Acquity BEH SEC 125Å, 1.7 μ m, 4.6 x 30 mm from Waters. For intact-level analyses performed on the 30 mm column, the flow rate was set to 0.250 mL/min for 0.75 min, decreased to 0.035 mL/min for 3.35 min, and raised to 0.250 mL/min for 0.9 min. The flow rate for middle-level analyses was set to 0.250 mL/min for 0.80 min, decreased to 0.035 mL/min for 3.8 min, and raised to 0.250 mL/min

for 0.4 min. 20 and 12 μg were injected for intact and middle level analyses, respectively.

The Synapt G2 was operated in sensitivity mode and positive polarity with a capillary voltage of 3.0 kV. Desolvation and source temperatures were set to 450 and 100 $^{\circ}\text{C}$, respectively. Desolvation and cone gas flow rates were 750 and 60 L/Hr, respectively. The cone voltage was fixed to 80 V to avoid in source ion activation while ensuring ion transmission. The backing pressure of the Z-Spray source was set to 6 mbar. The Ar flow rate was 5 mL/min. Ions were focused in the helium cell (120 mL/min), prior to IM separation. In the IM cell, the N_2 flow rate was 60 mL/min. The IM wave velocity and height were 800 m/s and 40 V, respectively. Data were acquired in the 1000 – 10000 m/z range, without ion selection in the quadrupole.

Parameters for CIU experiments were defined in the MS File of the MassLynx v4.1 software (Waters, Manchester, UK). Collision voltages in the trap cell were increased from 0 to 200 V in 10 V steps. Each replicate of CIU fingerprints was acquired in three runs, corresponding to different MS files each containing seven IM-MS functions (0 – 60 V, 70 – 130 V, 140 – 200 V). For each voltage step of intact mAbs experiments, the number of scans and scan time were set to 5 and 3 s, respectively. These 15 s acquisition slots, i.e. function slots, were aligned with the mAb elution, and the first and last functions were implemented at 1.75 and 3.37 min, respectively. For middle-level analyses, acquisition slots comprised 2 scans of 3 s each, with the first and last functions starting at 1.95 and 2.85 min, respectively. Consecutive IM-MS functions were separated by 1.2 s intervals to ensure effective application and stable CVs.

Native ESI-CIU experiments.

Samples were infused to the electrospray source using a syringe infusion pump (KD Scientific, Holliston, MA, USA) using 250 μL syringes (Hamilton, Bonaduz, Switzerland) at an infusion rate of 3.5 $\mu\text{L}/\text{min}$. Desolvation and source temperatures were set to 450 $^{\circ}\text{C}$ and 100 $^{\circ}\text{C}$, respectively. The Synapt G2 was set up with the parameters previously described. Trap CVs were increased manually from 0 to 200 V in 10 V steps with 1-min acquisitions.

Native nanoESI-CIU experiments.

The Synapt G2 was coupled to the automated chip-based nanoESI device (TriVersa NanoMate, Advion, Ithaca, USA). The capillary voltage and the pressure of the nebulizer gas were set at 1.75 kV and 0.55 psi, respectively. The source temperature was 100 $^{\circ}\text{C}$ while the desolvation gas was not heated. Other MS parameters were similar to those previously described. Trap CVs were increased manually from 0 to 200 V in 10 V steps with 1-min acquisitions.

Data treatment.

Chromatographic, IM and MS data were analyzed using MassLynx v4.1. CIU data were processed using the CIUSuite 2 v2.1 software³³. Data sets were collected in triplicate (with three SEC runs for each replicate) to generate averaged CIU fingerprints. ATDs were smoothed

using a Savitsky-Golay algorithm with a window length of 5 and a polynomial order of 2. Interpolation by a factor of 2 allowed to double the number of steps along the CV axis. Averaged and differential plots with their associated RMSDs were generated using the 'Basic Analysis' module. RMSDs under 10 % between technical replicates account for a good reproducibility of CIU data (Table S1). Transitions between CIU features were quantitatively assessed by their corresponding CIU50 values using the CIUSuite 2 'Stability Analysis' module. Parameters used for feature detection (along with their median drift times) and CIU50 analysis were as follows: standard mode for feature and CIU50 detections; minimum feature length = 2 steps; feature allowed width (in mobility axis units) = 0.75 ms; no CV gap length allowed within a feature; drift time spectrum = centroid at maximum value for each CV; transition region padding = 15 V. When features coexist at similar intensity across many voltages, the software only detects the most intense feature. In such cases, states can be better assessed and visualized by directly extracting ATDs at CVs of interest. Figures of stacked ATDs were generated with the ORIGAMI^{ANALYSE} v1.2.1.4 software³¹.

For mAbs subclass categorization, the centroid of the ATDs were standardized at CV = 0 V to ensure comparison solely on unfolding patterns, irrespective of drift time variations due to different masses. Adalimumab (IgG1), panitumumab (IgG2) and reslizumab (IgG4) were chosen as reference mAbs to build classification methods at intact and middle levels using the 'Classification' module of the CIUSuite 2 software^{33, 36}. Univariate feature selection (UFS) plots were used to select the most diagnostic voltages, i.e. with the higher scores, to classify clusterized mAbs (ofatumumab, trastuzumab and nivolumab).

RESULTS AND DISCUSSION

Development of the SEC-CIU workflow.

In CIU experiments performed on TWIMS instruments, ions are activated in the trap collision cell by increasing collision voltages from 0 to 200 V prior to IM separation. CVs are ramped either manually or sequentially using a sample list, resulting in a error-prone and highly time-consuming process (~75 minutes for each sample, in triplicate, with 10 V steps). Previous manual CIU studies have been performed in nanoESI mode using either glass capillary nanoemitters^{9, 15-17} or automated chip-based nanoESI devices^{18, 21, 27, 28}. Both techniques hardly maintain spray stability throughout the whole CV ramp due to clogging^{37, 38}, leading to a laborious process for the MS operator. Hence, hyphenation of SEC to native IM-MS is of major interest to automate CIU experiments while substantially reducing the overall time process. Since online SEC coupling ensures a continuous controlled flow rate with a stable electrospray, CIU data can be recorded automatically during the sample elution.

Optimization of SEC column.

First, the choice of the SEC column length was decisive to significantly reduce the acquisition time. We compared two SEC columns, an Acquity BEH200Å 4.6 $\times$ 300 mm and

an Acquity BEH125Å, 4.6 × 30 mm (Waters), requiring runs of 18 and 5 minutes, respectively. Although the longer elution on the 300 mm column affords an increased number of scans and improved desalting efficiencies³⁵, no significant difference was observed on CIU fingerprints (RMSD < 6%, Figure S1). Since data were of equal quality for both columns, the SEC-CIU methodology was developed and optimized on the 30 mm column to considerably speed up the process.

Optimization of SEC flowrate.

Automated SEC-native MS experiments for therapeutic proteins are usually performed at flow rates comprised between 0.10–0.25 mL/min³⁵. Online SEC-CIU first requires slowing down SEC flow rates in order to provide chromatographic peaks large enough to record several CV steps with a reasonable number of scans and scan time per CV. However, great care must be taken to guarantee good signal-to-noise ratio and high enough IM-MS intensities even at low flow rate. In the case of mAbs at both intact and middle levels, we determined that 0.035 mL/min was the best compromise between number of steps, scan parameters and IM-MS signal, leading to CIU data acquired for two minutes with enough MS intensity.

Optimization of CIU parameters.

CIU parameters, i.e. scan slots and trap CV, are defined by the user within a single MassLynx MS file (Figure 1). One IM-MS function is implemented for each CV. Considering that MassLynx limits the number of recorded IM-MS functions to fifteen for a single analysis, two or three runs are necessary to cover the 0 – 200 V range when using 10 V steps. Generally, CIU experiments are performed with 2, 5 or 10 V increments^{20, 22, 24, 39}. In our hands, 10 V steps were

enough to obtain well-resolved CIU fingerprints for an efficient identification of mAbs subclasses. After data processing in CIUSuite 2 (see Material and Methods), at least 8 specific diagnostic CVs were available for mAbs classifications. If needed, an additional run including appropriate CV values would help to better define highly populated regions or features that are only present in a narrow voltage range. Results and fingerprints presented thereafter were acquired with a three-runs method, which allows longer acquisition times for each CV and improves fingerprint resolution compared to two runs (Figure S2). In this method, runs contained seven IM-MS functions with 10 V steps to take full advantage of the chromatographic peak width while optimizing scan parameters. For intact mAbs, the acquisition time for each CV was set to 15 s (5 scans of 3 seconds) to improve the S/N ratio. Once the run has started, CVs are automatically raised following the user's method. Of note, CVs can also be ramped in decreasing order without altering CIU fingerprints (Figure S3). For each of the three runs (0 – 60 V, 70 – 130 V, 140 – 200 V), CIU data are stored within a single MassLynx.raw file compatible with the CIUSuite 2 software³³ for data analysis. Since ions are activated without prior selection in the quadrupole, different charge states are then available at once for fingerprint generation. The three output files are automatically combined to generate a complete CIU fingerprint (0 – 200 V) corresponding to the extracted charge state. It is worth noting that three unique MS files can be used for SEC-CIU experiments as intact mAbs all exhibit the same elution time on 30 mm columns, thus enabling high-throughput analyses.

Overall, SEC-CIU offers a fast-online desalting³⁵ compared to classical nanoESI-CIU experiments which

Figure 1. Schematic representation of the acquisition workflow of SEC-CIU data (0 – 60 V range) for intact mAbs. For each collision voltage, one IM-MS function (greyscale bar) is implemented in the MassLynx MS file along the sample elution. Scans parameters were set to 5 scans of 3 seconds each. A three-runs method was used to generate a complete CIU replicate (0 – 200 V). Averaged unfolding plots are generated from three replicates, illustrated here by the CIU fingerprint of the 26+ charge state of adalimumab.

require manual buffer exchange, and the possibility to automate data acquisition to further reduce the data collection time saving two hours on average (depending on the desalting procedure used).

Proof of concept of SEC-CIU for IgG1 analysis.

NanoESI-CIU experiments have previously been reported for the characterization of mAbs^{7, 21-23, 28}. We first evaluated our online SEC-ESI-CIU method on adalimumab, a therapeutic mAb which belongs to the IgG1 subclass, and compared obtained fingerprints with nanoESI-CIU ones²⁸ in order to assess whether CIU key features are retained with our SEC-CIU setup. As electrospray generates larger droplets with higher charge states than nanoESI³⁷, only one charge state (26+) was available for comparison between nanoESI- and SEC-ESI-CIU (Figure 2a). Both methods provided the same number of unfolding transitions (Figure 2b, c and e) and identical conformational states are obtained in both fingerprints (Figure S4c-e), but relative intensities between co-existing states slightly differ, which results in an apparent translation towards lower CVs for SEC-CIU (more obvious for transitions from state 0 to 1 and state 3 to 4) and leads to an RMSD = 15 % between the two conditions (Figure S4a). For the 26+ charge state of intact adalimumab, the first conformational state becomes the most intense at 20 V vs 30 V for nanoESI- and SEC- CIU, respectively (Figure 2e). While the third state was observed in both cases at the same voltage (50 V), the fourth conformational state again is already the main feature at 150 V for SEC-CIU but not until 190 V for nanoESI-CIU.

To rule out the possible effect of the SEC column to explain differences observed between SEC-ESI-CIU and nanoESI-CIU fingerprints, we recorded an additional CIU fingerprint with an ESI source under identical ESI conditions as for SEC-CIU (source and desolvation

temperatures of 100 °C and 450 °C, respectively) except for the flow rate. Overall, very similar CIU fingerprints were obtained in SEC-ESI and ESI conditions (RMSD < 8 %, Figure S4b and c), as depicted for the 26+ charge state of adalimumab at the intact level (Figure 2c, d and e), which demonstrates that the hyphenation of SEC to CIU does not significantly affect mAb CIU patterns.

MAbs being very stable molecules, we usually perform our native MS analyses in quite harsh temperature desolvation conditions, either in SEC-native MS³⁵ or nanoESI-CIU^{21, 28}, to achieve more efficient desolvation resulting in subsequent better mass accuracy. We thus next evaluated the influence of source and desolvation temperatures on the nanoESI- and SEC- CIU fingerprints. No significant difference (RMSD < 9 % for the 28+ charge state) was observed between SEC-ESI-CIU fingerprints generated at 150 °C or 450 °C desolvation temperature (same number of conformational transitions and states) independently of the mAb subclass (Figure S5). These experiments confirm that quite harsh ESI temperature conditions do not strongly alter CIU patterns, as only intensities/ratios of the different conformational states are affected (Figure S5). NanoESI-CIU fingerprints recorded at source temperatures ranging from 40 °C to 100 °C also exhibit very similar CIU patterns (as deduced from median drift times, Figure S6) and only differ in terms of absolute CIU₅₀ values, which are higher at 40 °C than 100 °C, in agreement with less ion activation at lower temperature. Our results highlight that in the particular case of highly stable mAbs, quite harsh ESI or nanoESI temperature conditions do not significantly affect CIU fingerprints (in terms of number of transitions and median drift time values of the conformational states).

Altogether, SEC-CIU fingerprints provide the same level of information as nanoESI- or ESI- CIU.

Figure 2. Comparison of nanoESI, SEC-ESI and ESI experiments. (a) Native mass spectra of intact adalimumab corresponding to nanoESI (red trace), SEC-ESI (blue trace) and ESI (black trace). Zooms on the 28+ charge state are provided in insets for SEC-ESI and ESI mass spectra. CIU fingerprints of the 26+ charge state of intact adalimumab obtained with (b) nanoESI, (c) SEC-ESI and (d) ESI infusion modes. (e) ATDs extracted at different voltages for nanoESI (red), SEC-ESI (blue) and ESI (black) are depicted in the right panel.

Figure 3. Intact and middle levels SEC-CIU experiments. (a) 27+ charge state fingerprints of our three reference mAbs, adalimumab, panitumumab and reslizumab. (b) Subclass classification ($z = 27+$) of ofatumumab (IgG1), trastuzumab (IgG1) and nivolumab (IgG4) obtained with our in-house intact level classification method. (c) 21+ charge state fingerprints of F(ab')₂ fragments corresponding to our three reference mAbs. (d) Middle level subclass classification ($z = 21+$) of ofatumumab (IgG1), trastuzumab (IgG1) and nivolumab (IgG4).

SEC-CIU for mAb classification.

Previous works have demonstrated that mAbs subclasses, which namely differ in their number and bridging of disulfide bonds, could be classified based on their unfolding patterns^{7, 28}. To benchmark our SEC-CIU strategy, we further applied it to the analysis of three mAbs belonging to different subclasses, adalimumab (IgG1), panitumumab (IgG2) and reslizumab (IgG4). At the intact level, all mAbs adopt a narrow charge state distribution (26–30+). As the 27+ charge state appears as the best compromise between native structure, signal intensity and CIU information content, it will be used thereafter for classification purposes (additional charge states are shown in supplementary data, Figures S7 and S8). SEC-CIU fingerprints obtained for the 27+ charge state reveal three conformational transitions for the IgG1 but only two transitions for IgG2 and IgG4 (Figure 3a). Although IgG2 and IgG4 exhibit the same number of transitions, mAbs subclasses could unambiguously be distinguished based on their specific ATDs profiles (with CIU₅₀ values of 36 V and 166 V for IgG2 vs 30 V and 117 V for IgG4) in agreement with Tian *et al.* observations⁷. The 27+ charge state univariate feature selection (UFS) plot obtained with CIUSuite 2 shows that one diagnostic CIU region to differentiate subclasses is comprised between 90 – 125 V (Figure S9a). We subsequently built a classification method based on these specific CVs using the three mAbs previously analyzed as references.

To validate SEC-CIU for mAb subclass identification, we analyzed three additional mAbs (trastuzumab and ofatumumab - IgG1s and nivolumab - IgG4) and subjected them to this automated classification. As already reported^{21, 28}, although unfolding patterns between mAbs from the same subclass are not strictly identical, probably

due to the contribution of the variable domains of F(ab')₂ regions, the CIUSuite 2 module unambiguously recognizes ofatumumab and trastuzumab as IgG1s (86.0 ± 4.3 % and 94.9 ± 1.2 % of confidence, respectively), and nivolumab as an IgG4 (75.0 ± 4.4 %) (Figures 3b and 3b). These results highlight the potential of our SEC-CIU workflow to classify mAbs at the intact level.

We next performed SEC-CIU experiments at the middle-level after IdeS digestion. Botzanowski *et al.* reported that CIU fingerprints of F(ab')₂ fragments are more informative than intact CIU fingerprints for mAbs subclass identification, whereas Fc subdomains do not allow clear-cut classification due to similar unfolding patterns²⁸. Since F(ab')₂ and Fc fragments do not co-elute on the 30 mm column and could thus not be activated simultaneously, we focused our study on F(ab')₂ subunits to optimize data acquisition time. For the 21+ charge state of the F(ab')₂ subdomain, only two conformational transitions are observed in the CIU fingerprint of the IgG2 (panitumumab), while three and five conformational transitions occur for IgG1 (adalimumab) and IgG4 (reslizumab), respectively (Figure 3c). Results obtained in terms of transitions for charge states 21 and 22+ (Figure S10) were consistent with IgG2s being less prone to unfolding compared to other subclasses due to two additional disulfide bridges in the hinge region. Again, mAb subclasses could clearly be differentiated by comparing their unfolding patterns (Figure S11). The UFS plot of the F(ab')₂ 21+ charge state pinpoints diagnostic CIU collision voltages in the 85 – 120 V range (Figure S9c). Our middle-level classification based on the three reference mAbs (adalimumab, panitumumab and reslizumab) unequivocally categorizes ofatumumab and trastuzumab as IgG1s (92.8 ± 2.7 % and 95.6 ± 1.2 %), and nivolumab as an IgG4 (92.2 ± 2.5 %) (Figures 3d and S9d). Scores

obtained were similar (> 90 %) to F(ab')₂-classification using manual CIU fingerprints²⁸. Higher scores achieved after IdeS digestion compared to the intact level confirm that mAb subclass categorization is more accurate at the middle level, in agreement with Botzanowski *et al.*²⁸.

Once full CIU-fingerprints (0 – 200 V) of reference mAbs have been generated and classification parameters optimized, we developed a targeted scheduled SEC-CIU based on the most diagnostic CVs extracted from UFS plots³³. This allowed us to propose a single-run SEC-CIU strategy for rapid IgGs classification (15 minutes for triplicate CIU maps) by recording only 7 diagnostic CVs (in 5 V steps, 90 to 120 V for *z* = 27 + at intact level, or 85 and 95 to 120 V for *z* = 21 + at middle level), with scan parameters identical to Figure 1. This method provides classification scores > 85 % at both intact and middle levels for trastuzumab and nivolumab (Figure S12). Targeted scheduled single-run SEC-CIU preserves key information for mAbs categorization, and also affords a drastic reduction of the overall acquisition time.

Altogether, our results highlight the suitability of SEC-CIU for fast mAb classification at both intact and middle level, while offering a characterization as precise as nanoESI-CIU experiments but with a considerable reduction of the acquisition time.

SEC-CIU for mAbs-multiplexed analyses.

An additional benefit of short SEC columns stems from the co-elution of intact mAbs that enables their simultaneous activation in the trap cell. Performing SEC-CIU experiments without prior ion isolation in the quadrupole thus allows to acquire CIU data for different proteins at once, while also covering their entire charge state distributions in the meantime. Of course, mAbs should feature distinct *m/z* ratio to avoid overlapping CIU fingerprints. The characterization of mAbs mixtures is of key interest, as co-formulated mAbs with synergic effects represent promising therapeutic entities^{40, 41}.

In order to fully exploit the potential of SEC-CIU for fast mAb classification, we subsequently aimed at analyzing the three mAbs subclasses in a single experiment. We thus evaluated our SEC-CIU strategy on a mixture containing intact ofatumumab (IgG1), panitumumab (IgG2) and nivolumab (IgG4). Online SEC desalting provided well-resolved MS peaks for the three mAbs (Figure 4a). In our case, MS peaks exhibit an average FWHM of 15 *m/z* for the 27+ charge state of intact mAbs, which corresponds to a resolution of ~310 over the 5300 – 5500 *m/z* range.

Consequently, in order to ensure enough separation of two consecutive peaks, we mixed deglycosylated mAbs with a mass difference higher than 450 Da to avoid fingerprint interferences. The quality of the separation allowed to extract ATDs at *m/z* ranges specific to each

Figure 4. MABs-multiplexed SEC-CIU experiments. (a) Native SEC-CIU mass spectrum of an equimolar (15 μ M) mAb mixture containing three intact mAbs, ofatumumab, panitumumab and nivolumab (trap CV = 90 V). (b) 27+ charge state SEC-CIU fingerprints of each intact mAb subclass obtained from the mAb mixture. (c, d, e) ATDs were extracted in 20 V steps from 0 to 200 V, allowing the comparison of unfolding processes of single-mAb (dotted lines) and mAbs-multiplexed SEC-CIU (solid lines) experiments at intact level.

mAb, resulting in well-defined CIU fingerprints which still afford an unambiguous subclass classification (Figure 4b). An issue of quadrupole non-selected CIU lies in possible interferences between species activated in the trap cell which might influence unfolding transitions^{20, 34}. Thereby, CIU data obtained from the mAb mixture were compared with those acquired using single-mAb experiments to assess whether multiplexing alters CIU profiles. For each of the three subclasses, the superposition of ATDs demonstrated that the presence of other mAbs in the sample matrix does not compromise the unfolding process (Figure 4c-e). Overall, multiplexed SEC-CIU experiments can easily be implemented for mAbs mixture analysis provided enough MS resolution.

Altogether, mAbs-multiplexed experiments provide significant improvements over standard CIU approaches. Our method offers a wide range of information, i.e. fingerprints of multiple proteins and their different charge-states, while reducing data acquisition time (same time for one to three mAbs analyses). As F(ab')₂ fragments also co-elute on the 30 mm SEC column, our multiplexing strategy can be further expanded to the middle-level for mAbs subclass classifications (Figure S13).

CONCLUSIONS

We have demonstrated here the potential of online hyphenation of SEC to native IM-MS for the automation of CIU experiments. In our hands, the combination of improved and fast online desalting and automated data collection afforded by our SEC-CIU workflow considerably shortens the overall time process (from 3 hours to 15 minutes for targeted scheduled SEC-CIU). SEC-CIU eases data acquisition while preserving the quality and key features (number of conformational transitions) of nanoESI-CIU fingerprints. Our method proved to be efficient for rapid mAb subclasses identification, providing specific signatures of each subclass at both intact and middle levels. In addition, either targeted scheduled CIU method relying on diagnostic trap CVs or mAb multiplexing allowed us to extend SEC-CIU to even more complex samples analyses, widening the scope of information available within one SEC-CIU experiment.

Altogether, we believe that online SEC-CIU will promote the integration of CIU in analytical workflows for routine biotherapeutics characterization. SEC-CIU appears as an asset to rapidly assess protein gas-phase unfolding. Therefore, we envision that further developments to automate data acquisition and interpretation will usher in the implementation of CIU approaches in R&D laboratories.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge at <http://pubs.acs.org>. Table S1, RMSDs of technical SEC-CIU replicates; Figure S1, SEC-CIU fingerprints on 300 vs 30 mm SEC columns; Figure S2, SEC-CIU fingerprints with 2 vs 3 runs; Figure S3, SEC-CIU fingerprints with increasing vs decreasing CVs; Figure S4, Comparisons between nanoESI-/SEC-ESI- CIU;

Figure S5, Influence of ESI desolvation temperature on SEC-CIU fingerprints; Figure S6, Influence of source temperature on nanoESI-CIU fingerprints; Figures S7 – S10, Additional SEC-CIU fingerprints and classifications; Figure S11, ATDs of F(ab')₂ for reference mAbs; Figure S12, Targeted scheduled SEC-CIU classifications; Figure S13, Middle-level mAbs-multiplexed SEC-CIU.

AUTHOR INFORMATION

Corresponding Author

*Phone: +33 (0)3 68 85 26 79. Fax: +33 (0)3 68 85 27 81.

*E-mail: sarah.cianferani@unistra.fr

Author Contributions

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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ABBREVIATIONS

ATD, arrival time distribution; CCS, collision cross section; CIU, collision induced unfolding; CV, collision voltage; ESI, electrospray; F(ab')₂, full fragment antigen-binding; Fc, fragment crystallizable; IgG, immunoglobulin G; IM, ion mobility; mAb, monoclonal antibody; MS, mass spectrometry; SEC, size exclusion chromatography; TWIMS, travelling wave ion mobility spectrometry.

REFERENCES

1. Leney, A. C.; Heck, A. J., Native Mass Spectrometry: What is in the Name? *J Am Soc Mass Spectrom* **2017**, *28* (1), 5-13.
2. Goth, M.; Pagel, K., Ion mobility-mass spectrometry as a tool to investigate protein-ligand interactions. *Anal Bioanal Chem* **2017**, *409* (18), 4305-4310.
3. Eschweiler, J. D.; Kerr, R.; Rabuck-Gibbons, J.; Ruotolo, B. T., Sizing Up Protein-Ligand Complexes: The Rise of Structural Mass Spectrometry Approaches in the Pharmaceutical Sciences. *Annu Rev Anal Chem* **2017**, *10*, 25-44.
4. Pringle, S. D.; Giles, K.; Wildgoose, J. L.; Williams, J. P.; Slade, S. E.; Thalassinou, K.; Bateman, R. H.; Bowers, M. T.; Scrivens, J. H., An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument. *Int J Mass Spectrom* **2007**, *261* (1), 1-12.
5. Gabelica, V.; Marklund, E., Fundamentals of ion mobility spectrometry. *Curr Opin Chem Biol* **2018**, *42*, 51-59.

6. Dixit, S. M.; Polasky, D. A.; Ruotolo, B. T., Collision induced unfolding of isolated proteins in the gas phase: past, present, and future. *Curr Opin Chem Biol* **2018**, *42*, 93-100.
7. Tian, Y.; Han, L.; Buckner, A. C.; Ruotolo, B. T., Collision Induced Unfolding of Intact Antibodies: Rapid Characterization of Disulfide Bonding Patterns, Glycosylation, and Structures. *Anal Chem* **2015**, *87* (22), 11509-15.
8. Tian, Y. W.; Ruotolo, B. T., Collision induced unfolding detects subtle differences in intact antibody glycoforms and associated fragments. *Int J Mass Spectrom* **2018**, *425*, 1-9.
9. Allison, T. M.; Reading, E.; Liko, I.; Baldwin, A. J.; Laganowsky, A.; Robinson, C. V., Quantifying the stabilizing effects of protein-ligand interactions in the gas phase. *Nat Commun* **2015**, *6*, 8551.
10. Shelimov, K. B.; Jarrold, M. F., Conformations, unfolding, and refolding of apomyoglobin in vacuum: An activation barrier for gas-phase protein folding. *J Am Chem Soc* **1997**, *119* (13), 2987-2994.
11. Shelimov, K. B.; Clemmer, D. E.; Hudgins, R. R.; Jarrold, M. F., Protein Structure in Vacuo: Gas-Phase Conformations of BPTI and Cytochrome c. *J Am Chem Soc* **1997**, *119* (9), 2240-2248.
12. Rabuck-Gibbons, J. N.; Lodge, J. M.; Mapp, A. K.; Ruotolo, B. T., Collision-Induced Unfolding Reveals Unique Fingerprints for Remote Protein Interaction Sites in the KIX Regulation Domain. *J Am Soc Mass Spectrom* **2019**, *30* (1), 94-102.
13. Mehmood, S.; Marcoux, J.; Gault, J.; Quigley, A.; Michaelis, S.; Young, S. G.; Carpenter, E. P.; Robinson, C. V., Mass spectrometry captures off-target drug binding and provides mechanistic insights into the human metalloprotease ZMPSTE24. *Nat Chem* **2016**, *8* (12), 1152-1158.
14. Hopper, J. T.; Oldham, N. J., Collision induced unfolding of protein ions in the gas phase studied by ion mobility-mass spectrometry: the effect of ligand binding on conformational stability. *J Am Soc Mass Spectrom* **2009**, *20* (10), 1851-8.
15. Rabuck, J. N.; Hyung, S. J.; Ko, K. S.; Fox, C. C.; Soellner, M. B.; Ruotolo, B. T., Activation state-selective kinase inhibitor assay based on ion mobility-mass spectrometry. *Anal Chem* **2013**, *85* (15), 6995-7002.
16. Niu, S.; Ruotolo, B. T., Collisional unfolding of multiprotein complexes reveals cooperative stabilization upon ligand binding. *Protein Sci* **2015**, *24* (8), 1272-81.
17. Fantin, S. M.; Parson, K. F.; Niu, S.; Liu, J.; Polasky, D. A.; Dixit, S. M.; Ferguson-Miller, S. M.; Ruotolo, B. T., Collision Induced Unfolding Classifies Ligands Bound to the Integral Membrane Translocator Protein. *Anal Chem* **2019**, *91* (24), 15469-15476.
18. Veale, C. G. L.; Mateos Jimenez, M.; Mackay, C. L.; Clarke, D. J., Native Ion Mobility Mass Spectrometry (IM-MS) reveals that small organic acid fragments impart gas-phase stability to carbonic anhydrase II. *Rapid Commun Mass Spectrom* **2019**.
19. Laganowsky, A.; Reading, E.; Allison, T. M.; Ulmschneider, M. B.; Degiacomi, M. T.; Baldwin, A. J.; Robinson, C. V., Membrane proteins bind lipids selectively to modulate their structure and function. *Nature* **2014**, *510* (7503), 172-175.
20. Liu, Y.; Cong, X.; Liu, W.; Laganowsky, A., Characterization of Membrane Protein-Lipid Interactions by Mass Spectrometry Ion Mobility Mass Spectrometry. *J Am Soc Mass Spectrom* **2017**, *28* (4), 579-586.
21. Hernandez-Alba, O.; Wagner-Rousset, E.; Beck, A.; Cianferani, S., Native Mass Spectrometry, Ion Mobility, and Collision-Induced Unfolding for Conformational Characterization of IgG4 Monoclonal Antibodies. *Anal Chem* **2018**, *90* (15), 8865-8872.
22. Campuzano, I. D. G.; Larriba, C.; Bagal, D.; Schnier, P. D., Ion Mobility and Mass Spectrometry Measurements of the Humanized IgGk NIST Monoclonal Antibody. **2015**, *1202*, 75-112.
23. Ferguson, C. N.; Gucinski-Ruth, A. C., Evaluation of Ion Mobility-Mass Spectrometry for Comparative Analysis of Monoclonal Antibodies. *J Am Soc Mass Spectr* **2016**, *27* (5), 822-833.
24. Watanabe, Y.; Vasiljevic, S.; Allen, J. D.; Seabright, G. E.; Duyvesteyn, H. M. E.; Doores, K. J.; Crispin, M.; Struwe, W. B., Signature of Antibody Domain Exchange by Native Mass Spectrometry and Collision-Induced Unfolding. *Anal Chem* **2018**, *90* (12), 7325-7331.
25. Pisupati, K.; Tian, Y.; Okbazghi, S.; Benet, A.; Ackermann, R.; Ford, M.; Saveliev, S.; Hosfield, C. M.; Urh, M.; Carlson, E.; Becker, C.; Tolbert, T. J.; Schwendeman, S. P.; Ruotolo, B. T.; Schwendeman, A., A Multidimensional Analytical Comparison of Remicade and the Biosimilar Remsima. *Anal Chem* **2017**, *89* (9), 4838-4846.
26. Tian, Y.; Lippens, J. L.; Netirojanakul, C.; Campuzano, I. D. G.; Ruotolo, B. T., Quantitative collision-induced unfolding differentiates model antibody-drug conjugates. *Protein Sci* **2019**, *28* (3), 598-608.
27. Botzanowski, T.; Erb, S.; Hernandez-Alba, O.; Ehkirch, A.; Colas, O.; Wagner-Rousset, E.; Rabuka, D.; Beck, A.; Drake, P. M.; Cianferani, S., Insights from native mass spectrometry approaches for top- and middle- level characterization of site-specific antibody-drug conjugates. *MAbs* **2017**, *9* (5), 801-811.
28. Botzanowski, T.; Hernandez-Alba, O.; Malissard, M.; Wagner-Rousset, E.; Deslignière, E.; Colas, O.; Haeuw, J.-F.; Beck, A.; Cianferani, S., Middle level IM-MS and CIU experiments for improved therapeutic immunoglobulin subclass fingerprinting. *Anal Chem* **2020**, *92* (13), 8827-8835.
29. Sivalingam, G. N.; Yan, J.; Sahota, H.; Thalassinou, K., Amphitrite: A program for processing travelling wave ion mobility mass spectrometry data. *Int J Mass Spectrom* **2013**, *345*, 54-62.
30. Eschweiler, J. D.; Rabuck-Gibbons, J. N.; Tian, Y.; Ruotolo, B. T., CIUSuite: A Quantitative Analysis Package for Collision Induced Unfolding Measurements of Gas-Phase Protein Ions. *Anal Chem* **2015**, *87* (22), 11516-22.
31. Migas, L. G.; France, A. P.; Bellina, B.; Barran, P. E., ORIGAMI: A software suite for activated ion mobility mass spectrometry (aIM-MS) applied to multimeric protein assemblies. *Int J Mass Spectrom* **2018**, *427*, 20-28.
32. Sivalingam, G. N.; Cryar, A.; Williams, M. A.; Gooptu, B.; Thalassinou, K., Deconvolution of ion mobility mass spectrometry arrival time distributions using a genetic algorithm approach: Application to alpha(1)-antitrypsin peptide binding. *Int J Mass Spectrom* **2018**, *426*, 29-37.
33. Polasky, D. A.; Dixit, S. M.; Fantin, S. M.; Ruotolo, B. T., CIUSuite 2: Next-Generation Software for the Analysis of Gas-Phase Protein Unfolding Data. *Anal Chem* **2019**, *91* (4), 3147-3155.
34. Vallejo, D. D.; Polasky, D. A.; Kurulugama, R. T.; Eschweiler, J. D.; Fjeldsted, J. C.; Ruotolo, B. T., A Modified Drift Tube Ion Mobility-Mass Spectrometer for Charge-Multiplexed Collision-Induced Unfolding. *Anal Chem* **2019**, *91* (13), 8137-8146.
35. Ehkirch, A.; Hernandez-Alba, O.; Colas, O.; Beck, A.; Guillaume, D.; Cianferani, S., Hyphenation of size exclusion chromatography to native ion mobility mass spectrometry for the analytical characterization of therapeutic antibodies and related products. *J Chromatogr B Analyt Technol Biomed Life Sci* **2018**, *1086*, 176-183.
36. Polasky, D. A.; Dixit, S. M.; Vallejo, D. D.; Kulju, K. D.; Ruotolo, B. T., An Algorithm for Building Multi-State Classifiers Based on Collision-Induced Unfolding Data. *Anal Chem* **2019**, *91* (16), 10407-10412.
37. Konermann, L.; Ahadi, E.; Rodriguez, A. D.; Vahidi, S., Unraveling the mechanism of electrospray ionization. *Anal Chem* **2013**, *85* (1), 2-9.
38. Kirby, A. E.; Jebrail, M. J.; Yang, H.; Wheeler, A. R., Folded emitters for nanoelectrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* **2010**, *24* (23), 3425-31.
39. Eschweiler, J. D.; Martini, R. M.; Ruotolo, B. T., Chemical Probes and Engineered Constructs Reveal a Detailed Unfolding Mechanism for a Solvent-Free Multidomain Protein. *J Am Chem Soc* **2016**, *139* (1), 534-540.
40. Liu, P.; Zhu, X.; Wu, W.; Ludwig, R.; Song, H.; Li, R.; Zhou, J.; Tao, L.; Leone, A. M., Subunit mass analysis for monitoring multiple attributes of monoclonal antibodies. *Rapid Commun Mass Spectrom* **2019**, *33* (1), 31-40.
41. Kim, J.; Kim, Y. J.; Cao, M.; De Mel, N.; Albarghouthi, M.; Miller, K.; Bee, J. S.; Wang, J.; Wang, X., Analytical characterization of coformulated antibodies as combination therapy. *MAbs* **2020**, *12* (1), 1738691.

Figure 1. Schematic representation of the acquisition workflow of SEC-CIU data (0 – 60 V range) for intact mAbs. For each collision voltage, one IM-MS function (greyscale bar) is implemented in the MassLynx MS file along the sample elution. Scans parameters were set to 5 scans of 3 seconds each. A three-runs method was used to generate a complete CIU replicate (0 – 200 V). Averaged unfolding plots are generated from three replicates, illustrated here by the CIU fingerprint of the 26+ charge state of adalimumab.

250x188mm (150 x 150 DPI)

Figure 2. Comparison of nanoESI, SEC-ESI and ESI experiments. (a) Native mass spectra of intact adalimumab corresponding to nanoESI (red trace), SEC-ESI (blue trace) and ESI (black trace). Zooms on the 28+ charge state are provided in insets for SEC-ESI and ESI mass spectra. CIU fingerprints of the 26+ charge state of intact adalimumab obtained with (b) nanoESI, (c) SEC-ESI and (d) ESI infusion modes. (e) ATDs extracted at different voltages for nanoESI (red), SEC-ESI (blue) and ESI (black) are depicted in the right panel.

291x135mm (150 x 150 DPI)

Figure 3. Intact and middle levels SEC-CIU experiments. (a) 27+ charge state fingerprints of our three reference mAbs, adalimumab, panitumumab and reslizumab. (b) Subclass classification ($z = 27+$) of ofatumumab (IgG1), trastuzumab (IgG1) and nivolumab (IgG4) obtained with our in-house intact level classification method. (c) 21+ charge state fingerprints of F(ab')₂ fragments corresponding to our three reference mAbs. (d) Middle level subclass classification ($z = 21+$) of ofatumumab (IgG1), trastuzumab (IgG1) and nivolumab (IgG4).

316x149mm (150 x 150 DPI)

Figure 4 : MAb-multiplexed SEC-CIU experiments. (a) Native SEC-CIU mass spectrum of an equimolar (15 μM) mAb mixture containing three intact mAbs, ofatumumab, panitumumab and nivolumab (trap CV = 90 V). (b) 27+ charge state SEC-CIU fingerprints of each intact mAb subclass obtained from the mAb mixture. (c, d, e) ATDs were extracted in 20 V steps from 0 to 200 V, allowing the comparison of unfolding processes of single-mAb (dotted lines) and mAbs-multiplexed SEC-CIU (solid lines) experiments at intact level.

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