

## Development of new methodologies for native mass spectrometry and ion mobility approaches to characterize multiprotein complexes and therapeutic proteins

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## Développement d'approches de spectrométrie de masse native et de mobilité ionique de nouvelle génération pour la caractérisation de complexes multiprotéiques et protéines thérapeutiques

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À mon frère,

À mes parents,

« Remember... The Force will be with you, always. » Obi-Wan Kenobi (Star Wars Episode IV)

Remerciements

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## Résumé

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## Développement d'approches de spectrométrie de masse native et de mobilité ionique de nouvelle génération pour la caractérisation de complexes multiprotéiques et protéines thérapeutiques

Thèse soutenue par : Evolène DESLIGNIERE Dirigée par : Dr. Sarah CIANFERANI

### Introduction

Les approches de spectrométrie de masse (MS) structurale s'inscrivent aujourd'hui dans une démarche analytique complémentaire à l'ensemble des approches classiques de biophysique pour la caractérisation tridimensionnelle de complexes multiprotéiques<sup>1</sup>. La MS structurale permet d'obtenir des informations allant de la structure primaire (modifications post-traductionnelles comprises) à la structure quaternaire des protéines, mais aussi d'étudier la dynamique, la conformation et les interactions entre sous-unités. Les techniques de MS structurale se divisent en deux catégories : les approches avec marquage (échange hydrogène-deutérium, pontage chimique, marquage radicalaire, etc.) et les approches sans marquage dites « natives », dont la MS native (nMS), la mobilité ionique (nIMS), et le *top-down* (nTD). Durant ma thèse, je me suis focalisée sur les méthodes de nMS et nIMS.

La nMS permet de transférer des complexes maintenus par des interactions non-covalentes vers la phase gazeuse du spectromètre de masse en conservant leur intégrité. Cette technique donne accès à certaines informations telles que l'homogénéité, la stœchiométrie, la stabilité ou l'affinité relative des interactions<sup>2</sup>. D'abord limitée aux complexes de petite taille, la nMS est désormais largement appliquée dans le domaine de la biologie structurale, pour le criblage protéines/ligands<sup>3</sup>, pour la caractérisation de complexes multiprotéiques<sup>4</sup>, allant même jusqu'à des capsides virales de plusieurs millions de Daltons<sup>5</sup>. Cette technique est aussi utilisée pour l'étude de protéines thérapeutiques, par exemple pour calculer le nombre moyen de drogues fixées sur un anticorps monoclonal (mAb)<sup>6</sup>.

La nMS peut également être couplée à la spectrométrie de mobilité ionique (nIMS-MS), ce qui apporte une dimension supplémentaire de séparation des ions en phase gazeuse, basée sur leur charge et leur conformation<sup>7</sup>. La distribution du temps d'arrivée (ATD) des ions dans la cellule IMS est alors convertie en section efficace rotationnellement moyennée (CCS), qui est étroitement liée à la conformation des ions et donne donc des informations sur la structure tridimensionnelle des édifices macromoléculaires analysés (protéines/ligands<sup>8</sup>, protéines membranaires<sup>9</sup>, mAbs<sup>10</sup>, etc.). Cependant, lorsque les variations de conformations sont très fines ( $\Delta$ CCS < 2%), une simple mesure de CCS par IMS-MS ne permet pas de discriminer les différentes espèces, en particulier du fait de la faible résolution des instruments IMS-MS de type *travelling wave* (TWIMS) de première génération. Pour

pallier ce manque de résolution, plusieurs axes ont émergé ces dernières années : des expériences de *collision-induced unfolding* (CIU ou « déploiement induit par collisions »)<sup>11</sup>, et tout récemment des nouvelles technologies IMS-MS de haute résolution<sup>12</sup>. Alors que l'apport de la haute résolution en IMS-MS pour l'analyse des protéines natives est encore très peu documenté, la CIU, qui consiste à suivre par IMS-MS les changements conformationnels d'un ion après activation énergétique par collisions au sein du spectromètre de masse, a déjà permis de mettre en évidence des différences de conformations très faibles dans le cas d'interactions protéines/ligands<sup>11</sup>, de variants fonctionnels de protéines membranaires<sup>13</sup>, ou de protéines thérapeutiques<sup>14</sup>.

C'est dans ce contexte d'amélioration de la préparation d'échantillon et des méthodologies de nMS et IMS-MS que se situe mon projet de thèse, qui est articulé autour de trois points principaux :

• L'amélioration des protocoles de préparation d'échantillon et l'intégration des données de nMS et nIMS-MS en biologie structurale pour différents projets collaboratifs ;

• L'évaluation d'un instrument IMS-MS de haute résolution, le TWIMS cyclique (cIMS-MS), pour la caractérisation conformationnelle de mAbs thérapeutiques ;

• Le développement d'approches CIU automatisées pour un débit d'analyse plus rapide des mAbs et leurs produits dérivés, et l'apport de la haute résolution en IMS-MS pour les expériences de CIU.

## Partie I. Introduction aux approches de MS structurale

La première partie de cette thèse propose une introduction bibliographique aux différentes techniques de MS structurale. Ces dernières consistent à analyser des protéines intactes en conditions dénaturantes ou natives (MS, IMS-MS, TD-MS), ou bien des peptides issus d'une digestion enzymatique (pontage chimique, et méthodes de marquages non-covalents). Une attention particulière est portée sur les méthodes de nMS et nIMS-MS, qui font l'objet de cette thèse. La complémentarité des techniques de MS structurale avec les approches biophysiques classiques est également illustrée.

Les méthodes ainsi que l'instrumentation utilisées durant cette thèse pour les expériences de nMS, nIMS-MS et CIU sont présentées dans un second chapitre.

## Partie II. Amélioration de la préparation d'échantillon et intégration des données de spectrométrie de masse native et de mobilité ionique en biologie structurale

La première partie de mon travail de thèse concerne l'amélioration de la préparation d'échantillon pour la nMS en évaluant la versatilité du couplage de la chromatographie d'exclusion stérique (SEC) à la nMS pour différents systèmes biologiques ainsi que l'apport d'approches combinées de nMS et nIMS-MS dans un projet de biologie structurale.

#### 1. Versatilité du couplage SEC-nMS

Dans un premier chapitre, je résume mes travaux de thèse concernant l'évaluation de la versatilité du couplage SEC-nMS pour l'analyse de différents types de complexes multiprotéiques de tailles et complexités croissantes. Une étape d'échange du tampon (« dessalage ») est généralement réalisée en amont de l'analyse en nMS afin d'échanger le tampon de formulation des protéines souvent incompatible avec la nMS contre un tampon volatil compatible avec la nMS (typiquement l'acétate d'ammonium, AcONH<sub>4</sub>). Cette étape est souvent chronophage (~½ journée), laborieuse et peut générer des artéfacts (agrégation, dissociation, etc.). Le couplage SEC-nMS s'est montré particulièrement prometteur pour s'affranchir de l'échange de tampon manuel dans le cas des mAbs, des protéines très stables, disponibles en grandes quantités (plusieurs mg) et dont la purification est bien maîtrisée<sup>15</sup>. Dans le cadre de ma thèse, j'ai pu élargir l'application du couplage SEC-nMS à différents systèmes biologiques moins stables, disponibles en plus faibles quantités et/ou plus hétérogènes :

• pour caractériser des **complexes protéine/acide nucléique** impliquant des récepteurs nucléaires (RNs), des protéines multi-domaines qui possèdent à la fois la capacité de fixer un ligand, des partenaires protéiques et/ou un ADN<sup>16</sup>. Bien souvent le manque de stabilité des complexes RN/ADN dans l'AcONH<sub>4</sub> rend l'analyse nMS délicate. La SEC-nMS a ici été la seule méthode permettant une détermination précise des masses et stœchiométries RN/ADN, et ceci malgré la présence d'une protéine contaminante.

• pour l'analyse d'**oligomères d'hélicases** RuvBL1/2 (R1/R2) impliquées dans les ribonucléoparticules (snoRNPs). Selon les partenaires/cofacteurs en présence, R1 et R2 s'associent soit sous forme d'anneau hexamérique (~300 kDa) soit de dodécamère<sup>17</sup> (~600 kDa). Cependant les complexes impliquant R1R2 sont souvent purifiés en faible quantité ou instables dans AcONH<sub>4</sub>. Chez la levure (yR1R2), le maintien des interactions yR1R2 est conditionné par la présence d'adénosine diphosphate (ADP) ajouté au tampon de purification. Avec un échange de tampon manuel, l'intégrité du complexe est maintenue uniquement dans le cas où de l'ADP est ajouté au tampon de dessalage (Figure 1A, B). Cependant, la qualité spectrale obtenue n'est pas satisfaisante (pics MS larges dus à la présence d'adduits d'ADP, Figure 1B). Là encore, la SEC-nMS a été la seule approche permettant de déterminer sans ambigüité l'effet de la fixation de cofacteurs d'ADP sur l'état d'oligomérisation de yR1R2 (Figure 1C).

En conclusion, ce premier chapitre de ma thèse m'a permis de mettre en avant les avantages de la SEC-nMS pour l'analyse de complexes biologiques difficiles à manipuler en AcONH<sub>4</sub>, à savoir : (i) la possibilité de réaliser le dessalage en ligne et rapidement, et (ii) l'apport d'une dimension supplémentaire de séparation couplée en ligne avec l'identification en nMS. Les développements

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réalisés dans le cadre de ma thèse ainsi que les avantages et limitations de la SEC-nMS ont fait l'objet d'une publication en première auteure dans *International Journal of Mass Spectrometry* (Deslignière E. *et al, Int J Mass Spectrom* **2021,** 461, 116502).



**Figure 1.** Spectres de masse en conditions natives obtenus pour yR1R2. **(A)** Après dessalage manuel, sans ADP dans le tampon de dessalage. **(B)** Après dessalage manuel, avec de l'ADP ajouté au tampon de dessalage. **(C)** Après dessalage en ligne *via* couplage SEC-nMS. Les masses des monomères mesurées en SEC-nMS sont 50 746 ± 1 Da et 51 481 ± 1 Da pour yR1 et yR2, respectivement.

## 2. Intégration des données nMS et nIMS-MS dans un projet de biologie structurale – Caractérisation de complexes de hauts poids moléculaires impliquant les hélicases RuvBL1 et 2

Dans un second chapitre, j'ai souhaité illustrer l'apport de la combinaison des données de nMS et nIMS-MS en complément des méthodes biophysiques plus classiques dans le cadre d'un projet de biologie structurale, pour mieux comprendre les interactions moléculaires mises en jeu dans la formation de trois complexes multiprotéiques en lien avec les snoRNPs.

Le complexe R2D, constitué des hélicases R1R2 (hR1R2 chez l'humain) et d'un partenaire d'interaction DPCD identifié par analyse protéomique et de fonction inconnue, sert de projet « modèle » pour exposer l'approche d'intégration des données nMS et nIMS-MS aux techniques plus classiques de biologie structurale. Une démarche similaire sera utilisée ensuite pour caractériser les complexes R2T'P' et R2S'P'.

Pour R2D, la nMS a d'abord montré que la présence de DPCD (23 kDa) déstabilise le dodécamère, avec une fixation sur l'hexamère en stœchiométrie 1:3 (383.6 kDa, Figure 2). Afin d'évaluer le changement de conformation globale induit par la fixation de DPCD, des analyses nIMS-MS ont été réalisées sur hR1R2, R2D et DPCD pour comparer les mesures de CCS expérimentales avec les CCS théoriques, calculées à partir de structures 3D de haute résolution, ou de modèles SAXS (diffusion des rayons X aux petits angles) de basse résolution proposés par nos collaborateurs. Les résultats obtenus pour DPCD révèlent une très bonne cohérence entre les valeurs expérimentale et théorique, montrant par ailleurs que la conformation présente en solution est conservée en phase gazeuse. Les données nIMS-MS obtenues sur R2D suggèrent que la fixation des trois molécules de DPCD n'a pas d'influence majeure sur la conformation globale de l'hexamère hR1R2. Les CCS expérimentales seront ensuite comparées aux CCS théoriques du modèle R2D qui sera prédit à partir des données SAXS.



Figure 2. Spectre nMS et représentation Driftscope des données nIMS-MS du complexe R2D.

Une approche similaire a été utilisée pour caractériser les complexes R2T'P' et R2S'P' impliquant hR1R2, la nMS permettant de déterminer sans ambiguïté la stœchiométrie de fixation des partenaires T'P' (RPAP3/PIH1D1) et S'P' (SPAG1/PIH1D2), fixés sur les dodécamères hR1R2 avec des stœchiométries 1:3 et 1:1, respectivement. Des mesures de CCS ont été réalisées, et seront comparées aux CCS théoriques de structures obtenues en cryo-microscopie électronique, qui sont en cours de résolution. Ces résultats seront intégrés dans une publication en préparation.

La combinaison d'approches nMS et nIMS-MS a permis d'obtenir des informations difficilement accessibles à partir des techniques de biophysique classiques, à savoir les stœchiométries de fixation des partenaires et l'impact de leur fixation sur la conformation de protéines de plusieurs centaines de kiloDaltons. Ainsi, l'usage de techniques de MS structurale orthogonales à d'autres approches biophysiques offre une vision plus large et une caractérisation approfondie de complexes biologiques hétérogènes.

## Partie III. Apports de la haute résolution en mobilité ionique : application à l'analyse des mAbs thérapeutiques

Le deuxième axe de recherche de mon travail de thèse est focalisé sur les approches IMS-MS de haute résolution et leur apport pour l'étude conformationnelle de mAbs à visée thérapeutique. La caractérisation conformationnelle des mAbs (Higher Order Structure, HOS) reste encore délicate, n'est pas routinière et nécessite l'utilisation de techniques orthogonales. L'IMS-MS a été proposée récemment comme l'une d'entre elles mais son application pour la caractérisation des mAbs reste limitée du fait de la faible résolution des instruments IMS-MS de première génération. Au début de mon travail de thèse, la résolution accessible sur un instrument commercial de type TWIMS (Synapt G2 HDMS) était limitée à 40 (CCS/ $\Delta$ CCS). Aujourd'hui, plusieurs technologies permettent d'atteindre une haute résolution supérieure à 200<sup>12</sup>. Cette partie est consacrée à l'évaluation des performances du nouvel instrument cIMS-MS en comparaison de la cellule de TWIMS linéaire disponible au sein du laboratoire, pour la caractérisation conformationnelle des mAbs et produits associés.

#### 1. Description de l'instrument cIMS-MS

Le premier chapitre de cette partie propose une description détaillée de l'instrumentation de haute résolution IMS utilisée dans le cadre de ce travail de thèse, à savoir la mobilité ionique à géométrie cyclique cIMS-MS, commercialisée en 2019 par Waters. Cette plateforme circulaire contient quatre tubes de dérive incurvés successifs pour une longueur totale de 98 cm, ce qui équivaut à doubler en un tour la résolution théorique du TWIMS linéaire<sup>18</sup>. Au-delà du premier tour en IMS, les ions peuvent être maintenus dans la cellule cIMS pendant plusieurs tours supplémentaires (*multipass*), offrant ainsi la possibilité d'augmenter considérablement la longueur disponible pour la dérive des ions pour obtenir un meilleur pouvoir séparatif. De plus, la géométrie particulière de la cellule cIMS permet d'accéder à des modes de séparation IMS plus avancés. Il est notamment possible de sélectionner une fenêtre IMS contenant les espèces d'intérêt, tandis que les autres populations sont éjectées. Ensuite, les espèces comprises dans cette fenêtre IMS sont réinjectées dans la cellule cIMS pour plusieurs tours afin d'améliorer leur séparation/définition (IMS<sup>n</sup>, par analogie aux expériences MS<sup>n</sup>).

Finalement, les potentialités de la haute résolution IMS-MS pour l'analyse des mAbs ont été illustrées au travers de deux projets.

# 2. Apport de la haute résolution IMS-MS pour la détermination de l'appariement de ponts disulfures au niveau peptidique

Dans le cadre du développement d'un IgG4 possédant une cystéine additionnelle dans sa chaîne lourde, une digestion à la trypsine a permis d'identifier un peptide portant deux ponts disulfures (masse mono-isotopique de 5187.27 Da). Afin de déterminer quels étaient les résidus Cys appariés, j'ai développé une approche innovante basée sur la comparaison des ATDs de ce dipeptide avec celles de dipeptides synthétiques correspondant aux différents isomères de ponts disulfures. Ces variants, dont la séparation était limitée sur l'instrument TWIMS-MS linéaire de première génération (Figure 3A), ont été clairement différenciés en cIMS-MS (Figure 3B). L'intérêt du mode *multipass* a été démontré grâce à l'utilisation de fits gaussiens, qui ont mis en évidence la détection d'un plus grand nombre de conformères après deux tours en IMS. Cette méthode alternative nécessite moins de retraitement des données que les techniques classiques *bottom-up* d'identification par MS/MS des Cys appariées, ce qui représente un atout considérable pour l'intégration de cet outil dans les entreprises biopharmaceutiques. Ces résultats ont fait l'objet d'une publication en première auteure dans *Journal of the American Society for Mass Spectrometry* (Deslignière E. *et al., J Am Soc Mass Spectrom* **2021**, *32* (10), 2505-2512).



**Figure 3.** Séparation d'isomères de ponts disulfures sur les cellules TWIMS **(A)** linéaire *versus* **(B)** cyclique. Un fit gaussien (traces colorées) a été réalisée sur chaque ATD.

## 3. Utilisation de la haute résolution en IMS-MS pour la caractérisation de différents formats de mAbs en conditions natives

• J'ai d'abord cherché à différencier les sous-classes de mAbs à partir de leur profil IMS. L'instrument TWIMS-MS utilisé au laboratoire (Synapt G2 HDMS) présente des limitations en terme de résolution IMS, illustrées notamment dans le cas des mesures de CCS sur différents mAbs intacts (150 kDa) ou après digestion partielle en sous-unités (fragments F(ab')<sub>2</sub> et Fc, 100 et 50 kDa) au niveau dit « middle ». En effet, les immunoglobulines (IgG) se divisent en plusieurs isotypes : les IgG1 et 4 possèdent deux ponts disulfures dans leur région charnière mais un appariement différent des chaînes lourdes et légères, tandis que les IgG2 comprennent quatre ponts disulfures dans leur région charnière. Or, sur le TWIMS de première génération, ces différences structurales subtiles ne donnent pas lieu à des variations significatives de <sup>™</sup>CCS<sub>N2</sub> ni au niveau intact (< 1%), ni sur les sous-unités digérées F(ab')<sub>2</sub> (< 2.6%) ou Fc (< 0.3%)<sup>19</sup>. Les analyses réalisées en cIMS-MS montrent qu'après un tour, un gain de résolution modéré est obtenu par rapport à la cellule TWIMS linéaire. Les IgG1 et 4 ne semblent pas pouvoir être clairement distinguées du fait d'appariement de ponts disulfures très proches, néanmoins les IgG4s présentent a priori une plus grande flexibilité conformationnelle, qui se traduit par davantage de conformères détectés par fits gaussiens que pour les IgG1s. Les IgG2s possèdent quant à elles un profil IMS-MS bimodal caractéristique, lié à la présence d'isomères de ponts disulfures spécifiques aux IgG2s. La haute résolution en IMS-MS permet donc d'observer la richesse conformationnelle propre aux IgG2s, qui n'est pas décelable sur les ATDs d'un instrument de première génération. Ces deux populations principales ont ensuite été isolées successivement, puis réinjectées afin (i) d'obtenir une meilleure définition des espèces d'intérêt, et (ii) de vérifier qu'il n'y a pas d'interconversion entre ces deux isoformes. L'absence d'interconversion corrobore la présence de deux variants disulfures, cependant des expériences supplémentaires en conditions réductrices sont nécessaires pour confirmer l'origine de ces deux pics IMS. Une unique population est attendue après réduction.

• J'ai ensuite caractérisé un **anticorps trispécifique** (tsAb, Figure 4A), dont l'analyse SEC-nMS a révélé la présence de deux pics correspondant à des monomères de masses identiques. De faibles différences de <sup>TW</sup>CCS<sub>N2</sub> (< 2%), dans l'erreur de mesure de l'IMS-MS de l'instrument TWIMS de première génération, ont été mesurées en SEC-nIMS-MS (Figure 4B), ne permettant pas de conclure de manière non ambiguë quant à l'existence de deux conformères. Le couplage SEC-nIMS-MS n'étant pas mis en place sur l'instrument cIMS-MS utilisé, l'analyse des conformères intacts à partir de la seule dimension de séparation IMS n'a pas permis une distinction des deux espèces (Figure 4C). En revanche, l'analyse cIMS des sous-unités de mAbs s'est montrée bien plus informative que dans le cas du TWIMS linéaire, avec deux populations IMS bien séparées, confirmant définitivement la co-existence de deux conformères de la région Fab (Figure 4D, E).



**Figure 4. (A)** Structure du tsAb étudié. Des expériences IMS-MS ont été réalisées au niveau intact sur **(B)** la cellule TWIMS linéaire et **(C)** la cellule TWIMS cyclique après 6 tours. Les ATDs ont également été enregistrées au niveau *middle* après digestion **(D)** sur la cellule TWIMS linéaire et **(E)** en cIMS, permettant de séparer deux conformères de la sous-unité Fab1.

En conclusion, si la plateforme de haute résolution IMS-MS cyclique semble bien adaptée à la caractérisation de peptides et petites protéines en conditions dénaturantes, certaines limitations subsistent pour les protéines intactes de haute masse analysées en conditions natives (IgG1s *versus* IgG4s) pour lesquelles il serait nécessaire de disposer de résolutions encore plus élevées.

# Partie IV. Développements d'approches CIU appliquées aux protéines thérapeutiques

Parmi les approches permettant de pallier le manque de résolution des instrumentations IMS-MS, les expériences de CIU peuvent constituer une alternative intéressante. Lors d'une expérience CIU, les ions sont progressivement accélérés dans la cellule *trap* d'un instrument de type TWIMS-Q-ToF avant leur séparation en IMS. Les collisions avec les molécules de gaz étant de plus en plus énergétiques, un déploiement de la structure des ions peut être observé. Ces transitions conformationnelles, qui se traduisent par des variations d'ATDs, sont représentées par une empreinte CIU appelée carte, spécifique à chaque protéine. Les approches CIU ont été mises en place au laboratoire par le Dr. Botzanowski, qui a évalué leur répétabilité, reproductibilité et robustesse. La CIU permet de distinguer des espèces possédant des conformations très proches lorsque l'IMS-MS seule ne le peut pas, par exemple pour la différentiation des sous-classes d'IgGs<sup>14,20</sup>. Dans ce contexte, les objectifs de cette

dernière partie de mon travail de thèse sont : (1) d'évaluer la versatilité des approches CIU pour différents formats de mAbs de taille et de complexité croissantes, (2) d'augmenter le débit d'analyse en automatisant les expériences de CIU ; et (3) d'évaluer l'apport de la haute résolution en IMS-MS pour ce type d'expériences.

#### 1. Influence de différents paramètres MS sur les cartes CIU des mAbs

Dans un premier temps, j'ai évalué l'influence de différents paramètres MS susceptibles d'altérer le déploiement des mAbs en amont de la cellule *trap*, à savoir la tension d'accélération en source, la température de la source, la pression au sein de l'interface, et la sélection des ions dans le quadripôle. J'ai notamment mis en évidence un effet important de la pression sur les empreintes CIU de mAbs intacts, avec une perte des états conformationnels les plus natifs à basse pression, due à des collisions plus énergétiques entre les ions et les molécules de gaz dans le spectromètre de masse. Une optimisation fine de ces paramètres est nécessaire afin de ne pas induire un déploiement prématuré des protéines.

#### 2. Versatilité de l'approche CIU pour les protéines thérapeutiques

J'ai ensuite montré la versatilité de l'approche CIU mise en place précédemment au laboratoire pour un grand nombre de protéines thérapeutiques, allant des IgGs intactes (150 kDa) aux sous-unités constitutives F(ab')<sub>2</sub> et Fc :

• J'ai contribué à la **mise en place d'outils de classification** à partir des cartes CIU, qui permettent une distinction des IgGs 1, 2 et 4 au niveau intact mais encore plus facile au niveau *middle* (après digestion du mAb en sous-unités), les différences d'empreintes CIU étant plus marquées qu'au niveau intact. Ces méthodes de classification se sont révélées particulièrement utiles pour la caractérisation d'un mAb hydride IgG2/4, eculizumab (Figure 5 ; Botzanowski T. *et al, Anal Chem* **2020**, 92(13), 8827-8835).



**Figure 5.** Cartes CIU d'eculizumab aux niveaux intact et middle. Les méthodes de classification développées permettent de déterminer l'isotype du mAb en comparaison à des cartes de référence. L'étude CIU sur les sousunités met en évidence le format hybride d'eculizumab (IgG2/4).

• Je me suis ensuite focalisée sur les **anticorps immunoconjugués** (ADC), notamment pour le suivi de la stabilité conformationnelle des intermédiaires de réaction impliqués dans la formation d'un ADC site-spécifique portant deux drogues (*drug-to-antibody ratio* DAR = 2). Dans ce dernier cas, la réaction de conjugaison a lieu sur les glycans des deux domaines Fc du trastuzumab (Figure 6). La première étape de déglycosylation induit une déstabilisation du produit. Les deux étapes suivantes permettent de fixer deux drogues sur le mAb, celles-ci conférant une stabilité accrue à l'ADC par rapport au mAb non conjugué (Figure 6). Ces résultats ont été publiés en première auteure dans *Pharmaceuticals* (Deslignière E. *et al., Pharmaceuticals* **2021**, 14 (6), 498).



**Figure 6.** Expériences CIU réalisées pour les différents produits obtenus durant la conjugaison de l'ADC sitespécifique T-GlyCLICK-DM1. Les cartes CIU et les valeurs CIU50 associées à chaque transition conformationnelle sont présentées pour l'état de charge 24+.

• Je me suis également intéressée aux cartes CIU de deux **ADCs à cystéine**, à savoir le brentuximab vedotin (DAR 0, 2, 4, 6, et 8) et le trastuzumab deruxtecan (DAR 8). La conjugaison des drogues sur les résidus cystéines nécessite une réduction des ponts disulfures interchaînes. Les empreintes CIU des deux ADCs à cystéine montrent que la réduction des liaisons disulfures entraîne une déstabilisation des mAbs en phase gazeuse se traduisant par l'apparition de nouveaux états conformationnels plus dépliés que ceux du mAb parent. Cette déstabilisation augmente progressivement des espèces DAR2 (un seul pont réduit) à DAR8 (où les quatre ponts sont réduits). Dans le cas d'un ADC à lysine « de contrôle » pour lequel les ponts disulfures restent intacts (trastuzumab emtansine, DARO à 8), la conjugaison n'entraîne pas de déstabilisation du mAb, confirmant ainsi le rôle clé des liaisons disulfures interchaînes dans le maintien de la structure des mAbs.

# 3. Automatisation des expériences de CIU – Développement du couplage SEC-CIU et application aux protéines thérapeutiques

Les expériences de CIU étant réalisées en grande partie de manière manuelle et forte de mon expérience en SEC-nMS, j'ai ensuite abordé le point de l'automatisation de l'ensemble des étapes de l'analyse CIU, de la préparation d'échantillon à l'acquisition des données. Pour cela, un couplage en ligne de la SEC à la CIU (SEC-CIU) a été développé : les acquisitions CIU sont ici réalisées automatiquement durant l'élution de la protéine d'intérêt (Figure 7A). Les cartes SEC-CIU, générées en ~45 min pour un triplicat, sont identiques à celles obtenues en 3 h par CIU « manuelle ». Enfin, différentes stratégies de multiplexage (mélange de mAbs) et/ou d'approches SEC-CIU ciblées (acquisition de tensions en *trap* signatures d'une IgG) ont permis une amélioration considérable des débits d'analyse (15 min pour trois mAbs). De plus, les performances du couplage ainsi développé ont été évaluées pour la classification de plusieurs IgG aux niveaux intact (Figure 7B, C) et *middle*, avec l'obtention de scores aussi élevés qu'en CIU manuelle. Ces développements ont fait l'objet d'une publication première auteure dans *Analytical Chemistry* (Deslignière E. *et al., Anal Chem* **2020**, 92(19), 12900-12908).



**Figure 7. (A)** Acquisition de données SEC-CIU (mAb intact). **(B)** Les cartes de référence générées permettent de créer une classification basée sur la région la plus discriminante (pointillés). **(C)** Classification obtenue par CIU ciblée.

Le couplage SEC-CIU mis en place sur le Synapt G2 (TWIMS linéaire) a ensuite été utilisé pour l'étude des deux isomères du tsAb précédemment analysés en cIMS-MS dans la partie III. Alors que la cIMS-MS n'a pas permis de mettre en évidence des différences de conformations au niveau intact, la SEC-CIU a quant à elle révélé des empreintes CIU légèrement distinctes pour les deux espèces, validant ainsi la tendance observée avec les mesures de <sup>TW</sup>CCS<sub>N2</sub> réalisées sur le TWIMS linéaire. Cet exemple illustre l'apport et la **complémentarité des nouvelles approches SEC-CIU (niveau intact) et cIMS-MS** (niveau middle) pour répondre à un problème concret rencontré dans l'industrie biopharmaceutique. L'orthogonalité des approches de haute résolution IMS-MS et CIU permet une caractérisation plus fiable de conformères possédant des propriétés d'interaction avec l'antigène très différentes.

#### 4. Apport de la haute résolution IMS-MS pour les expériences de CIU

J'ai ensuite pu évaluer l'apport de la haute résolution IMS-MS pour les expériences CIU appliquées aux mAbs au niveau intact. Une meilleure définition graphique des cartes est obtenue en cIMS-MS, permettant de mieux distinguer des états conformationnels qui coexistent, et d'ainsi révéler des états jusqu'ici invisibles sur les instruments TWIMS de première génération. L'avantage du *multipass* (deux tours) pour la génération de cartes CIU encore mieux résolues a également été démontré pour l'étude de mAbs intacts.

Ce gain de résolution IMS est particulièrement intéressant lorsqu'il s'agit de **discriminer les isotypes d'IgGs au niveau intact**. En effet, les cartes CIU des mAbs étant mieux résolues, il est plus facile de séparer et d'identifier les sous-classes d'IgGs, et notamment les IgGs 1 et 4 qui présentaient des traces CIU très similaires sur la cellule TWIMS linéaire. La figure 8 illustre les bénéfices de la haute résolution CIU-cIMS pour la classification des IgGs : plus la valeur de  $-\log_{10}(valeur-p)$  est élevée, plus la différence entre les cartes CIU des trois isotypes (1, 2 et 4) est marquée. Ainsi, pour l'état de charge 27+, des tensions en *trap* entre 125 – 155 V permettront de classer plus facilement les mAbs sur la cellule TWIMS cyclique que linéaire (Figure 8A). Ceci est encore plus flagrant sur l'état de charge 28+, qui présente une large région diagnostique entre 100 – 140 V contrairement à l'instrument TWIMS (Figure 8B). La haute résolution CIU-cIMS offre donc un avantage certain sur la CIU réalisée sur une cellule linéaire, pour laquelle il est souvent difficile d'obtenir rapidement un score de classification élevé (> 70%) au niveau intact (Figure 8C), à moins d'optimiser très finement les mAbs ont ainsi été classés soit à partir de cartes complètes, soit en appliquant la CIU ciblée sur un mélange de deux mAbs.



**Figure 8. (A-B)** Identification des zones les plus discriminantes entre les isotypes d'IgGs 1, 2 et 4 au niveau intact. Les mAbs de référence choisis sont le trastuzumab (IgG1), le panitumumab (IgG2) et le nivolumab (IgG4). **(C)** Classification d'elotuzumab (z = 27+) obtenue sur les instruments TWIMS linéaire et cyclique.

Enfin, grâce à la géométrie de l'instrument, j'ai ensuite pu étudier de manière plus poussée les deux isoformes de ponts disulfures séparées en cIMS-MS pour les IgG2s. L'analyse consiste ici à isoler et extraire une population résolue en cIMS-MS, puis à l'activer non pas en *trap* mais lors de sa réinjection

dans la cellule IMS, c'est-à-dire en sortie de la région *pre-store* (IMS-CIU-IMS). Les résultats très préliminaires obtenus sur le denosumab intact montrent une légère différence de comportement en phase gazeuse entre les deux variants disulfures lors de la première transition conformationnelle. Des expériences supplémentaires sont nécessaires pour confirmer ces premières tendances. Une piste d'amélioration serait ici de travailler au niveau *middle* : en effet, l'activation CIU en *pre-store* est moins énergétique que celle en *trap*, et il est donc plus difficile d'induire un déploiement pour des mAbs intacts, qui possèdent plus de degrés de liberté que des sous-unités digérées.

Ainsi, ces développements (automatisation/résolution) ouvrent la porte à l'intégration plus systématique de la CIU dans les stratégies de caractérisation de mAbs dans l'industrie pharmaceutique. Les nouvelles fonctionnalités de l'instrument cIMS-MS offrent également d'autres perspectives pour une étude plus poussée des traces CIU.

### **Conclusion générale et perspectives**

Mes travaux de thèse se sont articulés autour de développements méthodologiques en nMS et nIMS-MS, dans le but de répondre à différentes limitations identifiées au début de ma thèse : (i) le recours à un échange de tampon manuel en AcONH<sub>4</sub> problématique pour certains complexes, et (ii) le manque de résolution des données IMS et l'intégration de celles-ci en biologie structurale.

J'ai d'abord pu démontrer l'intérêt du couplage SEC-nMS pour différents complexes multiprotéiques. La SEC-nMS, qui fournit un dessalage rapide et efficace, et offre une dimension supplémentaire de séparation, est un nouvel outil pour répondre aux problématiques de biologie structurale. J'ai également illustré la complémentarité de la nMS et de la nIMS-MS avec d'autres techniques biophysiques classiques dans le cas de complexes multiprotéiques de hauts poids moléculaires.

J'ai ensuite pu évaluer l'apport d'un instrument de haute résolution en IMS, le TWIMS cyclique, développé pour pallier la faible résolution des cellules TWIMS linéaires de première génération. L'instrument cIMS-MS est particulièrement intéressant pour des petites protéines. Bien que le gain de résolution semble limité en mode *multipass* pour les protéines intactes de haute masse, d'autres modes sont exploitables (IMS<sup>n</sup>, activation) pour approfondir la caractérisation conformationnelle de ces protéines en conditions natives. Cette étude préliminaire mérite d'être approfondie sur la plateforme cIMS-MS mais aussi en évaluant les possibilités d'autres instruments IMS-MS de haute résolution récemment commercialisés tels que le SLIM (*structures for lossless ion manipulations*) proposé par MOBILion<sup>21</sup>, ou la technologie TIMS (*trapped IMS*) disponible sur des Q-ToF timsTOF Pro (Bruker) et qui se montre prometteuse pour l'étude de protéines natives<sup>22</sup>.

Finalement, j'ai pu poursuivre les développements en CIU initiés au laboratoire, notamment en proposant un couplage SEC-CIU pour l'automatisation des expériences CIU. L'émergence de nouveaux couplages mono-dimensionnels de chromatographies non dénaturantes à la nMS offre ici de nouvelles

perspectives : la SEC pourrait être remplacée par la chromatographie d'interaction hydrophobe (HIC), pour étudier l'influence du nombre de drogues conjuguées sur les profils CIU dans le cas d'ADCs à lysine, ou par la chromatographie échangeuse d'ions (IEX), pour déterminer l'impact de modifications post-traductionnelles sur la stabilité en phase gazeuse d'une protéine.

Enfin, d'autres problématiques qui devront être résolues dans le futur pour la caractérisation des protéines en nMS et nIMS-MS ont été identifiées en fin de thèse :

• Analyse de complexes de très hauts poids moléculaires : La détection de tels complexes est limitée en nMS classique (20 MDa)<sup>5</sup> car il est difficile de transmettre des édifices de hauts poids moléculaires en assurant une bonne désolvatation des ions, et donc une mesure de masse précise. Deux techniques basées sur la détection d'ions individuels, et non pas d'un ensemble d'ions, s'avèrent prometteuses pour l'étude de larges entités protéiques, et notamment les virus : la MS à détection de charge<sup>23</sup>, et les systèmes électromécaniques nanométriques<sup>24</sup>.

• Interprétation des données nIMS-MS : La modélisation et la prédiction de la structure des protéines lors de leur transfert de la phase liquide à la phase gazeuse représentent un véritable challenge. D'une part, les interactions électrostatiques doivent être considérées sur de plus longues distances dans le vide, ce qui ralentit considérablement les calculs. D'autre part, les champs de force couramment utilisés pour les études de dynamique moléculaire en phase liquide ne sont pas toujours représentatifs de la phase gazeuse. Enfin, un autre point essentiel pour la modélisation réside dans le placement des charges sur les protéines, qui est primordial pour simuler au mieux les répulsions électrostatiques, notamment lors des déploiements ou compactions qui ont lieu au cours des expériences CIU.

• Interprétation des données CIU – Apport des méthodes nTD-MS : Concernant l'interprétation des données CIU, il est désormais nécessaire de tendre vers une meilleure compréhension des mécanismes impliqués dans le déploiement protéique, afin de déterminer l'origine exacte des transitions observées sur les cartes CIU, voire de prédire les empreintes CIU. D'une part, le mode IMS<sup>n</sup> disponible sur l'instrument cIMS-MS apparaît ici intéressant pour suivre l'évolution d'un état conformationnel. D'autre part, les approches TD qui se sont développées ces dernières années et qui consistent à fragmenter la protéine intacte *via* différents modes de fragmentation, ont déjà été appliquées sur plusieurs protéines de référence afin de relier le déploiement CIU à la localisation des fragments nouvellement accessibles<sup>25</sup>. Le développement du nTD-MS est encore balbutiant car le recouvrement de séquence obtenu est souvent faible, peu de régions étant susceptibles d'être fragmentées. De nouvelles techniques de fragmentation plus énergétiques, comme la photodissociation ultraviolette (UVPD), permettent cependant d'accéder à des régions plus enfouies des protéines, et donc d'obtenir plus d'informations sur la séquence et les zones d'interactions entre les différents partenaires. Les études de dynamique moléculaire pourraient ainsi s'appuyer sur

l'ensemble de ces données pour améliorer la sélection des structures proposées pour la protéine et

ses états activés.

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## **Scientific Communications**

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- Duivelshof, B. L.; <u>Deslignière, E.</u>; Hernandez-Alba, O.; Ehkirch, A.; Toftevall, H.; Sjögren, J.; Cianférani, S.; Beck, A.; Guillarme, D.; D'Atri, V., Glycan-Mediated Technology for Obtaining Homogeneous Site-Specific Conjugated Antibody–Drug Conjugates: Synthesis and Analytical Characterization by Using Complementary Middle-up LC/HRMS Analysis. *Anal Chem* 2020, *92* (12), 8170-8177, DOI: 10.1021/acs.analchem.0c00282.
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#### **Book chapter:**

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#### Poster communications:

- <u>Deslignière, E.</u>; Ehkirch, A.; Botzanowski, T.; Beck A.; Hernandez-Alba, O.; Cianférani S., Towards automation of Collision Induced Unfolding experiments through online Size Exclusion Chromatography coupled to native Mass Spectrometry. Spectrométrie de Masse et Analyse Protéomique (SMAP), 16 – 19<sup>th</sup> September 2019, Strasbourg (France).
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## Manuscript

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## **Main Abbreviations**

AcONH4: Ammonium acetate	Fab: Antigen-binding fragment
ADC: Antibody-drug conjugate	FAIMS: High field asymmetric waveform ion mobility
ADP: Adenosine diphosphate	spectrometry
Ag: Antigen	Fc: Fragment crystallizable
ATD: Arrival time distribution	FDA: Food and Drug Administration
ATP: Adenosine triphosphate	FL: Full length
avDAR: Average drug-to-antibody ratio	FWHM: Full width at half maximum
bsAb: Bispecific antibody	HCD: Higher-energy collisional activation
BV: Brentuximab vedotin	HDX: Hydrogen/deuterium exchange
CCS: Collision cross section	HIC: Hydrophobic interaction chromatography
CDMS: Charge detection mass spectrometry	HRF: Hydroxyl radical footprinting
CDR: Complementarity-determining region	HSP: Heat shock protein
CID: Collision-induced dissociation	IEX: Ion exchange chromatography
cIMS-MS: Cyclic ion mobility spectrometry-mass	<b>IgG:</b> Immunoglobulin G
spectrometry	IMS: Ion mobility spectrometry
CIU: Collision-induced unfolding	IWMATD: Intensity weighted mean of an ATD
CODV: Cross-over dual variable domain arm	LBD: Ligand-binding domain
CQA: Critical quality attribute	LC: Liquid chromatography
CSD: Charge state distribution	LDA: Linear discriminant analysis
CV: Collision voltage (trap)	LiP: Limited proteolysis
DAR: Drug-to-antibody ratio	mAb: Monoclonal antibody
DBD: DNA-binding domain	MALDI: Matrix assisted laser desorption ionization
DC: Direct current	MD: Molecular dynamics
DMA: Differential mobility analyzer	MS: Mass spectrometry
Dn species: mAb linked to n drugs	MW: Molecular weight
DSC: Differential scanning calorimetry	nIMS: Native ion mobility spectrometry
dTD: Denaturing top-down	NMR: Nuclear magnetic resonance spectroscopy
DTIMS: Drift tube ion mobility spectrometry	nMS: Native mass spectrometry
ECD: Electron capture dissociation	NR: Nuclear receptor
EHSS: Exact hard sphere scattering	nTD: Native top-down
EM: Electron microscopy	oaToF: Orthogonal acceleration time-of-flight mass
ERRα: Estrogen-related receptor alpha	spectrometer
ESI: Electrospray ionization	<b>OMS:</b> Overtone mobility spectrometry
ETD: Electron transfer dissociation	PA: Projection approximation
ExD: Electron transfer/capture dissociation	Pi: Pressure in the interface region

#### Abbreviations

**PSA:** Projected superposition approximation **PTM:** Post-translational modification **R1/R2:** Human RuvBL1/RuvBL2 **RF:** Radio-frequency **RMSD:** Root-mean-square deviation rpLC: Reversed-phase liquid chromatography SAXS: Small angle X-ray scattering scFv: Single chain variable fragment **SEC:** Size exclusion chromatography SID: Surface-induced dissociation SLIM: Structures for lossless ion manipulations **SLS:** Static light scattering **SRIG:** Stacked ring ion guide TD: Top-down T-DM1: Trastuzumab emtansine T-DXd: Trastuzumab deruxtecan **TIMS:** Trapped ion mobility spectrometry **TM:** Trajectory method ToF: Time-of-flight tsAb: Trispecific antibody T-wave: Travelling wave **TWIMS:** Travelling wave ion mobility spectrometry **UFS:** Univariate feature selection UVPD: Ultraviolet photodissociation Vc: Sampling cone voltage WH: Wave height WV: Wave velocity

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# Introduction

Introduction

**General Introduction** 

## **General Introduction**

Analyzing protein complexes and protein interaction networks is an important endeavor as almost all biological processes rely on regulated cooperation between multiple protein subunits, cofactors, DNA, or even small messenger molecules. Structural and functional characterization of biomolecules involved in these machineries and how they interact is the key to understand biological processes<sup>1</sup>.

Different analytical and biophysical approaches are available to investigate protein topology and interactions. Among them, mass spectrometry (MS), and particularly structural MS techniques, have emerged over the last two decades to complement well-established biophysical methods, including X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and electron microscopy (EM), for the three-dimensional characterization of multiprotein complexes<sup>2,3</sup>. Structural MS provides diverse information ranging from primary to quaternary structures of proteins, and allows studying subunit dynamics/interactions, protein conformation, and (dis)assembly of biomolecules. Structural MS approaches are divided into two categories: peptide-centric strategies, such as hydrogen/deuterium exchange (HDX), protein painting, or chemical cross-linking, and protein-centric strategies, including native MS (nMS), ion mobility spectrometry (IMS) and top-down (TD). This thesis focuses on nMS and IMS approaches.

nMS enables the characterization of intact macromolecular assemblies by preserving noncovalent interactions, and gives access to sample homogeneity/heterogeneity, binding stoichiometry/affinity, and complex stability. First limited to small complexes, nMS is now widely applied in the field of structural biology<sup>4</sup>, for protein/ligand screening<sup>5</sup>, and analysis of increasingly larger multiprotein complexes, from GroEL<sup>6</sup> to megadaltons ribosomes<sup>7</sup> and viral capsids<sup>8</sup>. nMS has also become a pivotal tool for the study of therapeutic proteins like monoclonal antibodies (mAb) products<sup>9</sup>, which will be largely considered in the present manuscript.

The coupling of ion mobility to mass spectrometry (nIMS-MS) offers an additional dimension of ion separation in the gas phase, based on charge and conformation. The arrival time distribution (ATD) of ions resulting from IMS separation can be converted into a rotationally-averaged collision cross section (CCS), which reflects the global gas-phase conformation of ions. CCSs constitute structural constraints that can be useful for molecular modeling purposes<sup>10</sup>. However, standalone CCS measurements often cannot differentiate co-drifting species, mostly because of low resolving powers available on first generation IMS instruments<sup>11</sup>. In order to overcome these limitations, new strategies have arisen, among which collision-induced unfolding (CIU) experiments<sup>12</sup>, and high-resolution IMS-MS technologies<sup>13,14</sup>. CIU has already proved efficient to pinpoint subtle differences between species with very close conformations, including protein/ligand complexes<sup>15</sup>, diverse mAb formats<sup>16,17</sup>, and even membrane proteins' functional variants<sup>18</sup>. Conversely, the use of high-resolution IMS-MS to investigate native protein assemblies has been scarcely reported until now<sup>19,20</sup>.

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In this context, my PhD work has focused on methodological developments related to (i) the sample preparation step for nMS and nIMS-MS analysis of biological complexes, (ii) the application of high-resolution IMS-MS to therapeutic proteins, and (iii) the automation of CIU approaches and potentialities of high-resolution CIU-MS.

The manuscript is therefore structured in four parts summarized thereafter:

- The **first part** corresponds to an overview of state-of-the-art structural MS approaches for the study of noncovalent complexes. The instrumentation and methods used during this PhD work are also presented.
- The second part is dedicated to the integration of nMS and nIMS-MS data into structural biology studies. A first project consisted of expanding the coupling of size exclusion chromatography to nMS (SEC-nMS) to a broader range of biological samples, as SEC-nMS was mostly restricted to mAb-based products at the beginning of t<sup>2</sup>his thesis. The potential of nMS and nIMS-MS to complement classical biophysical techniques in structural biology programs is then illustrated through the characterization of high molecular weight multiprotein complexes involving human RuvBL helicases and different binding partners.
- The third part describes the use of high-resolution cyclic IMS-MS (cIMS-MS) for the analysis of biotherapeutics (mAb-based products), with the aim of evaluating advantages of cIMS over linear travelling-wave IMS (TWIMS) instruments. The cIMS-MS platform affords higher resolving powers owing to multipass separation, and new multifunctional capabilities to select IMS populations. cIMS-MS is applied at the peptide level for disulfide-bridged isomers, and on intact proteins to distinguish immunoglobulin G (IgG) subclasses, but also conformers from a trispecific antibody (tsAb).
- The fourth part presents the development of CIU methodologies for improved characterization
  of different mAb formats (IgGs, antibody-drug conjugates ADCs, tsAb). Available bioinformatics
  tools for data treatment were first evaluated in order to achieve more detailed classification of
  IgG subclasses. Next, a SEC-CIU workflow was developed to fully automate CIU experiments
  from sample preparation to data interpretation. The coupling of SEC to CIU allowed to drastically
  reduce CIU acquisition time, which was further enhanced by relying on targeted and/or
  multiplexing strategies. Lastly, benefits of high-resolution cIMS-MS for CIU approaches were
  demonstrated for in-depth analysis of IgGs.

# **PART I – Introduction to Structural MS Approaches**

The first part of this thesis provides a general introduction to structural MS approaches, with a more detailed description of nMS and nIMS-MS techniques which were the focus of my PhD work. Methodologies and instrumental parameters related to nMS, nIMS-MS, and CIU experiments, are also described.

#### **Chapter 1 – Structural MS Techniques**

Chapter 2 – Instrumentation and Methods for nMS and nIMS-MS Approaches

Part I – Introduction to Structural MS Approaches

# **Chapter 1 – Structural MS Techniques**

Over the past two decades, technological advancements in MS-based proteomics have enabled the direct analysis of intact native proteins and their complexes<sup>21</sup>. While conventional bottom-up approaches rely on protein digestion, structural MS techniques offer a view of proteins at the intact level, affording a better understanding of their structures and functions.

Structural MS has now become a pillar for integrative structural biology studies, as it provides a plethora of information from protein primary structure to higher-order quaternary structure, and interactions/dynamics of protein assemblies<sup>2,21</sup>. To achieve this range of information, several MS approaches have been developed to characterize proteins<sup>22</sup>:

- Peptide-centric approaches probe the structure of proteins by using classical bottom-up proteomics workflows (Figure 1). Proteins are first manipulated in their native states, directly in solution *via* surface labelling (HDX, protein painting, etc.), cross-linking, or limited proteolysis methods. Subsequent digestion and liquid chromatography (LC)-MS/MS analysis give information on proteins based on identified peptides.

- Protein-centric approaches consist of studying intact proteins or protein complexes (Figure 1). In native conditions, noncovalent assemblies are maintained and analyzed by using nMS, nIMS, or native top-down (nTD) methods. Analyses in denaturing conditions, for which noncovalent interactions are not preserved, provide additional information on intact species, *via* denaturing MS or top-down (dTD).



Figure 1. Illustration of main structural MS techniques. Adapted from Lössl et al., 2016 (ref. 21).

#### 1. Native mass spectrometry

#### 1.1. Evolution of nMS – Technological developments and biological applications

In the late 1980s, the revolutionary development of soft ionization techniques, such as matrix assisted laser desorption ionization (MALDI)<sup>23</sup> and electrospray ionization (ESI)<sup>24</sup>, allowed to analyze larger biomolecules. Because MALDI generates singly charged ions, their m/z values can span beyond the detection range of many mass spectrometers. Conversely, ESI produces multiply charged ions and is thus well adapted for mass spectrometers with limited upper m/z ranges. ESI generates a spray of charged droplets in presence of a strong electric field. The radii of droplets decrease *via* solvent evaporation/fission events as droplets pass down a potential and pressure gradient through the mass spectrometer inlet towards the analyzer region<sup>25,26</sup>. The development of nanoESI in 1996 allowed for lower sample consumption and higher tolerance to salts, and so nanoESI became the most popular ionization technique for proteins<sup>27</sup> (Figure 2).

After ESI introduction in 1989 by Fenn *et al.* for large molecules<sup>24</sup>, the groups of Chait and Henion performed pioneering works in nMS through studies of noncovalent protein/ligand complexes<sup>28,29</sup> (Figure 2). Intact protein/protein interactions were observed the following year<sup>30</sup>. At the same time, technological developments focused on improving different mass analyzers. Early quadrupole mass analyzers were limited to m/z < 3 000. This m/z range was increased thanks to the development of quadrupoles operating at lower radiofrequencies<sup>31,32</sup>. Yet, higher m/z suffered from a lack of resolving power. On the other hand, time-of-flight (ToF) analyzers have no theoretical upper mass limit, and provide high resolution/sensitivity. The ESI-orthogonal acceleration ToF configuration (ESI-oaTOF) was described in 1991<sup>33</sup>, and was later used to observe noncovalent interactions within an hexameric enzyme<sup>34</sup>. The first ESI-Q-ToF instrument was finally presented in 1996<sup>35</sup>, and rapidly became the most successful platform for nMS analysis.

A series of technological advancements to enhance the transmission and desolvation of high m/z ions on Q-ToF instruments pushed forward the applications of nMS<sup>36,37</sup>. The term "native MS" was ultimately coined in 2004<sup>38,39</sup>. Lastly, the introduction of Orbitrap mass analyzers, along with subsequent modifications for higher m/z ranges (up to m/z 80 000), opened new avenues for the characterization of large macromolecular complexes and their proteoforms, providing increased resolution, sensitivity, and better desolvation<sup>7,40-43</sup>.

Over the years, nMS has been used to study complexes of increasing complexity and heterogeneity, such as the GroEL chaperonin assembly<sup>6</sup>, mAb-based biotherapeutics<sup>44</sup>, the ribosome 30S subunit<sup>45</sup>, hemocyanines<sup>46</sup>, and virus capsids up to 18 MDa<sup>8,47</sup> (Figure 2). nMS also afforded the characterization of overexpressed proteins in crude cell lysates, overcoming the need for purification and buffer exchange<sup>48-51</sup>. Lastly, the analysis of membrane proteins has received a growing interest after the group of Robinson demonstrated that detergent micelles could be used to solubilize membrane proteins, micelles being removed through activation in the gas phase<sup>52</sup>. Nanodiscs and amphipols are alternative solubilization techniques<sup>53,54</sup>.

Recently, nMS offered further insights into interactions between the receptor binding domain of the SARS-CoV-2 spike protein and the ACE2 host cell receptor<sup>55,56</sup>. The SARS-CoV-2 main protease M<sup>pro</sup>, which is a key antiviral drug target, was also characterized, proving that nMS is mature enough to answer vital and urgent biological issues<sup>57</sup>.



Figure 2. Milestones for instrumental developments and biological applications in nMS.

#### 1.2. Information obtained using nMS

Mass measurements and binding stoichiometry represent the most common applications of nMS. Gas-phase stability can be assessed by determining the accelerating voltage needed to dissociate 50% of the complex ( $Vc_{50}$  experiments)<sup>58-60</sup>. Binding affinities and specificity can also be evaluated either through competition experiments, with an equimolar mixture of ligands that compete for the same binding site, or with titration experiments to obtain affinity ( $K_A$ ) and dissociation ( $K_D$ ) constants<sup>57,60-63</sup>.

Denaturing MS experiments are generally performed concomitantly with nMS. The use of organic solvents (acetonitrile, methanol, propanol) and acids disrupts the native state of proteins. As a result, unfolded proteins present a wide charge state distributions in low m/z ranges. This allows to measure the intact mass of each species involved in complex formation.

Masses of subunits can also be determined by partially dissociating the native complex in a controlled manner, without backbone cleavage. This approach is referred to as "complex-up" MS<sup>64</sup>. Collision-induced dissociation (CID) or surface-induced dissociation (SID) are classically employed to release subunits<sup>65-67</sup>.

#### 2. Ion mobility spectrometry-mass spectrometry

#### 2.1. Evolution of IMS

IMS measures ions' drift through a region filled with a background gas, under the influence of an electric field. IMS was described in 1898<sup>68</sup>, 15 years before MS<sup>69</sup>.

IMS was first coupled to a magnetic sector mass spectrometer by McDaniel *et al.* in 1961<sup>70</sup>. Soon after, McAfee and Edelson (1963) presented the first IMS-ToF instrument<sup>71</sup> (Figure 3). The hyphenation of IMS to a ToF mass analyzer is particularly appropriate because ToF operates on the microsecond scale while the IMS separation occurs on a millisecond timescale, and so ToF and quadrupole mass spectrometers rapidly replaced magnetic sectors ones for IMS experiments. These early IMS designs were based on the linear drift tube IMS (DTIMS).

By the 1970s, the first IMS-MS instrumentation was commercialized under the name Plasma Chromatograph, and used an ambient pressure IMS spectrometer interfaced to a quadrupole mass spectrometer<sup>72</sup>. However, it was not until the groundbreaking developments from the groups of Hill<sup>73,74</sup>, Bowers<sup>75,76</sup>, Jarrold<sup>77,78</sup>, and Smith<sup>79</sup> in 80s and 90s that the field of IMS expended. Significant improvements have occurred, notably the use of electrodynamic fields to confine, transfer and focus ions across regions of different pressures into high vacuum<sup>80</sup>, with the subsequent development of the ion funnel<sup>79</sup>. In the meantime, Dugourd *et al.* introduced the first high-resolution DTIMS instrument<sup>78</sup>.

In 2006, Waters released a TWIMS-based IMS-MS platform, which helped to put the technique in the hands of a broader research community<sup>81,82</sup>. Early-generation TWIMS instruments had limited resolving powers<sup>83</sup> (R ~ 40  $\Omega/\Delta\Omega$ , Synapt G2 HDMS), often preventing the separation of closely related conformations<sup>11</sup>. Different high-resolution technologies/platforms were developed after 2010, including trapped IMS (TIMS)<sup>84,85</sup>, the cIMS geometry<sup>14</sup>, as well as several structures for lossless ion manipulations (SLIM) designs<sup>86-88</sup> (Figure 3). Tandem IMS platforms have also been proposed, and can be of main interest to overcome the limitations of IMS<sup>1</sup> analyses, especially if ions are activated between IMS cycles<sup>89</sup>. This strategy was first applied on a tandem DTIMS instrument, where a specific conformation separated in the first dimension is selected and further activated at the entrance of the second drift tube<sup>90,91</sup>. A tandem TIMS was recently designed, allowing for mobility selection of subspecies and ion activation between the two TIMS analyzers<sup>92</sup>.



**Figure 3.** Milestones in the development of IMS instrumentation. Adapted from Eldrid *et al.* 2020, and May *et al.* 2015 (ref. 89 and 93).

Part I – Introduction to Structural MS Approaches

Current IMS techniques can be divided into three separation concepts<sup>93</sup> (Figure 4):

- Time-dispersive IMS generates an ATD profile, with all ions travelling along the same path. These methods include DTIMS, TWIMS, and overtone mobility spectrometry (OMS).

- Spatially-dispersive IMS separates ions along different paths, but without significant dispersion in time. These techniques comprise high field asymmetric waveform IMS (FAIMS) and differential mobility analyzers (DMA).

- Ion confinement and selective release methods consist of trapping ions in a pressurized region of the mass spectrometer, and ejecting them selectively based on their mobilities. TIMS, SLIM, and cIMS are based on this technique.



Figure 4. Schematic representation of the three categories of IMS techniques. From May et al., 2015 (ref. 93).

#### 2.2. Information obtained in (n)IMS-MS

IMS-MS experiments contain information regarding m/z ratio of species, ions' intensities, and drift times. Multiple features can be extracted:

- IMS separation generates an ion mobility spectrum, called ATD. This raw output already provides qualitative information on samples, as ATDs give a direct snapshot of conformational heterogeneity. Sharp peaks indicate a single conformer, while broad IMS profiles reflect rich conformational spaces. The impact of complex formation on a protein's conformational landscape can thus be assessed.

- Arrival times can be further converted into rotationally-averaged CCS values, which represent the 3D structures of proteins (Figure 5). nIMS-MS measurements proved that nMS mostly preserves native conformations of proteins<sup>94-96</sup>, even if gas-phase collapse can occur in some cases<sup>97-99</sup>. Thus, CCSs can be integrated as structural constraints to establish protein/complexes models, and can be compared to theoretical values obtained from resolved structures<sup>100</sup> (see part I, chapter 2 for more details).

- Lastly, IMS-based CIU experiments inform on the conformational stability of proteins upon gasphase activation. Apo/holo proteins, and even protein isoforms, are expected to yield distinct CIU behaviors.



Figure 5. Schematic representation of CCS.

#### 2.3. Principles of main IMS techniques available for CCS calculations

It is estimated that roughly 95% of reported CCSs were measured with DTIMS (85%) and TWIMS (10%), the remainder CCS values being obtained with TIMS or DMA<sup>101</sup>. These four methodologies will be described in the following paragraphs.

#### 2.3.1. DTIMS

DTIMS is the historical and simplest form of IMS. This time-dispersive IMS technology is composed of a drift tube filled with an inert buffer gas, generally N<sub>2</sub> or He<sup>102</sup>. Ions are directed through the stationary gas by a weak uniform electric field E. Ions are slowed down as they collide with the background gas<sup>103</sup> (Figure 6). Hence, for a given charge state, the drift time of compact ions will be shorter than elongated ones, considering that they undergo fewer collisions with gas particles. The separation of the ions occurs as a function of their shape, which is related to the ion mobility (K) or CCS. In DTIMS, CCS values can be directly determined without calibration, provided that all parameters are accurately known and carefully controlled (voltages, pressures, tube length, etc.)<sup>104</sup>. DTIMS is thus considered as a primary method for CCS measurements, and so it is the main IMS technique used to determine reference CCS values<sup>105,106</sup>. It is worth noting that early generation DTIMS instruments suffered from a low duty cycle (~1 %) because ions were injected into the tube in narrow pulses<sup>107</sup>. Recent studies have shown that ion losses could be reduced through ion accumulation and multiplexing schemes, leading to a duty of 60%<sup>108,109</sup>. DTIMS instruments are commercialized by several vendors, including Agilent Technologies<sup>110</sup>, Tofwerk<sup>111</sup>, and Excellims (Table 1).



Figure 6. Schematic representation of DTIMS separation and principle.

#### 2.3.2. TWIMS

TWIMS is another time-dispersive method, but contrary to DTIMS, it uses a dynamic electric field<sup>81</sup>. The drift cell of linear TWIMS instruments consists of a stacked ring ion guide (SRIG) filled with a static gas, typically He, N<sub>2</sub>, or Ar<sup>81,112</sup>. Radio-frequency (RF) voltages of opposite phases are applied to adjacent ring electrodes to radially confine ions. A direct current (DC) voltage is superimposed to the RF on an electrode and switched to the following electrode after a given time along the SRIG, creating travelling waves (T-waves) that propel ions trough the IMS cell<sup>81,82</sup> (Figure 7A). By surfing on the waves, larger ions experience more friction with the background gas and slip behind the waves (also called roll-over), and so their drift time is longer than smaller ions (Figure 7B). Because the electric field is not

static in TWIMS experiments, the determination of CCS values requires previous IMS calibration using reference ions of known CCSs<sup>106</sup>. TWIMS instruments are commercialized by Waters.



**Figure 7. (A)** Schematic representation of TWIMS separation and principle. **(B)** A DC voltage is applied to ring electrodes (blue) constituting the TWIMS cell to create the T-wave on which ions can surf.

#### 2.3.3. TIMS

TIMS belongs to the "confinement and selective release" category. Ions are radially confined in the ion tunnel by a RF voltage. TIMS uses a buffer gas (N<sub>2</sub>) to push ions into the drift cell, while a low non-uniform electric field (axial field gradient) is applied in the opposite direction, i.e. towards the tunnel entrance<sup>84,85</sup>. The force of the electric field counteracts the force of the gas flow, and so ions of different sizes are trapped at different regions along the tunnel<sup>113</sup> (Figure 8). More precisely, ions will be trapped at the place where the electric field strength is such that ions velocity (KE) equals the buffer gas velocity (v<sub>g</sub>). Ions with lower mobilities are trapped closer to the exit of the tunnel because higher fields strengths are required to counterbalance the drag from the buffer gas<sup>114</sup>. Following the trapping event, the electric field is gradually reduced, affording the sequential elution of trapped ions. Conversely to DTIMS and TWIMS, low-mobility ions are released first. TIMS is not a primary method for CCS measurement, and thus requires previous CCS calibration<sup>106</sup>. The TIMS technology has been commercialized by Bruker<sup>115</sup>.



Figure 8. Schematic representation of TIMS separation and principle.

#### 2.3.4. DMA

The DMA uses a uniform electric field applied between two parallel-plate electrodes. Ions enter the DMA through an inlet slit made in of the electrode. Ions migrate towards the other electrode under the influence of the applied field, while being transported in a stream of buffer gas which flows parallel

to the plates<sup>116</sup> (Figure 9). Only ions of a specific electrical mobility will exit the DMA through the outlet slit. Scanning the voltage between the two plates allows to sequentially sample ions of a given mobility. DMA can be used as a primary method for CCS measurements, provided that the applied voltage, temperature, pressure, and sheath gas flowrate are precisely known<sup>106</sup>. DMA devices are able to perform CCS measurements not possible with DTIMS, at it can detect very large analytes such as antibodies<sup>117</sup>, viruses, and other macromolecules<sup>118</sup>. DMA instruments are commercialized by SEADM and TSI.



Figure 9. Schematic representation of IMS separation and principle using a DMA.

#### 2.4. Towards high resolution IMS

Recent developments in IMS instrumentation aimed at improving available resolving powers. For DTIMS and TWIMS, R depends on the path length (L), the electric field (E), charge of ions (z), and buffer gas temperature (T) (Equation 1)<sup>119</sup>. Technological advances thus encompass increases of parameters  $L^{14,86}$  or  $E^{120}$ , and reduction of  $T^{121,122}$ .

$$R \sim \sqrt{\frac{zLE}{T}}$$
 (1)

The first device able to perform multipass IMS separation was the ion cyclotron mobility spectrometer, composed of four successive drift tubes<sup>123</sup>. High-resolution TWIMS-based instruments were later developed, a main advantage of TWIMS being that L can be extended without requiring additional voltage<sup>13</sup>. In addition, ions enter and exit the drift region at the same potential, which makes TWIMS particularly suited for closed-loops designs<sup>81,93</sup>.

In 2019, Giles *et al.* presented a cyclic TWIMS platform (cIMS-MS) in which the IMS cell is placed orthogonally to the axis of the mass spectrometer<sup>14</sup> (SELECT SERIES Cyclic IMS, Waters, U.K.). A resolving power of ~920 was achieved after 58 passes (~57 m) for carbohydrates<sup>124</sup> (Table 1).

The SLIM technology<sup>86</sup>, which was further explored to create a serpentine ultralong path with extended routing (SUPER-SLIM)<sup>87</sup>, also makes full use of the advantages of TWIMS by considerably extending L to 13.5 m. This approach allows to transmit ions while limiting losses in sensitivity, and resulted in a resolving power of ~1860 after a 540 m-long multipass separation (40 passes, Table 1)<sup>87</sup>.

Other IMS technologies have emerged to enhance IMS resolution, such as TIMS, whose resolving power goes up to 400<sup>84,125</sup> (Table 1).

	DTIMS	Linear TWIMS	Cyclic TWIMS	SLIM- TWIMS	TIMS
Commercial name	Agilent 6560 IM-QTOF <sup>♯</sup> <i>Tofwerk IMS-TOF</i> ▲	Synapt G2 HDMS	SELECT SERIES Cyclic IMS	MOBIE	timsTOF Pro
Release date	2014/2016	2009	2019	2021	2017
Vendor	Agilent Technologies <i>Tofwerk</i>	Waters	Waters	MOBILion Systems	Bruker
Path length L	0.80 m <i>0.20 m</i>	0.25 m	0.98 m	13.5 m (SUPER)	0.097 m
Multipass	No	No	Yes	Yes	No
Highest reported resolving power (Ω/ΔΩ) (for small molecules)	58 (ref. 110) <i>250 (ref. 111)</i>	40 (ref. 83)	920 after 58 passes ≈ 57 m (ref. 124)	1860 after 40 passes ≈ 540 m (ref. 87)	400 (ref. 125)

**Table 1.** Comparison of different IMS-MS platforms in terms of achievable resolving powers. <sup>#</sup>The drift tube operates at low pressure. <sup>A</sup>The drift tube operates at atmospheric pressure.

#### 2.5. IMS-MS applications

#### 2.5.1. IMS-MS for structural biology studies

While IMS was initially used for chemical warfare agents, successive instrumental developments pushed forward its integration into research and industrial environments<sup>93</sup>. The range of IMS-MS applications has now expanded, ranging from small molecules (lipids, glycans, etc.) to larger assemblies, including proteins and their complexes, even in the megadalton range<sup>126,127</sup>. For example, nIMS-MS has been employed to probe conformational changes upon ligand<sup>60,128</sup> or metal<sup>129,130</sup> binding, to characterize conformational differences across protein mutants involved in Parkinson's disease<sup>131</sup>, or to give further insight into aggregation process and oligomerization states of peptides implicated in amyloid assembly<sup>132-134</sup>.

#### 2.5.2. The special case study of mAb-based biotherapeutics

nIMS-MS has now become an essential tool for the analysis of biotherapeutics, and so this approach was extensively used during this PhD work for the characterization of different mAb formats.

#### 2.5.2.1. Introduction to mAbs

B lymphocytes play a key role in humoral immune response of vertebrates<sup>135</sup>. B cells produce antibodies, or IgGs, that recognize and bind foreign antigens (Ag). mAbs are able to bind to one unique epitope on a target Ag. Elimination of immune mAbs/Ag then occurs through different pathways<sup>136,137</sup>, among which antibody-dependent cell-mediated cytotoxicity (ADCC) involving natural killer cells<sup>138</sup>, complement-dependent cytotoxicity (CDC)<sup>139</sup>, or antibody-dependent cellular phagocytosis (ADCP)<sup>140</sup>. Due to their high specificity and efficiency, mAbs have become the new backbone of the pharmaceutical industry, with new mAb formats coming through company pipelines<sup>141</sup>. More than 100 mAb-based products are currently approved by the U.S. Food and Drug Administration (FDA) for the

treatment of various human diseases, including cancers, autoimmune disorders and inflammatory diseases<sup>142</sup>.

#### 2.5.2.2. Structure of IgG

IgGs are Y-shaped glycoproteins which comprise two heavy chains (~50 kDa each) and two light chains (~25 kDa each) linked *via* disulfide bonds (Figure 10A). Each IgG contains six different domains: one variable (VL) and one constant (CL) for light chains, and one variable (VH) and three constant (CH1, CH2, CH3) for heavy chains. Each variable domain in the antigen-binding fragment (Fab) region regulates antigen recognition *via* three hypervariable loops called complementarity-determining regions (CDR), which impart unique antigen specificity to mAbs. The dimeric structure of the two Fab arms connected through the hinge region is the F(ab')<sub>2</sub> domain (~100 kDa). The fragment crystallizable region (Fc) is responsible for effector functions<sup>143</sup>.



Figure 10. (A) Structure of IgG (exemplified for an IgG belonging to subclass 1). (B) Examples of mAb-based formats.

The vast majority of mAb-related products approved by the U.S. FDA are canonical IgGs. IgGs serve as basis for the development of several other types of formats, including truncated formats, such as single chain variable fragments (scFv), engineered bispecific antibodies (bsAb), or ADCs (Figure 10B). ADCs are empowered mAbs that consist of a tumor-targeting antibody covalently attached to highly potent cytotoxic drugs *via* a cleavable or non-cleavable chemical linker<sup>144</sup>. Depending on the conjugation process, species with different number of attached drugs, i.e. drug-to-antibody ratio (DAR), are obtained (Dn species). The type of conjugation determines the average drug-to-antibody ratio (avDAR) and the drug load distribution (DLD) of the ADC.

#### 2.5.2.3. Importance of nIMS-MS for the characterization of mAb-based products

Because of their structural complexity, thorough characterization of mAbs and their derivatives is essential along product development. The monitoring of critical quality attributes (CQAs), among which size- and charge-related variants, glycosylation, disulfide pairings, or other post-translational modifications (PTMs such as deamidation, oxidation, etc.), and avDAR/DLD for ADC products, is mandatory to meet regulatory agencies criteria, as these CQAs may impair the stability, immunogenicity and efficacy of the drug product<sup>145</sup>.

A first application of nIMS-MS is the assessment of mAbs' disulfide bonds heterogeneity. By using IMS-MS under denaturing and native conditions, Atmanene *et al.* highlighted heterogeneous disulfide pairings of a recombinant Ag<sup>146</sup>. In this work, IMS-MS was the sole analytical technique able to pinpoint differences in disulfide heterogeneity between two Ag preparations. As IMS-MS offers a direct snapshot of conformational spaces of species under study, using this technique is particularly interesting for preliminary investigations of mAb conformations.

Several groups aimed at measuring CCS values of mAbs and their related compounds<sup>98,147-149</sup>. Pritchard *et al.* applied nIMS-MS to three monomeric conformations, with CCSs ranging from 61 to 76 nm<sup>2</sup>, and two dimers (CCS between 106 and 115 nm<sup>2</sup>) for a commercial rhGH-specific antibody<sup>150</sup>. Elsewhere, CCS measurements were used to monitor the formation of a bsAb in real time, the CCS of the bsAb (66.9 nm<sup>2</sup>) being intermediate to those of its parental mAbs (65.6 and 69.6 nm<sup>2</sup>)<sup>151</sup>. CCS values of the different Dn species of lysine- and cysteine-based ADCs on the market<sup>152,153</sup>, as well as a site-specific ADC<sup>17</sup>, were also measured. nIMS-MS is useful to measure drug load profiles and calculate avDAR values. Even if the DAR calculation based on nIMS-MS results is not as straightforward as from nMS data, the comparison of drift times obtained for Dn populations allows for rapid semi-quantitative DAR determination.

In-depth characterization of mAbs conformations remains challenging through nIMS-MS, especially in native conditions. mAbs are highly dynamic molecules due to the inherent flexibility of the hinge region. Flexibility arises from rotation, waving or elbow bending of Fab arms, and wagging of the Fc domain<sup>154</sup>. In the gas phase, this flexibility is reflected by wide conformational spaces in IMS, with makes the resolution of distinct conformers difficult, as a continuum of conformational families is rather observed<sup>98</sup>. Molecular dynamics (MD) simulations also indicate that desolvation causes a contraction (collapse) in the hinge region<sup>98,99,155</sup>. Due to their dynamic nature, crystallization of mAbs remains difficult<sup>156</sup>, and structural information on IgGs is lacking, with only two crystal structures available for full length (FL) human antibodies<sup>157,158</sup>.

New high-resolution IMS-MS instruments offer new opportunities to tackle the conformational heterogeneity of mAb-based formats, and could even answer to the analytical challenge related to the detection of small PTMs at intact protein level, provided that these PTMs lead to structural variations.

#### 2.6. Collision-induced unfolding approaches

In some cases, species with very close conformations cannot be differentiated solely based on IMS-MS measurements<sup>148,149</sup>, mostly because of low resolving powers of first-generation TWIMS devices. CIU approaches offer an elegant alternative to overcome these limitations.

#### 2.6.1. Principle

CIU experiments are performed by sequential increase of an accelerating potential difference to induce ion activation before IMS separation<sup>15</sup>. As the accelerating voltage is raised, collisions with the buffer gas (generally  $N_2$  or Ar) become more energetic, which leads to a build-up of internal energy in

the ions. Upon activation, ions may cross energy barriers and undergo conformational transitions through different conformational states/intermediates related to the unfolding or compaction of the protein (without breaking noncovalent bonds)<sup>159</sup>. These conformational transitions are monitored through ATD changes. ATDs are recorded at each collision voltage (CV). For TWIMS instruments, the CV corresponds to the voltage applied to ions at their entrance into the trap cell (Figure 11). CVs translate into laboratory-frame collision energy when multiplied by the charge state of a precursor ion<sup>97</sup>. Unfolding plots, named CIU fingerprints, can be generated to better visualize CIU data (Figure 11). CIU fingerprints represent a unique signature of each species behavior upon activation, allowing the rapid detection of subtle changes of conformational state in the gas phase.



Figure 11. Principle of CIU experiments.

#### 2.6.2. Mass spectrometers available to perform CIU experiments

Most papers reporting on CIU use TWIMS instruments, with ion activation occurring in the trap cell, located upstream of the IMS region, through collisions with an inert background gas under low pressure conditions (Figure 11)<sup>15,17,20,160-162</sup>. On other types of IMS spectrometers (TIMS, DTIMS), insource activation is more common, but might generate different protein unfolding mechanisms because of the presence of residual solvent molecules that may lead to ion-molecule reactions<sup>163</sup>.

In spite of the high prevalence of TWIMS platforms for CIU experiments, CIU has also been performed on DTIMS spectrometers, where activation occurs either behind the source capillary<sup>163-165</sup> or in the trapping funnel<sup>163</sup>. In-source activation was utilized on a TIMS instrument to generate fingerprints for cytochrome *c*, allowing to observe more conformers as a result from higher TIMS resolving power compared to first-generation TWIMS<sup>166</sup>. Tandem DTIMS-DTIMS-MS devices were employed in early works on CIU. A specific conformation separated in the first dimension is selected and further activated at the entrance of the second drift tube. A tandem TIMS was developed recently, allowing for mobility selection of sub-species and ion activation between the two TIMS analyzers<sup>92</sup>. Multistage capabilities of the cIMS instrument can also be exploited to select/activate an ion population after initial IMS separation<sup>20</sup>.

It should be noted that fingerprints resulting from activation in different regions of the IMS-MS instrument cannot be directly compared. Indeed, temperatures, pressures, as well as the presence of residual solvent vapor, depend on the spectrometer region and might lead to alternative unfolding pathways<sup>163</sup>. Similarly, cross-platforms comparisons are far from being straightforward.

#### 2.6.3. CIU applications

The group of Ruotolo largely contributed to the development of CIU, with pioneering work performed on apo *versus* holo transthyretin, highlighting an increased resistance towards unfolding of the ligand-bound protein<sup>167</sup>. In a later study on kinase I and II inhibitors, Ruotolo and colleagues developed a classification method to identify the binding mode of a series of inhibitors, based on the fact that ligands with the same properties present similar CIU patterns<sup>15</sup>. Since then, multiple studies have reported on protein stabilization<sup>160,162,168-173</sup> or destabilization<sup>174</sup> upon ligand or metal binding. CIU can be described as a gas-phase analog of differential scanning calorimetry (DSC) or fluorimetry (DSF) to monitor changes in protein thermodynamics upon ligand binding<sup>162</sup>.

Membrane proteins represent another research area where CIU provides valuable information on the binding of drugs<sup>175</sup>, ligands<sup>159</sup>, or lipids<sup>176</sup>. Laganowsky *et al.* showed that CIU fingerprints reflect the selectivity of lipid binding to membrane proteins, with lipids of functional relevance conferring enhanced CIU stability compared to other bound lipids<sup>161</sup>. Recently, the classification strategy previously described was applied to distinguish the binding of lipids *versus* porphyrin, which connect to different sites of the membrane translocator protein (TSPO)<sup>174</sup>. Signature unfolding patterns were obtained for lipid and protoporphyrin TSPO binders (Figure 12), with protoporphyrin binding providing the highest degree of gas-phase stabilization (conformational transitions at higher CVs). The classification algorithm allowed to identify the binding location of several partners, as exemplified in Figure 12. This represents a promising screening technique to determine the binding site of drug targets for the discovery of new therapeutics. CIU experiments can also give useful information on membrane proteins themselves. Protein mutants of the KCNQ1 voltage-gated potassium channel voltage-sensing domain could be distinguished with CIU, each mutation being related to different disease and functional significance<sup>18</sup>.

Lastly, CIU has become an important tool for the characterization of mAb-based products<sup>12</sup>. This will be described in more details in part IV.



Figure 12. CIU experiments performed on the TSPO protein in apo and holo states. (A) CIU fingerprints obtained in absence or presence of lipids/porphyrin. (B) The classification of binders allows to accurately identify their binding site, including for unknown partners. Adapted from Fantin *et al.*, 2019 (ref. 174).

#### 3. Top-down MS

TD-MS approaches have attracted increasing attention in recent years. TD-MS provides primary sequence information and direct PTM assessment, by fragmenting intact proteins (MS/MS or MS<sup>n</sup>), which is of utmost interest to identify proteoforms<sup>177</sup>. The term "top-down" is often misused, and so Lermyte *et al.* have proposed a standardized lexicon to clarify TD-MS approaches<sup>64</sup>. True TD requires backbone fragmentation, and should not be mistaken with complex dissociation for intact mass measurements of subunits (complex-up)<sup>178</sup> (Figure 13). TD methods can be performed in denaturing or native conditions:

- dTD-MS consists of fragmenting denatured proteins;

- nTD-MS relies on the fragmentation of intact native assemblies.

- Complex-down MS breaks the native complex into subunits, which generally involves unfolding of the ejected subunit. Subsequent fragmentation of ejected unfolded subunits is then carried out.

 TD approaches = backbone fragmentation
 Other techniques \$ TD

 Denaturing TD
 Denaturing MS

 Complex-down
 Complex-up

 Native TD
 Native MS

**Figure 13.** Illustration of TD approaches, which involve backbone fragmentation, *versus* other techniques. Adapted from Lermyte *et al.*, 2019 (ref. 64).

Different fragmentation techniques can be employed for TD-MS approaches, among which CID, SID, electron transfer dissociation (ETD), electron capture dissociation (ECD), higher-energy collisional dissociation (HCD), and ultraviolet photodissociation (UVPD)<sup>179</sup>. Combining several fragmentation methods increases sequence coverage through complementary product ions<sup>180-183</sup>. CID yields *b/y* fragment ions, while ECD and ETD generate *c/z* fragment ions. *a/x*, *b/y* and *c/z* are obtained with higher energy activation techniques such as UVPD<sup>184</sup>.

Currently, the use of dTD-MS is more common than that of nTD-MS, with a growing interest for mAb characterization in recent years<sup>182,183,185</sup>. nTD-MS represents a real challenge, especially for large and heterogeneous proteins/complexes, because low sequence coverages are obtained for native folded species. nTD-MS is a promising method as it can provide valuable information on interaction regions and proteoform-dependent conformations. Secondary and tertiary structures of reference proteins (ubiquitin<sup>186</sup>, cytochrome *c*<sup>187</sup>, glutamate dehydrogenase<sup>188</sup>, etc.) were probed by ExD (this term encompasses ECD/ETD methods). ExD and UVPD were also used to identify metal or ligand binding sites, and allowed to locate NAD<sup>+</sup> on ADH<sup>188</sup>, small aggregation-inhibiting compounds on amyloid proteins<sup>189</sup>, heme on myoglobin<sup>190</sup>, or zinc ions in the insulin pentamer<sup>191</sup>. nTD-MS was also

employed to study RNA/proteins interactions<sup>192</sup>, or to determine aggregation region of amyloid protofibrils<sup>193</sup>. In addition, the nTD-MS approach is of interest to better understand CIU patterns, as it can pinpoint newly-unfolded regions, as exemplified by different groups on ADH<sup>194,195</sup>.

#### 4. Mass photometry

Although it is not a MS method, mass photometry is a recent addition to the field of structural biology that affords direct mass measurements of intact noncovalent assemblies. As it will be used in this thesis to complement nMS experiments, a description of this new approach is presented here.

Mass photometry is a label-free technique based on the detection of native proteins/complexes in solution through light scattering<sup>196</sup>. A small droplet (3 μL) of sample solution is deposited on a microscope cover glass<sup>197</sup>. The sample is irradiated with a laser operating in the visible spectrum (488 – 525 nm). Changes in reflectivity at the glass-water interface occur when an adhering biomolecule replaces water (Figure 14A). These binding events are then detected upon analysis of the interference between scattered and reflected lights. Variations in the reflectivity magnitude (image contrast) are directly proportional to the mass of the analyte, and can be converted into a molecular mass through calibration using biomolecules of known masses (Figure 14B).

Contrary to nMS, mass photometry is amenable to analyzing a broad range of volatile and nonvolatile buffer solutions. More importantly, buffer exchange is not required prior to mass photometry experiments, which is advantageous as proteins can be analyzed in their original buffers, and so the sample preparation step is considerably reduced. In addition, mass photometry is more sensitive than nMS, allowing to analyze proteins in a 100 pM – 100 nM concentration range, while micromolar concentrations (> 5  $\mu$ M) are necessary in nMS<sup>198</sup>. It could be argued that complex dissociation might occur as a result from sample dilution. However, because mass photometry experiments are rapid (< 1 min), measurements at low concentrations will still be valid as long as the associated off-rate is in the order of minutes<sup>198</sup>.

Even if mass measurements from mass photometry are not as resolved as nMS ones, this method offers a rapid insight into sample heterogeneity, affording determination of binding stoichiometries. The technique also appears to be sensitive to conformation, which might influence counts for each species, however this aspect needs to be further investigated<sup>198</sup>.

Mass photometry was used for the characterization of membrane proteins<sup>199</sup>, DNA<sup>200</sup>, to assess binding affinities of mAb/antigen complexes<sup>201,202</sup>, to monitor protein/protein heterodimerization<sup>203</sup>, and for the analysis of various large macromolecular assemblies<sup>196,198,204</sup> up to proteasomes 26S (~1.7 MDa) and 26S (~2.5 MDa)<sup>198</sup>.



**Figure 14.** Example of mass photometry experiment performed on a NADH:ubiquinone oxidoreductase complex. **(A)** Principle of mass photometry, based on interference between scattered and reflected lights. Binding events at the glass-water interface are recorded. The image contrast is related to the mass of the analyte. **(B)** After calibration, contrast values can be converted into molecular masses (top). Binding events are recorded along the duration of the experiment (bottom), allowing to count each species. Adapted from Sonn-Segev *et al.*, 2020 (ref. 198).

#### 5. Peptide-centric approaches

#### 5.1. Surface labelling

#### 5.1.1. Hydrogen/deuterium exchange-MS (HDX-MS)

HDX-MS is mostly used as a differential approach that compares the deuterium incorporation (uptake) within a protein in free *versus* bound states. HDX-MS targets solvent-exposed amide protons located in the protein backbone (Figure 15). Deuteration is performed by diluting samples in a deuterated solvent, and labile amide backbone hydrogens are exchanged with deuterium<sup>205</sup>. The deuteration reaction is quenched using acid pH (2.5) and low temperature (0 °C) to avoid back-exchange. The protein is then enzymatically digested, followed by LC-MS/MS analysis. The deuteration step is realized at different incubation times to compare deuterated profiles and deuterium incorporation in absence or presence of protein partners<sup>205</sup>. HDX-MS thus provides information on interaction regions between protein partners. It can also probe the conformational dynamics of a protein by assessing solvent accessibility at different deuteration times. HDX-MS is now a well-established method for the characterization of multiprotein complexes, intrinsically disordered proteins, membrane proteins, and for comparability/stability studies of biosimilar products<sup>206,207</sup>.

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Figure 15. Workflow used for HDX-MS experiments.

#### 5.1.2. Hydroxyl radical footprinting methods

Hydroxyl radical footprinting (HRF-MS) of proteins consists of exposing a protein in solution to hydroxyl radicals HO•, which can be generated by different means, such as radiolysis of water, gamma or X-rays radiations, or high voltage electrical discharge in water<sup>208</sup>. Among HRF techniques, fast photochemical oxidation of proteins (FPOP) is a popular method based on laser photolysis of peroxide H<sub>2</sub>O<sub>2</sub>, which occurs on the millisecond to second timescale<sup>209,210</sup>. Similar to HDX, HRF targets solvent-accessible regions, but hydroxyl radicals react primarily with side chains of amino acids, and not with the protein backbone<sup>211</sup>. The footprinting reaction is then quenched, and followed by enzymatic digestion prior to LC-MS/MS analysis. HRF-MS is also a differential approach (apo *versus* holo proteins) which looks for peptides that are differentially oxidized. This technique allows locating interaction regions within a complex<sup>212</sup>. Conformational dynamics information can also be inferred from HRF-MS experiments.

#### 5.1.3. Protein painting

Like HDX and HRF, protein painting is a differential approach. Protein painting consists of coating the solvent-accessible regions of a protein with small molecular dyes<sup>213</sup> (Figure 16). After painting, a classical bottom-up proteomics workflow is used. Dyes remain bound to the protein following denaturation, and block the tryptic digestion for painted cleavage sites. If a peptide is identified in an unpainted sample but is absent in a painted one, it means that the peptide has been protected against digestion, and it thus located in a solvent-exposed region (Figure 16). Peptides generated for apo *versus* holo proteins give information on interaction regions within a complex. Protein painting has been used to determine contact regions between a tripartite ligand-receptor-protein involved in interleukin signalling<sup>213</sup>.



Figure 16. Workflow of protein painting experiments. Adapted from Luchini et al., 2014 (ref. 213).

#### 5.2. Cross-linking MS

The chemical cross-linking of proteins or complexes in their native states creates new covalent bonds between proximal residues<sup>214</sup>. Cross-linkers are composed of two elements<sup>215</sup> (Figure 17):

- The spacer indicates the distance between the two targeted residues. Cross-linkers with short (< 10 Å) to medium spacer arms (10 - 30 Å) are well adapted for intramolecular cross-linking, and medium/long (> 30 Å) ones are more useful for intermolecular cross-linking. Using varying spacer lengths ensures a more comprehensive characterization of the protein structure.

- Reactive end groups dictate which amino acids are targeted. Primary amines are excellent candidates for cross-linking because of their high reactivity, and so the majority of cross-linking MS studies uses reagents that react with amines of lysine side chains and protein N-termini.

After the cross-linking reaction, an enzymatic digestion for subsequent MS/MS proteomic analysis is performed<sup>216</sup> (Figure 17). The identification of cross-linked peptides allows to determine spatial proximities either between residues located on two different proteins (interproteins cross-links) or within one protein (intraprotein cross-links). Dead-end cross-links also give information on solvent accessibility.

Chemical cross-linking MS is a powerful tool to map protein/protein or protein/ligand interactions and elucidate architectures of protein complexes, which is of utmost interest to refine 3D structural models generated from conventional biophysical techniques<sup>217</sup>.



Figure 17. Workflow of cross-linking experiments. Adapted from Götze et al., 2019 (ref. 216).

#### 5.3. Limited proteolysis MS (LiP-MS)

Proteolysis depends not only on the primary sequence, but also on the overall fold and dynamics of the protein<sup>218</sup>. Hence, if the protein is in its native state, peptides buried in the protein core are less susceptible to enzymatic cleavage. Limited proteolysis thus occurs at solvent-exposed and flexible regions, such as loops, providing information on surface residues and higher order structures of proteins<sup>219</sup>. Due to their unfolded nature, intrinsically disordered proteins are more prone to proteolysis than globular ones, and so limited proteolysis has especially been exploited for the

characterization of disorder in proteins<sup>220</sup>. In addition, LiP can provide valuable information to locate interaction regions between protein partners by comparing peptides obtained in apo *versus* holo states<sup>221</sup>, or to evidence conformational changes<sup>222</sup>. Although this method is typically used for purified proteins, Picotti and colleagues have recently extended LiP-MS to probe structural changes on a proteomic-wide scale in complex matrices<sup>223</sup>.

#### 6. Complementarity with other biophysical techniques

Protein- and peptide-centric MS approaches have played key roles in answering central biological questions, usually in combination with conventional biophysical methods such as X-ray crystallography, NMR and cryo-EM (Figure 18). Large, flexible, dynamic assemblies are often elusive to X-ray crystallography and cryo-EM mapping<sup>224</sup>. When both techniques fail to provide structural information, small angle X-ray scattering (SAXS) envelops are generally used to propose bead models. nMS provides a first insight into protein complex topology and dynamics. In addition, Marklund *et al.* showed that CCS measurements hold important structural information to support *ab initio* modelling, as it allows to complement SAXS data by filtering models to select the most accurate ones<sup>225</sup>. The complementarity of these techniques proved to be particularly powerful for the characterization of CRISPR-Cas systems. By combining masses measured in nMS for the complex and its subunits, and EM data, a topological model of the *E. coli* type I Cascade was proposed<sup>226</sup>, and was confirmed soon after by cryo-EM reconstruction<sup>227</sup>. This was the first CRISPR-Cas system to be structurally characterized. nMS, SAXS, and EM methods were later used as complementary methods to highlight high similarities between the *P. aeruginosa* Csy complex and the Cascade quaternary structure despite the lack of sequence homology<sup>228</sup>, which was further supported by nIMS-MS and molecular modelling<sup>229</sup>.

Peptide-centric strategies also afford valuable data for structural biology studies. HDX-MS provides dynamics and flexibility information, even for regions invisible to cryo-EM which instead gives static pictures<sup>230</sup>. More importantly, cross-linking MS has seen increased use in recent years because it can guide computational protein homology modelling and protein/protein docking, thus appearing as an essential tool to overcome positional ambiguity in cryo-EM maps<sup>231-235</sup>.



#### Integrative structural biology

Figure 18. Complementary structural MS and biophysical techniques for integrative structural biology studies.

#### 7. Conclusions

The rise of structural MS offers opportunities to probe the structure/function of biomolecular systems with increasing analytical depth. Structural MS approaches yield information that is complementary to what is obtained with classical biophysical techniques. While individual methods are often insufficient to understand highly complex and dynamically interacting machineries, their characterization can be achieved by merging the unique benefits of diverse analytical techniques (Figure 18)<sup>21</sup>.

nMS has clearly come of age for structural biology, and we are now entering a new era, where the future of nMS appears to be hybrid. nMS is being more and more combined with other techniques such as IMS and TD. Recent years have seen the development of different online nondenaturing LC-(IMS)-MS coupling, allowing to push forward the limits of nMS, not only for academic researchers but also for biopharmaceutical companies.

In this context, my PhD work has focused on methodological developments related to nMS and IMS-MS approaches.

#### Scientific communication

CIU approaches (instrumentation, applications, etc.) for protein analysis, as well as new combinations of LC and IMS-MS, have been reviewed in a book chapter.

Deslignière, E.; Hernandez-Alba, O.; Cianférani, S., Advanced IM-MS-based Approaches for Protein Analysis: Collision-Induced Unfolding (CIU) and Hyphenation of Liquid Chromatography to IM-MS. In A. E. Ashcroft & F. Sobott (Eds.), *Ion Mobility-Mass Spectrometry: Fundamentals and Applications*, The Royal Society of Chemistry, **2022**.

# **Book Chapter**

Advanced IM-MS-based Approaches for Protein Analysis: Collision-Induced Unfolding (CIU) and Hyphenation of Liquid Chromatography to IM-MS

Deslignière, E.; Hernandez-Alba, O.; Cianférani, S.

In *Ion Mobility-Mass Spectrometry: Fundamentals and Applications*, Eds. A. E. Ashcroft & F. Sobott, The Royal Society of Chemistry, **2022**.

Part I – Introduction to Structural MS Approaches

# CHAPTER 16

# Advanced IM-MS-based Approaches for Protein Analysis: Collision-induced Unfolding (CIU) and Hyphenation of Liquid Chromatography to IM-MS

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# 16.1 Introduction

Ion mobility (IM) spectrometry is a biophysical technique that has considerably evolved in the past ten years, especially since its combination with mass spectrometry (IM-MS). Up to now, this technique has been used for very different analytical purposes, ranging from the study of drug-like small molecules and peptides to the characterization of megaDalton protein

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complexes under native MS (nMS) conditions.<sup>1</sup> The combination of IM with nMS (nIM-MS) was a breakthrough towards the characterization of protein conformations in the gas-phase. The addition of the IM separation dimension enabled the conformational characterization of different biological complexes,<sup>2-6</sup> membrane proteins,<sup>7,8</sup> oligonucleotides,<sup>9-12</sup> intrinsically disordered proteins,<sup>13-15</sup> and viral capsids<sup>16,17</sup> among others (see also Chapters 14 and 15 in the present book). Furthermore, nIM-MS in combination with molecular dynamics (MD) allowed monitoring of the conformational changes of some biomolecules as a result of a self-solvation mechanism upon solvent evaporation during the ESI process.<sup>11,18,19</sup> It is estimated that roughly 95% of reported collision cross-sections (CCS) were measured with drift tube IM (DTIMS, 85%) and travelling-wave IM (TWIMS, 10%), the remainder of CCS values being obtained with trapped IM (TIMS) or a differential mobility analyzer (DMA).<sup>20</sup> The lower number of reported CCS with TIMS can be explained by its recent release on the market (2017) compared with other more classical IM devices (DTIMS, TWIMS), yet the role of TIMS in structural biology is increasing progressively (see also Chapter 5). The number of studies where field asymmetric waveform IM (FAIMS) is combined with nMS is very limited for the moment, even if FAIMS is able to maintain the noncovalent interactions of native proteins and protein complexes.<sup>21</sup> Indeed, this technology is predominantly used as an ion filter rather than for structural characterization, likely due to the fact that structural information cannot be inferred from the compensation voltages of molecular ions<sup>22</sup> along with the distortion of tertiary protein structures that can potentially occur during FAIMS separations as a consequence of the high electric field (see also Chapter 6).<sup>23,24</sup> Nevertheless, the design of new low-field differential IM devices has been recently proposed by Shvartsburg and Pathak<sup>25</sup> (between 1.0 and 4.0 kV) to avoid ion heating and allow this technology to be applied to different conformational studies.

CCS measurements are crucial to characterize biomolecules in the gasphase. Overall, this information is used in integrative structural studies to provide conformational constraints, in combination with other biophysical techniques and MD simulations to elucidate the 3D structure of proteins and complexes.<sup>26</sup> Otherwise, the CCS measurement, and hence, nIM-MS as a standalone characterization tool, does not usually provide enough structural information to obtain a detailed 3D structure. Furthermore, in some cases where quasi-iso-collisional cross-section proteins are analyzed, IM-MS only affords partially resolved or even co-drifting peaks due to the current low resolution of some IM platforms. Despite high resolution spectrometers with extended path lengths (e.g. structures for lossless ion manipulations (SLIM)<sup>27</sup> and the latest generation cyclic IM-MS, see also Chapter 4<sup>28</sup>) that have entered the field, alternative IM-based methods, such as collision-induced unfolding (CIU), have been developed to gain information on the structure of molecular conformers. Early studies on collisional activation were carried out by Shelimov et al. in 1997 to investigate the activation barriers related to the gas-phase conformers of apomyoglobin,<sup>29</sup> but also to assess the unfolding patterns of cytochrome *c versus* bovine pancreatic trypsin inhibitor, which bear a different number of disulfide bonds (none and three, respectively).<sup>30</sup> After the commercialization of quadrupole/TWIMS/time-of-flight mass spectrometers,<sup>31</sup> modern technological developments have helped to widen the range of CIU examples. Current applications of CIU include probing interactions within protein-ligand<sup>32</sup> complexes, binding of lipids to membrane proteins,<sup>33</sup> conformational characterization of therapeutic proteins,<sup>34</sup> and even analysis of crude extracts.<sup>35</sup>

In this chapter, we focus on CIU, its principles and technical implementation as well as its application to analysis of a broad diversity of proteins and protein complexes. Finally, we highlight the potential of coupling nondenaturing liquid chromatography (ndLC) to nIM-MS, especially for automation of CIU experiments.

# 16.2 CIU Experiments for Protein Analysis

## 16.2.1 CIU Principle

CIU experiments are performed by sequentially increasing an accelerating potential difference to induce ion activation before IM separation. As the accelerating voltage increases, collisions with the background gas (e.g. argon or nitrogen) become more energetic, leading to a build-up of internal energy in the ions. Activated ions may cross energy barriers and undergo conformational transitions through different intermediates related to the unfolding or folding of the protein (without dissociation of covalent bonds), referred to as conformational states/transitions. These conformational transitions are monitored through variations of arrival time distributions (ATD), which may be converted into CCS after calibration. ATDs are acquired at each collision voltage (CV), which corresponds to the voltage applied to ions at the entrance into the collision cell of TWIMS devices. The CV translates into laboratory frame energy when multiplied by charge states of precursor ions. Unfolding plots, named CIU fingerprints, can then be generated to better visualize CIU data. To generate CIU fingerprints, ATDs are extracted at a selected m/z range corresponding to the FWHM of the mass peak of interest for each applied CV, normalized to the highest intensity, smoothed, and stacked into a twodimensional plot (see Figure 16.1).

# 16.2.2 Sample Preparation for CIU Analysis

Manual desalting into a near-neutral pH and volatile buffer is necessary prior to nIM-MS injection, and sample preparation for CIU analysis thus remains labor-intense and time-consuming. Because CIU experiments require quite long acquisition times to record data along the whole CV ramp (that is, from 0 to 200 V with 2, 5 or 10 V steps), samples are usually injected *via* static nanospray needles<sup>34,36,37</sup> or microfluidic devices<sup>38-41</sup> (see Figure 16.1). More recently, size exclusion chromatography (SEC) coupled to CIU was also reported to increase the throughput of CIU experiments<sup>42</sup> (see more details in Section 16.5.3).



Figure 16.1 Schematic representation of workflows used for CIU experiments.

### 16.2.3 Instrumentation

TWIMS devices have pushed forward the development of CIU approaches,<sup>43</sup> from first-generation instruments commercialized in 2006 to the newest high-resolution cyclic IM-MS, and so the vast majority of studies published on CIU were carried out on TWIMS platforms.<sup>32,39,44–47</sup> In typical CIU experiments performed on TWIMS instruments, ion activation occurs in the trap cell (situated immediately prior to the IM region) through collisions with an inert buffer gas under low pressure conditions (see Figure 16.1). Conversely, in-source activation, which is more frequently used on other types of IM devices (TIMS, DTIMS), might lead to alternative protein unfolding mechanisms due to the presence of residual solvent molecules that can potentially lead to ion-molecule reactions.<sup>48</sup>

In spite of the high prevalence of TWIMS devices for CIU experiments, CIU has also been implemented on DTIMS spectrometers, with activation either behind the source capillary<sup>37,48,49</sup> or in the trapping funnel.<sup>48</sup> Recently, the use of TIMS has been reported using in-source activation for CIU fingerprinting

of cytochrome *c*, with a higher number of resolved conformers resulting from the improved resolving power of TIMS compared to linear TWIMS.<sup>50</sup> Tandem IM-IM-MS platforms with two successive drift tubes were employed in early works on CIU, offering the possibility for collisional activation in different regions of the interface, where ions activated in the first dimension may be selected for additional activation in the second dimension.<sup>51,52</sup> The latest generation high-resolution cyclic IM, which affords multistage IM-MS, can also be used to select an ion population after IM separation. Isolated ions are then activated and further separated by IM for CIU experiments.<sup>47</sup>

It should be noted that ion activation carried out in various regions of the IM-MS instrument, with very different instrumental parameters (pressure, temperature, residual solvent vapor) depending on the interface region,<sup>48</sup> might lead to alternative unfolding pathways. Thus, great care must be taken when comparing two CIU fingerprints of ions activated in different regions of the IM-MS instrument.

### 16.2.4 CIU Data Acquisition

On-line CIU data acquisition is usually performed in a semi-automatic way, meaning that the increase of CV is done using sequential data acquisition through a pre-programmed sample list. Technical replicates (*n* = 3 or higher, to rule out experimental errors) are recorded to generate a final averaged CIU fingerprint (see Figure 16.1). The CIU data acquisition pipeline results thus in a quite tedious and time-consuming process. Classical "semi-automated" experiments (manual buffer exchange followed by automated sequential acquisition) take around several hours ranging from sample preparation to data acquisition. An alternative to speed up the data acquisition process was reported by Vallejo *et al.* who suggested to record only IM-MS spectra at specific CVs (median voltages of CIU unfolding states and CIU transitions). However, this approach still requires one to previously record the whole CIU fingerprint to determine the diagnostic CVs.<sup>49</sup> Furthermore, software development (ORIGAMI<sup>MS</sup>) aimed at the automation of CIU experiments, albeit with still manual buffer exchange.<sup>53</sup>

CIU data can be acquired either with<sup>32</sup> or without<sup>39</sup> previous ion selection in the quadrupole. The influence of quadrupole-selection on CIU fingerprints has been investigated by the group of Ruotolo on monoclonal antibodies (mAbs).<sup>49</sup> The same number of features and transitions were observed with and without selection, except that the transition to the most unfolded state occurred at lower CVs without selection. This was attributed to charge stripping, that is, loss of charged adducts with increased CV from precursor ions with n + 1 charges. Charge stripping increases not only with collision energies but also with charge states, as higher z+ exhibit lower barriers to charge-stripping reactions. Conversely, when precursor ions are selected in the quadrupole, charge-stripping is limited as only one population enters the trap cell, thus reducing possible ion-ion interactions between ions of different charge states. The authors demonstrated that mAb ions produce similar,
low levels of charge stripping without selection. Overall, although one should be aware of a potential charge stripping effect on CIU fingerprints, direct comparison of CIU fingerprints acquired without selection is still possible as long as experiments are performed under identical conditions.<sup>40–42</sup> Moreover, recording data without prior quadrupole selection provides access to more charge states at once, reducing the acquisition time while widening the information content available compared to experiments with quadrupole selection, for which only the most native and intense charge states are favored.

### 16.2.5 Software for Data Interpretation

Several software packages allow the generation of CIU fingerprints (Amphitrite,<sup>54</sup> CIUSuite,<sup>55</sup> PULSAR,<sup>33</sup> ORIGAMI<sup>ANALYSE</sup>,<sup>53</sup> Benthesikyme<sup>56</sup> and CIU-Suite 2;57 see also Chapter 7). CIUSuite and CIUSuite 2 also provide automated tools for features detection of unfolding transitions, including CIU50 values, which correspond to the voltage necessary to convert 50% of a protein conformation to the following conformational state.<sup>55,57</sup> Additionally, root-mean-square deviation (RMSD) plots are available on different programs for direct comparison of unfolding pathways.33,53,55,57 Small variations between CIU technical replicates can be assessed through RMSDs, with RMSD values <10% indicating a good reproducibility. RMSD values between fingerprints of separate experiments greater than those of technical replicates might then lead to the conclusion that gas-phase unfolding pathways of the two proteins are significantly different. Benthesikyme generates summary plots which capture the variations in average arrival time and in the FWHM of the ATD along the CV ramp to compare proteins.<sup>56</sup> Altogether, CIU fingerprints offer a unique signature of the behavior of each protein or protein complex upon activation (see Figure 16.1), allowing the rapid detection of subtle changes of conformational state in the gas-phase.

# 16.3 Applications of nanoESI-CIU Experiments for Protein Analysis

### 16.3.1 Protein/Ligand Complexes

Characterizing the binding of ligands to proteins and/or protein complexes is an area of utmost relevance in drug discovery. nMS-based approaches have been integrated into protein/ligand validation phases, as the information content of such experiments can be used to rapidly provide binding stoichiometries, specificities or affinities, inform on the nature of ligand attachment, and elucidate the location of binding.

As ligand binding to a protein may alter its conformation/unfolding pathway, CIU patterns can serve for qualitative classification of protein/ ligand systems, with CIU acting as a gas-phase analog of differential scanning calorimetry (DSC) or fluorimetry (DSF) to assess variations on protein thermodynamics upon ligand binding (T<sub>m</sub>-shift like). Pioneering work has been performed by the group of Ruotolo. Early examples of CIU fingerprints were already reported in 2009 with the first "contour plots" (former name of CIU fingerprints) of apo- and holo-transthyretin (apo-TTR and TTRthyroxine).<sup>58</sup> These CIU maps revealed a common global unfolding pathway for both apo- and ligand-bound TTR forms, with the same number of main peaks appearing in the same order and with identical drift times. It is worth noting that noncovalent bonds between the protein and its ligand may be dissociated early on, with further unfolding thus related to the apo form of the protein. In-depth analysis of CIU data revealed several subtle differences between the two protein complexes, the main one being that each conformational ensemble appears at higher voltage values for TTR-thyroxine relative to apo-TTR, which was interpreted as an increased stability conferred to the TTR tetramer upon ligand binding. Similarly, Hopper et al. employed CIU to study the stability of compact protein conformations of FK-binding protein, hen egg-white lysozyme, and horse heart myoglobin in the presence and absence of bound ligands.<sup>44</sup> Again, the degrees of unfolding and dissociation induced by these defined collision energies were related to the stability of noncovalent intra- and intermolecular interactions within protein complexes, highlighting the additional conformational stability of protein ions in the gas-phase upon ligand binding. More recently, a CIU-based assay to determine kinase inhibitor binding modes has been reported.<sup>32</sup> CIU fingerprints were used to identify regions of maximum difference between the Abelson kinase bound to eleven type I or II inhibitors, and a scoring metric that clusters these data in a manner precisely correlated with their known binding modes was developed, paving the way for the use of CIU as a screening tool. Despite the small structural changes that exist between active and inactive kinases, CIU was shown to be an excellent method for differentiating type I and II inhibitors, requiring relatively small amounts of unmodified protein. Similar CIU clustering methods were used on the kinase-inducible domain (KIX) of the CBP protein to assess KIX:peptide interactions in mixed-lineage leukemia (MLL)- and phosphorylated KIX (pKID)-binding sites.<sup>59</sup> Gas-phase stabilization upon ligand binding was evidenced, and fingerprints pinpointed different unfolding patterns depending on the binding-site within KIX (see Figure 16.2A-D). Indeed, the fourth state reached 100% relative intensity for the KIX:MLL complex ( $\Delta$ drift time ~5 ms), but only accounted for 50% in the case of KIX:pKID (\drift time ~6 ms), and was absent for the ternary complex (see Figure 16.2B-D). Based on solution structures and electron capture dissociation (ECD) experiments, the authors hypothesized that the highest energy CIU transition corresponds to the unfolding of the KIX  $\alpha$ 3-helix. Indeed, all studied peptides contact  $\alpha$ 3 when bound to KIX, likely conferring additional stability to this region, which would explain the absence of the fourth feature for the ternary complex. Diagnostic CV regions for complex differentiation allowed for clustering of peptides bound to the MLL interface (MLL and E2A, see Figure 16.2E) and/or the pKID interface (pKID and c-Myb, see Figure 16.2E) against averaged reference fingerprints, proving that CIU can discriminate ligand binding regions (see Figure 16.2F).



Figure 16.2 CIU for protein/ligand complexes. CIU fingerprints of (A) apo KIX, (B) KIX:MLL complex, (C) KIX:pKID complex and (D) KIX:MLL:pKID complex. (E) NMR structure of KIX (grey) with four peptides: MLL (red), E2A (pink), pKID (dark blue) and c-Myb (light blue). (F) Clustering of KIX:peptide complexes. MLL-like complexes are circled in red, pKID-like complexes in blue, and ternary-like complexes in purple. Reproduced from ref. 59 with permission from American Chemical Society, Copyright 2018.

CIU experiments can also serve for functional point mutation screening in proteins, as reported by Rochel *et al.* on the PPAR $\gamma$  (peroxisome proliferatoractivated receptor gamma) nuclear receptor.<sup>60</sup> Again, significant differences were observed in CIU patterns of wild-type (WT) *versus* T475M PPAR $\gamma$  mutant, leading to the conclusion that the T475M mutant strongly stabilizes PPAR $\gamma$ coactivator peptide interactions, in agreement with hydrogen/deuterium exchange MS (HDX-MS) and MD simulations.

Altogether, all protein/ligand CIU studies report increased resistance to unfolding for ligand bound-proteins compared to their apo forms. In addition, CIU is a versatile and universal approach that can be applied to any type of protein/ligand system,<sup>33,46</sup> and even to tackle cooperative or allosteric ligand binding,<sup>61</sup> with a relatively high throughput. Development of dedicated algorithms for building multi-state classifiers based on CIU data,<sup>57,62</sup> along with automation possibilities using SEC,<sup>42</sup> will foster further implementation of CIU in MS-based drug discovery and validation pipelines.

### 16.3.2 Biotherapeutics

CIU approaches have extensively been reported for the characterization of a particular class of therapeutic proteins called mAbs (see also Chapter 13). Over the last 20 years, mAbs and their related compounds have evolved into the largest class of human therapeutics due to their high efficiency and specificity to treat various diseases such as cancers and auto-immune diseases. More than 100 mAb-based biotherapeutics, including antibody-drug conjugates (ADC) and bispecific antibodies (bsAb), are currently approved by regulatory agencies.<sup>63</sup> CIU appears as a rapid and highly reproducible tool to gain further insight into the gas-phase conformations of a large variety of mAbs-products, and represents an appealing approach for future biopharma quality control workflows.<sup>64</sup>

CIU was first employed for mAb analysis in 2015 to explore the unfolding pathway of the NISTmAb standard.<sup>65</sup> CIU was then applied to distinguish immunoglobulin (IgG) subclasses, which present a high sequence similarity but exhibit different numbers or patterns of disulfide linkages. While classical nIM-MS experiments failed to separate IgGs due to the low resolution of linear TWIMS instruments, CIU revealed distinct unfolding pathways for each subclass at the intact level, highlighting that CIU fingerprints are sensitive to disulfide bridges present within a protein.<sup>34</sup> The development of automated classification tools later helped to efficiently categorize IgGs based on CIU fingerprints.<sup>57,62</sup> This classification strategy was further expanded to the middle-level characterization of mAbs after enzymatic digestion, which eases subclass differentiation due to the presence of additional excited unfolding states for  $F(ab')_2$  fragments compared to the intact level<sup>41</sup> (see Figure 16.3A).



**Figure 16.3** CIU for mAbs analysis. (A) Middle-level CIU fingerprints of mAbs belonging to different subclasses. Fingerprints correspond to  $F(ab')_2$  fragments. (B) UFS plots for mAbs classification at intact (black) and middle (red) levels. (C) Middle-level subclass categorization for  $F(ab')_2$  fragments (z = 21+) of two mAbs, ipilimumab (IgG1) and reslizumab (IgG4).

Consequently, univariate feature selection (UFS) plots provide more diagnostic CVs (see Figure 16.3B), and thus higher mAb classification scores were obtained at the middle-level when using categorization algorithms<sup>41</sup> (see Figure 16.3C). Benefits of IdeS digestion prior to mAbs CIU fingerprinting were also demonstrated in the case of domain-exchange occurring between the variable domains from the two heavy chains of an IgG1.<sup>36</sup>

Differences between antibody glycoforms,<sup>66</sup> and glycosylated *versus* deglycosylated mAbs<sup>34,66</sup> were detected using CIU, with glycosylated mAbs unfolding at higher CVs than their deglycosylated analogues, confirming that glycoforms confer additional resistance to gas-phase unfolding to mAbs. CIU fingerprints were able to evidence even more subtle conformational behaviors that could not be probed by low-resolution IM cells for mAbs belonging to two different types of IgG4s, that is, WT and hinge-stabilized, which differ in their hinge region by an amino acid mutation that stabilizes Fab arm exchange.<sup>40</sup> Hinge-stabilized IgG4s displayed a better resistance to gas-phase unfolding than WT formats. Overall, these examples highlight the potential of CIU to tackle minor conformational variations on large proteins that may be difficult to characterize otherwise.

A wide range of mAbs-derived products can be examined using CIU. Studies conducted on nanobodies (also called Variable Heavy Homodimers or single Variable domain of Heavy-chain antibodies, VHHs) before and after disulfide bond reduction confirmed the influence of disulfide bridges on CIU, suggesting an increased resistance to gas-phase unfolding in presence of disulfide bridges<sup>67,68</sup> (see Figure 16.4A). The gas-phase behaviors of other mAbs formats, including mAbs biosimilars,<sup>69</sup> bsAbs,<sup>40</sup> or larger formats such as ADCs<sup>39,70</sup> were also assessed. Botzanowski et al. compared CIU fingerprints of a site-specific ADC versus naked parental mAb and evidenced that drug conjugation also had the effect of stabilizing the mAb<sup>39</sup> (see Figure 16.4B). Another study performed on a mAb conjugated with biotin via its native lysine residues highlighted a shift towards lower unfolding voltages as the number of conjugated biotins increased, suggesting a destabilization of the model ADC in agreement with DSC data.<sup>70</sup> An increased sensitivity to the conjugation levels of the ADC was achieved using CIU compared to classical nIM-MS and DSC experiments. Although very few papers have reported the characterization of ADCs using CIU, most likely because of the heterogeneity of first-generation ADCs, these compounds are one of the fastest growing class of oncology therapeutics, and CIU will undoubtedly prove useful in the ADC development pipeline.

#### 16.3.3 Membrane Proteins

Membrane proteins, which represent strategic current therapeutic targets in many diseases, still remain under-characterized due to their low yields of production, expression and purification along with the necessity to solubilize them in membrane mimics (detergent micelles, liposomes and nanodiscs, *etc.*) which are not MS compatible.<sup>71</sup> However, over the past decades,



**Figure 16.4** CIU for nanobodies and ADCs. (A) CIU fingerprints of an anti-HER2 VHH before/after disulfide bond reduction (z = 7+). (B) CIU fingerprints of a site-specific ADC *versus* its parental unconjugated mAb. The CIUSuite 2 differential plot between the two conditions highlights an increased resistance to unfolding upon conjugation; the centroids of ATDs (left hand axis) were standardized at CV = 0 V (difference on right hand axis equal to 0 for centroids of ATDs at CV = 0 V) to ensure comparison solely on unfolding patterns, irrespective of ATD variations due to different masses.

the group of Robinson has done pioneering research in the field of nMS and nIM-MS-based membrane protein analysis (see also Chapter 14).<sup>72,73</sup>

The first CIU studies on membrane proteins have focused mainly on the gas-phase stability of different membrane proteins/ligand systems.<sup>33,45,74</sup> The work from Laganowsky et al. highlights how CIU fingerprints reflect selectivity of lipid binding to membrane proteins, with lipids of functional relevance being found to have enhanced CIU stability compared to other lipids that were bound.<sup>45</sup> Current limitations of membrane protein/lipid interaction investigation by CIU arise from the fact that most experiments are carried out on a mixture of ligand-bound states that cannot even be MS-resolved (heterogeneity in binding stoichiometries), which leads to uncertainty about the role of individual lipid bound states in contributing to the overall protein stability. In a recent work, Laganowsky and coworkers utilized increasing source temperature to induce in-source detergent micelle release from different lipid-bound states of a model integral membrane protein (ammonia channel AmtB). The in-source collisional activation step allowed to isolate specific lipid-bound states of the protein prior to the CIU experiments (MS-IM-MS) to assess the variation of AmtB thermodynamics upon lipid binding. An overall gas-phase stabilization of the membrane protein was observed upon addition of two lipid molecules. Isolation of lipid-membrane protein complexes prior to CIU analysis affords a more precise evaluation of protein thermodynamics in the gas-phase, avoiding interference of CIU profiles due to complex neutral loss.<sup>75</sup>

Distinguishing the binding of ligands to membrane proteins from that of lipids, detergents and cofactors remains challenging, as broad peaks may be obtained. The effect of drug-binding on the gas-phase behaviors of membrane proteins was assessed in the case of the human membrane metallo-protease ZMPSTE24 bound to different HIV protease inhibitors, including ritonavir (see Figure 16.5A) and lopinavir.<sup>74</sup> Protein-drug conjugates were found to be more resistant to gas-phase unfolding than apo ZMPSTE24 with greater effect for lopinavir, consistent with more favorable  $K_{\rm D}$  value evaluated for this drug (see Figure 16.5B).



Figure 16.5 CIU for membrane proteins. (A) Native mass spectrum (top) of ZMP-STE24 (yellow) with ritonavir (black). Corresponding ATDs (bottom) with 11+ charge states selected for CIU circled in white. (B) Comparison of CIU fingerprints for apo, ritonavir-bound and lopinavir-bound ZMPSTE24. Reproduced from ref. 74 with permission from Springer Nature, Copyright 2016. (C) CIU fingerprints of KCNQ1 VSD variants: WT and mutants R231C, E115G and H126L. The first transition occurs at lower CVs for R231C. (D) Classification of KCNQ1 VSD functional variants. Probabilities of assignment for each class, WT (grey), gain of function (blue) and loss of function (red) are displayed in bar charts. (E) CIU50 stability analysis of KCNQ1 VSD variants for the first CIU transition. Reproduced from ref. 77 with permission from American Chemical Society, Copyright 2020.

CIU experiments can also give valuable information on membrane proteins themselves. Fanti et al. have reported on a specific denoising protocol to achieve CIU experiments for membrane proteins solubilized in detergents.<sup>76</sup> Their approach is illustrated through the quantification and classification of ligand binding associated with a 36 kDa dimeric protein, the translocator protein (TSPO). Separate gas-phase CIU unfolding signatures for lipid and protoporphyrin TSPO binders, known to bind at two separate sites, were obtained. Protoporphyrin binding provided a greater degree of gas-phase stabilization for TSPO than any of the lipids tested. CIU-classification schemes further revealed successful differentiation of lipid binders from protoporphyrin as well as subclasses of lipids based on their levels of chain saturation. CIU was also reported by the same group to distinguish classes of membrane protein variants associated with different diseases.<sup>77</sup> CIU experiments were performed on three membrane protein mutants of the KCNQ1 voltage-gated potassium channel voltage-sensing domain (KCNQ1 VSD, with four transmembrane helices), each point mutation having different disease and functional significance. Automated feature detection using CIUSuite 2 and comprehensive difference analysis of the CIU datasets showed that the variants were grouped by function and disease association (see Figure 16.5C). They also constructed a classification scheme based on the CIU data sets, which is able to differentiate the variant functional groups and classify a recently characterized variant (H126L) to its correct grouping (see Figure 16.5D). Lastly, they explored the stability differences associated with these variants using CIU50 analysis and found evidence that the "gain of function" mutant is destabilized relative to both WT and "loss of function" variants for the KCNQ1 VSD (see Figure 16.5C and E).

## 16.4 Toward More Detailed Understanding of Protein/Protein Complexes' Gas-phase CIU Pathways

Although CIU has been successfully applied to a large variety of proteins and macromolecular complexes, a gap remains in the detailed understanding of unfolding pathways and structural identification/modeling of conformational states. Several studies aimed at explaining in more detail the conformational intermediates observed during protein unfolding in order to improve the amount of useful information that can be extracted from CIU experiments.

The interactions regulating both gas-phase unfolding and compaction of proteins were first evidenced by probing the activation barriers of apomyoglobin<sup>29</sup> and cytochrome c.<sup>30</sup> For low charge states obtained *via* charge stripping, the activated protein adopted a compact conformation to maximize intramolecular interactions. For high charge states, unfolding driven by Coulombic repulsions occurred upon activation. For intermediate charge states, the protein favored an elongated conformation to accommodate both Coulombic repulsion and intramolecular interactions. More recent studies on protein folding/unfolding and dissociation underlined the important role of salt bridge rearrangement to explain gas-phase behaviors of activated complexes.<sup>78,79</sup>

Zhong *et al.* demonstrated a strong correlation between the number of CIU features and native domain structures in solution for sixteen proteins from 8 to 78 kDa, which contained one to four domains.<sup>80</sup> Following these observations, the detailed mechanism related to the unfolding of a multi-domain protein, the human serum albumin (HSA), was described.<sup>81</sup> Comparison of collision-induced dissociation (CID) breakdown curves for HSA-ligand complexes and CIU data revealed a clear correlation between the threshold voltage associated with CID-based ligand ejection and the CIU of individual domains within a protein. Additional CIU/CID analyses performed on HSA constructs built as noncovalent complexes composed of individual HSA domains helped to conclude on the domain associated to each CIU transition. The latter experiments also suggested that surface charges are redistributed during the CIU of multi-domain proteins.

Over the last years, advances in native top-down (TD) strategies, which afford information on the flexible region through different fragmentation techniques such as ECD, electron transfer dissociation (ETD) and newlyemerging ultraviolet photodissociation (UVPD), have led to apply native TD to CIU intermediate species.<sup>82-85</sup> Lermyte and Sobott employed ETD to evaluate changes in fragmentation patterns resulting from in-source CIU of various tetrameric proteins.<sup>83</sup> Higher fragment intensity and larger fragments were obtained with increasing unfolding of ions. Parts of the sequence exposed to ETD upon activation provided information on CIU pathways. Disruption of  $\beta$ -strands in alcohol dehydrogenase (ADH) could thus be monitored with additional fragments detected in the N-terminal region. Combining fragmentation techniques yields additional structural information, as each technique generates specific types of ions. Therefore, both ECD and UVPD were used to characterize protein complexes after in-source activation on a modified Orbitrap.<sup>84</sup> Solvent-exposed regions were identified based on increased ECD product ions upon activation, while UVPD enabled observation of charge migration to the unfolded regions. The analysis of UVPD fragment ions along the CV ramp allowed proposing a charge-directed mechanism, which involves partial refolding of the ADH N-terminus after its initial unfolding. Very recently, TD approaches for CIU experiments have been implemented on a drift tube platform.<sup>85</sup> The authors reported improved sequence coverage of tetrameric ADH by combining ECD and CID product ions that resulted from fragmentation at different CVs.

In spite of these recent developments, a clear understanding of intermediate features and CIU transitions is still lacking, but computational approaches could eventually bridge this knowledge gap. MD simulations can help to predict the behavior of proteins and their complexes in the gas-phase, however, these approaches are still in their early stages regarding ion activation,<sup>79,86,87</sup> and a complete model able to infer unfolding pathways based on the structure of the protein remains to be found.

# 16.5 Combining Liquid Chromatography (LC) with IM-MS and CIU Analysis

### 16.5.1 Denaturing LC-IM-MS for Proteomic Applications

Since IM operates in the millisecond time scale, this technique is particularly well suited to be implemented in LC-MS or LC-MS/MS experiments. IM affords an additional dimension of separation, and hence, improves the identification and characterization of gas-phase ions. The IM separation can be used in different ways depending on the purpose of the LC-IM/MS coupling. For instance, several IM-MS devices (DTIMS,<sup>88</sup> TWIMS,<sup>89</sup> FAIMS,<sup>90,91</sup> and TIMS<sup>92,93</sup>) in combination with reversed-phase LC have been successfully implemented in high-throughput proteomics to decrease the mass spectral background noise, and enhance the separation of co-eluting peptides leading to an improved peak capacity, selectivity and sensitivity, in both datadependent acquisition and data-independent acquisition workflows.<sup>94,95</sup> The same experimental set-up can be also used for other sample-digested analysis such as crosslinking-mass spectrometry analysis, where the CCS of the digested peptides can be used to more confidently identify and characterize crosslinks while discarding monolinked peptides from sequencing;<sup>96</sup> or HDX experiments to further discriminate overlapping isotopic profiles of different deuterated ions and increase the number of identified peptides.<sup>97,98</sup> Recent publications highlight also the benefits of the IM dimension in other -omics fields such as metabolomics (see also Chapter 12)<sup>99,100</sup> and lipidomics,<sup>101-103</sup> to increase confidence in ion annotation by using either the drift time or CCS alignments.

### 16.5.2 Non-denaturing LC-nIM-MS for Intact Protein CCS Calculation

Even though IM is progressively appearing in different LC-MS and LC-MS/ MS set-ups under denaturing conditions for different analytical purposes, the addition of this separation step is scarce when ndLC is combined with nMS. SEC was the first ndLC dimension hyphenated to nMS<sup>104</sup> to perform "in-line" buffer exchange and monitor protein/protein and DNA/protein interactions. Since the automation of nMS through its coupling with size exclusion chromatography (SEC-nMS),<sup>95</sup> several studies have implemented this strategy to the analysis of mAb and related products,<sup>39,105</sup> including forced-degradation studies,<sup>106</sup> structural characterization of proteins and protein complexes,<sup>107-109</sup> quantification of protein-ligand affinity,<sup>110</sup> and natural product screening.<sup>111</sup> Despite SEC-nMS being widely accepted as a mature method to analyze proteins and their interactions under native conditions, very few examples can be found where nIM-MS is also included to further characterize the global conformation of biomolecules. Van der Rest and coworkers developed a SEC-nIM-MS workflow to differentiate reference proteins with an experimental set-up based on their hydrodynamic volume, CCS and molecular mass.<sup>107</sup> Building on the oligomeric state and the CCS values, it was concluded that quaternary structure and the folded conformation of proteins were not impacted by the addition of the SEC dimension (see Figure 16.6A). Ehkirch *et al.* widened the applications of SEC-nIM-MS to study the conformational heterogeneity and relative quantitation of mAb size variants upon thermal stress.<sup>106</sup> The reported CCSs for therapeutic mAbs and ADCs in this study showed a good agreement with those obtained through direct infusion nMS (see Figure 16.6A). These results clearly demonstrated that SEC-nIM-MS was ready to integrate high-throughput analytical workflows to provide structural information of therapeutic proteins, including the conformational characterization of all SEC-separated species. The synergic effect of the SEC and nIM dimensions is clearly evidenced through the structural analysis of A $\beta$ (1–42) oligomers in a membrane-mimicking environment by Ciudad *et al.*<sup>112</sup>. While the different oligomers of A $\beta$ (1–42) cannot be fully



**Figure 16.6** Combination of SEC-nIM-MS. (A) Comparison of CCS values obtained in direct infusion nIM-MS and SEC-nIM-MS. Adapted from ref. 107 with permission from American Chemical Society, Copyright 2017. (B) Analysis of  $A\beta(1-42)$  octamer and tetramer with SEC-nIM-MS. Ions are unambiguously identified upon analysis of the evolution of the drift time as a function of the m/z (Driftscope). (C) Combination of HIC × SEC-IM × MS to separate, identify, quantify and characterize the global conformation of the different brentuximab vedotin (BV) populations. Reproduced from ref. 114 with permission from American Chemical Society, Copyright 2018.

characterized with the sole use of nMS, SEC-nIM-MS allows the differentiation of tetramer and octamer by analyzing the trend of the IM drift time as a function of the m/z signal (see Figure 16.6B). Furthermore, the CCS values obtained from IM measurements were essential in the integrative structural study of A $\beta$ (1–42) oligomers, thus allowing to select the most suitable structures among all the putative candidates resulting from the MD simulations.

A more complex 2D ndSEC × SEC-IM × MS workflow was also developed by Ehkirch *et al.*<sup>113</sup> to afford the use in the first chromatographic dimension of nonvolatile conventional SEC buffers (e.g. 50 mM of phosphate buffer and 250 mM potassium chloride, pH 6.8) instead of ammonium acetate to reduce the nonspecific interactions with the stationary phase of the column. SEC × SEC provides the best mAb size variant separation in the 1st SEC dimension while the 2nd SEC dimension was performed in 100 mM ammonium acetate for fast desalting prior to online nIM-MS analysis. Different mAbs were subjected to the 4D SEC  $\times$  SEC-IM  $\times$  MS analysis leading to particularly interesting results in the case of thermal-stressed pembrolizumab (Keytruda, MSD). The first SEC dimension exhibited three different size variant populations suggesting the presence of different oligomers (dimer or trimer) along with the presence of the mAb monomer. However, nMS revealed that those peaks correspond to different types of monomers with different degrees of oxidation. In this case, IM data highlighted that the most oxidized monomer (t = 36 min) exhibited the most compacted conformation (78.9 nm<sup>2</sup> instead of 79.5 nm<sup>2</sup>), suggesting that oxidation may have a straight impact on mAbs' CCS. The same experimental design can also be used with other ndLC chromatography, including hydrophobic interaction chromatography (HIC) or ion exchange chromatography (IEX), in the first separation dimension as illustrated by the 4D HIC × SEC-IM × MS coupling.<sup>114</sup> The contribution of IM allowed not only to quantify the CCS increase of each individual DAR as a result of the addition of the payloads, but also to confirm that thermal stress did not affect the global conformation of the different BV DARs populations (see Figure 16.6C).

Coupling of ndLC to nIM-MS is of benefit at different levels: keeping separation capabilities of ndLC upstream of the IM-MS instrument, quantification of the relative species either by ndLC, nMS or even nIM-MS along with unambiguous mass identification by nMS and conformational characterization by nIM-MS.

### 16.5.3 LC-nIM-MS for CIU Experiments

As one of the remaining, major bottlenecks of CIU workflows is its lack of automation for sample preparation (buffer exchange) and on-line data acquisition, SEC appeared as an attractive approach also for CIU experiment automation. In a recent publication, Deslignière *et al.* were able to reduce the overall CIU acquisition time by threefold with a fully automated SEC-nCIU

workflow.<sup>42</sup> In this case, CV ramping was synchronized with the SEC elution time of the analytes (mAbs at the intact and middle levels). SEC flowrate was decreased to 35 µL min<sup>-1</sup> to broaden the chromatographic peaks in order to increase the number of scans and improve S/N ratio for IM-MS signal averaging. Under these conditions, seven CVs were contained in a single SECnIM-MS run, which means that three different runs were required to record a full CIU fingerprint (from 0 to 200 V) with 10 V per run. This experimental design allowed to classify therapeutic mAb isotypes (IgG1, IgG2, and IgG4) at both intact- and middle-level as previously reported in direct infusion nanoESI-CIU experiments, albeit avoiding nano-electrospray clogging and drastically reducing the acquisition time (less than one hour analysis for a complete CIU dataset). Targeted scheduled SEC-CIU with acquisitions focused only on the most diagnostic CVs for isotype classification allowed an additional reduction of the overall time process to 15 minutes for triplicate experiments. These advantages afforded with the implementation of SEC in CIU workflows can pave the way to widen the use and applications of CIU experiments and ease the integration of this technique in more R&D laboratories and biopharma companies.

## 16.6 Concluding Remarks

CIU has gained in maturity step-by-step and is now implemented in most analytical workflows for the characterization of a broad diversity of protein/ protein or protein/ligand systems, from soluble multiprotein complexes to therapeutics like monoclonal antibodies and membrane proteins. Ligand binding assays and characterization of biotherapeutics are the most highly achieved applications of CIU in a biopharma context, which will be further strengthened by ongoing automation efforts (*e.g.* using LC upstream or by performing targeted scheduled CIU on defined collision energies of interest). For example, LC-CIU will open new doors for the characterization of charge or hydrophobic variants when coupled to IEX or HIC. We expect hyphenation of LC to CIU to even afford TD-CIU approaches to be developed, in order to tackle for instance the effects of post-translational modifications on gasphase unfolding.

CIU analyses have gained in accuracy, reproducibility and repeatability, which will foster their integration into gas-phase protein analyses, as well as adaptation to different IM-MS platforms. The development of outputs from CIU experiments and their translation into mechanistic or structural information through modeling of the conformational states is still ongoing. As CIU data interpretation improves, CIU will provide greater detail on the structures of gas-phase biomolecules, improving our understanding of gas-phase protein biophysics. CIU approaches represent a promising and amazing tool for expanding needs in biomolecule structural investigation.

# List of Abbreviations

ADC	Antibody-drug conjugate		
ADH	Alcohol dehydrogenase		
ATD	Arrival time distribution		
bsAb	Bispecific antibody		
CCS	Collision cross section		
CID	Collision-induced dissociation		
CIU	Collision-induced unfolding		
CV	Collision voltage		
DMA	Differential mobility analyzer		
DTIMS	Drift tube ion mobility spectrometry		
DSC	Differential scanning calorimetry		
DSF	Differential scanning fluorimetry		
ECD	Electron capture dissociation		
ETD	Electron transfer dissociation		
$F(ab')_2$	Full fragment antigen-binding		
FAIMS	Field asymmetric waveform ion mobility spectrometry		
HDX	Hydrogen/deuterium exchange		
HIC	Hydrophobic interaction chromatography		
HSA	Human serum albumin		
IEX	Ion exchange chromatography		
IgG	Immunoglobulin		
IM	Ion mobility spectrometry		
IM-MS	Ion mobility coupled to mass spectrometry		
KCNO1 VSD	Voltage sensor domain of the KCNO1 voltage-gated		
	potassium channel		
KIX	Kinase-inducible domain of the CPB protein		
LC	Liquid chromatography		
mAb	Monoclonal antibody		
MD	Molecular dynamics		
MLL	Mixed lineage leukemia peptide		
ndLC	Nondenaturing liquid chromatography		
nMS	Native mass spectrometry		
nIM-MS	Ion mobility coupled to native mass spectrometry		
nKID	Phosphorylated kinase-inducible domain peptide		
PPAR <sub>V</sub>	Peroxisome proliferator-activated receptor gamma		
RMSD	Root-mean-square deviation		
SEC	Size exclusion chromatography		
SEC-nMS	Size exclusion chromatography coupled to native mass		
	spectrometry		
SLIM	Structures for lossless ion manipulations		
TD	Top-down		
TIMS	Trapped ion mobility spectrometry		
TSPO	Translocator protein		
TTR	Transthyretin		
T T T/			

TWIMS	Travelling wave ion mobility spectrometry
UFS	Univariate feature selection
UVPD	Ultraviolet photodissociation
VHH	Single variable domain of a heavy-chain antibody
WT	Wild-type

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# Chapter 2 – Instrumentation and Methods for nMS and nIMS-MS Approaches

#### 1. Sample preparation – Buffer exchange

nMS analyses require the use of near-neutral pH and volatile buffers compatible with electrospray ionization and able to preserve weak noncovalent complex assemblies in solution (typically ammonium acetate AcONH<sub>4</sub>)<sup>236</sup>. Sample preparation generally consists of manual buffer exchange, called desalting, using a variety of devices such as microconcentrators, gel filtration devices, or dialysis units<sup>4</sup>. This process can alter proteins, leading to their aggregation, precipitation, denaturation, or even complex dissociation<sup>4</sup>. This step is tedious and time-consuming, and so manual buffer exchange remained for a long time a major bottleneck of the nMS workflow, hindering its throughput increase and automation. In 2003, Cavanagh et al. used self-packed cartridges to achieve fast desalting through SEC-nMS<sup>237</sup>. The SEC stationary phase consists of porous particles with controlled pore size, through which molecules diffuse based on their molecular size and hydrodynamic volumes, allowing to separate not only small nonvolatile salts from proteins, but also high versus low mass species<sup>238</sup>. Cavanagh's work opened up new opportunities for high-throughput buffer exchange, and online SEC-nMS finally started to emerge ten years later<sup>239-241</sup>, supported by the commercialization of inert stationary phases that were lacking until then. The development of new SEC column technologies, with reduced particle sizes (from 1.7 to 2.7 µm), allowed to improve separation and column efficiency while significantly reducing the analysis time up to five-fold with runs performed in dozens of minutes<sup>242</sup>.

#### 2. Instrumentation

During my PhD work, nMS and nIMS-MS experiments were mainly performed on a Synapt G2 HDMS (Waters, U.K.) described thereafter. Other instruments were also used for nMS analyses, but to a lesser extent. The LCT (Micromass, U.K., upgraded for high masses by MS Vision) and Exactive Plus EMR Orbitrap (Thermo Fisher Scientific, Germany) will be presented in the experimental section of the manuscript (p. 187).

#### 2.1. Description of the Synapt G2 HDMS mass spectrometer

The Synapt G2 HDMS is a Q-IMS-ToF mass spectrometer equipped with the T-Wave technology for TWIMS separation. This instrument comprises five main regions (Figure 19):

- The Z-spray source produces an electrospray orthogonal to the MS inlet. Ions enter the mass spectrometer with a Z-shaped trajectory, which deflects neutral molecules so that they diffuse away towards the primary pump. This design allows to improve the tolerance of the mass spectrometer to nonvolatile salts. The pressure in the interface region (Pi) can be modulated between 2 and 8 mbar *via* an external valve that reduces the suction of the primary pump<sup>243</sup>.

- A first SRIG serves to focalize ions. A RF-confining voltage is applied to adjacent electrodes, and a DC voltage is superimposed on the RF, creating a T-wave that propels ions into the quadrupole.

- The quadrupole analyzer is composed of four conducting rods connected to RF and DC generators (adjacent rods are of opposite RF phase)<sup>244</sup>. In RF-only mode, the quadrupole acts as an ion guide, and a wide m/z range travel through the quadrupole<sup>245</sup>. When a DC voltage is also applied, the quadrupole can be tuned so that only ions of a specific m/z are transmitted, other m/z species being lost through collisions with rods<sup>246</sup>.

- The TriWave comprises three T-Wave SRIGs, with superimposed RF and DC voltages<sup>82,247</sup>.

• In MS mode, all three SRIGs function as ions guides. Trap and transfers cells are filled with argon (10<sup>-2</sup> mbar), and a residual argon pressure (10<sup>-4</sup> mbar) is delivered to the IMS cell. Pressures and collision energies in trap/transfer regions can be optimized to provide a better transmission of ions.

• In IMS mode, the trap cell accumulates ions while the previous IMS cycle occurs. Ions are then released as a packet into the IMS cell for separation. The IMS region comprises two parts (1 - 6 mbar). A chamber filled with helium ensures the thermalization and focalization of ions before entering the main IMS cell<sup>83</sup>. The latter is filled with nitrogen, which serves as buffer gas for IMS experiments. Separated ions then travel through the transfer cell towards the ToF region.

- Lastly, ions are separated as a function of their flight time (proportional to their m/z ratio) in the ToF analyzer  $(10^{-6} \text{ mbar})^{248}$ . In order to minimize ion spreading and improve the resolution, the reflectron corrects the kinetic energy dispersion of ions having the same m/z. Ions with higher kinetic energies reach deeper into the reflection region, and arrive at the detector at the same time than less energetic ions of identical m/z.



Figure 19. Schematic representation of the Synapt G2 HDMS (Waters).

The instrument resolution depends on the operating mode, namely sensitivity, resolution, or highresolution modes. In the first two modes, ions move in a "V" trajectory into the ToF region, with a FWHM resolution of 10 000 (sensitivity) to 20 000 (resolution) for bovine insulin (m/z 956). Conversely, the high-resolution mode allows for a "W" trajectory, where the flight path is doubled, yielding an enhanced FWHM resolution of 40 000 at m/z 956. However, this gain of resolution is detrimental to sensitivity, and so the sensitivity mode is preferred for nMS and nIMS-MS experiments.

#### 2.2. Key instrumental parameters

The optimization of MS parameters is crucial in nMS and nIMS-MS experiments. Parameters should be tuned to ensure an efficient desolvation and transmission of ions, while preserving noncovalent interactions and avoiding the dissociation of complexes (Figure 20). The internal energy of ions in the mass spectrometer is dictated by two main parameters<sup>249</sup>:

- The accelerating voltage (cone voltage Vc on a Q-ToF instrument) confers kinetic energy to ions at the entrance of the interface region. Applying high voltages provides enough desolvation, but may break noncovalent assemblies. Conversely, when voltages are too low, noncovalent interactions are maintained, but insufficient desolvation prevents accurate mass measurements.

- The pressure Pi has a role similar to that of the accelerating voltage<sup>243</sup>. At high pressures, the mean free path of ions is reduced. Ions undergo more frequent but less energetic collisions with gas molecules, effectively retaining the native conformation of species. Elevated pressure regimes provide sufficient focusing of the ion beam, ensuring the transmission of high m/z ions. Lowering the pressure leads to more energetic collisions that can disrupt noncovalent interactions and induce complex dissociation.

Altogether, a compromise between desolvation/transmission/complex integrity should be found *via* an optimized combination of Vc/Pi parameters (Figure 20).



Figure 20. Influence of MS parameters on nMS experiments. Adapted from Sanglier et al., 2008 (ref. 249).

#### 3. IMS-MS measurements

#### **3.1. Optimization of TWIMS separation**

The quality of the TWIMS separation relies on three parameters that should be carefully optimized: the wave velocity (WV), the wave height (WH), and IMS gas flowrates (He and N<sub>2</sub>). WV and WH parameters influence the number of roll-over events that occur during an IMS cycle<sup>250,251</sup> (Figure 21):

- If the WV is too low, ions will traverse the IMS cell without being caught up by the T-wave, and so the IMS separation will not be efficient as ions cannot surf on the T-wave. If the WV is too high, ions undergo numerous roll-over events. Extended ions will be significantly slowed down, even possibly to the point that the next ion packet will be released from the trap cell before slower ions have been delivered to the pusher. In addition, for high WV values, ion activation may occur, broadening IMS peaks.

- If the WH is too high, ions will not be able to surf on the T-wave, preventing their separation. If the WH is too low, the number of roll-over events increases, leading to higher drift times. Because ions will interact more with the T-wave, diffusion can occur, resulting in large IMS peaks.

- IMS gas flowrates are also important to provide a correct IMS resolution. First, for a constant N<sub>2</sub> flowrate, the He flowrate in the He cell should be kept high enough to ensure the focusing and thermalization of ions. Otherwise, broad IMS peaks may be observed. The gas pressure in the TWIMS cell mediates the number of collisions between ions and gas particles, and so increasing the N<sub>2</sub> gas flowrate can increase the IMS resolution. Of note, the nature of the drift gas (He, N<sub>2</sub>, CO<sub>2</sub>, Ar, etc.) also plays a role on the IMS separation<sup>252</sup>.

A compromise should be found between these three parameters to optimize the IMS separation (Figure 21).



Figure 21. Influence of WV and WH on IMS separation.

The quality of the IMS separation can be determined by calculating the time-based IMS resolution (or resolving power), determined as follows on a TWIMS instrument<sup>253</sup> (Equation 2).  $t_D$  corresponds to the drift time of the analyte, and  $\Delta t_D$  is the FWHM of the peak.

$$R = \frac{t_D}{\Delta t_D}$$
(2)

The valley separation is another metric that can be used to assess the separation between two distinguishable peaks (Equation 3)<sup>253</sup>. H corresponds to the height of the major IMS peak, and  $H_{valley}$  is the height of the valley with respect to the baseline. The valley separation is 100% for baseline-resolved peaks ( $H_{valley} = 0$ ).

%valley separation= 
$$\frac{H - H_{valley}}{H} \times 100$$
 (3)

#### 3.2. CCS measurements and calibration

#### 3.2.1. Mason-Schamp equation

In IMS experiments, ions migrate through a buffer gas with a velocity  $v_D$  (m.s<sup>-1</sup>) related to the analyte's mobility K (m<sup>2</sup>.V<sup>-1</sup>.s<sup>-1</sup>) under the influence of an electric field E (V.m<sup>-1</sup>). The ion velocity can be deduced from the time spent in the IMS cell  $t_{D'}$  (s) and length L of the drift cell (m) (Equation 4)<sup>254</sup>:

$$K = \frac{v_D}{E} = \frac{L}{E \times t_{D'}}$$
(4)

The ion mobility K depends on experimental gas temperature T and pressure P. In order to compare experiments performed on different instruments, it is better to use the reduced mobility K<sub>0</sub>, for which T and P are normalized to standard conditions ( $T_0$  = 273.15 K and P = 760 Torr) as follows<sup>254</sup> (Equation 5):

$$K_0 = K \times \frac{P}{P_0} \times \frac{T_0}{T} = K \times \frac{P}{760} \times \frac{273.15}{T} = \frac{L}{E \times t_{D'}} \times \frac{P}{760} \times \frac{273.15}{T}$$
(5)

The reduced mobility K<sub>0</sub> serves to calculate the CCS value (momentum transfer cross section  $\Omega$  in m<sup>2</sup>) via the Mason-Schamp equation<sup>255</sup>. The parameters of this equation 6 are e – charge of an electron (1.6 × 10<sup>-19</sup> C), z – ion charge, N – number density of the buffer gas (m<sup>-3</sup>), k<sub>B</sub> – Boltzmann's constant (1.38 × 10<sup>-23</sup> J.K<sup>-1</sup>), T – drift region temperature, M<sub>gas</sub> and M<sub>ion</sub> – buffer gas and ion masses (kg.mol<sup>-1</sup>):

$$K_{0} = \frac{3 \times e \times z}{16N} \times \sqrt{\frac{2\pi}{k_{B}T}} \times \left(\frac{1}{M_{gas}} + \frac{1}{M_{ion}}\right) \times \frac{1}{\Omega}$$
(6)

The CCS value is then determined by combining equations 5 and 6:

$$\Omega = \frac{3e}{16N} \times \frac{T}{273.15} \times \frac{760}{P} \sqrt{\frac{2\pi}{k_B T}} \times \sqrt{\left(\frac{1}{M_{gas}} + \frac{1}{M_{ion}}\right)} \times \frac{z \times t_{D'} \times E}{L}$$
(7)

The Mason-Schamp equation (Equation 7) is only valid for homogeneous, constant, low electric fields, which enables DTIMS to measure K as a primary method<sup>254</sup>.

#### 3.2.2. Calibration on TWIMS instruments and conversion into CCS

Because the electric field is not static in TWIMS, CCS values cannot be obtained directly from the Mason-Schamp relationship. Calibration with standards of known CCS values, primarily derived from DTIMS measurements (in He or N<sub>2</sub>), is required. Over the years, different groups have published CCS tables for peptide and protein ions to calibrate IMS-MS experiments<sup>256-262</sup>. Reference values from the work of Bush *et al.*, which comprises a set of native protein calibrants, were used during this thesis<sup>257</sup>. Calibrants and proteins should be analyzed in strictly identical conditions.

#### 3.2.2.1. Drift time correction

A first step consists of correcting the drift time  $t_D$  measured at the ToF detector (also called arrival time) to determine the effective drift time  $t_{D'}$ , i.e. time taken to travel through the IMS cell. Travel times in the transfer cell and in the ToF analyzer are not significant. The arrival time is corrected for the *m/z*-dependent flight time from the transfer cell to the pusher<sup>263</sup> (Equation 8):

$$t_{D'} = t_D - C \frac{\sqrt{m/z}}{1000}$$
(8)

Where c is the enhanced duty cycle (EDC) delay coefficient. On the Synapt G2 HDMS, the EDC equals 1.41 for m/z < 5000, and 1.57 for m/z > 5000.

#### 3.2.2.2. Calibration

The Mason-Schamp equation is first expressed using corrected CCS values from calibrants ( $\Omega'$ , DTIMS) as a function of their corrected drift time ( $t_{D'}$ , TWIMS) (Equations 9 – 10):

$$\Omega' = \frac{\Omega}{z \times \sqrt{\frac{1}{M_{gas}} + \frac{1}{M_{ion}}}} = A \times \frac{t_{D'} \times E}{L}$$
(9)

Where constant A is defined as:

$$A = \frac{3e}{16N} \times \frac{T}{273.15} \times \frac{760}{P} \sqrt{\frac{2\pi}{k_{B}T}}$$
(10)

Equation 9 can be fitted to a power law<sup>264</sup> (Equation 11):

$$\Omega' = \mathsf{A}' \times \mathsf{t}_{\mathsf{D}'}^{\mathsf{B}} \tag{11}$$

This power law can be linearized<sup>263</sup> (Equation 12):

$$\ln(\Omega') = \ln(A') + B \times \ln(t_{D'})$$
(12)

Plotting  $ln(\Omega')$  versus  $ln(t_{D'})$  allows for the determination of constants A' and B. A' accounts for the correction of pressure, temperature and electric field parameters, while B' compensates for the nonlinear effect of the TWIMS cell<sup>265</sup>. Finally, the linear calibration plot is obtained by combining equations 9 and 11, as follows (Equation 13). The charge state, constants, and masses are known, the drift time  $t_{D'}$  is measured, and so the CCS value  $\Omega$  of the ion of interest can be calculated.

$$\Omega = A' \times t_{D'}^{B} \times z \times \sqrt{\left(\frac{1}{M_{gas}} + \frac{1}{M_{ion}}\right)} = A' \times t_{D''}$$
(13)

The choice of calibrants is crucial to ensure accurate CCS measurements<sup>106</sup>. Several groups have showed that it is better to match the chemical class, mass, and charge state of calibrants to those of the analyte<sup>258,266-268</sup>. Thus, during my PhD work, calibrants were chosen to match species under study, as indicated in recommendations from Gabelica *et al.* on how to perform/report IMS-MS experiments<sup>106</sup>. Very recently, Richardson *et al.* introduced a new TWIMS calibration procedure that takes into account the radial distribution of ions and the effects of velocity relaxation<sup>269</sup>. The authors suggested that a minimal set of calibrants, composed of large multiply charged ions and peptides/small molecules of low charge states (for example, native BSA and peptides SDGRG/GRGDS), is enough to achieve CCS deviations < 4% for all classes of analytes from metabolites to native proteins.

#### **3.3. Theoretical CCS calculations**

Experimental IMS-MS values can be compared to theoretical ones, using structural coordinate files generated by high-resolution techniques such as NMR, cryo-EM, and X-ray crystallography. Algorithms to calculate theoretical CCS values are divided into three main categories (Figure 22):

- **Projection Approximation (PA)**: The PA method considers atoms as hard spheres. The calculated CCS is the rotationally averaged projected area of the analyte, i.e. its projected shadow averaged over all possible orientations, also taking into account the radius of the buffer gas<sup>270</sup> (Figure 22A). The PA algorithm ignores gas scattering effects, which makes it the fastest calculation method. However, this leads to a systematic underestimation of CCS values<sup>271</sup>. The improved projected superposition approximation (PSA) model developed later includes a shape-factor to correct for concave surfaces<sup>272-</sup><sup>275</sup>. Different software packages are available for PA calculations: CCSCalc<sup>276</sup>, IMoS<sup>277</sup>, IMPACT<sup>225</sup>, MOBCAL<sup>278,279</sup>, and Sigma<sup>270</sup> (Table 2).

- Exact Hard Sphere Scattering (EHSS): The EHSS model only considers hard-sphere collisions between buffer gas and analyte atoms, and does not take into account long range interactions<sup>279</sup> (Figure 22B). Gas scattering angles are calculated from multiple collisions, allowing to achieve more accurate CCSs. Nonetheless, the EHSS method is more computationally expensive than PA<sup>225</sup>, and is known to overestimate CCS values<sup>271</sup>. EHSS calculations can be performed using several software packages: EHSSrot<sup>280</sup>, IMoS, and MOBCAL<sup>279</sup> (Table 2).

- **Trajectory Method (TM)**: TM is considered to be the most accurate and realistic method for CCS calculations, as it includes short and long range interactions by taking into account temperaturedependent Lennard-Jones potentials between gas molecules and atoms<sup>254</sup> (Figure 22C). Because of this, the TM algorithm is extremely time-consuming. A faster alternative introduced in the IMPACT software infers pseudo-TM values based on PA calculations<sup>225</sup>. The TM algorithm is implemented in several programs: Collidoscope<sup>281</sup>, CoSIMS<sup>282</sup>, IMoS, and MOBCAL<sup>278</sup> (Table 2).

During my PhD work, I used three different software for CCS calculations: IMoS, IMPACT, and MOBCAL.

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**Figure 22.** Schematic representation of the three methods used to calculate theoretical gas-phase CCS values: **(A)** PA, **(B)** EHSS, and **(C)** TM. Adapted from D'Atri *et al.*, 2017 (ref. 126).

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		Algorithm for CCS calculations			
		РА	EHSS	ТМ	
Software package	CCSCalc	$\checkmark$	×	X	
	Collidoscope	×	×	$\checkmark$	
	CoSIMS	×	×	$\checkmark$	
	EHSSrot	×	$\checkmark$	×	
	IMoS	$\checkmark$	$\checkmark$	$\checkmark$	
	IMPACT	$\checkmark$	×	×	
	MOBCAL	$\checkmark$	$\checkmark$	$\checkmark$	
	Sigma	$\checkmark$	×	×	

 Table 2. Programs available for CCS calculations using either PA, EHSS, or TM algorithms.

CCS values can also be roughly estimated in the case of spherical proteins, based on a relationship between mass and CCS proposed by Ruotolo *et al.*<sup>263</sup> (Equation 14), which can be useful when no high-resolution structure is available:

$$\Omega = 2.435 \times M^{2/3} \tag{14}$$

#### 4. CIU experiments

#### 4.1. Generation of CIU fingerprints

On the Synapt G2 HDMS, CIU experiments are performed by activating ions in the trap cell, prior to IMS separation. The trap CV is ramped from 0 to 200 V, either in 2, 5 or 10 V steps depending on the desired graphical resolution of CIU maps. It is worth mentioning that in the case of protein/ligand interactions, complex dissociation may occur early on, with further unfolding thus related only to the apo form of the protein. ATDs are then extracted at a specific m/z range for each applied CV, normalized to the highest intensity, smoothed, and represented as a two-dimensional CIU fingerprint (Figure 23A).

#### 4.2. Software for data interpretation

Several programs are available for the generation of CIU fingerprints (Amphitrite<sup>283</sup>, CIUSuite<sup>284</sup>, PULSAR<sup>159</sup>, ORIGAMI<sup>ANALYSE 285</sup>, Benthesikyme<sup>286</sup> and CIUSuite 2<sup>287</sup>). CIUSuite and CIUSuite 2 afford

automated tools to detect features of unfolding states (mean drift times) and transitions (CIU50 values). The CIU50 corresponds to the voltage required to convert 50% of a protein conformation to the following conformational state<sup>284,287</sup> (Figure 23B).

Moreover, root-mean-square deviation (RMSD) plots can be obtained on various software packages to compare unfolding patterns of two species<sup>159,284,285,287</sup> (Figure 23C). This allows to assess variations between technical CIU replicates (n = 3), RMSD < 10% indicating a good reproducibility<sup>288</sup>. If RMSD values between fingerprints of separate experiments are greater than those of technical replicates, gas-phase unfolding behaviors of the two species are significantly different. Pinpointing small changes solely based on RMSD plots is sometimes challenging, and so Benthesikyme generates summary plots able to capture variations in both average arrival time and FWHM of the ATD upon activation for a more precise comparison of proteins<sup>286</sup>. Figure 23D represents the intensity weighted mean of each ATD (IWM<sub>ATD</sub>) along the CV ramp.

During this thesis, different software packages were used to fully exploit CIU data: CIUSuite 2, Benthesikyme, and ORIGAMI<sup>ANALYSE</sup>.



**Figure 23.** Representations used for CIU experiments. **(A)** CIU fingerprints of proteins A and B. **(B)** CIU50 values of conformational transitions. **(C)** Differential plot between proteins A and B, and associated RMSD value. **(D)** IWM<sub>ATD</sub> of proteins A and B along the CV ramp.

Part I – Introduction to Structural MS Approaches

# Results

Results

# PART II – nMS and nIMS-MS: From Technological Improvement to Methodological Integration in Structural Biology Projects

The second part of this thesis focuses on the use of nMS and nIMS-MS for structural biology studies. A first objective of my PhD work consisted of broadening the scope of applications for the SEC-nMS coupling previously implemented mainly for mAb analysis in our laboratory. In a second chapter, nMS and nIMS-MS were used to characterize a series of large multiprotein complexes involving human RuvBL helicases. In this context, the complementarity of nMS-based techniques with several other biophysical approaches was explored.

#### Chapter 1 – Versatility of the SEC-nMS Coupling

Chapter 2 – Integration of nMS and nIMS-MS for Structural Characterization of Human RuvBL1-2 Complexes

Part II – nMS and nIMS-MS for Structural Biology Projects

### Chapter 1 – Versatility of the SEC-nMS Coupling

#### 1. Analytical context

In our team, the SEC-nMS coupling has been developed and implemented during the PhD work of Dr. Anthony Ehkirch, who focused on mAb products<sup>289</sup>. In fact, SEC-nMS has been employed mainly for the characterization of mAb-based therapeutic proteins<sup>17,240,289-293</sup>. Few papers have been dedicated to other types of proteins<sup>59,133,294,295</sup>, but these first studies show that SEC-nMS represents a promising technique for rapid desalting and assessment of biomolecules and their complexes, even with small sample quantities available  $(1 - 5 \mu g)^{294}$ .

#### 2. Objectives

The aim of this chapter is to evaluate the potential of SEC-nMS for a larger scope of biological applications, including protein/nucleic acids complexes and high molecular weight species. Comparisons between manual desalting followed by nMS analysis *versus* online SEC-nMS are presented. These new applications illustrate the benefits of the coupling to avoid the generation of artefacts that may occur using manual buffer exchange.

#### 3. SEC-nMS for nuclear receptor/DNA complexes

#### 3.1. Nuclear receptors

Nuclear receptors (NRs) represent the largest known superfamily of eukaryotic transcription factors that regulate the expression of genes involved in numerous physiological processes, including reproduction, growth, and homeostasis<sup>296,297</sup>. Transcriptional regulation occurs through ligand-mediated recruitment of coregulator proteins (coactivator or corepressor), which trigger induction or repression of target genes<sup>298</sup>. NRs contain the following domains<sup>299</sup> (Figure 1):



Figure 1. Schematic representation of NRs' domains. Adapted from Huss et al., 2015 (ref. 312).

- the N-terminal A/B domain that comprises the ligand-independent activation function 1 (AF-1)<sup>300</sup>;

- the DNA-binding domain (DBD), which recognizes specific DNA sequences known as hormone response elements<sup>301</sup>;

- the hinge domain, a short region whose main role is to connect adjacent domains;

- the ligand-binding domain (LBD), which binds to ligands and interacts with coregulator proteins<sup>302</sup>. The LBD contains the ligand-dependent activation function 2 (AF-2)<sup>300</sup>.
NRs are generally found as monomers in solution but can form homo- or heterodimers upon DNA binding<sup>303</sup>. The LBD also contributes to receptor dimerization.

nMS has been used to characterize either DBD or LBD of various NRs, and also FL NRs<sup>169,304-306</sup>. Yet, structural analysis can be more difficult for FL samples because N-terminal and hinge domains are intrinsically disordered and highly flexible<sup>307,308</sup>. In addition, the presence of co-purified species like heat shock proteins (HSP)<sup>309</sup> can result in ionization competition between the different proteins. The presence of cations associated to oligonucleotide binding also leads to low quality nMS signals with broad peaks, low mass spectral resolution, and difficult data interpretation<sup>310</sup>. Lastly, the manual buffer exchange step can induce complex dissociation, aggregation, or denaturation.

In the following paragraphs, I aimed at characterizing the FL estrogen-related receptor alpha (ERR $\alpha$ ), as well as its complex with DNA. ERR $\alpha$  is an orphan receptor, which means that its endogenous ligands have not been identified yet<sup>311,312</sup>. Samples were provided by the team of Dr. Isabelle Billas (IGBMC, UMR7104, Strasbourg).

### 3.2. nMS results

## 3.2.1. Direct nMS injection

We first performed manual desalting followed by direct nMS analysis using the TriVersa NanoMate (Advion, U.S.) coupled to a ToF instrument (LCT). The initial nMS spectrum obtained for ERR $\alpha$  exhibits a single species corresponding to the mass of an HSP70 (71 423 ± 7 Da, Figure 2A), suggesting that the presence of HSP70 which may be preferentially ionized over ERR $\alpha$  prevents the detection of the protein of interest. In order to reduce amounts of HSP70 in the samples, FL ERR $\alpha$  samples were subjected to affinity chromatography on a heparin column during the purification step. The resulting nMS spectrum for this new ERR $\alpha$  sample preparation indicates that most of HSP70 proteins have been removed, allowing to observe a charge state distribution (CSD) corresponding to dimeric ERR $\alpha$  (93 012 ± 20 Da, Figure 2B). However, the quality of the signal obtained using manual desalting is poor, with extremely low intensities (S/N = 13).

Next, the FL ERR $\alpha$ /DNA complex was reconstituted (1 hour at 4°C, ratio 1:1) after manual buffer exchange. A shift towards higher *m*/*z* confirms the formation of the complex, with a mass of 108 663 ± 13 Da, which corresponds to dimeric ERR $\alpha$  linked to one DNA fragment (Figure 2C). Still, the intensity remains very low (S/N = 16), and the quality of the signal could undoubtedly be improved.



**Figure 2.** Mass measurements on the LCT after manual desalting. **(A)** nMS spectrum obtained for FL ERR $\alpha$  without prior affinity chromatography **(B)** nMS spectrum generated for FL ERR $\alpha$  with affinity chromatography during the purification step, and **(C)** resulting nMS spectrum after incubation with DNA (Vc = 200 V; Z69307/69314/69330).

# 3.2.2. SEC-nMS coupling

We next moved to online desalting *via* the SEC-nMS coupling implemented on the Synapt G2 HDMS instrument. As we wanted to separate FL ERR $\alpha$  from residual HSPs to improve the quality of the signal, we chose a 150 mm SEC column (Acquity BEH200, 4.6 x 150 mm, 1.7  $\mu$ m). Shorter SEC columns may not provide enough separation of the two species, while using longer ones would increase the analysis time and could lead to complex dissociation because of dilution in the column.

Two peaks are observed on the SEC-UV chromatogram of FL ERR $\alpha$  (Figure 3A). The first peak at ~6.8 min corresponds to dimeric ERR $\alpha$  (93 089 ± 6 Da), while HSP70s are found in the second peak at ~7.7 min, in good agreement with elution based on MWs. Thanks to the additional dimension of separation provided by SEC, the protein of interest could be efficiently separated from co-purified HSP70s, allowing to improve the signal intensity (S/N = 126) and to achieve more accurate mass measurements.

Analysis of a preformed FL ERR $\alpha$ /DNA complex (fraction collected from gel filtration) reveals three chromatographic peaks (Figure 3B). The peak at 6.8 min corresponding to ERR $\alpha$  has now disappeared, suggesting that the formation of ERR $\alpha$ /DNA species has been successful. Indeed, a shift towards shorter elution time is observed (peaks 1 and 2 at ~6.2 and 6.5 min, respectively), indicating the presence of new species of higher molecular weights (MW), which nMS identifies as dimeric FL ERR $\alpha$ 

bound to one DNA fragment. Different FL ERR $\alpha$ /DNA populations ranging from 110 kDa (peak 1, major species) to 103 kDa (peak 2, minor ones) are detected as a result from DNA heterogeneity. The third chromatographic peak at ~9.0 min corresponds to DNA fragments (13 – 18 kDa).

Overall, these results emphasize benefits of using SEC-nMS for the characterization of protein/DNA interactions in the case of a FL NR sample.



**Figure 3.** SEC-nMS experiments on the Synapt G2, with corresponding UV chromatograms and nMS spectra, for (A) FL ERR $\alpha$  (Vc = 180 V; O65626) and (B) FL ERR $\alpha$  in presence of DNA (Vc = 180 V; O65630).

# 4. SEC-nMS for high molecular weight multiprotein complexes

# 4.1. Yeast Rvb

Yeast Rvb1 and Rvb2 (~50 kDa each) are members of the AAA+ family (<u>A</u>TPases <u>a</u>ssociated with diverse cellular <u>a</u>ctivities)<sup>313</sup>. The biological significance of these complexes will be presented in detail in the next chapter. Rvbs predominantly assemble as heterohexameric rings (> 300 kDa), with a small proportion of dodecamers<sup>314,315</sup>. This equilibrium can be shifted depending on protein partners, as exemplified by the dodecameric Rvb/INO80 complex<sup>316</sup>. Rvb assemblies are stabilized by adenosine diphosphate (ADP)<sup>316</sup>, and are thus purified with buffers containing ADP in order to preserve the integrity of complexes<sup>317</sup>.

nMS analysis of Rvb samples can be challenging as complexes are often produced in small quantities, and may be unstable in AcONH<sub>4</sub>. The potential of SEC-nMS for the characterization of high molecular weight complexes was thus evaluated. Samples were provided by the team of Dr. Xavier Manival (IMoPA, UMR7365, Nancy).

# 4.2. Direct versus SEC-nMS injection

nMS analyses were first performed *via* direct injection after manual desalting (Zeba 7 kDa, AcONH<sub>4</sub> 150 mM, pH 7.5). Monomers are mostly detected (Figure 4A). Only low intensity signals with S/N = 5 are observed for hexamers even after careful optimization of crucial parameters (Vc and Pi), suggesting that the complex was disrupted during manual buffer exchange. ADP (0.5 mM) was next added to the AcONH<sub>4</sub> solution prior to manual desalting. The resulting nMS spectrum shows that the equilibrium has been shifted towards the hexameric form, but the intensity of the signal remains low (S/N = 15) (Figure 4B). Besides, nMS peaks are broad (FWHM = 149, and Rs @*m*/*z* 8199 = 55) because of the presence of multiple ADP adducts on Rvb1/2 species, avoiding accurate mass measurements.

In order to improve the quality of the desalting and subsequent mass determination, online SECnMS experiments were next conducted on a SEC column dedicated to high molecular weight proteins, i.e. with pore particles of 450 Å (BEH450, 4.6 x 150 mm, 2.5  $\mu$ m), using an ADP-free mobile phase composed of 150 mM AcONH<sub>4</sub> at pH 7.5. SEC-nMS provides high resolution (Rs @*m/z* 8106 = 811) and signal intensity (S/N = 71) for Rvb1/2 hexamers (Figure 4C). The main hexameric population bears three ADP (307 973 ± 8 Da). Another minor population bound to two ADP (307 563 ± 8 Da) is detected.

These results underline the potential of SEC-nMS to maintain noncovalent interactions within the hexameric Rvb1/2 ring. In addition, due to increased desalting efficiency, different ADP stoichiometries could be identified without the need to access instruments of higher MS resolution (Orbitrap).



**Figure 4.** nMS spectra of yeast Rvb samples **(A)** after manual gel filtration, without ADP in the desalting buffer (Vc = 200 V; O66227), **(B)** after manual gel filtration, with ADP in the desalting buffer (Vc = 200 V; O66251), and **(C)** using online SEC-nMS (Vc = 200 V; O66274). Masses of monomers in SEC-nMS are 50 746  $\pm$  1 Da and 51 481  $\pm$  1 Da for Rvb1 and Rvb2, respectively.

# 5. Concluding remarks

In this chapter, we have shown that SEC-nMS is amenable to analyzing various types of proteins, from protein/DNA interactions to large macromolecular assemblies. SEC-nMS offers multiple advantages over classical nMS experiments (i.e. manual desalting followed by direct injection), of which the most important are:

- Rapid online buffer exchange with better desalting efficiency, allowing to avoid complex dissociation or aggregation;

- High-throughput analysis (7 to 18 min depending on the inner diameter for 150 mm SEC columns, and even < 5 min if shorter columns are used);

- Separation of low and high molecular weight species contained within a mixture, based on their hydrodynamic volumes, leading to more straightforward nMS spectra interpretation;

- Separation of co-purified proteins.

Of course, several drawbacks related to this coupling have to be taken into account. Although this has not been reported yet, it is possible that dissociation of unstable and/or low affinity complexes might occur because of dilution along elution in the SEC column. In addition, higher amounts of material can be required compared to nanoESI injection.

However, benefits of SEC-nMS largely outweigh these potential limitations. While initially limited to mAb samples, the coupling is mature enough to tackle a wider range of proteins, and could be further extended to even more complex samples such as membrane proteins or crude extracts.

# Scientific communication

Advantages/drawbacks and new applications of the SEC-nMS coupling have been reviewed in the following paper.

## Peer-reviewed article

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# Publication 1

# Pushing the limits of native MS: Online SEC-native MS for structural biology applications

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Part II – nMS and nIMS-MS for Structural Biology Projects

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# Pushing the limits of native MS: Online SEC-native MS for structural biology applications



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#### ABSTRACT

Native mass spectrometry (nMS) is now widely applied to investigate non-covalently assembled biomolecule complexes. nMS requires the use of near-neutral pH and volatile buffers to preserve the native state of proteins. However, buffer exchange into nMS-compatible solvent is usually performed manually, which results in a time-consuming and tedious process, thus appearing as a major drawback for nMS analysis. Conversely, online coupling of size exclusion chromatography (SEC) to nMS affords a fast-automated and improved desalting, but also provides an additional dimension of separation for complex protein mixtures. We illustrate here the benefits of SEC-nMS compared to manual offline desalting for the characterization of a wide variety of biological systems, ranging from multiprotein assemblies, protein—ligand and protein—nucleic acid complexes, to proteins in a detergent environment. We then highlight the potential of the coupling to further integrate ion mobility while preserving the native conformations of proteins, allowing for rapid collision cross section measurement and even collision-induced unfolding experiments. Finally, we show that online SEC coupling can also serve as the basis for multidimensional non-denaturing liquid chromatography (LC) workflows, with the SEC acting as a fast desalting device, helping to achieve first dimension LC separation in optimal chromatographic conditions while being compatible with further nMS analysis.

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#### 1. Introduction

Native MS (nMS) remained for a long time the prerogative of few academic expert laboratories. The recent growing interest of biopharma companies for this methodology, especially for the characterization of therapeutic proteins like monoclonal antibodies (mAbs), prompted manufacturers to develop new instrumentations adapted to biopharma specific requirements, from sample preparation to data acquisition and treatment.

nMS was first introduced in the early 90s by the groups of Katta&Chait and Henion for the analyses of myoglobin [1] and kinase/substrate [2] complexes. The validity of gas phase nMS to conclude about solution binding stoichiometries and even more for affinities was for a long time a question of heated debates: "Do gas phase native MS spectra really reflect solution phase behavior?"; "Are gas phase data reliable to extrapolate solution phase

\* Corresponding author. E-mail address: sarah.cianferani@unistra.fr (S. Cianférani). behavior?". Major advances in studying macromolecular interaction by nMS have been performed by the groups of Carol Robinson and Albert Heck, and helped converting early skepticism into competence and consistency of practical use of nMS. nMS provides unambiguous determination of subunit stoichiometry, the most straightforward and important application of nMS, on a variety of biological assemblies ranging from multiprotein, e.g. transthyretin, retinol binding [3], GroEL [4], or HSP [5], to protein-ligand and protein-nucleic acid assemblies, including RNA editing complexes like the CRISPR-Cas systems [6], RNA polymerase II [7], and ribonucleoparticles [8]. Applications of nMS also encompass highly complex membrane proteins such as TRP channels [9], GPCR [10] or ABC transporters [11], and binding of lipids [12] or other small molecules [13] to membranes for which high resolution instrumentation is a real breakthrough. Even MegaDaltons ribosomes [14], hemocyanines [15], COP9 signalosome [16], dynactin complex [17] and virus particles with masses up to 18 MDa [18] can be measured by nMS.

From 2005, hyphenation of ion mobility (IM) spectrometry to nMS added a new dimension of gas-phase data interpretation,

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affording conformational characterization along with stoichiometry determination. Ruotolo et al. first described a new type of instrumentation combining nMS and ion mobility (nIM-MS) and demonstrated that the overall topology of a ring-shaped protein complex could be retained in the gas phase [19]. More advanced strategies combining gas phase activation to nIM-MS, termed collision-induced unfolding (CIU), allow assessing differential gas phase unfolding behavior of proteins. Overall, nIM-MS demonstrated that most solution structures are retained into the gas phase [19,20].

However, despite all those technological breakthroughs, nMS was still poorly implemented in industrial environments in 2015, mostly due to its lack of automation. Indeed, prior to nMS, sample preparation consisting of buffer exchange has to be performed manually, hampering throughput necessary in companies. nMS analyses have to be performed in a volatile buffer, compatible with electrospray ionization and able to preserve weak non-covalent complex assemblies in solution [21]. Ammonium buffers are thus classically used for nMS analyses. Sample preparation usually consists of manual buffer exchange, or desalting, into ammonium acetate using a variety of devices such as gel filtration devices, microconcentrators or dialysis units, a step which is guite timeconsuming, labor intensive and in some cases delicate. It can also lead to alteration of the sample, including aggregation, precipitation, or chemical modifications. Hence, this manual buffer exchange process appears as a major drawback for nMS analysis automation and throughput increase. The size exclusion chromatography (SEC), for which separation is based on the differences in hydrodynamic volumes, is achieved through a column packed with particles having precise pore sizes, which makes SEC interesting for fast buffer exchange, that is, separation of small nonvolatile salt molecules from protein species, and/or separation of high versus low mass proteins contained in a mixture.

Even if already suggested in early 2003 by Cavanagh et al. using self-packed gel filtration cartridges [22] and few years later (2008) by Waitt et al. with commercial SEC columns [23], the online coupling of SEC to nMS only became reality five years later for reference proteins [24,25], soluble protein/protein complexes [26] and biotherapeutics [27–32] analysis. Several reasons may account for the delay between first experiments and its effective wider application, among which: (i) the fact that older generation of MS instruments were not sufficiently tolerant to high salt contents necessary for nMS analysis, even volatile salts such as ammonium acetate, (ii) the lack of chemical inertness of SEC columns leading to poor peak shapes with volatile salts, and (iii) the amount of starting biomaterial required for the analysis. SEC afforded first a way to automate nMS experiments providing rapid assessment of proteins and complexes integrity of large numbers of samples, using small sample quantities (between 2 and 5  $\mu$ g).

The benefit of SEC-nMS has been broadly documented by our group [27,28,33] and others [29–32] for therapeutic proteins characterization, more particularly for mAb-based product analysis. In the present work, we aim at widening the scope of SEC-nMS use for different systems of interest we had in the lab. After first presenting the SEC-nMS technical parameters to be optimized for each system, several examples will serve to discuss benefits but also limitations of online SEC-nMS. We then show the efficiency of the coupling to further integrate IM for fast collision cross section measurement and CIU experiments. Lastly, we demonstrate how online SEC coupling can also be used as the basis to develop multidimensional non-denaturing liquid chromatography (LC) setups, with the SEC column acting as a fast desalting device, helping to achieve first dimension LC separation in optimal chromatographic conditions while being compatible with nMS analysis.

#### 2. Practical aspects, advantages and drawbacks of online SECnMS

**Columns** – Several types of SEC columns are currently available on the market from different manufacturers for the analysis of proteins and macromolecular complexes. They offer a wide variety in terms of column dimensions, stationary phase chemistry, particle sizes and porosities. More particularly, the development of sub-3  $\mu$ m (from 1.7 to 2.7  $\mu$ m) particle size SEC columns, also considered as the new generation SEC columns, allowed to improve separation and column efficiency while significantly reducing the analysis time between three- and five-fold with runs performed in dozens of minutes [34] (Figure S1). As expected, the highest resolution is achieved with the lowest SEC particle size (<2  $\mu$ m). SEC columns are also available with different lengths, from 30 to 300 mm, that will affect the separation of the different protein populations and their nMS signal.

Mobile phase – Among the different parameters that have to be optimized to perform online SEC-nMS, the ionic strength and the pH of the mobile phase are critical to maintain the native conformation of the proteins. Those parameters mainly depend on the nature of the analyte and are the same as for offline nMS. SEC-nMS experiments can be performed with ammonium acetate, bicarbonate or formate mobile phases. Ventouri et al. conducted indepth investigation on the ability of SEC-nMS to preserve the native fold of reference proteins depending on the nature, pH and ionic strength of the solvent [35]. Ammonium acetate was more effective to retain the native protein conformation under nearphysiological pH conditions, although adsorption and peak tailing could be observed especially at lower ionic strength, which was also reported for mAb analysis [36]. Ammonium bicarbonate and formate may lead to higher fractions of denatured populations [35,37]; however, the presence and level of denatured species depend on the analyte, and need to be evaluated for other types of proteins.

Online SEC-nMS for high throughput online buffer exchange For online desalting purposes, the focus will be on using short SEC columns (30 - 50 mm), as increased runtimes will be obtained with longer columns [24]. A compromise between short time of analysis, keeping efficient separation of low molecular weight nonvolatile salts and proteins, limited protein adsorption on the stationary phase and acceptable MS intensities has to be found. Several short new-generation SEC columns between 30 and 50 mm are currently available from different manufacturers (Thermo-Fisher, Waters, Agilent, Phenomenex, etc.). Online SEC-nMS analysis of therapeutic mAbs could be successfully achieved with any of those columns, but in our hands, Waters BEH200 30 mm and Agilent AdvanceBio SEC 50 mm afford the best compromise for therapeutic protein analysis and are used at first line. However, protein adsorption could be different for other types of proteins. The flowrate also plays a critical role on SEC-nMS data: even if flowrates ranging between 100 and 250  $\mu$ L/min can be used, lower elution flowrates (100 µL/min) usually provide increased nMS signal-tonoise ratio (Figure S2) and are thus better suited [28]. To conclude, for high throughput purposes resulting in <5 min runs, the shortest columns (30 - 50 mm) with smallest particle size  $(<3 \mu m)$  should be preferentially selected (Figure S1).

SEC is particularly well-adapted to perform online buffer exchange for proteins for which manual desalting may induce the precipitation of the sample or does not completely remove the non-volatile salts of the original buffer. The benefits of online SEC-nMS is clearly exemplified with a mAb-RNA conjugate (Fig. 1A). After 6 cycles of Vivaspin, only minor noisy MS signal that could correspond to the mAb-RNA conjugate is observed in the *m/z* range 6000 - 7500 (Fig. 1 A1), avoiding any mass calculation and

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**Fig. 1. SEC-nMS for efficient desalting and improved protein separation**. Native mass spectrum of mAb-RNA conjugate obtained with (A1) manual desalting and (A2) online SEC-nMS. (B1) Chromatographic separation and (B2) corresponding native mass spectra of a mixture of four reference proteins (PK, ADH, ConA, and deglycosylated NISTmAb) obtained with a 300 mm SEC column (Waters BEH125). (C1) Chromatogram of nonstressed (black line) and thermally-stressed (red line) bevacizumab. (C2) Native mass spectra corresponding to the high molecular weight species (HMWS) and low molecular weight species (LMWS) of bevacizumab after thermal stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

determination of RNA distribution and binding stoichiometry. Conversely, SEC-nMS analysis affords efficient sample desalting and detection of several species with accurate mass measurements (Fig. 1 A2).

**Online SEC-nMS for improved protein separation** – One of the main issues in analyzing complex protein mixtures is the difference in ionization efficiencies of the constituents that might lead to an overwhelming MS signal of the protein with the highest ionization efficiency, and very minor signals detected in the background of the native mass spectrum. In such cases where several species may hamper the detection of each other, SEC-nMS might be an interesting alternative to simultaneously afford sample desalting and separation of proteins based on their hydrodynamic volume, leading to an easier and straightforward native mass spectra

interpretation. For separation purposes, longer columns (150 or 300 mm) with enhanced chromatographic separation capabilities but also longer run times will be preferred [28]. A mixture composed of four reference proteins, ConA, ADH, NISTmAb and PK, was analyzed with an Acquity BEH200 ( $4.6 \times 30 \text{ mm}$ ,  $1.7 \mu \text{m}$ ) column (Fig. 1B). Reference proteins were efficiently separated in the chromatographic dimension as depicted in Fig. 1 B1. Only NISTmAb and ADH show two overlapping peaks since they exhibit very similar hydrodynamic volumes. In this case, the mass spectra associated with both peaks allowed the identification of both proteins (Fig. 1 B2). Thereby proteins with different sizes can be separated with long SEC columns (300 mm) avoiding ionization competitions between the analytes, which enables a more reliable identification and quantification of different populations.

Another application of SEC-based protein separation is the separation and quantification of LMWS and HMWS [38] in forced degraded studies. When using nMS as a standalone technique, the relative quantification of protein size variants can be misleading due to the modification of the intensity of the different oligomers that may arise at the interface of the mass spectrometer as a consequence of the ionization process (disruption of noncovalent bonds, formation of nonspecific interactions, ionization competition between HMWS and LMWS). Upon thermal stress, the intensity of HMWS and LMWS increases compared to the nonstressed mAb sample (Fig. 1 C1), and the relative quantitation of each population can be assessed based on the areas of chromatographic peaks. However, the relative quantification of HMWS and LMWS significantly varies when using nMS as standalone technique (Figure S3). In this case, the relative intensity of the dimer is estimated to 21.5% (Figure S3A) compared to 14.5% (Figure S3B) with online SEC-nMS data, which corresponds to a variation of 50%. These differences stem on the formation of nonspecific HMWS in the interface of the mass spectrometer during the ionization process compared to nanoESI-nMS. For this reason, online SEC-nMS is particularly well-suited to assess protein stability through forced degraded studies by comparing the relative intensity of the size variant populations. Online SEC-nMS enables an efficient separation, a more reliable quantification and a simultaneous identification of high- and low-molecular species to afford a precise characterization of protein degradation.

**Sensitivity of SEC-nMS** – Overall, SEC-nMS coupling often requires larger amount of sample compared to nanoESI-nMS. Even though the sensitivity of the technique depends on several factors such as the ionization efficiency of the analyte or the type of mass spectrometer used, injected material can vary from 2 to 5  $\mu$ g. However, mass spectra with a suitable S/N ratio can be obtained with less material. The limit of detection of our online SEC coupled to a Synapt-G2 HDMS mass spectrometer with glycosylated pertuzumab was established to 1  $\mu$ g (Figure S4), where the S/N ratio (80) and the resolution of the MS peaks (Rs @m/z 5290 = 587) allow an accurate mass measurement and assignment of all mAb populations. Lower amounts could be injected in other SEC-nMS setups [26], nevertheless 1  $\mu$ g of loaded mAb offers an adequate trade-off between the amount of sample and mass spectrum quality.

**Main SEC-nMS drawbacks** – The use of volatile salts in the mobile phase of SEC-nMS systems may induce further interactions with the stationary phase of the column, leading to the coelution of different protein populations [36] or providing some discrepancies between the elution volumes and the hydrodynamic volume of the oligomers [24]. Since SEC separation is based on the hydrodynamic volume, only proteins with different sizes will be efficiently separated, otherwise, only coeluting or partially resolved chromatographic peaks will be obtained with this kind of chromatography. In addition to possible interactions with the stationary phase, sample dilution in the mobile phase will occur due to diffusion in the column, which may also dissociate unstable and/or low affinity complexes.

Normally, nMS is performed in the nanoESI regime to reduce the amount of injected sample. Conversely, SEC-nMS experiments are performed in ESI ionization mode and thus require higher amounts of starting material. Upon addition of SEC dimension, the flowrate is increased significantly (between 100 and 300  $\mu$ L/min) implying the use of higher desolvation gas and source temperatures to improve solvent evaporation and hence, enhance nMS data. The latter parameter has been recently reported in the literature to have an impact on mAb gas-phase energetics by increasing the internal energy of the ions [33]. These results suggest that source temperatures, and desolvation gas temperatures, need to be carefully adjusted to avoid further source activation and/or fragmentation. In

spite of these potential drawbacks, this review illustrates numerous examples where SEC in combination with nMS has been used to provide further insights into the characterization of therapeutic proteins and macromolecular complexes, and hence, showing the suitability of this coupling for structural biology.

# 3. SEC-nMS is suitable for a variety of non-covalent complexes analyses

**Protein/ligand and protein/metal interactions** – SEC-nMS can help to probe non-covalent interactions between proteins and small molecules. nMS has been reported for high-throughput screening of ligands libraries against enzymes or receptors, the latter being extensively applied in drug discovery. nMS has already been reported as a straightforward, fast and reproducible method to detect ligand binding to specific targets, but also to determine binding affinity, stoichiometry and specificity [39], thus appearing as an attractive technique to complement more common approaches including nuclear magnetic resonance, or surface plasmon resonance [40]. However, the use of SEC coupled to nMS has been scarcely reported in literature.

Quinn's group has presented an online SEC-ESI-FTICR-MS approach to detect protein-ligand non-covalent complexes and to screen natural product extracts [41]. A second study details the technical developments and optimizations of SEC-nMS as a robust, quantitative, and automated platform to measure affinities of noncovalent protein-small molecule interactions on an Orbitrap instrument using indoleamine 2,3-dioxygenase 1 (IDO1), a catabolic enzyme, and inhibitory ligands as case study [42]. The scarce use of SEC coupled to nMS for protein-ligand screening may be explained by the risk of cross contamination, as high affinity ligand absorption on the SEC column may prevent further detection of binding of a lower affinity molecule to the target protein. Alternative nanoESI microfluidic devices, e.g. the Triversa Nanomate from Advion, have been developed, combining capabilities of sample preparation such as ligand addition to the protein and temperature-controlled incubation to reproducible nanoESI injection and nMS analysis [43]. Benefits of such systems is that using individual nozzles for each ligand/mixture of ligand prevents from cross contamination of capillaries or columns.

SEC-nMS can also be of interest to investigate protein—metal interactions, as reported by Jia et al. for [2Fe–2S] cluster-bridged complexes, for which offline nanoESI-nMS failed to detect clusterbound species [44]. As iron-sulfur clusters are sensitive to oxygen, SEC-nMS helps to minimize the possibility to oxidize unstable analytes due to reduced sample preparation and shorter time of analysis. Here, SEC-nMS performed under inert atmosphere (injection valve, sample syringe and samples flushed with argon) revealed the presence of a GLRX5 homodimer with one [2Fe–2S] cluster. This setup also allowed to monitor cluster transfer reactions to get better insight into intermediate [2Fe–2S] species, proving that SEC-nMS can be used as a robust and fast technique to elucidate both the cluster and protein components.

**Protein/protein interactions** – SEC is commonly used to investigate protein oligomerization state or more generally the distribution of size variants of a sample [45]. It is also often the last step of protein/complex purification. We thus performed systematic comparison of manual desalting followed by nMS analysis versus online SEC-nMS for different systems of increasing complexity. The first example relies on the oligomeric state assessment of a homo-oligomeric soluble protein PRMT2, a member of the protein arginine methyltransferase family that has diverse roles in transcriptional regulation [46]. Fig. 2 A1-2 presents the nMS mass spectra obtained after manual desalting (two cycles of Zeba 7 kDa, AcONH<sub>4</sub> 500 mM, pH 7.0) or SEC- nMS analysis of

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Fig. 2. Offline nanoESI-nMS (A1, B1, C1, D1) versus online SEC-nMS experiments (A2, B2, C2, D2) with corresponding SEC-UV chromatograms at 280 nm depicted in insets. Native mass spectra of (A) PRMT2, (B) an equimolar 1:1 P1:P2 mixture, (C) HMW Rvbs complexes, and (D) nuclear receptors RXR and NR7 bound to DNA.

mouse PRMT2. Except differences in the charge state distributions (CSD) due to the use of nanoESI versus ESI, similar mass spectra were obtained, highlighting the presence of two CSDs, a minor one with a mass of 50 726  $\pm$  1 Da corresponding to monomeric (PRMT2)<sub>1</sub> and a major one of 101 517  $\pm$  3 Da corresponding to dimeric (PRMT2)<sub>2</sub>. The PRMT2 example shows the potential of SEC-nMS to preserve the oligomeric state of a protein known as dimeric. Additionally, SEC-nMS ensures the spray stability at high concentration of AcONH<sub>4</sub> (500 mM) contrary to nanoESI-nMS.

Benefits of SEC is further illustrated on the multiprotein P1–P2 complex system. The nMS spectrum (Fig. 2 B1) obtained after manual desalting is highly complex and presents several CSDs. The first one between m/z 1000 and 2500 corresponds to partially unfolded monomeric P1 and P2; from m/z 2500 to 3500, the most intense CSDs correspond to folded monomeric P1 and P2 species; finally, in the m/z range 3700 – 5000, minor signals of homo/heterodimers are detected. Conversely, online SEC-nMS analysis of an equimolar mixture of P1 and P2 (100  $\mu$ M of each, 1-h room temperature incubation) revealed three partially resolved chromatographic peaks (inset, Fig. 2 B2). The most intense chromatographic peak corresponds to (P1)<sub>2</sub> homodimers (64 914 ± 1 Da), followed by a second peak with a mass of 59 973 ± 2 Da in agreement with a 1:1 stoichiometric (P1)<sub>1</sub>(P2)<sub>1</sub> heterodimer (Fig. 2 B2) and finally a peak at 7.8 min corresponding to (P2)<sub>2</sub> homodimers (55 030 ± 1 Da). This

example illustrates the benefits of using SEC separation capabilities (even if partial) to compensate for overlapping CSDs, "polishing" the differences in ionization efficiencies of monomers and dimers and resulting in better quality nMS spectra.

Finally, SEC-nMS is also adapted to the characterization of HMW complexes (>100 kDa). Yeast Rvb1 and Rvb2 are involved in various cellular processes ranging from ribonucleoprotein complex biogenesis to chromatin remodeling [47]. Rvbs predominantly assemble into hexameric rings >300 kDa [47], and are generally purified with buffers containing ADP, which helps to maintain the integrity of the hexamers [48]. The nMS spectrum obtained after manual desalting (Zeba 7 kDa, AcONH<sub>4</sub> 150 mM, pH 7.5) shows that monomers are mainly detected, and only low intensity signals with S/N = 5 are observed for hexamers, suggesting that complex stabilization via ADP was not retained with manual buffer exchange (Fig. 2 C1). Online SEC-nMS experiments performed using 150 mM ACONH<sub>4</sub> as mobile phase demonstrate the potential of the coupling to preserve non-covalent interactions within the hexameric ring of Rvbs, while improving the resolution (Rs @m/z 8106 = 811) and the intensity of the signal (71 S/N) (Fig. 2 C2). SEC-nMS highlights the presence of two populations of hexamers bearing 5 and 6 ADP respectively, the predominant one being bound to 6 ADP  $(307\ 973 + 8\ Da).$ 

Protein/nucleic acid interactions - For protein-nucleic acid

complexes with either RNA or DNA, an additional challenge has to be faced related to the presence of cations associated to oligonucleotide binding which in turn often leads to low quality nMS signals with broad peaks, low mass spectral resolution, and difficult data interpretation. Interactions of nuclear receptors with their cognate DNA response elements serve to illustrate this point. NR7 and RXR are transcription factors that can bind a variety of ligands which can further interact with specific DNA sequences, known as response elements, either as monomers or hetero/homo-dimers. nMS is often used to assess the binding stoichiometry of complexes involving nuclear receptors [49,50]. In this example, nMS was employed to uncover the dimerization properties of NR7 and RXR upon DNA binding. The nMS spectrum obtained after manual buffer exchange (Zeba 7 kDa, AcONH<sub>4</sub> 150 mM, pH 8.0) followed by complex reconstitution of NR7, RXR and DNA (1 h at 4 °C) reveals that the most intense CSD corresponds to the binding of one monomer of NR7 to a DNA fragment of 15 940 Da (Fig. 2 D1). Low intensity signals corresponding to HMWS are also observed in the 5300 - 5800 m/z region, but do not allow accurate mass measurements and proper mass-based identification. Conversely, the online SEC-nMS analysis (150 mM ACONH<sub>4</sub> pH 8.0 after the complex reconstitution) exhibits very different results. The first chromatographic peak at 6.8 min reveals the coexistence of different dimers each bound to one DNA fragment: (NR7)<sub>2</sub> homodimers (102 411  $\pm$  1 Da), (RXR)<sub>2</sub> homodimers (103 944  $\pm$  2 Da) and 1:1  $(NR7)_1(RXR)_1$  heterodimers  $(103\ 177 \pm 2\ Da)$  (Fig. 2 D2). The second chromatographic peak at 9.3 min corresponds to the binding of NR7 to DNA as a monomer (59 178  $\pm$  2 Da), while the last peak at 10.5 min shows the presence of isolated DNA species. Thus, online SEC-nMS allowed to unambiguously characterize the binding combinations between NR7, RXR and the DNA, highlighting the fact that these nuclear receptors predominantly bind DNA as dimers. Here, these results emphasize the potential of SEC-nMS to preserve and transmit heterogeneous protein-nucleic acids complexes.

SEC-nMS for proteins in a detergent environment - Membrane protein analysis by nMS is still challenging as it needs to be performed in a detergent environment. Online SEC-nMS can be applied also in presence of detergents, as depicted with the determination of the oligomeric species constituting amyloid-beta  $(A\beta)$  pores that form in the membrane of neurons to explain neurotoxicity in Alzheimer's disease [51]. Fig. 3A presents the nMS spectrum of the  $\beta$ -sheet pore-forming ( $\beta$ PFO) A $\beta$ (1–42) after manual desalting in C8E5 detergent. Several species ranging from monomers of high intensity to possible octamers of very low intensity were detected, precluding to conclude about the main species present in solution. To circumvent these limitations, SECnMS was performed in 200 mM ammonium bicarbonate pH 9.0 with 14.2 mM C8E5 detergent as mobile phase to benefit from SEC separation capabilities. SEC-nMS analysis of  $\beta PFOs_{A\beta(1-42)}$  resulted in two partially separated chromatographic peaks: the peak at 6.3 min corresponds to octamers (36 114  $\pm$  1 Da) due to the presence of intense 9+ and 7+ ions of the octamer, while the peak at 7.0 min could be attributed to tetramers (18 056  $\pm$  1 Da) (Fig. 3B). Comparison of SEC-nMS analysis of low versus high concentrations of A $\beta$ (1–42) revealed that the former was enriched in A $\beta$ (1–42) tetramers and the latter in octamers (inset, Fig. 3B) [51]. SEC-nMS presented a unique opportunity to establish the stoichiometry of the potentially distinct oligomer species of  $\beta PFOs_{AB(1-42)}$ , revealing that the main species present in the  $\beta PFOs_{A\beta(1-42)}$  sample were  $A\beta(1-42)$  tetramers and octamers [51]. In addition, since no charge states specific for other oligomer stoichiometries between tetramers and octamers were detected, these results suggested that tetramers were the building block for octamer formation. Overall, SEC-nMS appears promising to characterize membrane proteins in detergent environments. However, further developments are still

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necessary, as higher amounts of non-volatile detergents are required compared to nanoESI-nMS. Besides, higher flowrates used in online SEC-nMS compared to nanoESI-nMS may reduce MS sensitivity, and dissociation of unstable membrane protein complexes may occur [52].

# 4. Hyphenation of ion mobility to SEC-nMS for conformational characterization of protein and protein complexes

nIM-MS is now broadly used in structural biology to study the gas-phase conformations of protein and their non-covalent complexes [53,54]. IM separates ions based on their size, shape and charge under the influence of an electric field as they drift through an inert buffer gas in the mobility cell. Drift times can be further converted into rotationally averaged collision cross sections (CCS), which correspond to projected areas of ions. IM affords an additional level of gas-phase characterization, and can help not only to separate isomers but also to examine conformational changes upon complex formation. Hence, the hyphenation of SEC to nIM-MS, which was first reported by Van der Rest et al. [24], is of main interest to rapidly gain further insight into proteins conformations.

SEC-nIM-MS for spectrum cleaning and unambiguous CSDs attribution of oligomers - When several oligomerization states of a protein coexist, unambiguous peak assignment may not be achieved with SEC-nMS due to both low intensities or lack of isotopic resolution for higher oligomeric states. The benefit of SEC-nIM-MS for the separation of isobaric species along with unambiguous peak attribution of oligomers is clearly evidenced in the case of A $\beta$ (1–42) s, which form monomers, dimers, trimers, tetramers and octamers [51]. For instance, mass spectra obtained with SEC-nMS (Fig. 3B) exhibit a peak at m/z 3010 that could correspond to either dimeric (z = 3+), tetrameric (z = 6+) or octameric (z = 12+) species. With the addition of IM, the arrival time distribution (ATD) corresponding to m/z 3010 highlights the presence of two different populations at 8.55 and 12.01 ms (inset, Fig. 3C) which were identified as tetramers (z = 6+) and dimers (z = 3+), respectively. Since ions belonging to the same series are diagonally aligned on bidimensional drift time vs m/z plots, tetrameric 5+ ions and octameric 8+ ions could also be assigned (Fig. 3C). This example demonstrates the potential of SEC-nIM-MS to assess the coexistence of oligometric states for identical m/z while ensuring unambiguous CSDs attributions.

**SEC-nIM-MS for high-throughput CCS measurements of biotherapeutics** – While the use of nIM-MS is commonplace in academic research laboratories, it remains scarce in biopharmaceutical companies. Although nIM-MS has already proven valuable to characterize biotherapeutic proteins such as mAbs, bispecific antibodies (bsAb) and antibody–drug conjugates (ADC) [55], bot-tlenecks hampering its routine use in biopharma include (i) relatively manual experiments that require highly skilled operators performed with nanoelectrospray capillaries of microfluidic devices and (ii) lack of automation of nIM-MS data processing.

Van der Rest et al. first demonstrated that CCS values of reference proteins obtained using SEC-nIM-MS were in good agreement with nanoESI-nIM-MS values [24]. With therapeutic mAbs as model proteins, we compared <sup>TW</sup>CCS<sub>N2</sub> obtained with nanoESInIM-MS after manual desalting and SEC-nIM-MS analysis [28,33]. Different points should be considered when performing SEC-nIM-MS for CCS calculation. As SEC-nIM-MS experiments are performed in ESI mode, CSDs in SEC-ESI are centered on higher charge states than in nanoESI. Dipole-dipole interactions and coulombic repulsions increasing with charge state, higher charge states might be more activated, which might result in slight protein unfolding. As shown in Fig. 4A, similar IM calibration equations were obtained

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**Fig. 3. NanoESI- and SEC-ESI- nIM-MS experiments of**  $A\beta(1-42)$ . (A) Native nanoESI mass spectrum obtained after manual desalting. (B) Native SEC-ESI mass spectrum corresponding to the chromatographic peak of the octamer (yellow slice of UV chromatogram shown in inset). Species were identified as monomers (white), dimers (orange), trimers (green), tetramers (pink) and octamers (yellow). (C) 2D SEC-nIM-MS Driftscope plot with charge state distributions of the different oligomers. The extracted ATD at *m*/*z* 3010 is shown in inset. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for <sup>TW</sup>CCS<sub>N2</sub> measurements performed in SEC-nIM-MS and offline injections of manually desalted proteins. Keeping that in mind, CCS were measured for a series of mAbs and show good correlation with <sup>TW</sup>CCS<sub>N2</sub> measured in nanoESI-nIM-MS (Fig. 4B). In this context, SEC-nIM-MS appears as an attractive setup to automate sample preparation while widening the information content available in a single few-minutes experiment as illustrated by Ehkirch et al. for the characterization of different therapeutic mAbs formats, including IgGs, ADCs and bsAbs [28]. Additionally, SEC-nIM-MS affords further characterization of HMWS in stressed mAbs samples, revealing a significant conformational compaction upon mAb aggregation [24].

Altogether, our results highlight that online coupling of SEC to nIM-MS does not significantly influence the global conformation of mAbs, which of course needs to be checked for other types of proteins.

**SEC coupled to Collision-Induced Unfolding** – CIU is an IMbased approach used to probe ion gas-phase unfolding. In travelling-wave IM spectrometry (TWIMS) instruments, CIU experiments are performed by raising collision voltages (CVs) in the trap cell before IM separation, subsequently activating ions that can undergo conformational changes [57]. While CIU appears as an

elegant alternative to circumvent low IM resolution of linear TWIMS cells, lack of CIU automation in both buffer exchange and data acquisition has precluded its wide adoption. In order to fully automate CIU experiments from sample preparation to data interpretation, we have developed a fast-online SEC-CIU coupling using short SEC columns, where CVs are automatically raised along sample elution (Fig. 4C) [33]. CIU fingerprints are generated with the CIUSuite 2 software to better visualize ATDs variations corresponding to conformational transitions (Fig. 4D). This SEC-CIU setup is particularly interesting to rapidly distinguish mAbs subclasses, as the latter exhibit different unfolding patterns at both intact [58] and middle [59] levels. Diagnostic CVs regions allow to identify mAbs subclasses with scores >80% (Fig. 4D). Targeted scheduled SEC-CIU, that is, acquisition of solely the most diagnostic CVs, helps to further reduce the data collection time while retaining clear-cut mAbs classifications.

Altogether, the combination of improved high-throughput desalting and automated data collection afforded by SEC drastically shortens the overall time process of CIU experiments, from 3 h for classical CIU experiments with manual buffer exchange to 15 min for targeted scheduled SEC-CIU.



**Fig. 4. SEC-nIM-MS experiments for CCS measurements and CIU approaches.** (A) Calibration curves for SEC-nIM-MS (grey) and offline injection (blue). ConA, ADH and PK were used as external calibrants as reported by Bush et al. [56]. (B) Table summarizing <sup>TW</sup>CCS<sub>N2</sub> measurements at intact level obtained with direct nanoESI versus SEC injections. <sup>¶</sup>All products were deglycosylated except for NISTmAb. (C) Schematic representation of SEC-CIU acquisitions. (D) SEC-CIU fingerprint and subclass classification obtained for intact ofatumumab. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

# 5. Hyphenation of non-denaturing LC through a SEC-based multidimensional methodology

SEC can be used for two main purposes: (i) separation and relative quantification of protein oligomers and (ii) to perform online buffer exchange prior to the analysis of proteins by nMS. However, as a consequence of the latter functionality, SEC can pave the way to allow the hyphenation of non-denaturing LC with nMS.

Coupling HIC to nMS: Development of HICxSEC-nMS strategy – Hydrophobic interaction chromatography (HIC) is the reference technique in guality control laboratories to separate and quantify the different drug-antibody ratio (DAR) populations of ADCs [60,61], especially in the case of cysteine-linked ADCs for which maintaining non-covalent interactions is critical for the analysis of the whole ADC scaffold. However, the analysis of the different populations of highly heterogeneous ADCs can lead to ambiguous interpretations when this analysis is based solely on the HIC dimension. To this end, nMS can afford additional information through the mass measurement of the intact species in their native state to enable the identification of the DAR populations. Since conventional HIC solvents are not compatible with nMS, several approaches have been proposed in the literature allowing the straight hyphenation of HIC with nMS. According to the main drawbacks associated with this coupling, the different experimental strategies were based on the modification of the composition of HIC mobile phase [62], the adoption of new HIC stationary phase materials [63] or the addition of a makeup flow followed by a splitting flow [64]. However, these strategies require the use of nMS-compatible mobile phases, that is, ammonium acetate, along with the addition of organic modifiers, which leads to the reduction of the peak separation and the potential denaturation of proteins. To this end, Ehkirch et al. designed and adapted a 2D LC system where the SEC cartridge

(AdvanceBio SEC,  $4.6 \times 50$  mm,  $2.7 \mu$ m, 300 Å) is implemented within the interface of the HIC-MS coupling enabling online buffer exchange in the front end of the mass spectrometer [65] (Fig. 5A). This experimental setup was used to characterize the different populations of a conjugated mAb (Fig. 5B). In this case, only five ADC populations were expected (D0, D2, D4, D6, and D8), however one additional peak is observed at 40 min in the HIC profile (Fig. 5B). The mass measurement enables the assignment of the additional HIC feature as a DAR4 positional isomer (Fig. 5C). The all-in-one combination of HICxSEC-nMS provided a comprehensive and streamlined characterization of all the species observed within the first LC dimension without compromising neither the chromatography separation nor the native structure of the proteins.

2D SECxSEC-nMS setup for improved SEC performances – The first non-denaturing HICxSEC-nMS setup can be considered as the basis for the conception of new MS-based multidimensional LC coupling strategies [65]. The idea is to benefit from synergic effects of performing first dimension non-denaturing LC without compromising chromatographic performances while being compatible with nMS. The implementation of SEC for fast desalting between the first analytical dimension of separation and the mass spectrometer has thus a great potential to overcome the limitations of these techniques when used as standalone methods. Indeed, SEC-UV is usually performed with high concentration of nonvolatile salts (typically around 100-500 mM) to reduce the nonspecific interactions between the stationary phase and the size variant species [66]. When SEC is coupled to nMS and hence, classical SEC mobile phase is replaced by MS-compatible solvents, proteins may undergo more interaction with the stationary phase (ionic and hydrophobic interactions), especially those with a pI greater than 7.0, giving rise to broader peaks with larger retention times, and leading to an underestimation of the relative intensity of size variants [36]. This is illustrated by the example of the



Fig. 5. 2D HICxSEC-nMS and SECxSEC-nMS experiments. (A) HICxSEC-nMS setup. (B) HIC profile of an in-house investigational ADC. (C) Deconvoluted mass of the highlighted HIC peak. (D) SEC profile of intact pembrolizumab with non-volatile and volatile salts in the mobile phase. (E) Comparison of SEC profile of intact pembrolizumab (solid line) and thermally-stressed pembrolizumab (dotted line).

characterization of HMWS and LMWS in the case of the therapeutic mAb pembrolizumab (Fig. 5D). Ehkirch et al. thus developed an experimental setup based on comprehensive 2D SECxSEC coupled to nMS to determine HMWS and LMWS of non-stressed and thermally-stressed mAbs [67]. The particular case of pembrolizumab pinpoints that the use of phosphate buffer in the first SEC dimension and the online combination of nMS are essential to provide baseline chromatography resolution and unambiguously assign mAb populations, respectively (Fig. 5D-E). The co-elution of the two chromatographic peaks when performing SEC with ammonium acetate mobile phase hinders the relative quantitation of both species. Furthermore, three distributions can be observed in pembrolizumab's chromatographic profile upon thermal stress (Fig. 5E), which may suggest the presence of different oligomer populations, that is, trimer, dimer, and monomer. However, nMS not only revealed that different chromatographic peaks corresponded to different types of monomeric populations (Fig. 5E), but also that those monomers had different degrees of oxidation.

The results obtained with these multidimensional LCxLC-IMxMS for therapeutics analysis under non-denaturing conditions pinpoints the complementarity of these techniques to expand the capabilities of non-denaturing LC and nMS techniques. In one hand, LC dimensions allow the separation and quantification of the different mAb populations based on the apparent hydrophobicity or size. On the other hand, nMS plays a pivotal role to unravel complex or unexpected chromatographic profiles affording a precise mass measurement of each individual chromatographic distribution.

#### 6. Concluding remarks

In the present study, we exemplify the use of online SEC-nMS for a broader range of biomolecules ranging from homo-oligomeric proteins to proteins with nucleic acid, but also protein-small molecule complexes or even larger assemblies up to hundreds of kDa. SEC-nMS is suitable for most soluble protein-protein complexes and can also be adapted for a selected series of detergents for membrane protein analysis. Online SEC-nMS in most cases not only affords rapid and improved desalting efficiency but also separation of co-eluting/overlapping species. Online nMS is quite versatile as many types of commercial columns, but also manually packed ones, and LC systems are available that can be coupled to any nMS compliant mass spectrometer. In addition, the SEC-nMS coupling provides enough sensitivity to analyze low amounts of material (few micrograms) with accurate mass measurements. This increase in sensitivity could be of major interest to tackle low affinity  $(K_a > 100 \mu M)$  assemblies or complexes maintained by weak interactions.

SEC-nMS was also the basis for the development of more complicated multidimensional 2D LCxLC workflows in which the SEC acts as fast desalting device, allowing to achieve first dimension LC separation in optimal chromatographic conditions while being compatible with downstream nMS analysis. The combination of multidimensional LC with nMS provides a synergic effect for the comprehensive characterization of mAb-based therapeutics in one single run without limiting the technical capabilities of these techniques when used as standalone techniques.

Online SEC does not hamper further IM measurements affording

conformational characterization of samples either through automated CCS measurements or even for CIU experiments. In addition, further MS/MS experiments (reported for the moment only with collision-induced dissociation (CID) fragmentation) are possible. We expect ultraviolet photodissociation (UVPD) and electrontransfer dissociation (ETD) being possible also, opening the way for automatic top-down nMS.

#### **CRediT** authorship contribution statement

**Evolène Deslignière:** Formal analysis, Writing - original draft, Writing - review & editing. **Marie Ley:** Formal analysis. **Maxime Bourguet:** Formal analysis. **Anthony Ehkirch:** Formal analysis. **Thomas Botzanowski:** Formal analysis. **Stéphane Erb:** Formal analysis. **Oscar Hernandez-Alba:** Formal analysis, Writing - original draft, Writing - review & editing. **Sarah Cianférani:** Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.

#### **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare no competing financial interest.

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#### Appendix A. Supplementary data

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# SUPPLEMENTARY INFORMATION

# Pushing the limits of native MS: Online SEC-native MS for structural biology applications

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Figure S1. SEC chromatograms obtained with different SEC columns for glycosylated trastuzumab.



**Figure S2.** Online SEC-nMS spectra of tetrameric ADH obtained with (A) constant 250  $\mu$ L/min flowrate and (B) reduced 100  $\mu$ L/min flowrate.



**Figure S3.** Identification and quantification of thermally-stressed trastuzumab oligomers obtained with (A) offline nMS, and (B) online SEC-nMS. The inset in the right-hand side of the figure corresponds to the mass spectrum of the main chromatographic peak.



**Figure S4.** (A) Native mass spectrum of intact pertuzumab obtained with online SEC-nMS at constant 100  $\mu$ L/min flowrate. The corresponding UV chromatogram is shown in inset. The amount of loaded mAb sample was 1  $\mu$ g. (B) Variation of MS intensity of SEC-nMS as a function of injected pertuzumab sample.

# Chapter 2 – Integration of nMS and nIMS-MS for Structural Characterization of Human RuvBL1-2 Complexes

# 1. Biological and scientific contexts

Human RuvBL1 (R1) and RuvBL2 (R2) are two highly conserved AAA+ ATPases that are essential in a wide range of cellular processes, such as chromatin remodeling, transcriptional regulation, maturation/stabilization phosphatidylinositol 3-kinase-like kinases (PIKK), and small nucleolar ribonucleoproteins (snoRNPs) assembly<sup>318,319</sup>. RuvBL subunits assemble into heterohexameric rings, and can further associate as double rings to form dodecamers<sup>320-325</sup>. Each subunit is composed of different domains<sup>320,323</sup>: DI and DIII constitute the core of the hexamer, while DII protrudes from one side of the ring (Figure 5). These domains define two distinct faces on the hexamer, the AAA-face where the ATPase activity is located, and the DII-face, which acts as a platform for docking and interaction with protein partners (Figure 5). Binding to clients influences the conformation of DII domains, and subsequently regulates R1R2 ATPase activity as a result of nucleotide-binding pockets opening<sup>326</sup>.



**Figure 5.** Structure of R1 and R2. Monomeric R1 (PDB code: 2C9O) and R2 (PDB code: 6H7X) assemble into hexamers or dodecamers. Adapted from Dauden *et al.* 2021 (ref. 319).

R1R2 are involved in various multi-protein complexes, including chromatin remodelers from the INO80 family<sup>327-329</sup> and the TIP60 histone acetyltransferase complex<sup>329</sup>. R1R2 are also part of the R2TP complex, an HSP90 co-chaperone implicated in the assembly and maturation of numerous protein complexes<sup>325,326,330-333</sup>. R2TP comprises proteins RPAP3 and PIH1D1 (T and P, respectively)<sup>331</sup>. Disruption of R1R2 dodecamers into hexamers occurs upon binding of TP, resulting in a hexameric R2TP complex whose structure has been recently solved by cryo-EM<sup>332</sup>. Maurizy *et al.* identified a new R2TP-like chaperone, called R2SP and composed of proteins SPAG1 and PIH1D2 (S and P, respectively)<sup>325</sup>. R2SP has been found to facilitate quaternary protein folding, however, an in-depth functional and structural characterization of the complex has not been conducted yet. R1R2 connects with a large variety of clients, evidenced by proteomics and interactomics studies<sup>334-336</sup>, but structural insights into these protein assemblies are often lacking.

Together with the teams of Dr. Edouard Bertrand (IGMM, UMR5535, Montpellier), Pr. Bruno Charpentier (IMoPA, UMR7365, Nancy), Dr Célia Plisson-Chastang (LBME, UMR5099, Toulouse), Dr.

Philippe Meyer (LBCME, UMR8226, Paris), and Dr. Tiago Bandeiras (iBET, Lisbon, Portugal), our laboratory aims at providing a better understanding of interactions between R1R2 and their different partners, through structural characterization of various complexes. These structural information are obtained using several biophysical techniques, including SEC coupled to static light scattering (SEC-SLS), MS, SAXS, NMR, cryo-EM, and X-ray crystallography.

### 2. Objectives

This chapter describes the structural characterization of R1R2 complexes bound to different protein partners. More precisely, my PhD work focused on the following complexes:

- FL R1R2 and ΔDII R1R2;
- R2D, which comprises the DPCD protein;
- R2TP, which contains RPAP3:PIH1D1 (TP) noncovalent heterodimers;
- R2SP, which involves SPAG1:PIH1D2 (SP) noncovalent heterodimers;

Here, I will show how nMS and nIMS-MS experiments can complement and validate data gathered across other biophysical techniques.

# 3. Characterization of FL and $\Delta$ DII R1R2 constructs

A first study consisted of analyzing two different R1R2 constructs, either FL or without their DII domains ( $\Delta$ DII), expressed in *E. coli*. These complexes will serve as core-platform for assembly of other protein partners, described later.

## 3.1. Presence and nature of nucleotides in binding pockets of R1/R2 monomers

R1 and R2 monomers (~50 kDa each) possess an adenosine triphosphate (ATP, 507 Da) or ADP (427 Da) binding pocket, and so we aimed at determining whether R1 and R2 are in a nucleotide-free or ATP/ADP-bound state.

MS analysis on FL R1R2 samples was first performed in denaturing conditions, which allows to break noncovalent interactions between R1, R2, and possible nucleotides. Two species corresponding to monomers R1 (51 631.4 ± 0.9 Da) and R2 (51 760.9 ± 0.5 Da) were detected (Figure 6A). Of note, comparison with theoretical masses (R1 = 51 760 Da and R2 = 51 886 Da) shows that the N-terminal methionine residue has been cleaved on both monomers (–131 Da), which typically occurs during protein expression in *E. coli*<sup>337</sup>. In addition, an intense peak is observed at *m/z* 428.0, suggesting the presence of ADP in the sample (data not shown). This hypothesis is strengthened by the absence of the *m/z* 507 peak that would correspond to ATP.

In native conditions, the nMS spectrum exhibits two populations corresponding to R1 (52 057  $\pm$  1 Da) and R2 (52 188  $\pm$  1 Da) (Figure 6B). Increases of +428 Da compared to masses obtained in denaturing conditions indicate the presence of one molecule of ADP on each monomer. In our case,

nucleotides were detected despite the absence of ADP molecules in purification and analysis buffers, suggesting that nucleotides originate directly from *E. coli*.

These first results unequivocally demonstrate that FL R1 and R2 are in an ADP-bound state. Identical conclusions were drawn from R1 and R2  $\Delta$ DII (data not shown). This is in good agreement with previous studies reporting the presence of ADP in R1/R2 binding pockets<sup>320,326,338,339</sup>. In particular, the N-terminal segment of R2 has also been found to block nucleotide exit and exchange from ADP to ATP<sup>326,339</sup>.



**Figure 6.** Mass measurements of R1 and R2 obtained **(A)** in denaturing MS (Vc = 40 V; O64062) and **(B)** in nMS (Vc = 200 V; O63491). \* = monomers R1 (dark blue) and R2 (light blue) without ADP molecules. Data were acquired on the Synapt G2 (Q-ToF).

### 3.2. Comparison of FL and ΔDII R1R2 complexes

### 3.2.1. nMS analyses

We next moved to the characterization of R1R2 complexes, in order (i) to determine their oligomerization states and (ii) to establish whether ADP molecules are retained upon oligomerization.

Even if complexes were clearly transmitted/identified on a ToF instrument (LCT), analyses on an Orbitrap instrument of higher MS resolution (Exactive Plus EMR) were preferred to assess ADP stoichiometries more accurately (Rs @m/z 8240 = 405 versus 555 on ToF and Orbitrap, respectively, Figure 7A).

For FL R1R2, two co-existing species corresponding to hexameric (313 086  $\pm$  59 Da) and dodecameric R1R2 (625 789  $\pm$  169 Da) are detected (Figure 7B). Masses indicate the presence of 6 – 7 ADP molecules on the hexamer, while 12 – 14 ADP are bound to the dodecamer, proving that nucleotides are preserved along the formation of FL R1R2 complexes<sup>320,326</sup>. Regarding R1R2  $\Delta$ DII

complexes, a mixture of hexamers (245 281  $\pm$  66 Da, bound to 6 – 7 ADP) and dodecamers (490 444  $\pm$  116 Da, with 12 – 14 ADP) is also present (Figure 7C).



**Figure 7.** nMS results obtained **(A)** on the LCT instrument for FL R1R2 (Vc = 140 V; Z69361). Inset: Comparison between FWHMs obtained for the 38+ charge state on the LCT *versus* EMR. High-resolution MS data were generated on the EMR Orbitrap instrument for **(B)** FL R1R2 and **(C)** R1R2  $\Delta$ DII (CID/CE = 25/200 V, Rs = 8.75; EM04295/EM04299).

A major difference between both constructs lies in the fact that the hexamer/dodecamer ratio significantly varies across samples. Indeed, while hexamers are the main population for FL R1R2 (66  $\pm$  1%), only a small proportion of hexameric R1R2  $\Delta$ DII is observed (13  $\pm$  2%). These results show that DII domains are not essential for the assembly of dodecamers. The crystal structure of truncated dodecameric R1R2  $\Delta$ DII suggest that interactions between the two rings are mediated by residual segments of the DII internal region (PDB code: 2XSZ)<sup>338</sup>.

The oligomerization state of R1R2 constructs has been studied by many groups, and reported as hexameric and/or dodecameric, with discrepancies most likely due to different purification methods<sup>317,338</sup>. Gorynia *et al.* showed using SAXS experiments that FL R1R2 contains a significant

fraction of hexamers in solution, while R1R2  $\Delta$ DII are mostly dodecameric<sup>338</sup>, in line with our observations.

# 3.2.2. Mass photometry analyses

In order to confirm the hexamer/dodecamer equilibrium revealed by nMS analyses, mass photometry experiments were conducted by Dr. Tomás de Garay (Refeyn, Oxford, U.K.). This technique quantifies light scattering from individual biomolecules in solution, and uses this signal to count molecules and measure their mass<sup>198</sup> (see part I, chapter 1, section 4). Contrary to nMS, it does not require any sample preparation (i.e. buffer exchange) prior to analysis, avoiding the generation of artefactual species, and low analyte concentrations are used (100 pM – 100 nM).

Mass photometry highlights the co-existence of three populations for both FL and truncated R1R2 (Figure 8). Even if this method has a low resolution compared to what can be achieved through Orbitrap mass measurements, species could be clearly identified as monomers, hexamers and dodecamers. It is worth noting that free monomers were seen on the Q-ToF (Synapt G2, Figure 6B), but not on analyses performed on the Exactive Plus EMR because the transmission is not linear on Orbitrap instruments<sup>340</sup>, and so only  $m/z > 6\,000$  were transmitted with our parameters. For straightforward comparison with nMS data, monomers were not taken into account for ratio calculations. The hexamer/dodecamer equilibrium for FL R1R2 is 64/36% (Figure 8A), and is shifted towards dodecamers when DII domains are removed (9/91%, Figure 8B). These values are extremely close to those generated with nMS (66/34 ± 1% and 13/87 ± 2% for FL and  $\Delta$ DII samples, respectively).

Overall, these experiments strongly corroborate the co-existence of single and double R1R2 rings. More importantly, nMS data correlate well with results obtained in solution, proving that nMS retains consistent proportions between oligomeric states in the gas phase.



**Figure 8.** Mass photometry experiments performed on **(A)** FL R1R2 and **(B)** R1R2 ΔDII. Analyses were performed in monoplicate.

# 3.2.3. nIMS-MS measurements

nIMS-MS data were also recorded on the Synapt G2 to gather conformational information on both complexes. <sup>TW</sup>CCS<sub>N2</sub> measurements are reported in Table 1 for the most native charge state.

For FL R1R2, <sup>TW</sup>CCS<sub>N2</sub> values of 13 081 ± 10 Å<sup>2</sup> and 21 961 ± 10 Å<sup>2</sup> were measured for hexamers and dodecamers, respectively, which represents an increase of 68% upon assembly of the double ring. These experimental results were compared to CCS values estimated from structures obtained with cryo-EM or X-ray crystallography. Calculations in the IMoS software were performed in He environment using PA and EHSS algorithms (see part I, chapter 2, paragraph 3.3). As a reminder, for large complexes (in He), PA tends to underestimate CCS values, whereas EHSS overestimates CCSs. CCS values of 14 483 ± 12 Å<sup>2</sup> (hexamer) and 24 656 ± 31 Å<sup>2</sup> (dodecamer) were obtained using the EHSS algorithm, which corresponds to an increase of 70% between the two oligomers, in good agreement with nIMS-MS data (Table 1). PA also yields an increase of 68% from hexamer to dodecamer. It is worth mentioning that the hexamer has been solved in the context of the INO complex<sup>327</sup>, i.e. with straight DII domains, and that the dodecameric structure comes from *Chaetomium thermophilum*<sup>322</sup>. These models fit well with nIMS-MS results.

Table 1. Comparison of IMS-MS an	cryo-EM/X-ray crystallography	/ structures for FL R1R2	and $\Delta DII R1R2$
complexes. *CCS = 2.435 x $MW^{2/3}$ fr	m Ruotolo <i>et al.,</i> 2008 (ref. 26	53). ▲CCS calculations are	reported as an
average of n = 6 replicates.			

Technique	Physical quantity	FL R1R2 Hexamer (PDB: 50AF <sup>327</sup> )	FL R1R2 Dodecamer (PDB: 5FM6 <sup>322</sup> )	R1R2 ΔDII Hexamer (PDB: 2XSZ <sup>338</sup> )	R1R2 ΔDII Dodecamer
	Measured MW (Da)	313 086 ± 59	625 789 ± 169	245 281 ± 66	490 444 ± 116
IMS-MS	MW-based prediction of CCS* (Å <sup>2</sup> )	11 227	17 815	9 541	15 143
	Measured CCS (Å <sup>2</sup> )	<b>13 081 ± 10</b> (z = 36+)	<b>21 961 ± 10</b> (z = 53+)	<b>10 953 ± 10</b> (z = 32+)	<b>18 673 ± 10</b> (z = 47+)
Cryo-EM or	CCS <sub>PA</sub> (Ų)▲	10 706 ± 44	17 988 ± 18	8 728 ± 55	
X-ray crystallography	CCS <sub>EHSS</sub> (Ų)▲	14 483 ± 12	24 656 ± 31	11 522 ± 4	Not available

For R1R2  $\Delta$ DII, <sup>TW</sup>CCS<sub>N2</sub> of 10 953 ± 10 Å<sup>2</sup> and 18 673 ± 10 Å<sup>2</sup> were measured for hexameric and dodecameric forms, respectively (+70% upon formation of the dodecamer). The CCS calculated from the hexameric R1R2  $\Delta$ DII structure is 11 522 ± 4 Å<sup>2</sup>, in line with nIMS-MS values (Table 1).

Lastly, increases of +59% from hexamers to dodecamers were expected from mass-based CCS predictions<sup>263</sup> for both constructs (Table 1), which is slightly lower than what is experimentally obtained. This may indicate that conformational variations occur upon assembly of dodecameric rings.

Overall, these comparisons demonstrate that conformations of R1R2 complexes are maintained in the gas phase, proving the validity of nIMS-MS to probe native conformations of large protein complexes.

# 4. Characterization of R2D complex

DPCD is a ~23 kDa protein that might play a role in cilia formation<sup>341,342</sup>. Cilia can be divided into two major types: motile cilia, which serve as flagella, and non-motile primary cilia, which function as sensory organelles. Defective cilia cells lead to primary ciliary dyskinesia (PCD), a recessive disorder caused by genetic mutations and characterized mainly by respiratory diseases<sup>343</sup>. DPCD was found to associate with human R1R2<sup>325,334</sup>, however, no in-depth structural investigation of DPCD at protein level was reported so far.

# 4.1. DPCD protein

# 4.1.1. Denaturing MS and nMS analyses

DPCD was first analyzed after manual desalting with AcONH<sub>4</sub> 150 mM (pH 7.5) supplemented with 0.5 mM of TCEP. In both denaturing and native conditions, a single species of 23 504  $\pm$  1 Da was detected, corresponding to monomeric DPCD (Figure 9A, B). These results are in line with the oligomeric state observed in solution with SEC-SLS experiments. Of note, when no reducing agent was added to the desalting buffer, two CSDs were observed, reflecting the co-existence of dimeric (47 004  $\pm$  1 Da) and monomeric DPCD populations (Figure 9C, D). This strongly suggests that dimerization occurs through an intermolecular disulfide bond. Supplementing the buffer with TCEP prevents the formation of multimeric forms.



**Figure 9.** Mass measurements of DPCD obtained on the EMR Orbitrap instrument. (A) Denaturing and (B) native MS spectra, with 0.5 mM TCEP in the AcONH<sub>4</sub> desalting buffer. (C) Denaturing and (D) native MS spectra, without TCEP in the AcONH<sub>4</sub> desalting buffer (EM04591/EM04602/EM05202/EM05212; CID/CE = 40/0 V in denaturing conditions and 25/100 V in native conditions, Rs = 8.75).

# 4.1.2. nIMS-MS for conformational characterization of DPCD

As no 3D structural data are available for DPCD, one of the key objectives was to gather information across different techniques to propose a DPCD 3D model. High-resolution X-ray crystallography experiments were not conclusive, and NMR gave access to limited structural information. In such cases where high-resolution techniques fail to provide enough structural data, combining approaches of lower resolution is of utmost interest to uncover evidences regarding the protein's structure/conformation and build a first 3D model.

nIMS-MS experiments were thus carried out on DPCD (Synapt G2 HDMS). In order to validate results obtained in the gas phase, nIMS-MS data were first compared with those obtained by SAXS and SEC-UV experiments. SAXS gives access not only to the radius of gyration ( $R_g$ ) and the molecular envelope of the molecule of interest, but also to estimations of the 3D structure of the analyte based on rigid-body modelling. Regarding SEC, calibration with well-adapted standards allows determination of the hydrodynamic Stokes radius ( $R_s$ ). Both  $R_g$  and  $R_s$  are sensitive to the global shape/size of the molecule in solution. <sup>TW</sup>CCS<sub>N2</sub> were converted into an  $R_{IMS-MS}$  radius, with the following formula, which assumes a spherical shape of the protein<sup>95</sup> (Equation 1):

$$R_{\rm IMS-MS} = \sqrt{\frac{\rm CCS}{\pi}}$$
(1)

Table 2 summarizes the outcomes of these calculations, with consistent radii obtained across the three different techniques.

Table 2. Comparison of IMS-MS, SEC and SAXS experiments for DPCD. *CCS = 2.435 x MW <sup>2/3</sup> from Ruotolo et al.,
2008 (ref. 263). A CCS calculations are reported as an average of n = 6 replicates. IMPACT infers TM values from
its PA calculations, using CCS <sub>TM</sub> = 0.843 x CCS <sub>PA</sub> <sup>1.05</sup> (Marklund <i>et al.</i> , 2015, ref. 225).

Technique	Physical quantity	DPCD
		<b>27 ± 2</b> (z = 9+)
IMS-MS	R <sub>IMS-MS</sub> (Å)	<b>28 ± 2</b> (z = 10+)
		<b>29 ± 2</b> (z = 11+)
SEC	R <sub>s</sub> (Å) 24	
SAXS	R <sub>g</sub> (Å) 22	
IMS-MS	Measured MW (Da)	23 504 ± 1
	MW-based prediction of CCS* (Å <sup>2</sup> )	1 998
		<b>2 356 ± 10</b> (z = 9+)
	Measured <sup>™</sup> CCS <sub>№2</sub> (Å <sup>2</sup> )	<b>2 505 ± 10</b> (z = 10+)
		<b>2 663 ± 10</b> (z = 11+)
SAXS▲	CCS <sub>TM</sub> from MOBCAL (Ų)	2 605 ± 37
	CCS™ from IMoS (Ų)	2 533 ± 11
	CCS™ from IMPACT (Ų)	2 555 ± 3

Experimental <sup>TW</sup>CCS<sub>N2</sub> were then compared to CCS values estimated from a 3D model generated through rigid-body modelling and fitting to SAXS data. Calculations were performed in He environment using the TM algorithm, which is well adapted for small proteins, and provides more accurate CCS

measurements by considering all interactions between ions and gas molecules. Three different software (MOBCAL<sup>278,279</sup>, IMoS<sup>344</sup> and IMPACT<sup>225</sup>) were used for calculations, with similar CCS values of 2 500 – 2 600 Å<sup>2</sup>. More importantly, values derived from the 3D model agree well with experimental nIMS-MS data. Indeed, <sup>TW</sup>CCS<sub>N2</sub> of 2 356 ± 10 Å<sup>2</sup> were measured for the most native charge state of DPCD (z = 9+) (Table 2). The DPCD model proposed here comprises a disk-like shaped domain with an extended tail, and flexible/unstructured regions, which also explains why obtaining crystals was difficult (Figure 10). Because of the non-globular shape of the protein, measured <sup>TW</sup>CCS<sub>N2</sub> are slightly higher than mass-based estimations proposed by Ruotolo *et al.*<sup>263</sup> assuming spherical analytes (1 998 Å<sup>2</sup>, Table 2).

Overall, nIMS-MS results are in good correlation with SEC and SAXS. These comparisons show that DPCD retains a memory of its solution structure upon transfer into the gas phase. nIMS-MS also corroborates the first model ever proposed for DPCD.



Figure 10. 3D model of DPCD obtained after rigid body modelling and fitting to SAXS data.

# 4.2. R2D complex

# 4.2.1. nMS analysis

We then analyzed the tripartite complex formed by FL R1, FL R2 and DPCD (R2D). The nMS spectrum shows two populations corresponding to hexameric FL R1R2 bound to two or three DPCD molecules (360 082 ± 77 Da and 383 611 ± 66 Da, respectively), with 1:3 being the major stoichiometry (Figure 11A). The dodecamer is no longer observed, indicating that interactions between the two R1R2 rings are disrupted upon DPCD binding, in favor of a stable R2D hexamer. Interestingly, the formation of the R2D complex only occurs in presence of DII domains, as evidenced by SEC-UV experiments followed by SDS-PAGE analysis, suggesting that DPCD connects to FL R1R2 *via* DII domains (Figure 11B).



**Figure 11. (A)** nMS spectrum of R2D on the EMR Orbitrap instrument (EM04294, CID/CE = 50/200 V, Rs = 8.75). **(B)** Chromatographic co-elution assay followed by SDS-PAGE analysis shows that DPCD does not interact with R1R2 in absence of DII domains.

# 4.2.2. nIMS-MS measurements

As for DPCD, we performed nIMS-MS measurements and compared them with data generated in solution for R2D. An experimental radius  $R_{IMS-MS} = 70$  Å is obtained, in adequacy with  $R_s = 73$  Å determined with SEC experiments (Table 3). Again, this indicates that the native global conformation of R2D is preserved in the gas phase.

nIMS-MS measurements yield a <sup>TW</sup>CCS<sub>N2</sub> value of 15 225  $\pm$  34 Å<sup>2</sup> for R2D (Table 3). An increase of 15% from hexameric FL R1R2 (13 081  $\pm$  10 Å<sup>2</sup>) to R2D is predicted for mass-based calculations, which is in good agreement with the experimental +16% obtained from nIMS-MS data (Table 3). This suggests that the binding of the three DPCD molecules does not significantly influence the overall conformation of R2D. These results will be compared to the R2D model once available.

Technique	Physical quantity	FL R1R2 Hexamer	R2D (1:3)	
IMS-MS	R <sub>IMS-MS</sub> (Å)	<b>65 ± 2</b> (z = 36+)	<b>70 ± 3</b> (z = 41+)	
SEC	R <sub>s</sub> (Å)	-	73	
IMS-MS	Measured MW (Da)	313 086 ± 59	383 611 ± 66	
	MW-based prediction of CCS* (Å <sup>2</sup> )	11 227	12 856	
	Measured CCS (Å <sup>2</sup> )	<b>13 081 ± 10</b> (z = 36+)	<b>15 225 ± 34</b> (z = 41+)	

**Table 3.** Comparison of IMS-MS, SEC and SAXS experiments for the R2D complex.  $*CCS = 2.435 \times MW^{2/3}$  from Ruotolo *et al.*, 2008 (ref. 263).

# 5. Monitoring R2TP-related complex assembly

The structure of R2TP has been documented in several studies<sup>204,325,326,332</sup>. RPAP3 contains two tetratricopeptide repeat (TPR) domains at the N-terminal half part of the protein that are able to recruit HSP chaperones (Figure 12A). The C-terminal domain plays a key role in binding of RPAP3 to the ATPase face of the hexameric ring. PIH1D1 possesses an N-terminal PIH domain (protein interacting with <u>H</u>SP), and a CS C-terminal domain which binds to RPAP3 (Figure 12A). R2SP has been recently identified as a R2TP-like complex<sup>325</sup>: SPAG1 contains a RPAP3-like C-terminal domain, and PIH1D2 comprises a PIH1D1-like PIH domain (Figure 12B).

Here, we studied three complexes, R2T'P', R2S'P' and R2S'P, for which flexible regions were removed to ease purification and obtain stable complexes (Figure 12C). The aim is to determine whether the stoichiometries and conformations of R2:TP and R2:SP are different.



**Figure 12.** Architecture of **(A)** human RPAP3 and PIH1D1 from the R2TP complex, and **(B)** human SPAG1 and PIH1D2 from the R2SP complex. **(C)** Constructs used to build complexes R2T'P', R2S'P', and R2S'P. Adapted from Maurizy *et al.*, 2018 (ref. 325).

# 5.1. nMS analyses of R2TP and R2SP complexes

All complexes were analyzed on the Synapt G2 as noncovalent interactions were not maintained on the EMR Orbitrap, even after careful optimization of instrumental parameters.

R2T'P' comprises the RPAP3<sub>396-665</sub>:PIH1D1<sub>199-290</sub> noncovalent heterodimer (42 975  $\pm$  1 Da). nMS experiments reveal the presence of three R2T'P' populations, with dodecameric R1R2  $\Delta$ DII bound to one (531 648  $\pm$  17 Da), two (575 176  $\pm$  10 Da) or three (618 642  $\pm$  24 Da) T'P' partners. The major stoichiometry is 1:1 (Figure 13A).

R2S'P' involves the SPAG1<sub>622-926\_Flag</sub>:PIH1D2<sub>213-315</sub> noncovalent heterodimer (46 761 ± 4 Da). The nMS spectrum exhibits a unique R2S'P' population, with one S'P' bound to dodecameric R1R2  $\Delta$ DII (537 585 ± 24 Da) (Figure 13B). A significant amount of free dodecamer is also detected.

These first results evidence distinct behaviors for R2T'P' versus R2S'P'. Indeed, T'P' and S'P' both connect to the R1R2  $\Delta$ DII dodecamer, but present different stoichiometries, with up to three bound T'P' partners, but only one S'P'.



**Figure 13.** nMS spectra obtained on the Synapt G2 for **(A)** R2T'P' (Vc = 200 V; O62965), **(B)** R2S'P' (Vc = 80 V, O62977), and **(C)** R2S'P (Vc = 200 V; O63499). Masses indicate the presence of 6 - 8 ADP molecules for R2T'P' and R2S'P', and 12 - 14 ADP for R2S'P.

R2S'P is composed of the SPAG1<sub>622-926</sub>:PIH1D2<sub>1-315</sub> noncovalent heterodimer (71 027  $\pm$  3 Da). nMS analysis shows the presence of two R2S'P species, where hexameric FL R1R2 binds one (384 692  $\pm$  13 Da) or two (456 039  $\pm$  22 Da) S'P proteins (Figure 13C). 1:1 is the main stoichiometry. A CSD corresponding to free hexameric FL R1R2 is also observed.

Collectively, these data indicate that when N-terminal domains of SPAG1/RPAP3 and PIH1D2/PIH1D1 are both truncated (R2S'P' and R2T'P'), the dodecameric assembly is preserved. Conversely, the binding of SPAG1- $\Delta$ N-ter and FL PIH1D2 breaks the dodecamer, as previously reported by Martino *et al.* for RPAP3- $\Delta$ N-ter and FL PIH1D1<sup>332</sup>. It was suggested that PIH1D2 is located in internal

DII regions<sup>345</sup> (like PIH1D1), and so the insertion of FL PIH1D2 (36 250  $\pm$  2 Da) between the two rings may explain why the dodecamer is disrupted.

# 5.2. nIMS-MS measurements for R2TP and R2SP complexes

Lastly, we performed nIMS-MS experiments on R2T'P' and R2S'P'. Data could not be acquired for R2S'P because of low IMS intensities.

Experimental <sup>TW</sup>CCS<sub>N2</sub> values of 19 733 ± 10 Å<sup>2</sup> and 20 049 ± 10 Å<sup>2</sup> were obtained for R2T'P' (major form 1:1) and R2S'P', respectively (Table 4). These data indicate that the addition of one T'P' (+5%) or S'P' (+7%) does not significantly influence the global conformation of the dodecamer R1R2  $\Delta$ DII. In both cases, increases of experimental <sup>TW</sup>CCS<sub>N2</sub> upon complexation are in agreement with mass-based predictions (+6%, Table 4).

As of today, no high-resolution structures have been proposed for R2T'P' and R2S'P' complexes. nIMS-MS data will be integrated to models established from other biophysical techniques once further structural information are available.

**Table 4.** Comparison of IMS-MS, SEC and SAXS experiments for R2T'P' and R2S'P' complexes.  $*CCS = 2.435 \times MW^{2/3}$  from Ruotolo *et al.*, 2008 (ref. 263).

Technique	Physical quantity	R1R2 ADII Dodecamer	R2T'P' (1:1)	R2S'P' (1:1)
IMS-MS	Measured MW (Da)	488 575 ± 8	531 648 ± 17	537 585 ± 24
	MW-based prediction of CCS* (Å <sup>2</sup> )	15 105	16 060	16 099
	Measured CCS (Å <sup>2</sup> )	18 725 ± 10	19 733 ± 10	20 049 ± 10
		(z = 48+)	(z = 48+)	(z = 48+)

# 6. Ongoing structural MS studies/Perspectives

In order to help gaining structural data, cross-linking MS experiments are ongoing. This will allow to refine SAXS-derived models. In addition, cryo-EM experiments are being performed to propose more accurate models for R2D and R2SP complexes. However, cryo-EM maps do not always provide clear structural information. For example, the precise location of PIH1D2 in R2SP complexes could not be precisely determined with first cryo-EM results. This underlines the importance of using complementary and orthogonal biophysical/MS techniques for structural biology programs.

# 7. Conclusions

In this chapter, the characterization of a series of large multiprotein complexes involving ATPases R1 and R2 was conducted using nMS and nIMS-MS experiments. First, nMS evidenced a mixture of hexameric and dodecameric R1R2 assemblies, with hexamers being the predominant FL form, while dodecamers appear to be the main population in the absence of DII domains. These results were confirmed by mass photometry experiments. Then, nMS highlighted different stoichiometries depending on the R1R2 client:
- Three DPCD are bound to the FL R1R2 hexamer. Binding of DPCD disrupts the dodecamer.
- Up to three truncated T'P' binds on dodecameric R1R2 ΔDII.
- When both SPAG1 and PIH1D2 are truncated, only one S'P' binds on the R1R2 ADII dodecamer.
- With FL PIH1D2, the dodecamer is broken, and two S'P are attached to the FL R1R2 hexamer.

Next, nIMS-MS experiments gave access to conformational information. Results were in good agreement with SEC-UV and SAXS data, confirming that proteins retain a memory of their structure in solution after transfer in the gas phase. This was particularly obvious for the DPCD protein, where only  $\sim$ 1% difference was obtained between the CCS calculated from the 3D model on IMoS and experimental averaged <sup>TW</sup>CCS<sub>N2</sub>. For the different complexes, addition of protein partners did not appear to affect the overall conformation of R1R2, which should be confirmed by additional cross-linking MS experiments.

Altogether, nMS and nIMS-MS efficiently complement biophysical techniques such as SAXS, SEC, and are of interest when atomic structures cannot be resolved. Structural MS techniques, including cross-linking MS, will help to validate models generated from SAXS envelopes, in order to establish a solid basis for structural characterization of new R1R2-related complexes.

#### Scientific communication

These data will be included in two articles currently in preparation.

# Part II – Conclusions

In this first part, we have seen through different applications that nMS and nIMS-MS approaches represent valuable techniques in the analytical toolbox of structural biology studies.

nMS and nIMS-MS have long been used to characterize a large variety of proteins and their complexes<sup>4</sup>. Until mid-2010s, the main drawback of nMS remained the time-consuming and laborious manual buffer exchange step. The hyphenation of SEC to nMS allowed to automate the process. The coupling offers numerous benefits over manual desalting. Two major advantages have been illustrated in the first chapter: SEC-nMS offers (i) a fast online buffer exchange with improved desalting efficiency, and (ii) separation of low and high molecular weight species for easier nMS spectra interpretation. While early applications of SEC-nMS were restricted mostly to mAbs and reference proteins<sup>240,241</sup>, I have shown that the coupling can be routinely employed for a wider range of biological complexes, to tackle proteins/DNA interactions involving NRs, or to preserve the integrity of large multiprotein assemblies (Rvb complexes)<sup>346</sup>. This could ultimately be extended to membrane proteins, with promising results obtained in our laboratory in detergent environments<sup>133</sup>. In addition to desalting/separation purposes, another point of interest that was not exploited in these examples is the possibility to achieve more accurate quantification of co-existing populations in a mixture by relying on the SEC-UV signal.

Hyphenation of other non-denaturing LC to nMS, including hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX), have emerged in the last few years. Advanced bidimensional couplings such as SECxSEC, HICxSEC, and SECxIEX have been developed, but cannot be used routinely because they are difficult to implement<sup>347-349</sup>. Recent publications thus tend to favor simpler unidimensional setups<sup>350-352</sup>. Although these new technological developments have been intended mainly for mAb analysis<sup>353</sup>, it could be useful for a broader range of proteins. For instance, IEX-nMS could separate DNA/NR species, allowing to assess the influence of different DNA sequences on NR dimerization in a single run.

In the second chapter, I have demonstrated the interest of nMS and nIMS-MS for the characterization of several high molecular weight complexes that involve human RuvBLs. Distinct behaviors were clearly pinpointed depending on the attached protein client, both in terms of binding stoichiometry and resulting oligomeric states for R1R2 rings. Comparisons of experimental nIMS-MS measurements with CCS calculated from high-resolution structures or SAXS-derived models confirm that conformations are retained in the gas phase. <sup>TW</sup>CCS<sub>N2</sub> measurements validated SAXS and SEC data, and *vice versa*. nIMS-MS is useful to probe the conformation of protein assemblies, and becomes even more powerful in combination with other biophysical techniques.

Overall, nMS can be considered as a bridge between interactomics using quantitative proteomics, and structural biology.

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Part II – nMS and nIMS-MS for Structural Biology Projects

# PART III – High-Resolution Cyclic IMS-MS for Conformational Characterization of Biotherapeutics

The third part of this thesis will focus on the use of high-resolution cIMS-MS for improved characterization of biotherapeutics. I will first present the cIMS-MS instrument and describe its multifunction capabilities. These advanced modes of acquisition will then be exploited to analyze different mAb formats either at peptide or protein levels.

# Chapter 1 – Description of the cIMS-MS Instrument

Chapter 2 – High-Resolution cIMS-MS to Assign Disulfide Bridges in Complementarity-Determining Regions of an IgG4

Chapter 3 – High-Resolution cIMS-MS for Improved Characterization of Biotherapeutics in Native Conditions

# **Chapter 1 – Description of the cIMS-MS Instrument**

# 1. Analytical context

As of today, the cIMS-MS platform remains the only commercially-available instrument able to perform dual mass- and mobility-selection. By analogy with tandem MS experiments, tandem IMS is of main interest to overcome limitations of single stage IMS<sup>89</sup>. During this thesis, capabilities of the cIMS platform (multipass/IMS<sup>n</sup>) were evaluated for different projects at peptide and protein levels, and will therefore be presented in more details hereafter.

# 2. cIMS-MS instrument

# 2.1. Description of the instrument

# 2.1.1. Before the cIMS region

The SELECT SERIES Cyclic IMS instrument (Waters, U.K.) has been constructed based on the Synapt G2-Si design (Figure 1). The StepWave  $^{TM}$  ion guide technology helps to maximize ion transmission after the source and remove neutral contaminants (Figure 1). Ions are accumulated in the trap cell ( $10^{-2}$  mbar N<sub>2</sub>) while the previous mobility separation phase occurs. Ions are then released as packets and go through a SRIG series ( $10^{-3}$  mbar N<sub>2</sub>), and a He cell ( $\sim$ 2 mbar) to refocus and thermalize ions just before entering the cIMS region.



Figure 1. Schematic overview of the SELECT SERIES Cyclic IMS from Waters.

#### 2.1.2. cIMS separator

The cIMS separator comprises two elements<sup>14</sup> (Figure 2A, B): (i) the main body of the cIMS separator that operates orthogonally to that of the ion beam (Figure 2A), and (ii) the ion entry/exit region (= array) located on the axis of the mass spectrometer (Figure 2B). These two regions provide L = 98 cm for IMS separation. This part of the instrument is isolated into a chamber filled with N<sub>2</sub> (~2 mbar)<sup>14</sup>.

- The main body comprises over 600 electrodes (Figure 2A, C). Opposite phases of RF voltages are applied to consecutive electrodes, providing a confinement in the z-direction (analogous to ring electrodes in linear TWIMS<sup>81</sup>). A DC voltage is applied to repeller electrodes in order to confine ions in the x-direction (Figure 2C). In order to create the T-wave, a DC potential is superimposed on the RF and switched sequentially to the adjacent plate (+y-direction) along the racetrack, which propels ions for IMS separation<sup>14,81</sup> (Figure 2C, D).

As the duration of separation phases increases with path length, storage times in the trap cell are extended (ion accumulation occurs during the on-going separation phase, providing duty cycle  $\sim 100\%$ )<sup>14</sup>. Consequently, more ions are present in packets released for IMS separation. The rectangular geometry of the ion transmission channel (surface = 2.5 cm<sup>2</sup> between inner and outer electrodes) provides a higher charge capacity compared to SRIGs from the linear TWIMS device (surface = 0.20 cm<sup>2</sup>), allowing to limit space charge effects, i.e. IMS peak broadening resulting from repulsions between ions<sup>354</sup> (Figure 2C).

- The array region has independent T-waves, and regulates ion entry, separation, and exit depending on applied potentials (see paragraph 2.2. for detailed potential energy diagrams). Array T-waves are controlled to achieve the desired function, oriented either in the x-direction for injection/ejection, or in the +y-direction, synched with T-waves of the main body for IMS separation (Figure 2D).

#### 2.1.3. Pre-array and post-array stores

Two series of SRIGs are present upstream (prestore) and downstream (poststore) of the cIMS separator (Figure 1). These regions, located in the same N<sub>2</sub> chamber as the cIMS separator, can act:

- as ion guides, transporting ions to and from the cIMS separator;

- as storage regions for selection of IMS-separated populations (IMS<sup>n</sup> slicing, described later).

#### 2.1.4. After the cIMS region

After the poststore, ions travel through a series of SRIGs ( $10^{-3}$  mbar N<sub>2</sub>), and a transfer ion guide ( $10^{-2}$  mbar N<sub>2</sub>), which can be used to fragment ions prior to the ToF detector (Figure 1).



**Figure 2. (A)** Photograph of the cIMS separator. Red arrows indicate the ion path. **(B)** Photograph of the array region (side view). Electrodes are 5 cm-long plates. Outer and inner electrodes are separated by 0.5 cm. One face of the array is presented in inset: array electrodes (seen in the middle) are segmented. **(C)** Structure of cIMS electrodes. **(D)** T-waves in the array can be oriented in the x-direction for ion injection/ejection (top) or in the +y-direction for IMS separation (bottom). Adapted from Giles *et al.*, 2019 (ref. 14).

# 2.2. cIMS operation

IMS experiments on the cIMS-MS instrument are carried out through a series of carefully tuned sequences containing different events, or functions, described by Giles *et al.*<sup>14</sup>. Events can be summarized as injection/separation/ejection, and are presented hereafter. Transitioning from one event to another relies on the control of voltages in the array region, whose DC bias and T-waves settings are independent from those of the main cIMS body.

# 2.2.1. Injection

The ion packet is transported from the trap to the array, where they are held until all ions have entered the array region. During the injection phase (5 - 15 ms), DC voltages in the cIMS region need to be optimized so that ions are maintained in the array (Figure 3):

- In order to prevent ions from starting IMS separation too early, the array offset is held below DC voltages from the prestore and cIMS electrodes, which creates a barrier of potential that effectively retains ions in the array region.

- In addition, to avoid the instantaneous exit of ions directly through the poststore, the array exit aperture is set above the array offset.



**Figure 3.** Schematic representation of potential energy in the cIMS region for injection. Voltages are tunable for each function (blue) or sequence (red). Blue arrows indicate the T-waves direction in the array. Energies modified for the injection event are circled in dotted lines. Adapted from Giles *et al.*, 2019 (ref. 14).

# 2.2.2. Separation

During the separation phase, potentials are raised so that array voltages are aligned with those of the cIMS racetrack, allowing to transfer ions for IMS separation/manipulation (Figure 4):

- The DC offset from the array is increased to match values from cIMS electrodes;

- Voltages from the array entrance/exit apertures are raised to match values of repeller electrodes to avoid exit of ions from the cIMS racetrack.

T-waves are oriented in the orthogonal +y-direction, to send ions sideways for IMS separation. The separation time can be tuned so that ions undergo either a single pass or multiple passes due to the geometry of the instrument. The duration of the separation phase determines the number of passes *n* and subsequent IMS resolution ( $R \sim \sqrt{n}$ ). Ion transmission has been shown to drop by 2.4% per pass for small peptides (GRGDS)<sup>14</sup>. Of course, losses in ion transmission are sample-dependent, and are expected to be significantly higher for larger proteins.



**Figure 4.** Schematic representation of potential energy in the cIMS region for separation. Voltages are tunable for each function (blue) or sequence (red). Blue arrows indicate the T-waves direction in the array. Energies modified for the separation event are circled in dotted lines. Adapted from Giles *et al.*, 2019 (ref. 14).

# 2.2.3. Ejection

# 2.2.3.1. Ejection to ToF

Once the separation phase has been completed, the array offset is decreased below cIMS electrode values, but maintained above that of the poststore so that ions can exit towards the ToF detector (Figure 5A). The exit aperture is lowered to ease the transport of ions outside of the array. T-waves (+y direction) of the cIMS racetrack continue to operate to ensure the delivery of all ions into the array, while T-waves in the array are oriented in the +x direction to propel ions forward. The final ejection event ends when all ions have been ejected from the racetrack.

# 2.2.3.2. Ejection to prestore

Due to the orthogonal arrangement of the cIMS region, ions can also be sent backwards to the prestore between consecutive separation stages. The array offset is thus reduced below the cIMS electrodes DC bias and above values from the prestore (Figure 5B). T-waves in the array are applied in the –x direction to deliver ions towards the prestore, and the entrance aperture is decreased to allow for efficient ion transport. The axial electric field in the prestore is set to zero. The "ejection to prestore" event is of particular interest for IMS<sup>n</sup> experiments presented thereafter.



**Figure 5.** Schematic representation of potential energy in the cIMS region for ejection events. Voltages are tunable for each function (blue) or sequence (red). Blue arrows indicate the T-waves direction in the array. Energies modified for ejection events are circled in dotted lines. Adapted from Giles *et al.*, 2019 (ref. 14).

#### 2.3. Multifunction capabilities

Events can be mixed and rearranged into complex sequences to access to advanced IMS experiments, taking advantage of the cyclic geometry of the IMS cell.

#### 2.3.1. IMS<sup>n</sup> slicing

During the IMS separation phase (steps 1–2, Figure 6A), the "eject-to-prestore" event can be applied to slice-out a specific population (step 4), while the remaining ions are ejected to the ToF detector without triggering data acquisition (steps 3 and 5). "Hold and eject" avoids ions leaking from the prestore, thus preventing losses of intensity (step 5). Species of interest are then reinjected into the cIMS racetrack for further separation by reapplying the axial field in the prestore (steps 6–8). Because populations are sent back to the prestore, the separation acquired in the IMS<sup>1</sup> stage is lost considering that ions are removed from the racetrack. This means that the number of passes is reset to zero at the start of the IMS<sup>2</sup> stage.

In theory, this can be done an infinite number of times, allowing to perform IMS<sup>n</sup> experiments. In practice, this will depend on ions losses occurring over multiple passes. Of note, it is possible to generate CIU/CID upon re-entry to the array. This point will be developed in part IV, chapter 4.

#### 2.3.2. Isolation

This sequence is used to reduce mobility windows, in order to narrow the range of mobilityseparated ions. These experiments consist of ejecting "unwanted" populations towards the ToF detector (steps 3 and 5, Figure 6B), while ions of interest are subjected to additional passes to achieve an enhanced resolution (steps 4 and 6). The total number of passes first includes the initial separation (*n* passes), with an additional pass needed to perform the isolation step. Indeed, as seen on Figure 6B, during step 4, isolated populations (black/red) necessarily start a new pass in order for low-mobility ions (blue) to be extracted. Once this pass has been completed, the next separation phase begins (*m* passes, Figure 6B). Overall, at the end of the sequence, ions have undertaken n + 1 + m passes.





This isolation approach is of main interest to avoid the "wrap-around" effect in the cIMS. Indeed, as the number of passes increases, ions span broader mobility ranges (i.e. the temporal separation between successive ATDs decreases, Figure 7), higher-mobility ions will eventually catch up with lower-mobility ions after multiple passes<sup>14,355,356</sup> (step 2 on Figure 6B, and Figure 7). This means that the fastest ions will be one pass ahead of the slowest ones.



**Figure 7.** Illustration of the wrap-around effect. After multiple passes, mobility ranges increase. In this example, after eight passes, high-mobility ions denoted with ♦ have caught up less mobile ions. As a result, species ♦ have started pass 10 while remaining ions are still in pass 9. Adapted from Giles *et al.* 2019 (ref. 14).

# 3. Objectives

Biotherapeutics are inherently complex proteins due to their structural heterogeneity, PTMs and flexibility. Hence, the characterization of these compounds requires advanced analytical tools. With its high resolving power and multifunction capabilities, the cIMS-MS instrument is ideally positioned to separate and characterize therapeutically-relevant molecules that were indistinguishable with early-generation TWIMS instruments.

The next chapters thus focus on the use of high-resolution cIMS-MS for improved analysis of a variety of mAb formats.

# Chapter 2 – High-Resolution cIMS-MS to Assign Disulfide Bridges in Complementarity-Determining Regions of an IgG4

## 1. Analytical context

As mAbs are highly disulfide-bonded proteins, it is crucial to ensure the correct assembly of disulfide connectivity and to verify the presence/absence of mispaired disulfide linkages<sup>357</sup>. Assessment of disulfide bridges remains challenging as (i) disulfide scrambling can occur during mAb production, sample preparation or in the mass spectrometer<sup>358-360</sup>, and (ii) possible disulfide-bridged isomers increase significantly with the number of Cys residues<sup>361</sup>. The combination of enzymatic digestion with LC-MS and/or LC-MS/MS using CID and/or ETD, is commonly used for disulfide bond analysis in proteins<sup>362</sup>. However, bottom-up approaches are traditionally time-consuming and labor-intensive in data processing and interpretation. In addition, fragmentation techniques lack the information of low abundant disulfide variants.

IMS has started to emerge as a rapid tool to characterize peptides containing isomeric disulfide linkages. De Pauw and co-workers conducted several studies on this particular subject, either using standalone IMS<sup>363,364</sup>, or by integrating it into CID/ETD workflows<sup>365</sup>. Although most isomers bearing two intramolecular disulfide bonds could be partially separated using linear TWIMS, structures with very close CCS result in ambiguous identification of disulfide networks because of poor IMS resolution, especially for highly constrained disulfide variants for which Coulombic repulsion is prevented<sup>363,364,366</sup>. Disulfide pairings assignment can be further completed by molecular modelling in order to predict structural constraints related to different disulfide connectivity, allowing to infer theoretical CCS values and subsequent arrival time order<sup>366</sup>. More recently, advances in high resolution IMS provided new opportunities to distinguish isomeric peptides. Performances of TIMS were highlighted for lasso peptides, whose C-terminal tail is trapped within an N-terminal macrocycle with strong disulfideconstraints (one or two disulfide bonds)<sup>367</sup>. Lasso peptides could be separated from their branchedcyclic analogs (R ~ 90 – 250  $\Omega/\Delta\Omega$ )<sup>368,369</sup>. The coupling of LC to TIMS-MS offered clear differentiation of disulfide-bridged peptide conformers based on their individual IMS profiles, even if analyzing a mixture of isomers still requires access to reference profiles of each variant, as each component may produce several features at once<sup>370</sup>.

In this context, high-resolution cIMS-MS appears to be promising for improved separation and identification of disulfide-bonded isomeric peptides in mAbs, which could be of main interest to ensure product quality along mAb manufacturing process.

#### 2. Objectives

This chapter focuses on the identification of disulfide bridges of a humanized IgG4 mAb, which contains two putative additional cysteine residues in the CDR3 of its light chain (positions 91 and 100,

Figure 8A). Peptide mapping highlighted that these Cys form two disulfide bonds contained within peptide T2-T7 (Figure 8A). Three possible disulfide isomers, which will be referred to as P1, P2 and P3, can be expected from the amino acid sequence of T2-T7 (Figure 8B). The aim of this project is to assign disulfide pairings using IMS-MS measurements. Experiments were carried out on two platforms, a classical linear TWIMS-MS device and a higher resolution cIMS-MS instrument, with the following objectives:

- Assess benefits of high-resolution IMS for improved separation of disulfide isomeric peptides;
- Evaluate the interest of multipass cIMS for better characterization of disulfide variants;
- Determine disulfide pairings based on IMS profiles.

This work was done in collaboration with Dr. Alain Beck's team (IRPF, Centre d'Immunologie Pierre Fabre, Saint-Julien-en-Genevois, France) and Dr. Dale Cooper-Shepherd (Waters, Wilmslow, U.K.).



**Figure 8. (A)** Light chain sequence from the humanized IgG4 mAb, with CDRs underlined. The CDR3 contains two additional Cys (91 and 100). The peptide T2-T7 is highlighted in blue/green. **(B)** Representation of the three possible disulfide-bonded isomeric variants of T2-T7.

## 3. Results

# 3.1. Benefits of multipass cIMS-MS to tackle the conformational landscape of disulfidebridged peptides

Instead of conventional peptide mapping approaches, we intended to use IMS-MS to characterize T2-T7. The T2-T7 fraction was manually collected, and first reinjected on a linear TWIMS-MS instrument ( $R \sim 40 \ \Omega/\Delta\Omega$ ). The 4+ charge state presents a single broad ATD centered on ~6.50 ms (FWHM = 0.77 ms), which could suggest the presence of multiple conformers (Figure 9A). In order to confirm this hypothesis and improve the separation of co-existing populations, we next moved to a higher resolution cIMS-MS platform. After one pass ( $R \sim 65 \ \Omega/\Delta\Omega$ ), T2-T7 reveals a highly heterogeneous ATD, with two main features (~24.56 and 25.92 ms) previously overlapped within a single peak using linear TWIMS (Figure 9B). Although advantages of cIMS over linear TWIMS for the resolution of conformational families are already obvious after one pass, populations may be better separated with more passes. After two passes ( $R \sim 92 \ \Omega/\Delta\Omega$ ), new features are indeed revealed, with two additional shoulders that can now be distinguished on the peak at ~37.06 ms (Figure 9C). These

results highlight the benefits of multipass cIMS for better conformational characterization. However, IMS patterns generated with cIMS-MS appear to be complex, and so we next aimed at performing Gaussian fitting to illustrate the conformational heterogeneity of samples.



**Figure 9.** IMS-MS experiments for the T2-T7 peptide. Results were obtained on **(A)** a linear TWIMS-MS instrument, and **(B)** on a cIMS-MS platform after one pass or two passes (\* = newly-detected peak shoulders).

#### 3.2. Determination of FWHM values expected for Gaussian fittings of single species

First, in order to calibrate the Gaussian fitting process for cIMS-MS data, IMS<sup>n</sup> slicing was carried out to determine FWHMs expected for single conformer at different passes. Selection and reinjection of a specific feature allows to achieve a better definition of ATDs, considering that fewer species are present in the cIMS cell upon reinjection from the prestore. This provides more accurate FWHMs measurements of the main feature contained within the slice. These experiments were conducted on the most homogeneous sample (synthetic peptide P2, described more in detail later).

The main population at ~43 ms after two passes presents a sharp profile, with high intensities, making it the first candidate for IMS<sup>n</sup> (Figure 10A). After one pass, the major species exhibits a FWHM of 0.87 ms. In multipass mode, the expected FWHM of a peak containing a single conformer should scale with the square root of the number of passes (Equation 1):

FWHM at *n* passes = FWHM at 1 pass 
$$\times \sqrt{n}$$
 (1)

Here, discrepancies between theoretical (1.23 ms) and experimental (1.34 ms) values were observed after two passes, which suggest that different conformers are comprised within the selected slice. The shape of the resulting ATD is asymmetric, further strengthening this conclusion.

Another feature, at  $\sim$ 30 ms after one pass, was then selected (Figure 10B). The main population has a FWHM of 0.65 ms at one pass. The FWHM measured at two passes (0.94 ms) is in good agreement with the theoretical value (0.92 ms), meaning that the selected feature corresponds to a single

conformer. These FWHMs were thus used as references to fit single species: FWHM<sub>1 pass</sub> = 0.65 ms, FWHM<sub>2 passes</sub> = 0.92 ms, FWHM<sub>4 passes</sub> = 1.45 ms. Peak width tolerance was set to  $\pm$  0.05 ms.



**Figure 10.** IMS<sup>2</sup> experiments to determine FWHM values expected for single species. **(A)** Selection of the main peak from P2. Upon reinjection of the selected population, the FWHM of the main feature is 0.87 ms after one pass. In theory, if this slice contains only one conformer, its FWHM at *n* passes =  $0.87 \times \sqrt{n}$ . For two passes, the measured FWHM (1.34 ms) is slightly higher than what is expected, meaning that several conformers are present. **(B)** Selection of a second feature from P2. Experimental and theoretical FWHM values after two passes are in good agreement, and were subsequently used as references for Gaussian fitting of single conformers.

# 3.3. Separation/identification of disulfide isomeric variants using multifunction cIMS-MS

#### 3.3.1. Multipass cIMS for unambiguous assignment of T2-T7 disulfide bonds

The IMS profile of T2-T7 was then compared to those obtained from synthetic peptides variants (P1, P2 and P3) corresponding to the possible combinations of disulfide bonds (Figure 8A). IMS-MS experiments were first carried out on a linear TWIMS-MS instrument (Figure 11A). For the 4+ charge state, IMS profiles for both P1 and P3 are close to T2-T7, with a single broad feature centered on ~6.50 and 6.28 ms, respectively. Conversely, P2 exhibits a bimodal distribution. Gaussian peak-fitting was used to roughly estimate conformers present within the broad ATD of each peptide (Figure 11A). For T2-T7, P1, and P3, two main populations were detected, along with a minor one. These results suggest that T2-T7 is similar to P1, although P3 cannot be definitively ruled out (different peak shapes, but very close arrival times and same number of conformers).

As the low resolving power of the Synapt G2 (~  $40 \Omega/\Delta\Omega$ ) does not allow for separation of co-existing conformers, we moved to a higher resolution cIMS-MS instrument (Figure 11B). Gaussian fitting was used as a means to assess conformational heterogeneity from complex IMS profiles. Nonetheless, the deconvolution of ATDs strongly depends on fitting parameters. Consequently, the number of detected features rather serves as an indication of sample heterogeneity, in order to (i) illustrate how high-resolution can enhance the separation of multiple conformers, and (ii) decipher differences that arise between the various isomeric peptides. T2-T7 unveils new features after one pass, with five

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conformers that were previously indistinguishable on the linear TWIMS-MS instrument (see supplementary data of publication 2). After two passes, new contributions to the main peaks could be uncovered, among which six major species with relative intensities > 45%, and several minor features of lower relative intensities (< 20%). For all synthetic peptides, IMS profiles display additional features, reflecting broad conformational landscapes (Figure 11B). T2-T7 and P1 exhibit highly similar profiles in terms of arrival times and relative intensities for all fitted conformers. Both peptides comprise two main populations (> 65%) centered on ~37.31 and 39.66 ms. On the contrary, P2 and P3 present only one major feature in their ATD profiles, and several minor conformers. Fewer species contribute to the main peak of P2 and P3 (two and four conformers, respectively) compared to T2-T7 and P1, which matches well with preliminary FWHMs measured on the linear TWIMS-MS device (Figure 11A). These results suggest that disulfide connectivities of T2-T7/P1 confer a higher degree of freedom for conformational changes than those of P2 and P3.



**Figure 11.** IMS-MS experiments performed on two different IMS-MS platforms. ATDs were obtained for the 4+ charge state of T2-T7 (black), and synthetic peptides corresponding to disulfide variants P1 (blue), P2 (green) and P3 (red). **(A)** Extracted ATDs (left) on a linear TWIMS-MS instrument (Synapt G2). Gaussian fitting (right) highlights the presence of multiple conformers. Grey dots correspond to experimental data. Thin black lines represent combined fits. **(B)** Results obtained on a cIMS-MS device after two passes with corresponding Gaussian fits (right).

## 3.3.2. Isolation of T2-T7/P1 features

As a final proof for the assignment of disulfide connectivity, isolation experiments were carried out on the two main peaks of T2-T7 and P1, while remaining ions were ejected to the ToF. Populations were isolated after one pass and subjected to four additional passes (Figure 12A). After five passes ( $R \sim 145 \Omega/\Delta\Omega$ ), both peptides yield strictly identical isolated-profiles, which definitely confirms the identification of disulfide bridges proposed after two passes. The sequential isolation of windows I and II revealed that selection I consists of four main ion populations, with relative intensities > 50%, whereas selection II comprises two dominant features (Figure 12B). For both isolated windows, conformers detected through Gaussian fitting are coherent with those obtained at two passes (same color code in Figures 11B and 12B), which indicates that no activation towards more compact/extended species occurs along the additional passes. Of note, no major interconversion between the two main conformers is observed.



**Figure 12.** Isolation experiments. **(A)** Populations of interest (windows I and II) of T2-T7 and P1 were isolated after one pass and sent for four additional passes. Resulting ATDs for each mobility window were extracted after five passes. **(B)** Gaussian fitting of T2-T7 isolated features at five passes. When already identified after two passes (Figure 11B), the same color code as in Figure 12B is used. One additional conformer is detected after five passes, represented with a dashed line (dark grey).

In conclusion, disulfide pairings of T2-T7, corresponding to Cys23-Cys88 and Cys91-Cys100 (in agreement with peptide mapping, see supplementary data of publication 2), could be established unequivocally based on the sole use of IMS profiles, with further insight into the conformational heterogeneity of the peptide using multipass cIMS-MS.

## 4. Conclusions

In this chapter, benefits of high-resolution cIMS-MS to separate isomeric disulfide-containing peptides have been highlighted. cIMS offers enriched conformational information compared to linear TWIMS, with (i) straightforward identification of disulfide variants and (ii) resolution of new species, including low abundant ones, especially after multiple cIMS passes. Disulfide linkages could be

successfully assigned by relying solely on IMS profiles (peak shapes and arrival times), without needing further CCS calculations. As the main bottleneck of peptide mapping remains the tedious and timeconsuming data processing, cIMS-MS has the potential to become a qualitative tool that could complement bottom-up approaches, allowing to minimize laborious manual data treatment.

#### **Scientific Communication**

This project has been published, and presented through poster communication.

#### Peer-reviewed article

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#### Poster communication

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# Publication 2

High-Resolution IMS-MS to Assign Additional Disulfide Bridge Pairing in Complementarity-Determining Regions of an IgG4 Monoclonal Antibody

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Research Article

# High-Resolution IMS-MS to Assign Additional Disulfide Bridge Pairing in Complementarity-Determining Regions of an IgG4 Monoclonal Antibody

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**ABSTRACT:** Monoclonal antibodies (mAbs) have taken on an increasing importance for the treatment of various diseases, including cancers and immunological disorders. Disulfide bonds play a pivotal role in therapeutic antibody structure and activity relationships. Disulfide connectivity and cysteine-related variants are considered as critical quality attributes that must be monitored during mAb manufacturing and storage, as non-native disulfide bridges and aggregates might be responsible for loss of biological function and immunogenicity. The presence of cysteine residues in the complementarity-determining regions (CDRs) is rare in human antibodies but may be critical for the antigen-binding or deleterious for therapeutic antibody development. Consequently, in-depth characterization of their



disulfide network is a prerequisite for mAb developability assessment. Mass spectrometry (MS) techniques represent powerful tools for accurate identification of disulfide connectivity. We report here on the MS-based characterization of an IgG4 comprising two additional cysteine residues in the CDR of its light chain. Classical bottom-up approaches after trypsin digestion first allowed identification of a dipeptide containing two disulfide bridges. To further investigate the conformational heterogeneity of the disulfide-bridged dipeptide, we performed ion mobility spectrometry–mass spectrometry (IMS–MS) experiments. Our results highlight benefits of high resolution IMS–MS to tackle the conformational landscape of disulfide peptides generated after trypsin digestion of a humanized IgG4 mAb under development. By comparing arrival time distributions of the mAb-collected and synthetic peptides, cyclic IMS afforded unambiguous assessment of disulfide bonds. In addition to classical peptide mapping, qualitative high-resolution IMS–MS can be of great interest to identify disulfide bonds within therapeutic mAbs.

## ■ INTRODUCTION

Monoclonal antibodies (mAbs) are 150 kDa tetrameric glycoproteins that have expanded the treatment of various diseases (cancer, arthritis, asthma, and diabetes).<sup>1</sup> Posttranslational modifications (PTMs) such as deamidations, disulfide bridges, or glycosylation play crucial roles in mAb structure and activity. Among them, inter- and intrachain disulfide connectivity ensures proper mAb folding and stabilizes their native high-order conformations and consequently their biological function.<sup>2-4</sup> Disulfide bonds can also lead to structural isoforms.<sup>5</sup> mAbs are highly disulfide-bridged molecules constituted of two light (LC) and two heavy chains (HC), linked by 16 disulfide bonds for IgG1 and IgG4, 18 for IgG2, and 25 for IgG3. The LC is connected to the HC by one disulfide bond. HCs are linked by two (for IgG1 and IgG4), four (for IgG2), and 11 (for IgG3) disulfide bridges located in a short hinge region. Each IgG contains 12 intrachain bonds located in six different domains: one variable (VL) and one constant (CL) for the LCs and one variable (VH) and three

constant (CH1, CH2, CH3) for the HCs. Each variable domain mediates antigen recognition via three hypervariable loops called complementarity-determining regions (CDR), which interact with antigens and govern the specificity and potency of the mAb. Conversely to sharks and camels (scFv, VHH), the presence of cysteine residues in the CDRs is rare in human antibodies.<sup>6,7</sup> However, some human antibodies have been reported to possess either one or two cysteine residues in the CDRs.<sup>7–9</sup> To consider such antibodies with two Cys in the CDRs as potential drug candidate, full disulfide bridge pairing assessment is necessary.

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As mAbs are highly disulfide-bonded therapeutics, it is crucial to ensure the correct assembly of disulfide connectivity and to evaluate the formation of mispaired disulfide linkages or chemical modifications to native disulfide bonds in the protein.<sup>10</sup> For this, in-depth characterization of mAb-based products is required to ensure their safety and efficacy. Indeed, disulfide bridges represent important critical quality attributes (CQAs) that need to be closely monitored along the biotherapeutics' development to guarantee product quality, as scrambling or reduction of disulfide bonds can occur during manufacturing<sup>11,12</sup> and storage.<sup>13,14</sup> In particular, the presence of free cysteines, that is, in the reduced forms, may induce protein aggregation or non-native disulfide pairings,<sup>15-</sup> resulting in a potential loss of potency or immunogenicity of the biotherapeutic. These cysteine residues can be of concern when located in the CDRs of the antibody<sup>18</sup> since their solvent exposure makes them more inclined to undergo PTMs.<sup>6</sup> As an example, single unpaired cysteine in the CDR3 of the LC has been reported as cysteinylated or modified by oxidation,<sup>19</sup> leading to complete inactivation of the antibody when both LCs were cysteinylated. As a result, IgGs with additional noncanonical cysteine are considered at risk during early developability assessment.

Assigning disulfide bridges is challenging as possible disulfide-bonded isomers increase significantly with the number of Cys residues. Hence, there is an increasing demand for efficient analytical methods for accurate characterization of disulfide bonds along the development process in order to (i) confirm the correct disulfide connectivity and (ii) verify the presence/absence of disulfide bond variants (non-native disulfide bonds). In this context, mass spectrometry (MS)based techniques, and particularly liquid chromatography coupled to mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) methods, have self-settled as most attractive to tackle disulfide bond assessment.<sup>20</sup> However, MS characterization of disulfide bridges is not trivial and great care should be exercised to prevent disulfide bond scrambling during sample preparation and/or fragmentation in the mass spectrometer.<sup>20</sup> Bottom-up approaches based on enzymatic digestion followed by collision induced dissociation (CID) lead to the cleavage of the peptide backbone amide bonds but maintain the disulfide bond intact, hence producing diagnostic b and y ions comprising the disulfide bond, as well as ions that do not contain the disulfide bond. Electron transfer dissociation (ETD), which generates c and z ions, is also widely used for PTMs localization. However, ETD is not well adapted for disulfide connectivity assessment as cleavage of the disulfide bond is preferred (most intense ions in the MS/MS spectrum) over backbone fragmentation (minor c/z peaks that may contain or not the intact disulfide bond). Combination of both fragmentation techniques using MS<sup>3</sup> look appealing and more successful, but are time-consuming.<sup>21,22</sup> Both CID and ETD methods lead to poorly fragmented precursors for peptides with at least two intramolecular disulfide bonds, requiring further time-consuming manual MS/MS assignment.

However, all fragmentation techniques lack the information on low abundant disulfide variants/positional isomers. Ion mobility spectrometry coupled to mass spectrometry (IMS– MS), which separates ions based on their charge, shape and size in the gas phase, has been described in few papers as a promising approach to tackle disulfide-variant heterogeneity. IMS has proved valuable for the characterization of therapeutic mAbs, allowing to differentiate several isoforms of intact

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antibodies<sup>23</sup> and to monitor batch-to-batch heterogeneity of disulfide pairings in antigens.<sup>24</sup> IMS has also been employed to determine topologies of disulfide-constrained isomeric peptides.<sup>25</sup> The group of De Pauw conducted several studies to investigate peptides bearing disulfide bridges, either by integrating IMS into CID and ETD workflows,<sup>26,27</sup> or by using standalone IMS–MS.<sup>28,29</sup> They first highlighted the structuring effect of disulfide bonds by comparing collision cross section (CCS) values of peptides bearing one to three intramolecular disulfide bridges with reduced reference peptides.<sup>28</sup> They demonstrated that classical traveling wave ion mobility spectrometry (TWIMS) can partially separate isomers bearing two intramolecular disulfide bonds. Molecular modeling can help to predict structural constraints related to different disulfide variants, which allows to infer theoretical CCS values and estimate the arrival time order.<sup>29</sup> However, structures with very close CCS, including highly constrained disulfide isomers for which Coulombic repulsion is prevented, lead to ambiguous disulfide pairing assignments due to insufficient resolution, the latter being limited by the length of linear TWIMS cells.<sup>28,29</sup> Advancements in high resolution IMS offer new opportunities to tackle isomeric peptides, as exemplified by the clear separation of lasso peptides and their branched-cyclic analogs obtained on a trapped IMS (TIMS) instrument with a resolving power R  $\sim$  90–250  $\Omega/\Delta\Omega.^{30,31}$ Another study using high-resolution TIMS showed that the differentiation of disulfide-bridged peptide isomers was possible based on their individual IMS profiles, however determining the isomer composition of a mixture remains challenging without reference to a profile library, as each isomer may produce several features at once.<sup>32</sup> Recently, Giles et al.<sup>33</sup> introduced a TWIMS-based high-resolution multipass cyclic ion mobility spectrometry (cIMS) instrument with increased path length allowing selectable resolving power. With the cIMS, R ~ 750  $\Omega/\Delta\Omega$  has been obtained after 100 passes for two isomeric pentapeptides. This type of cIMS device was shown to maintain native gas-phase structures of common proteins, including cytochrome c and concanavalin A.<sup>34</sup> Sisley et al.35 illustrated the benefits of high-resolution cIMS to increase the number of detected proteins from mouse and rat tissues via liquid extraction surface analysis. Multipass highresolution cIMS appears as an attractive technique to circumvent limitations of classical TWIMS cells for the elucidation of disulfide networks in peptides and proteins.

In this context, we report here on the disulfide identification of an IgG4 antibody containing two additional cysteine residues in the CDR of the LC at positions 91 and 100 (Figure S1). Peptide mapping first identified that the two additional Cys form two disulfide bridges. In order to assign correct disulfide bridge pairing, we performed IMS–MS on two platforms, a classical linear TWIMS platform and a higher resolution cyclic TWIMS instrument. We demonstrate the benefits of high-resolution cIMS–MS for the identification of two supplementary disulfide bonds in a humanized IgG4 mAb that contains two putative additional cysteines in the CDR3 of its LC. Finally, we highlight the advantages of multipass cIMS–MS for a better separation of conformers, with newly resolved species.

#### MATERIALS AND METHODS

Antibody and peptides production and purification. The recombinant antibody was expressed in transiently transfected HEK293 cells at the Centre d'Immunologie Pierre



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Figure 1. Bottom-up LC-MS experiments. LC-MS of the mAb tryptic digest was first carried out under nonreducing conditions, illustrated by (A) a UV chromatogram with an identified T2-T7 peak and (B) a MS spectrum zoomed on the 4+ charge state of T2-T7, indicating the presence of two disulfide bonds. (C) Representation of the three possible disulfide-bridged positional isomers of the T2-T7 peptide.

Fabre (Saint-Julien-en-Genevois, France) and purified using standard manufacturing procedures, including a protein A affinity chromatography step lead selection.<sup>24</sup> The synthetic peptides were produced and purified by standard manufacturing procedure.3

**Trypsin digestion.** 100  $\mu$ g of mAb were solubilized in 120  $\mu$ L Tris Base 50 mM, CaCl<sub>2</sub> 1 mM, 0.1% RapiGest (Waters, Wilmslow, UK), pH 7.1. Sample was incubated for 15 min at 80 °C under agitation (750 rpm). Fifteen  $\mu$ L of acetonitrile (ACN) were added, before digestion with 5  $\mu$ L of 1  $\mu$ g/ $\mu$ L trypsin solution (i.e., enzyme/substrate ratio 1:20 (w:w)) for 3h30 at 37 °C under agitation (750 rpm). The sample was split in two aliquots of equal volume. One aliquot was reduced by adding 2.5  $\mu L$  DTT 500 mM. The reduction was performed at 56 °C for 45 min under agitation (750 rpm). The reaction was stopped in both samples by adding 1  $\mu$ L of trifluoroacetic acid (TFA). RapiGest was eliminated by heating at 37 °C for 30 min and by centrifugation at 10 000 g for 5 min.

LC-MS/MS. Samples were analyzed on an ACQUITY UPLC H-Class system (Waters, Wilmslow, UK) coupled to a Synapt G2-Si quadrupole/TWIMS/time-of-flight (Q-TWIMS-ToF) mass spectrometer (Waters). The system was fully controlled by MassLynx v4.1. 50 pmol of sample preparation were injected on an ACQUITY UPLC BEH C18 column, 130 Å, 1.7  $\mu$ m, 2.1  $\times$  300 mm (Waters) set at 80 °C. The gradient was generated at a flow rate of 250  $\mu$ L/min using 0.1% TFA for mobile phase A and ACN containing 0.08% TFA for mobile phase B. Mobile phase B was raised from 5 to 43% in 38 min and to 80% in 2 min. For synthetic peptides, mobile phase B was raised from 25 to 40% in 15 min. Eluted samples from the column were detected by UV at 214 and 254 nm, and by MS. The Synapt G2-Si was operated in positive ionization mode with a capillary voltage of 3 kV and a sample cone

voltage of 40 V. For tandem MS experiments performed in data-dependent acquisition mode, the system was operated with automatic switching between MS (1 s/scan on m/z range [300;1800]) and MS/MS modes (1 s/scan on m/z range [50;2000]). The two most abundant peptides were selected on each MS spectrum for further isolation, and CID fragmentation using a collision energy ramp with the following settings: low mass (200 m/z) collision energy ramp from 6 to 20 eV, high mass (2000 m/z) collision energy ramp from 45 to 100 eV. Fragmentation was performed using argon as the collision gas. Glu-FibrinoPeptide was used for the ToF calibration and as lock-mass correction.

The T2-T7 peptide was manually collected in a time window of 30 s, corresponding to a fraction of ~125  $\mu$ L. CID experiments on the collected and synthetic peptides were carried out by direct infusion on the same mass spectrometer at a collision energy of 38 eV. The fragmentation spectra were deconvoluted with MaxEnt3 algorithm (Waters) and manually interpreted.

IMS-MS experiments on Synapt G2 HDMS. Samples were diluted to 10  $\mu$ M with a 50/49/1 (v/v/v) water/ACN/ formic acid solution before IMS-MS experiments. IMS-MS measurements were performed using a Synapt G2 Q-TWIMS-ToF instrument (Waters, Wilmslow, UK) coupled to an automated chip-based nanoESI device (TriVersa NanoMate, Advion, Ithaca, USA). The Synapt G2 was operated in positive ionization mode. The cone voltage of the source interface was fixed to 40 V. The TWIMS wave height and velocity were adjusted to 40 V and 1200 m/s, respectively. The cooling (He) and IMS separation  $\left(N_2\right)$  gas flow rates were set to 150 and 50 mL/min, respectively, giving a pressure of 3.3 mbar in the linear TWIMS cell. The IMS resolving power of this instrument is ~40  $\Omega/\Delta\Omega$ .



Figure 2. IMS–MS experiments performed on two different IMS instruments. ATDs were obtained for the 4+ charge state of the collected T2-T7 peptide (black), and the three synthetic peptides corresponding to disulfide-bridged variants P1 (blue), P2 (green), and P3 (red). (A) Results obtained on a linear TWIMS instrument. Extracted ATDs (left) were further investigated using Gaussian fitting (right), highlighting the existence of multiple conformers. Gray dots correspond to experimental data, while thin black lines represent combined fits. (B) Extracted ATDs (left) obtained on a cIMS platform after two passes, and their corresponding Gaussian fits (right).

IMS-MS experiments on cIMS. Samples were analyzed under the same solution conditions as the SYNAPT G2. Cyclic IMS-MS measurements were performed on a SELECT SERIES Cyclic IMS instrument<sup>33</sup> (Waters, Wilmslow, UK) which has a Q-cyclicTWIMS-ToF geometry. Peptide solutions were electrosprayed in positive ionization mode from PicoTipTM GlassTipTM borosilicate glass nanoflow capillaries (NewObjective, Woburn, MA, USA). The cone voltage was set at 40 V. The TWIMS wave height was set at 22 V with a velocity of 375 m/s. The helium cell and IMS separation gas flow rates were 150 and 45 mL/min, respectively, giving rise to a cIMS cell pressure of 1.7 mbar. Data were acquired with either one or two passes of the cyclic device. For higher mobility resolving power, ions corresponding to the front or rear portions of the arrival time distributions after one pass were selectively retained in the cyclic device for an additional four passes (for a total of five passes) by tuning the timings and voltages of the multifunctional ion entry/exit array.<sup>33</sup> All data were acquired on the quadrupole-isolated 4+ charge states of the fractionated and synthetic peptides  $(m/z \ 1298)$ .

**IMS–MS data treatment and Gaussian fitting.** IMS– MS data were analyzed using MassLynx v4.1. Arrival time distributions (ATDs) were extracted in the m/z range [1297;1301]. Gaussians were fitted to the extracted ATDs using the "Gaussian Fitting" module of the CIUSuite 2

software.<sup>37</sup> Gaussian fitting was performed in the protein only mode, with all signals considered to be related to the analyte. The following parameters were used for all ATDs: minimum peak amplitude = 0.05; maximum protein components fitted = 10; peak overlap penalty mode = relaxed, which penalizes overlaps >  $\sim$  85%, but allows overlapping peaks to be chosen if other solutions are poorly fitted. In order to "calibrate" the Gaussian fitting process, the expected fwhm of fits was determined using slicing experiments performed on a synthetic peptide<sup>33</sup> (Figure S2A). Briefly, one population was selected and ejected to the prestore while other populations were ejected to the ToF. This allows to have a better definition of the selected slice upon reinjection into the cIMS cell, providing an accurate fwhm measurement of the main feature contained within the slice (Figure S2A, B). After one pass, the major feature of our isolated slice exhibits a fwhm of 0.65 ms. In multipass mode, the expected fwhm of a peak containing a single conformer should scale with the square root of the number of passes.<sup>33</sup> At two passes, the measured fwhm (0.94 ms) matches well with the theoretical value (0.92 ms), meaning that the selected feature does correspond to a unique conformer. These fwhm values were subsequently used as references to fit single species: fwhm(1 pass) = 0.65 ms, fwhm(2 passes) = 0.92 ms, fwhm(4 passes) = 1.45 ms. Peak



Figure 3. cIMS isolation experiments. (A) After one pass, the main features of T2-T7 (gray boxes) and P1 (blue boxes) were sequentially isolated in the cIMS device and subjected to four additional passes. ATDs of isolated features were extracted after a total of five passes. (B) Gaussian fitting of the two T2-T7 selected windows after five passes. Gray dots correspond to experimental data, while thin black lines represent combined fits. When already identified after two passes (Figure 2B), the same color code as in Figure 2B is used. One additional conformer is detected after five passes, represented with a dashed line (dark gray).

width tolerance was set to  $\pm 0.05$  ms. Combined fits were in good agreement with experimental data ( $r^2 > 0.99$ ).

#### RESULTS

Peptide mapping and identification of disulfidecontaining peptides. Classical MS strategies rely on indirect proof of disulfide bridge existence by comparing reduced and nonreduced samples. We thus first performed peptide mapping after trypsin digestion, one of the common techniques used for disulfide bridge pairing assessment (Figure 1). Differential analysis of peptide maps obtained under reducing and nonreducing conditions allowed identification of a dipeptide of the CDR3 (Figure S1) of the LC (peptide T2-T7, Figure 1A) bearing two additional disulfide bonds (experimental monoisotopic mass 5187.41 Da) in agreement with the presence of four cysteine residues (Cys91/100/23/88). Careful investigation of the isotopic distribution of the 3+ and 4+ charge states of T2-T7 peptide allowed to rule out the presence of species with only one disulfide bridge, whose isotopic distribution would overlap with the dipeptide containing 2 S-S bridges (Figure 1B and S3). Three possible isomeric disulfide variants can be expected from the amino acid sequence of T2-T7 (Figure 1C).

IMS-MS analysis to establish T2-T7 disulfide pairings conformational heterogeneity. Instead of conventional

peptide mapping approaches, we intended to use IMS–MS, which has already proved to be a valuable and efficient tool to differentiate disulfide-bridged protein and peptide isomers, <sup>23,24,28,29,32</sup> to identify T2-T7 disulfide bridge pairing. To this aim, the T2-T7 fraction was manually collected, and its ATD compared to those obtained from synthetic peptide isomers (P1, P2 and P3) corresponding to the possible combinations of disulfide bonds (Figure 1C), on two IMS–MS platforms with different IMS resolutions.

IMS-MS experiments were first performed on the Synapt linear TWIMS instrument (Figure 2A). The 4+ charge state of T2-T7 exhibits a broad IMS ATD centered on ~6.50 ms. While a bimodal distribution is observed for P2, both P1 and P3 present primarily broad ATD features, centered on ~6.50 and 6.28 ms, respectively. As broad ATDs might suggest coexistence of multiple conformers, we then used Gaussian fitting (see Materials and Methods) as indication of multiple populations present for each peptide. For T2-T7, P1, and P3, Gaussian detection allows the identification of two main populations along with a minor one of lower mobility (relative intensity <10%). Overall, based on arrival time and ATD profile, these IMS-MS results might suggest that T2-T7 is similar to P1, even if P3 cannot be definitively ruled out (different peak shapes but very close ATDs and same number of conformers). Of note, P1 and P3 were also found to coelute in reversed-phase liquid chromatography (rpLC), avoiding the

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use of rpLC in front of IMS-MS to separate isomeric peptides (Figure S4).

As the different conformers were not resolved at the linear TWIMS resolving power of around 40  $\Omega/\Delta\Omega$ , we next performed IMS experiments on the quadrupole-isolated 4+ charge state of each peptide on the higher resolution cyclic IMS instrument (Figure 2B). As cIMS generates complex ATD profiles, we used Gaussian fitting as an indicative tool for conformer heterogeneity assessment from IMS patterns. As the deconvolution of ATDs strongly depends on fitting parameters, the number of detected features was used as an indication of the conformer heterogeneity to illustrate how highresolution can improve the separation of conformers, and help to understand differences that appear between the various isomeric peptides. After one cIMS pass (R ~ 65  $\Omega/\Delta\Omega$ ), T2-T7 reveals a highly heterogeneous ATD, with at least five conformers (fwhm = 0.65 ms) previously overlapped within a broad ATD on the linear TWIMS platform (Figure S5). Although the benefits of cIMS-MS over linear TWIMS to detect multiple conformers are already clear after one pass, populations could potentially be better resolved with more passes. Indeed, after two cIMS passes (R ~ 92  $\Omega/\Delta\Omega$ ), new features are uncovered, highlighting the advantages of multipass cIMS-MS for improved conformational characterization (Figure 2B). Additional contributions to the main peaks could be detected based on our fitting parameters (fwhm = 0.92 ms), with six major species with relative intensities estimated >45%. Several minor conformers with lower relative intensities (<20%) were also fitted.

After two passes, ATDs of all synthetic peptides show additional features, unveiling broad conformational spaces (Figure 2B). T2-T7 and P1 present highly similar profiles with equivalent arrival times and relative intensities for all detected conformers. Overall, both peptides comprise two main contributions (>65%) centered on ~37.31 and 39.66 ms. Conversely, P2 and P3 exhibit only one major peak in their ATD profiles, and several minor populations. Fewer features are contained within the main peak of P2 and P3 (two and four conformers, respectively) compared to T2-T7/P1, in agreement with preliminary fwhm's obtained using linear TWIMS (Figure 2A), suggesting that T2-T7 possesses a higher degree of freedom for conformational changes.

As species of interest can be isolated based on their mobility in the cIMS device while the rest of the ions are ejected,<sup>33</sup> the two main peaks of T2-T7 peptides and P1 were further investigated. Ions in each mobility window, depicted I and II in Figure 3, were selectively isolated after one pass and subjected to four more passes, leading to a total of five passes ( $R \sim 145$  $\Omega/\Delta\Omega$ ). Strictly identical isolated-profiles were obtained after five passes for the synthetic peptide P1, which strongly corroborates the assignment of disulfide bonds established after two passes (Figure 3A). The sequential isolation of windows I and II revealed that selection I comprises four main ion populations >50%, whereas selection II consists of two dominant features (Figure 3B). For both isolated windows, the detected conformers (fwhm = 1.45 ms) appear to be coherent with those obtained at two passes (same color code in Figure 2B and 3B), indicating that no activation toward more extended/compact species occurs within the time frame of the four additional passes. No interconversion between the two main conformers is observed.

Altogether, based on the sole use of IMS-MS data, these results allowed to unambiguously conclude that peptides T2-

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T7 and P1 bear similar cysteine connectivities, with disulfide bonds corresponding to Cys23-Cys88 and Cys91-Cys100 in agreement with classical peptide mapping results (Figure S6). In addition, our results highlight the benefit of high resolution IMS instrumentation for more accurate qualitative identification of disulfide peptides compared to linear TWIMS, relying on peak shapes and arrival times. cIMS offers more definition of the ATDs with several newly resolved species apparent (including low abundant ones) compared to the lower resolution linear TWIMS. Multipass cIMS allowed a better separation of conformers, identification of low abundant ones and finally better assignment/identification of the disulfide connectivities.

#### CONCLUSIONS

We have presented here the potential of high-resolution IMS for rapid and unambiguous profiling of disulfide pairings in an IgG4 with two additional Cys residues in the CDR of its light chain. First, a classical bottom-up approach after tryptic digestion allowed identification of a peptide containing two disulfide bonds. A comparison between ATDs obtained on the cIMS device for the mAb-collected and synthetic peptides provided unequivocal determination of disulfide bridges. While linear TWIMS results were ambiguous, benefits of higher IMS resolutions obtained on a cyclic instrument were clearly illustrated. Indeed, the gas-phase conformational heterogeneity of this disulfide-bridged peptide investigated through cIMS-MS experiments revealed broader conformational landscapes than on a linear TWIMS. Our work illustrates the ability of high resolution IMS to solve tricky analytical issues related to the identification of disulfide conformers through high resolution separation of isomeric peptides.

Multipass high resolution cIMS helps to circumvent current analytical limitations related to the elucidation of cysteine connectivity and conformational heterogeneity. Indeed, the main bottleneck of classical bottom-up approaches used for disulfide bonds characterization remains the tedious treatment of MS<sup>n</sup> data. ETD and CID both require extensive and manual data analysis, leading to highly time-consuming processes, especially if the protein is cysteine-rich. While IMS appears as an elegant technique to complement bottom-up strategies, the resolution inherent to linear TWIMS cells sometimes prevents the separation of disulfide-bridged isomers. Our study highlights the benefits of multipass high resolution cIMS to clearly differentiate isomeric disulfide-bonded peptides based solely on ATD profiles. By minimizing the need for laborious manual data analysis, the cyclic IMS approach described here thus has the potential to become a straightforward qualitative tool for accurate characterization of low abundant conformers in biological samples.

In particular, multipass cIMS could be of great interest for a rapid in-depth disulfide identification of a wide range of mAbrelated products, as incorrectly folded forms need to be closely monitored during biotherapeutic development to ensure drug quality. We envision that multipass cIMS will help to decipher disulfide networks not only for peptides but also for large intact mAb formats comprising more complex cysteine connectivity.

#### ASSOCIATED CONTENT

#### **3** Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.1c00151.

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Amino acid sequence corresponding to the light chain of mAb hzIgG4 (Figure S1); determination of the expected fwhm for a single conformation (Figure S2); theoretical and experimental isotopic distributions for the 4+ charge state of T2-T7 peptide (Figure S3); extracted ion chromatograms obtained in rpLC for the three synthetic peptides P1–P3 (Figure S4); cIMS–MS experiments after one pass (Figure S5); deconvoluted CID MS/MS fragment-ion spectrum obtained for the collected fraction of T2-T7 (z = 4+, m/z 1297.86), with deduced fragmentation sequence (Figure S6) (PDF)

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#### Notes

The authors declare no competing financial interest.

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#### ABBREVIATIONS

ATD, arrival time distribution; CCS, collision cross section; CDR, complementarity-determining region; CH, heavy-chain constant domain; CID, collision-induced dissociation; cIMS, cyclic ion mobility spectrometry; CL, light-chain constant domain; CQA, critical quality attribute; ETD, electron-transfer dissociation; HC, heavy chain; LC, light chain; IMS–MS, ion mobility spectrometry–mass spectrometry; LC–MS, liquid chromatography coupled to mass spectrometry; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; mAb, monoclonal antibody; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PTM, post-translational modification; rpLC, reversed-phase liquid chromatography; TIMS, trapped ion mobility spectrometry; TWIMS, traveling wave ion mobility spectrometry; VH, heavy-chain variable domain; VL, light-chain variable domain

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# SUPPORTING INFORMATION

# High Resolution IMS–MS to Assign Additional Disulfide Bridge Pairing in Complementarity-Determining Regions of an IgG4 Monoclonal Antibody.

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**Figure S1.** Amino acid sequence corresponding to the light chain of mAb hzIgG4. Complementaritydetermining regions (CDRs) are underlined. The CDR3 contains two additional cysteines, Cys 91 and 100 (bold red). The peptide T2-T7 is highlighted in blue (T2) and green (T7).



**Figure S2.** Determination of the expected fwhm for a single conformation. **(A)** Schematic representation of the cIMS sequence. Ion paths are represented by gray arrows. 1-2: Ions are injected from store into the array for IMS separation. 3-5: Ions of interest are ejected and maintained in the pre-store while other populations are ejected to ToF. 6-8: Isolated ions are reinjected for IMS separation (single or multipass mode). **(B)** cIMS sequence applied to P2. Upon reinjection of the isolated slice, the fwhm of the main feature is 0.65 ms after one pass. In theory, if this feature contains only one conformer, its fwhm at *n* passes =  $0.65 \times \sqrt{n}$ . For two passes, the measured fwhm is 0.94 ms, which is close to the expected value (0.92 ms). These fwhm's are thus used as references for Gaussian fitting of single species.



**Figure S3.** Theoretical and experimental isotopic distributions for the 4+ charge state of T2-T7 peptide. Theoretical profiles correspond to the presence of one (orange) or two (purple) disulfide bonds within T2-T7.



**Figure S4.** Extracted ion chromatograms obtained in rpLC for the three synthetic peptides corresponding to disulfide-bridged variants P1 (blue), P2 (green) and P3 (red).



**Figure S5.** cIMS-MS experiments after one pass. (A) Extracted ATDs of the collected T2-T7 peptide (black), and the three synthetic peptides corresponding to disulfide-bridged variants P1 (blue), P2 (green) and P3 (red). (B) Gaussian-fitting was performed for each peptide. Gray dots correspond to experimental data and thin black lines represent combined fits. The color code for conformers is identical to Figure 2 in the main text.



**Figure S6.** Deconvoluted CID MS/MS fragment-ion spectrum obtained for the collected fraction of T2-T7 (z = 4+, m/z 1297.86), with deduced fragmentation sequence. Blue y-ions correspond to diagnostic ions containing the disulfide information.
# Chapter 3 – High-Resolution cIMS-MS for Improved Characterization of mAb-based Biotherapeutics in Native Conditions

#### 1. Analytical context

Because of the structural complexity and heterogeneity of mAbs and their derivatives, low resolution IMS often fails to capture conformational variations originating from disulfide networks or PTMs. For example, IgG subclasses present different numbers and patterns of interchain disulfide bridges, and could not be distinguished through IMS-MS measurements on a linear TWIMS-MS platform<sup>16,148,149</sup>. In addition, positional isomers commonly exist in ADC drug substances as a result of varying positions of conjugated drugs. While IMS-MS measurements of cysteine-linked brentuximab vedotin (BV) and lysine-linked trastuzumab emtansine (T-DM1) were able to differentiate between Dn species<sup>152,153</sup>, Marcoux *et al.* estimated that an IMS resolving power of ~300 would be required to achieve separation of T-DM1 positional isomers<sup>153</sup>.

Even with the recent release of instruments with higher IMS resolution, conformational characterization of mAb-based products has not been widely reported on such platforms. Dn species on heavy and light chains of a model ADC were separated with SLIM-MS, allowing for avDAR determination in denaturing conditions<sup>371</sup>. Jeanne Dit Fouque *et al.* benchmarked the TIMS capabilities for a series of native proteins, among which intact bevacizumab whose heterogeneous distribution was evidenced by Gaussian fitting<sup>372</sup>. High resolution cIMS-MS could thus open new avenues to investigate conformational landscapes of mAb formats in native conditions.

As of today, few papers have dealt with native proteins on the cIMS-MS instrument<sup>19,20</sup>. It was demonstrated that native conformations of reference proteins (cytochrome *c*,  $\beta$ -lactoglobulin, conA) were retained even after prolonged exposure to T-waves after multiple passes (up to 360 ms)<sup>20</sup>. Similarly, increased storage times in the trap cell resulting from longer IMS cycles do not significantly alter conformations. One important point underlined in the work from Eldrid *et al.* is that ATDs of cytochrome *c* (z = 7+) get broader when the number of cIMS passes increases, but without further improvement of resolution between overlapping features, indicating that native proteins consist of a very large number of co-existing conformers<sup>20</sup>. This observation may be further enhanced for mAb products due to their dynamic nature.

#### 2. Objectives

In this chapter, we present innovative cIMS-MS approaches to explore the conformational heterogeneity of mAb-based biotherapeutics. cIMS-MS was used to tackle concrete issues that can be encountered along the development of mAb-related products in biopharmaceutical companies.

Advantages of high-resolution cIMS-MS compared to linear TWIMS-MS were assessed (i) for straightforward identification of IgG subclasses based on their IMS profiles and (ii) to distinguish conformers of a tsAb sample. These experiments were carried out at the BIBS platform (INRAE, Nantes, France) in collaboration with Dr. Hélène Rogniaux and Simon Ollivier.

#### 3. Conformational spaces of native proteins on the cIMS-MS instrument

In order to get a better feeling on native proteins' analyses on the new cIMS-MS platform, we subjected three tetrameric reference proteins (conA, ADH, PK), and an intact deglycosylated mAb to multiple cIMS passes. Recorded ATDs first illustrate ion losses after multiple passes (Figure 13A). After the second pass, at least half of the transmitted ions are lost for all proteins under study (we assume 100% of ions were transmitted during the first pass, Figure 13A, B). On average, transmission drops by at least 50% at each additional pass (Figure 13B). While Giles *et al.* obtained an overall signal decline of 20% from one to six passes for the GRGDS peptide (m/z 491.2)<sup>373</sup>, our results clearly evidence that heavier ions undergo much greater losses during multipass separation.

We then compared the gas-phase conformational heterogeneity of the different proteins (Figure 13A). For conA (102 kDa), a single population is detected even after five passes, with a minor increase of FWHM (+2.2 ms), indicating a narrow conformational space. Conversely, IMS profiles of elotuzumab (146 kDa) and PK (226 kDa) broaden along passes. Increases of +9.5 and +7.9 ms at FWHM are obtained after five passes for elotuzumab and PK, respectively, but no additional peaks are detected. ADH, a protein with a mass close to a mAb (147 kDa), is the only analyte for which three features are clearly separated.



**Figure 13. (A)** Extracted ATDs for a series of intact native proteins at one to five passes. **(B)** Ion transmission as a function of the number of passes. The transmission is considered to be 100% after one pass.

Broadening of IMS peaks were observed for all mAbs studied in this chapter. These results may suggest that mAbs possess a wider conformational space compared to other high molecular weight proteins, with a continuum of conformers that appear to be difficult to separate using multipass cIMS. This is consistent with previous comparisons obtained on mAbs *versus* similarly-sized protein complexes, reflecting the dynamic flexibility of mAbs<sup>98</sup>.

#### 4. High-resolution cIMS-MS to differentiate mAb isotypes

#### 4.1. Linear TWIMS does not allow distinguishing mAb isotypes

Therapeutic mAbs are selected from three IgG subclasses, namely IgG1, 2 and 4<sup>374,375</sup>. IgG isotypes exhibit distinct disulfide linkages. While IgG2s possess six interchain disulfide bonds, both IgGs 1 and 4 only have four disulfide bridges, although with different connectivities (Figure 14A). In addition, the IgG2 subclass is the sole one to present three disulfide isoforms, termed A, A/B and B<sup>376</sup> (Figure 14A). IgG2A is considered to be the canonical IgG2 structure, with four disulfide bridges in the hinge region. The IgG2B isoform has both of its Fab domains connected to the hinge *via* disulfide bonds, whereas only one Fab is disulfide-linked to the hinge for the IgG2A/B isoform.

IMS-MS measurements on a linear TWIMS-MS platform (Synapt G2 HDMS, Waters, U.K.) provide  $\Delta^{TW}CCS_{N2} < 1\%$  at the intact level between the three subclasses, which falls within the error of <sup>TW</sup>CCS<sub>N2</sub> measurements for this instrument<sup>149</sup> (Figure 14B). Yet, as the charge state increases, a trend seems to emerge for the IgG2, with higher <sup>TW</sup>CCS<sub>N2</sub> values than the IgG1 and IgG4, suggesting that the IgG2 isotype adopts a gas-phase behavior distinct from other subclasses.



**Figure 14. (A)** Schematic representation of IgG subclasses. The IgG2 isotype has three disulfide isoforms, called A, B and A/B. **(B)** <sup>TW</sup>CCS<sub>N2</sub> measurements obtained on a linear TWIMS-MS instrument at the intact level, for different charge states of intact deglycosylated mAbs. Charge states 21-25+ were acquired in nanoESI mode, and charges states 26-29+ in ESI mode. Standard deviations between triplicate measurements are  $\leq 0.2$  nm<sup>2</sup>.

Experiments performed at the middle level after IdeS digestion (below the hinge region) show only very minor improvements in terms of subclass differentiation, with  $\Delta^{TW}CCS_{N2}$  up to 2.6% for F(ab')<sub>2</sub> subunits containing both the hinge region and variable domains. For Fc subunits, quasi-iso-cross sectional <sup>TW</sup>CCS<sub>N2</sub> are obtained ( $\Delta^{TW}CCS_{N2} < 0.5\%$ ), which is consistent with ~95% sequence similarity (see publication 5 in appendix). IgG isotypes cannot be distinguished based on <sup>TW</sup>CCS<sub>N2</sub> measurements due to the low resolving power of the linear TWIMS-MS instrument, hence the need to access platforms with higher IMS resolution.

# 4.2. High-resolution cIMS-MS provides better separation of intact mAbs than linear TWIMS-MS

In order to evaluate the potential of high-resolution cIMS-MS for the separation of intact native mAbs, we first compared IMS profiles obtained on a linear TWIMS-MS instrument and a cIMS-MS platform, either after one or multiple passes.

The three mAbs under investigation belong to distinct IgG subclasses (elotuzumab IgG1, panitumumab IgG2, nivolumab IgG4) (Figure 15A). On the linear TWIMS-MS device, all three ATDs extracted for the 27+ charge state overlap due to poor IMS resolution, with  $\Delta t_D \sim 0.8$  ms between the fastest mAb (nivolumab) and the slowest one (panitumumab) (Figure 15B). On the cIMS-MS instrument, a slight separation is already observed after one pass, and  $\Delta t_D$  (panitumumab-nivolumab) increases to 2.7 ms. After five passes, ATD apexes could be clearly distinguished, with  $\Delta t_D \sim 7.0$  and 5.7 ms between consecutive IMS peaks. IMS profiles appear to be broader for all mAbs, reflecting wide conformational spaces which most likely result from their inherent flexibility. Beyond five passes, wrap-around effect occurs.

These results illustrate benefits of high-resolution cIMS-MS for enhanced separation of intact mAbs (~145 kDa), especially when using multipass capabilities. Although differences in terms of arrival times seem to be mainly mass-related, at least for elotuzumab (IgG1) and nivolumab (IgG4), panitumumab (IgG2) appears as an exception, which might suggest a particular gas-phase behavior for the IgG2 subclass.



**Figure 15. (A)** nMS spectra of intact deglycosylated elotuzumab, panitumumab and nivolumab obtained on the cIMS-MS platform (Vc = 150 V; CI0002583/CI0002584/CI0002548). **(B)** Extracted ATDs for the 27+ charge state of elotuzumab (grey), panitumumab (orange) and nivolumab (blue) obtained on the linear TWIMS-MS instrument, and on the cIMS-MS instrument after one or five passes.

# 4.3. Can IgG subclasses be differentiated based on their IMS profiles?

#### 4.3.1. IgG1 versus IgG4

Separating IgGs 1 and 4 with first-generation IMS-MS instruments has been particularly challenging, considering that these two subclasses differ solely in terms of interchain disulfide connectivity, as they otherwise have the same number of disulfide bonds (inter- and intrachain). We recorded ATDs for a series of IgGs 1 and 4 to see if trends allowing for differentiation of these two isotypes could be observed on the high-resolution cIMS-MS platform, with the ultimate goal of classifying IgGs directly based on their IMS profiles.

Gaussian fits will be used as a means to illustrate the conformational heterogeneity of mAbs and to provide a comparison between subclasses. As already mentioned in the previous chapter, the number of detected features depends on fitting parameters. In the following experiments, we have estimated FWHM values based on the diffusion-limited theoretical formula (Equation 1):

FWHM at *n* passes = FWHM at 1 pass 
$$\times \sqrt{n}$$
 (1)

Of course, we are well aware of diffusion effects that can occur for high mass species such as intact mAbs<sup>377</sup>, however at this point we are not able to take into account this phenomena to correct FWHMs after *n* passes on the cIMS-MS device. Hence, the number of conformers might be over-estimated, but relative subclass comparison remains relevant since the same bias is introduced for all mAbs.

### 4.3.1.1. IgG1 – Intraclass cIMS variability

For the IgG1 subclass, 27+ charge states of trastuzumab, elotuzumab and ofatumumab all present a single IMS peak after one pass, with two main contributions detected using Gaussian fitting (Figure 16A). The resolution at one pass is not sufficient to separate trastuzumab and elotuzumab because of very close masses (145 903  $\pm$  6 Da and 145 911  $\pm$  6 Da, respectively). Differences between trastuzumab/elotuzumab remain subtle even after five passes, but ofatumumab (146 540  $\pm$  5 Da) could be slightly separated from the two other mAbs (Figure 16B). New features are uncovered at five passes, highlighting once again the benefit of multipass cIMS to explore conformational landscapes of biotherapeutics. Three major populations (relative intensity > 50%), along with several minor ones (< 10 %), could be fitted for each mAb (Figure 16B). Overall, all IgG1s under study exhibit very similar asymmetrical profiles, with tailing on the right side of the IMS peak.



**Figure 16.** cIMS profiles and associated Gaussian fits obtained for three IgG1s, trastuzumab, elotuzumab, and ofatumumab, after (A) one pass and (B) five passes (z = 27+).

### 4.3.1.2. IgG4 – Intraclass cIMS variability

Regarding the IgG4 subclass, nivolumab (144 056  $\pm$  7 Da), reslizumab (144 263  $\pm$  2 Da) and natalizumab (146 649  $\pm$  5 Da) also have a single IMS peak comprising two major populations after one pass (Figure 17A). After five passes, nivolumab and reslizumab could not be differentiated because of close masses, while the arrival time of natalizumab correlates with its higher mass (Figure 17B). Additional conformers are detected at five passes using Gaussian fitting, with four main features (> 50 %) for each mAb, and several low-intensity populations.



**Figure 17.** cIMS profiles and associated Gaussian fits obtained for three IgG4s, nivolumab, reslizumab, and natalizumab, after (A) one pass and (B) five passes (z = 27+).

# 4.3.1.3. IgG1 vs IgG4 – Interclass cIMS comparison

Interestingly, FWHM values at five passes are higher for the IgG4 subclass compared to IgG1s (+5 ms on average, Figure 18), also reflected by more fitted conformers > 50% for IgG4s, indicating an increased gas-phase flexibility. This is in good agreement with trends underlined by Pacholarz *et al*<sup>98</sup>. The authors suggested that IgG4s are more flexible than IgG1s because of the light chain being linked further to the center of mass. Indeed, disulfide bonds connect to the cysteine residue of the heavy chain located at position 220 *versus* 131 for IgGs 1 and 4, respectively (Figure 14A), which confers more movement and accessible space to IgG4s' light chains. The wider conformational heterogeneity of IgG4s may also be explained by possible Fab arm exchange resulting from serine residues in the hinge region of wild-type IgG4s at position 228 on heavy chains.



**Figure 18.** Overlay of ATDs (z = 27+) obtained after five passes for two IgG1s (elotuzumab, ofatumumab) and two IgG4s (nivolumab, natalizumab).

Although these observations provide first hints to distinguish IgGs 1 and 4, it is still tricky to identify IgGs 1 or 4 by relying solely on their IMS patterns at the intact level. While nivolumab and reslizumab exhibit broad ATDs that significantly differ from those of IgG1s, the IMS profile of natalizumab may be falsely identified as IgG1-like, due to its asymmetry and right-side tailing. In conclusion, differences remain too subtle to achieve a straightforward IgG subclass categorization from the sole use of cIMS-MS profiles. Although we initially decided to focus on intact species to avoid sample pretreatment (IdeS digestion) that might influence mAbs' conformation, IMS-MS measurements at the middle level may be more informative to differentiate IgGs 1 and 4.

#### 4.3.2. IgG2 isoforms

As previously described, IgG2s possess three disulfide variants. IMS profiles were recorded on the linear TWIMS-MS and cIMS-MS instruments for two IgG2s, panitumumab (144 762  $\pm$  4 Da), and denosumab (145 202  $\pm$  5 Da). As the number of approved IgG2s is limited, we also include an hybrid IgG2/4, eculizumab (145 850  $\pm$  2 Da), whose F(ab')<sub>2</sub> domain and interchain disulfide bonds are IgG2-like, while its Fc subdomain is IgG4-like<sup>149</sup>. On the linear TWIMS-MS instrument, a single IMS peak is observed for the three mAbs, although with a small right-side shoulder for eculizumab, indicating that disulfide isoforms cannot be differentiated because of low TWIMS resolution (Figure 19A). Conversely, on the cIMS platform, two peaks are already clearly observed after one pass for denosumab (Figure 19B). The IMS profile of panitumumab shows an asymmetry on the left side of the main peak, with two conformers > 50% detected with Gaussian fitting. Eculizumab also has a left-side shoulder. After five passes, all mAbs present a bimodal distribution, although the separation is less obvious for panitumumab (Figure 19C).

Bagal *et al.* showed that nIMS could separate isoforms A and B for intact IgG2s<sup>378</sup>. Conversely, Cys  $\rightarrow$  Ser mutants IgG2s display a single IMS peak, providing strong evidence that multiple conformers in IgG2s are disulfide-related. nIMS was also used elsewhere to tackle the disulfide bond heterogeneity of a series of wild-type and mutant IgG2s<sup>292</sup>. In these studies, only two isoforms were separated by nIMS<sup>292,378</sup>. Based on these previous works, the slower and more extended species that we observe most probably correspond to isoform B which has a constrained structure<sup>292,378,379</sup>, however the faster one could be either isoform A or A/B. Jones *et al.* suggested that variants A and A/B may co-drift<sup>292</sup>.

FWHMs and Gaussian fits further evidence the conformational heterogeneity of IgG2s. Of note, the IMS pattern of eculizumab resemble that of a "pure" IgG2 at the intact level, in line with trends observed on our linear TWIMS instrument<sup>149</sup>.



**Figure 19. (A)** ATDs recorded on the linear TWIMS-MS instrument for intact deglycosylated IgG2s (panitumumab, denosumab) and hybrid IgG2/4 eculizumab (z = 22+, nanoESI mode). **(B-C)** cIMS profiles and associated Gaussian fits obtained at one pass and five passes. ATDs were extracted for the 27+ charge state.

We next performed IMS<sup>n</sup> experiments on denosumab and panitumumab to ensure that no interconversion between the two peaks (I and II) identified as isoforms has occurred. Indeed, interchain disulfide isoforms cannot spontaneously rearrange in the gas-phase (disulfide scrambling is rather promoted in alkaline pH conditions<sup>380</sup> or induced by CID). Windows I and II were selectively isolated in the prestore after four passes (Figure 20). After reinjection from the prestore, isolated species were subjected to four additional cIMS passes. Population I reveals for both mAbs an interconversion of the main species (for panitumumab, 432.2 ms) towards a more extended conformation (440.9 ms). No major interconversion occurs for population II, although a peak tailing can be observed for denosumab. More importantly, all features have different arrival times, meaning that the two main conformers are not in equilibrium, which is consistent with our isoform hypothesis. Additional experiments in reducing conditions would help to definitely conclude on the origin of these two populations, with one homogeneous population expected.



**Figure 20.** IMS<sup>2</sup> experiments performed on panitumumab and denosumab. Slices I and II were reinjected for four passes to check for possible interconversion between the two main features.

# 4.3.3. Conclusions

Overall, IgG2s exhibit signature gas-phase profiles contrary to IgGs 1 and 4, originating from different interchain disulfide networks. This allows for direct identification of this specific subclass, based solely on qualitative analysis of IMS profiles. High-resolution cIMS-MS would thus be of a valuable method to assess the presence of A, B, and/or A/B forms in IgG2s, which is of particular interest as IgG2 isoforms differ in their biological activity and binding ability<sup>379</sup>. In conclusion, cIMS-MS offers clear advantages over linear TWIMS for mAbs characterization and subclass determination, even if trends between IgG1s and 4 remain subtle.

# Scientific communication

A scientific publication reporting on these results is currently in preparation.

#### Oral presentation at international conference

<u>Deslignière, E.</u>; Ollivier, S.; Beck, A.; Giles, K.; Richardson, K.; Hernandez-Alba, O.; Ropartz, D.; Rogniaux, H.; Cianférani, S., High-resolution cyclic ion mobility-mass spectrometry for improved conformational characterization and subclass differentiation of native intact therapeutic monoclonal antibodies. 69<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, October 31-November 4<sup>th</sup> 2021 (Philadelphia, PA, USA).

#### 5. High-resolution cIMS-MS to distinguish isomers of an anti-HIV tsAb

#### 5.1. Presentation of the tsAb under study

The human immunodeficiency virus (HIV) continually evolves, leading to extensive viral diversity<sup>381</sup>. Hence, the development of therapeutics capable of preventing infection across multiple strains or subtypes is a real challenge. Broadly neutralizing antibodies provide a new approach to HIV-1 prevention and treatment to bind diverse strains of the virus<sup>382</sup>. Sanofi researchers engineered an anti-HIV tsAb that simultaneously target three epitopes (two epitopes on Fab1 and one on Fab2, Figure 21) on the virus, the CD4 binding site, the GP41 membrane proximal external region (MPER) and the V1V2 glycan site<sup>383</sup>. Combining specificities against different epitopes within a single molecule would improve HIV-1 neutralization and simplify treatment, as only one protein would need to be administered<sup>384</sup>. However, these next-generation products are increasingly complex in terms of structure, and their in-depth analytical characterization requires state-of-the-art analytical tools. In this part, we aimed at analyzing a tsAb using nIMS-MS measurements on the cIMS-MS instrument. The tsAb sample was obtained through a collaboration with the BioAnalytics department from Sanofi Vitry-sur-Seine (France).



**Figure 21.** Schematic representation of the anti-HIV tsAb under study. Adapted from Xu *et al.*, 2017 (ref. 383). The tsAb comprises variable domains from three different mAbs arranged in an IgG1 scaffold: one classical Fab arm (VRC01) and a cross-over dual variable (CODV) domain arm.

#### 5.2. SEC-nMS highlights the presence of two tsAb isomers

Preliminary SEC-nMS analyses were performed during the PhD work of Dr. Anthony Ehkirch. At the intact level, two peaks were observed on the SEC chromatogram (Figure 22A). Surprisingly, both species correspond to isomeric monomers (182 086 ± 1 Da), indicating an atypical behavior of the tsAb on the SEC column (Figure 22B). Masiero *et al.* attributed these isomers to the YPP motif in the CDR3 of the heavy chain in the CODV arm, for which isomerization of the proline residue occurs, leading to a mixture of YP<sub>trans</sub>P<sub>cis</sub> and YP<sub>trans</sub>P<sub>trans</sub> forms<sup>385</sup>. In order to confirm that the Fab1 domain is responsible for the co-existence of the two isomers, middle-level experiments were next carried out. After digestion above the hinge region using the FabALACTICA<sup>®</sup> enzyme (Genovis, Sweden), three peaks were detected on the SEC chromatogram (Figure 22C). nMS allowed to identify the different species as Fab1 subunit (peak 1, 79 301 ± 1 Da), co-eluted Fc and Fab2 subdomains (peaks 2/3, 52 859 ± 1 Da)

and 49 945  $\pm$  1 Da, respectively), and another Fab1 subunit (peak 4, 79 301  $\pm$  1 Da) (Figure 22D). It is worth noting that the second Fab1 subdomain eluted significantly later compared to species of lower masses, highlighting secondary interactions with the SEC material<sup>386</sup>. These results corroborate the fact that the presence of tsAb isomers is related to the YPP motif located on the Fab1 subunit.



**Figure 22. (A)** SEC-UV chromatogram of intact glycosylated tsAb and **(B)** SEC-nMS spectra of the two separated monomers C1 (blue) and C2 (red) (Vc = 180 V; O57191). **(C)** SEC-UV chromatogram of FabALACTICA-digested tsAb and **(D)** SEC-nMS spectra of each species at middle level (Vc = 180 V; O50499).

# 5.3. IMS-MS to differentiate tsAb isomers

#### 5.3.1. Linear TWIMS-MS results

nIMS-MS experiments were then carried out to determine whether isomerization leads to different conformations. We first exploited the SEC-nIMS-MS coupling that is routinely used on our linear TWIMS-MS system (Synapt G2 HDMS) to record IMS profiles of the two isomers, with ATDs extracted as a function of m/z and retention time. For the 29+ charge state at intact level, a difference of 0.6 ms was observed between arrival times of both conformers, which corresponds to  $\Delta^{TW}CCS_{N2} = 0.6 \text{ nm}^2$  (<sup>TW</sup>CCS<sub>N2</sub> = 91.6 ± 0.1 nm<sup>2</sup> versus 92.2 ± 0.1 nm<sup>2</sup>, Figure 23A). Similar trends were obtained for the 28+ charge state.

At the middle level, the Fab1 subunit of conformer C1 was also found to be more compact (<sup>TW</sup>CCS<sub>N2</sub> = 50.1 nm<sup>2</sup> for the 19+ charge state) than for conformer C2 (<sup>TW</sup>CCS<sub>N2</sub> = 50.5 nm<sup>2</sup>, Figure 23B). These results are in good agreement with CCS<sub>N2</sub> calculated from the crystal structure of the CODV Fab1 (PDB

code: 5WHZ). Theoretical CCS<sub>N2</sub> values obtained with the IMoS software were  $43.0 \pm 0.2 \text{ nm}^2$  and  $54.8 \pm 0.1 \text{ nm}^2$  using PA and EHSS methods, respectively. Although slight differences are observed between the two isomers at both intact and middle levels, these small variations fall within the error of IMS measurement of the low resolution TWIMS platform.

#### 5.3.2. cIMS-MS results

We next moved to the cIMS-MS instrument with the aim of gathering further evidence to distinguish conformational behaviors of tsAb isomers. As the SEC coupling has not been implemented on our cIMS-MS platform yet, the differentiation of conformers relies solely on the IMS dimension.

At the intact level, a single ATD is observed for the 29+ charge state after six passes (Figure 23C). Because the SEC dimension is not used to provide a first dimension of separation, the IMS peak of the major conformer C2 most likely overlaps with the signal of the minor conformer C1, preventing the distinction of both isomers.

Nonetheless, at the middle level, two species are clearly separated for Fab1 subunits (Figure 23D). After four passes, a difference of 9 - 10 ms between C1 and C2 is obtained for both 19+ and 20+ charge states. These results definitely confirm the presence of two conformations related to the Fab1 domain of the CODV arm.



**Figure 23.** IMS profiles of tsAb isomers. ATDs obtained **(A)** at the intact level (z = 28+/29+) and **(B)** at the middle level (z = 19+) on the linear TWIMS-MS instrument, using the SEC-nIMS-MS coupling. **(C)** ATDs obtained at the intact level after six passes on the cIMS-MS platform (z = 29+). **(D)** Extracted ATDs of Fab1 subunits after four passes on the cIMS device (z = 19+/20+).

Lastly, IMS<sup>2</sup> experiments were performed on the cIMS-MS instrument to investigate Fab1 subunits separated at the middle level. After three passes (IMS<sup>1</sup>), each species were sequentially sliced-out, and reinjected from the prestore for three passes, which can be summarized as: IMS<sup>1</sup>(3 passes)  $\rightarrow$  Slicing  $\rightarrow$  IMS<sup>2</sup>(3 passes) (Figure 24). A slight interconversion between the two conformers occurs, in agreement with slow conformational isomerization previously reported for the C1 YP<sub>trans</sub>P<sub>trans</sub>  $\Rightarrow$  C2 YP<sub>trans</sub>P<sub>cis</sub> equilibrium<sup>385</sup>.



**Figure 24.** IMS<sup>2</sup> experiments performed on the cIMS-MS instrument. After three passes, conformer C1 (blue) or C2 (red) is sliced-out, and reinjected for three passes, showing an interconversion between both populations.

# 5.4. Conclusions

Altogether, cIMS-MS results clearly evidence the co-existence of two isomers originating from two YPP configurations on the CODV subdomain of the tsAb. While the differentiation of conformers was not clear-cut on the linear TWIMS-MS instrument, the use of high-resolution cIMS emphasizes distinct gas-phase behaviors for C1/C2 at the middle level.

#### Scientific communication

These data will be presented in a publication currently in preparation.

#### 6. Conclusions of chapter 3

We have demonstrated the advantages of multipass and IMS<sup>n</sup> capabilities available on the cIMS-MS platform to characterize several mAb products. While IgG subclasses could not be unambiguously distinguished with the low resolving power of first-generation TWIMS-MS devices, high-resolution cIMS-MS provides new insights into conformational ensembles of mAbs, yielding better identification of IgG isotypes from IMS data. In particular, cIMS-MS separates disulfide isoforms of IgG2s, providing a bimodal IMS signature pattern for this subclass. cIMS-MS is also amenable to analyzing more complex mAb formats, and allowed for differentiation of conformational isomers of a tsAb sample.

# **Part III – Conclusions/Perspectives**

In this third part, advantages of high-resolution cIMS-MS for enriched characterization of biotherapeutics have been underlined. Analyzing mAbs is difficult because of their high complexity and flexibility. State-of-the-art tools are thus needed to explore broad conformational spaces of these products. cIMS affords an improved definition of ATDs, and multipass cIMS further enhances the separation of co-existing features.

First, I have demonstrated benefits of high-resolution cIMS-MS for identification/separation of disulfide variants at peptide level. cIMS-MS proved to be efficient to decipher disulfide networks not only for peptides, but also for larger intact mAb formats comprising more complex cysteine connectivities. Indeed, IgG subclasses could be better distinguished compared to results obtained on a linear TWIMS-MS instrument. Disulfide variants of IgG2s were well separated, offering new opportunities for in-depth analysis of isoforms' gas-phase behaviors. In particular, we envision that the latest integration of an ExD cell able to perform ECD into the cIMS-MS platform will foster complex biomolecules investigation<sup>387</sup>. This cell, located either after or before the IMS cell, would be of utmost interest to perform nTD-MS on the different separated mAb species<sup>182,388</sup>. We expect IgG2s isoforms to exhibit distinct fragmentation patterns. While IgG2s present specific IMS patterns, IgGs 1 and 4 possess similar profiles, preventing direct mAb isotyping. IgG4s appear to be slightly more flexible than IgG1s in the gas phase, which needs to be confirmed through middle-level analyses. CIU approaches have already emerged as an alternative technique to distinguish mAb subclasses, and will be exploited in the next part of this thesis<sup>16,149</sup>.

cIMS-MS has the potential to address conformational heterogeneity that could arise from increasing complexity of innovative mAb formats<sup>141</sup>. High-resolution cIMS-MS was able to pinpoint small conformational differences between *cis/trans* isomers of an anti-HIV tsAb. Multifunction capabilities of the cIMS-MS device will undoubtedly prove useful to support the development of next-generation products.

Being far larger, more heterogeneous, flexible, and produced as a result of batch processing, biotherapeutics provide a significant regulatory challenge compared to small molecules. Latest advancements to achieve higher IMS resolution have focused on extending path lengths with compact designs. The newest SLIM-based instrument commercialized in June 2021 (MOBIE<sup>®</sup>, MOBILion Systems, U.S.), has been intended for biotherapeutics analysis at peptide and protein levels<sup>371,389</sup>. In the long term, these high-resolution IMS-MS platforms could enter into R&D laboratories of biopharmaceutical companies, however at this point a high level of expertise is required to operate these instruments, hindering their integration into high-throughput automated biopharma environments.

# PART IV – Development of CIU Approaches for Therapeutic Protein Characterization

The fourth part of this thesis deals with CIU approaches applied to a variety of mAb formats. In the following chapters, I aimed at developing an automated CIU workflow as a means to increase the throughput of these experiments. A second research axis consisted of evaluating the interest of high-resolution cIMS-MS and IMS<sup>n</sup> to gather new information on mAbs' unfolding patterns in the gas phase.

# Chapter 1 – Influence of a Series of MS Parameters on CIU Patterns

Chapter 2 – Applications of CIU Approaches for Better Characterization of Different mAbrelated Formats

Chapter 3 – Coupling of SEC to CIU

Chapter 4 – Exploring the Capabilities of the High-Resolution cIMS-MS Instrument to Characterize mAbs' Unfolding Patterns

# Chapter 1 – Influence of a Series of MS Parameters on CIU Patterns

#### 1. Analytical context

Overcoming limitations in IMS resolution remains challenging for intact native proteins, especially as the size of complexes under investigation increases. CIU approaches represent an elegant alternative to circumvent the lack of TWIMS resolution, allowing to probe the gas-phase behaviors of activated ions<sup>15,18,148</sup>. As a reminder, CIU experiments consist of accelerating ions in the trap cell of TWIMS instruments prior to IMS separation by raising trap CVs. As ions get accelerated, their collisions with the background gas become more energetic, which leads to a buildup of internal energy, and so ions may cross energy barriers and undergo conformational changes<sup>12</sup>. Subtle conformational differences between co-drifting IMS species translate into distinct unfolding patterns, providing signature CIU fingerprints for each protein complexes. While early examples of gas-phase unfolding have focused on small native proteins (< 17 kDa)<sup>390,391</sup>, modern implementations of CIU methods have been extended well beyond these demonstrations to include in-depth characterization of proteinligand complexes, membrane proteins, and biotherapeutics<sup>12</sup>. Pioneering work has been performed by the group of Ruotolo, both in terms of applications<sup>15,16</sup> and software developments<sup>284,287</sup>. In particular, the CIUSuite program released in 2015 has ushered in more comprehensive analyses of CIU fingerprints, with precise feature detection (centroid drift time and CV range), differential plots and associated RMSDs, but also early stages of clustering scores based on discriminating CV regions<sup>284</sup>. It is in this context that CIU methods were implemented in our laboratory by Dr. Thomas Botzanowski.

#### 2. Preliminary results

Several metrics were first evaluated during the PhD work of Dr. Botzanowski using the CIUSuite software on mAb samples<sup>392</sup>. The RMSD across technical replicates of a CIU fingerprint was found to be < 3%, indicating a good repeatability of CIU experiments. These values are in line with the recommendations of the U.S. FDA (RMSD < 10%)<sup>288</sup>. In addition, the reproducibility of CIU experiments was assessed by comparing fingerprints from two different operators (distinct sample preparations), yielding a RMSD < 3% (Figure 1A) which agrees well with U.S. FDA requirements (RMSD < 15%)<sup>288</sup>. The robustness of the technique was also underlined, allowing to retain unfolding states and their associated transitions (RMSD < 5%). Of note, the formula from CIUSuite underestimates RMSD values, hence calculations were modified in the next-generation software CIUSuite 2 to achieve more accurate RMSDs (Figure 1B)<sup>287</sup>. Despite a slight increase in CIUSuite 2 (+2% in this example), RMSD values are still compliant with U.S. FDA recommendations. Fingerprints presented in the following paragraphs and chapters were generated using CIUSuite 2.



**Figure 1**. Reproducibility evaluated on the 22+ charge state of intact deglycosylated reslizumab with differential plots and corresponding RMSD values generated using **(A)** CIUSuite and **(B)** CIUSuite 2.

Overall, the focus of these preliminary CIU studies has been put mainly on small experimental variations to evaluate reproducibility and robustness metrics. Crucial MS parameters known to influence gas-phase activation/dissociation of native proteins upstream of the trap cell have not been explored yet, although their impact on unfolding behaviors could be substantial.

# 3. Objectives

In order to better understand implications of MS settings on the activation process, I investigated different MS parameters that play a key role to maintain native conformations, among which Vc, Pi, and the source temperature. Results presented thereafter illustrate the extent to which these parameters may alter unfolding patterns. Examples are based on mAb samples, which were the main focus of CIU experiments conducted during this PhD work.

#### 4. Influence of MS parameters on gas-phase activation

#### 4.1. Cone voltage Vc pre-activation

First, the voltage of the sampling cone Vc should be tuned to avoid unfolding or fragmentation of the protein while ensuring ion transmission. For <sup>TW</sup>CCS<sub>N2</sub> measurements of intact mAbs, setting Vc to 20 V generally fulfills both criteria. However, for CIU experiments, such low values sometimes provide poor information regarding the gas-phase behavior of mAbs upon activation. Indeed, a single transition is detected for the 24+ charge state of glycosylated trastuzumab, whereas for Vc = 80 V, two transitions are obtained (Figure 2). When Vc = 20 V, less kinetic energy is imparted to ions as a consequence of reduced ion acceleration, and so further collisional activation is required to generate excited states (+24.5 V to reach CIU50 of the first transition). The most unfolded state would also be observed even with low Vc values, although higher trap energies (> 200 V) would be required. As trap CV values are limited to 200 V on the Synapt G2 HDMS, 80 V appears as a good compromise to initially maintain the native state of intact mAbs while giving access to several exploitable unfolding transitions. Of course, the value of Vc should be adapted depending on the size and fragility of the mAb format. For instance, a lower Vc may be preferred for IdeS-digested F(ab')<sub>2</sub> subunits<sup>149</sup>, which have been shown to be slightly

pre-activated at 80 V, or in the case of ADCs for which too high Vc values might induce drug fragmentation.



**Figure 2.** Influence of Vc on unfolding patterns of glycosylated trastuzumab (z = 24+). Fingerprints were acquired in nanoESI mode with Vc set to (A) 20 V and (B) 80 V. Transitions occur at lower CIU50 values when Vc increases.

#### 4.2. Interface pressure Pi

Another parameter of utmost importance in nIMS-MS is the pressure Pi in the interface region, which influences the mean free path of ions. Operating at low gas pressures allows the ion to be more accelerated between collisions, resulting in an energy transfer that increases the internal energy of ions (ion heating). Conversely, high interface pressures induce more frequent but less energetic collisions, subsequently reducing the internal energy of ions (ion cooling)<sup>393,394</sup>. Simulations of insource activation as a function of Pi (up to 3 mbar) and Vc have been performed by Wilson et al.<sup>395</sup>. The authors demonstrated that ions exhibit similar levels of activation at low Vc values, regardless of interface pressures. The effect of the Pi parameter is more obvious at higher Vc, for which ion activation increases as the pressure diminishes. CIU fingerprints presented in Figure 3 clearly illustrate the influence of Pi on gas-phase activation. At 6 mbar, the ground state is centered on ~10.5 ms (<sup>TW</sup>CCS<sub>N2</sub> = 79.1  $\pm$  0.1 nm<sup>2</sup>), whereas at 2.6 mbar the initial feature has been shifted to a higher drift time of ~13.3 ms corresponding to a more extended/unfolded conformation ( $^{TW}CCS_{N2}$  = 82.0 ± 0.3 nm<sup>2</sup>). Consequently, the first two transitions are not detected at 2.6 mbar. The remaining transitions occur at lower CIU50 values than at 6 mbar. In addition, the signal intensity of ions rapidly decreases along the CV ramp: while high Pi values ensure enough transmission even at high CVs, it is not the case for low Pi values (loss of MS signal for CVs > 170 V, Figure 3). These results emphasize the need to acquire CIU fingerprints at pressures that minimize ion activation and maintain native conformations.



**Figure 3.** Influence of the pressure Pi in the interface region on the gas-phase unfolding of intact deglycosylated trastuzumab (z = 28+). Fingerprints were acquired in ESI mode at **(A)** 6 mbar and **(B)** 2.6 mbar.

### 4.3. Source temperature

The influence of the source temperature on unfolding patterns was next evaluated. Fingerprints of intact adalimumab were acquired at source temperatures ranging from 40 to 100°C (Figure 4). Similar CIU patterns are obtained in terms of number of transitions and median drift time values of the conformational states, albeit transitions occur at different CVs. Increasing the temperature confers more thermal and therefore internal energy to ions<sup>394</sup>, resulting in pre-activation reflected by lower CIU50 values ( $\Delta$ CIU50<sub>100-40°C</sub> = -26.3 V for the first transition, and -18.4V for the second one). In the case of highly stable mAbs for which unfolding plots are not altered, quite harsh temperature conditions ~100°C can thus be used, also as a means to improve desolvation of low-intensity charges states (for example, the 26+ charge state is no longer observed at 40°C).



**Figure 4.** Influence of the source temperature on the nanoESI-CIU fingerprint of intact deglycosylated adalimumab (z = 23+). Transitions occur at lower CIU50 values with higher source temperatures.

#### 4.4. Quadrupole selection and available charge states

Although the quadrupole selection usually does not appear to be crucial for nIMS-MS experiments, isolating a precursor ion prior to the trap cell has been shown to influence gas-phase unfolding. CIU datasets can be acquired either with or without previous ion selection in the quadrupole, but most CIU experiments rely upon the MS-based selection of a single protein charge state to avoid chemical noise related to the presence of other populations within the trap cell. In order to illustrate this phenomenon, fingerprints were recorded with and without isolation (Figure 5). The number of features and transitions is maintained, however, conformational transitions occur at lower CVs without quadrupole selection. Similar behaviors were observed by Vallejo *et al.* in the case of mAb samples<sup>164</sup>. The authors attributed the preemptive unfolding to the charge-stripped 24+ precursor ion (i.e. generation of a 23+ ion due to the loss of a charged adduct following collisions with neutral gas particles in the trap cell), which interferes with the actual 24+ charge state. The amount of chargestripping increases with collision energies and charge states, as higher charge states present lower barriers to charge-stripping reactions. Of note, mAbs generate low levels of charge stripping. Another explanation could be that the larger number of ions present in the trap cell induce more Coulombic repulsions, leading to pre-activation of non-isolated species. On the contrary, when ions are selected in the quadrupole, ion-ion and ion-neutral interactions are limited as only one population enters the trap cell.

Even if such interactions may occur, we found that the direct comparison of non-quadrupole selected CIU fingerprints is still valid<sup>148,149</sup>, provided that data are acquired under the same conditions. Besides, recording data without prior quadrupole selection gives access to more charge states in a single experiment, shortening the overall procedure while broadening the information content available compared to experiments with quadrupole selection, for which only the most native and intense charge states are favored.



Figure 5. Influence of quadrupole-selection on the unfolding pattern of intact deglycosylated trastuzumab (z = 24+). Fingerprints were acquired (A) without quadrupole selection and (B) with isolation of the 24+ ion precursor.

# 5. Conclusions

CIU is amenable to monitoring gas-phase activation of proteins, and in particular for therapeutic mAbs. Instrumental parameters that were most likely to influence ion unfolding have been reviewed, highlighting a major contribution of the interface pressure on ion activation, reflected by a loss of native states for low pressures. Conversely, source temperature and voltage do not have a significant effect on unfolding patterns as conformational features are preserved, at least in the case of intact mAbs. As we now have a better understanding of the impact of MS parameters on unfolding behaviors, we next moved to in-depth conformational characterization of different mAb formats through CIU experiments.

# Chapter 2 – Applications of CIU Approaches for Better Characterization of Different mAb-related Formats

#### 1. Analytical context

CIU approaches have recently extended into valuable tools to investigate therapeutically-relevant proteins, offering more conformational insights into a wide range of mAb-based products, from nanobodies<sup>396</sup> to bsAbs<sup>148</sup>, and even larger formats such as ADCs<sup>17,397</sup>. Many of these applications illustrate how CIU can differentiate closely related conformations of co-drifting species when IMS fails to do so<sup>12</sup>. Perhaps one of the most striking examples is the ability of CIU to distinguish mAb isotypes contrary to nIMS-MS measurements. As described in part III (chapter 3), IgG subclasses, whose numbers and patterns of interchain disulfide bonds differ, cannot be separated due to poor linear TWIMS resolution<sup>149</sup>. While high-resolution cIMS-MS affords improved identification of IgG2s, clearcut differentiation of IgGs 1 and 4 remains challenging. In such cases where conformational differences are very subtle, CIU represents an elegant alternative for improved characterization. Tian et al. demonstrated that intact nontherapeutic mAb subclasses generate distinct gas-phase CIU fingerprints, highlighting that unfolding behaviors are most likely driven by disulfide connectivities present within protein ions<sup>16</sup>. This pivotal work paved the way for further characterization of mAbs and their associated products. CIU has proved to be efficient to tackle small alterations in mAbs' structures, among which antibodies' glycoforms<sup>16,398</sup>, or sequence mutations in the hinge region of IgG4s to avoid Fab arm exchange<sup>148</sup>. The influence of conjugation on mAbs' unfolding patterns has also been explored, although only in few papers<sup>17,397</sup>, probably because of the heterogeneity of secondgeneration ADCs conjugated on lysine or cysteine residues<sup>144</sup>. CIU can afford valuable information to assess gas-phase stabilization/destabilization as a result of drug conjugation process, as exemplified by increased resistance to unfolding of a site-specific DAR4 ADC compared to its parent mAb<sup>17</sup>.

As biotherapeutics have become a multibillion dollar industry, their analytical characterization through CIU received proportional interest<sup>12</sup>. Notably, the U.S. FDA has pushed forward the technique for better conformational profiling of mAbs, to tackle batch-to-batch variations<sup>288</sup>, and more recently to determine the effect of accelerated storage conditions on mAb stability<sup>399</sup>, which represents a CQA that needs to be closely monitored to ensure proper folding of the protein<sup>400</sup>. Overall, CIU approaches hold a great potential for future integration into development pipelines of mAb-based products, especially with recent software improvements that will promote faster data treatment.

#### 2. Objectives

In this chapter, I aimed at developing innovative CIU methods for improved characterization of biotherapeutics' gas-phase behaviors. We first evaluated new bioinformatics tools for automated and quantitative identification of IgG subclasses. CIU approaches were then applied to larger and more

complex ADC samples as a means to (i) tackle subtle conformational differences occurring along the conjugation and (ii) assess the influence of the different conjugation strategies on gas-phase stability.

#### 3. Evaluation of bioinformatics tools for better differentiation of IgG subclasses

# 3.1. Dataset generation - CIU fingerprints of mAb isotypes at intact and middle levels

Building on the work of Tian *et al.* to distinguish intact mAb subclasses<sup>16</sup>, we aimed at expanding the strategy to middle-level CIU, where the mAb scaffold is IdeS-digested<sup>401</sup>, for improved differentiation of mAb isotypes. These experiments were performed on three mAbs (adalimumab, panitumumab, reslizumab) by Dr. Botzanowski, and further exploited in this PhD work after the release of the CIUSuite 2 program in 2019<sup>287</sup>.

At the intact level, fingerprints generated for the 22+ charge state show that all subclasses exhibit the same number of conformational states, which prevents a straightforward isotype identification, even if CIU50 values ultimately pinpoint different unfolding behaviors (Figure 6A). Conversely, at the middle level, F(ab')<sub>2</sub> subdomains provide specific unfolding patterns for each isotype, both in terms of number of conformational states and CIU50 values (Figure 6B). As the redistribution of vibrational energy is more efficient upon collision with the trapping gas for small F(ab')<sub>2</sub> subunits than for intact mAbs, ions can populate additional excited states, offering better fingerprinting of each mAb subclass. In particular, IgGs 1 and 4 could be clearly distinguished despite possessing close disulfide connectivities, with four and six features, respectively. This represents a major asset of middle-level CIU compared to high-resolution cIMS-MS measurements that fail to separate these two subclasses. Of note, the IgG2 displays few transitions, with only three conformational states, most likely due to a higher number of disulfide bonds in the hinge region that confer an increased resistance to unfolding to the F(ab')<sub>2</sub> subunit. For Fc subdomains, very similar patterns were detected, which can be attributed to both high sequence similarities across Fc regions of the three subclasses (> 90%), and the absence of interchain disulfide bridges to connect Fc dimers (Figure 6C).



**Figure 6.** CIU experiments on adalimumab, panitumumab and reslizumab, each belonging to a different IgG subclass (left – disulfide patterns of isotypes are represented in orange). CIU fingerprints and their associated CIU50 values were generated for **(A)** intact mAbs, **(B)** F(ab')<sub>2</sub> subunits, and **(C)** Fc subunits.

# 3.2. UFS plots for better mAbs differentiation

These first results confirm that unfolding behaviors between mAb isotypes mainly stem from distinct disulfide networks, which lead to F(ab')<sub>2</sub> fingerprints being more informative than the intact level for IgG identification. However, these experimental observations would benefit from a quantitative tool to evaluate how significant differences between CIU patterns really are. The CIUSuite 2 software package includes a module that allows to compare fingerprints in a more quantitative manner through univariate feature selection (UFS) plots, which help to identify the most diagnostic energies<sup>287</sup>. UFS analysis is performed using ANOVA F-tests to assess intra-class (technical replicates) and inter-class (IgG isotypes) variances for each CV<sup>402</sup> (Figure 7A). High –log<sub>10</sub>(p-value) values indicate that unfolding patterns significantly differ between mAb isotypes (Figure 7B, C). The UFS plot obtained for F(ab')<sub>2</sub> subunits pinpoints a large discriminating region between 45 and 145 V, whereas at the intact level and for Fc subunits, very few diagnostic CV regions are detected (Figure 7B). This quantitative analysis definitely corroborates that the identification of mAbs is easier based on IdeS-digested F(ab')<sub>2</sub>

regions. At this point, the differentiation of mAb subclasses has been exemplified on three samples, based on their unfolding patterns and CIU50 values. However, to screen and classify additional mAbs, an efficient categorization method cannot rely solely on these two criteria, which is particularly obvious for Fc regions for which no meaningful data can be extracted without further analysis. First, CIUSuite 2 sometimes fails to determine CIU50 values if several features co-exist, as only the most intense transition is detected (for example for states 2 and 3 of reslizumab F(ab')<sub>2</sub>, Figure 6B). In addition, intra-class differences in terms of primary sequence lead to variations in CIU50 values, and sometimes even in numbers of conformational states<sup>148</sup>, and so the subclass determination is not straightforward. A more reproducible and robust classification method is thus required.



**Figure 7.** Univariate feature selection process. **(A)** ANOVA analysis illustrated on two groups, IgGs 1 and 4, with F-test statistics calculated for each dataset in each group shown by dotted lines. **(B)** UFS plot obtained for adalimumab, panitumumab and reslizumab at the intact level (z = 22+, orange), for F(ab')<sub>2</sub> subunits (z = 21+, red) and Fc subdomains (z = 12+, blue). Vertical lines represent standard deviations. **(C)** ATDs of F(ab')<sub>2</sub> subdomains extracted at 125 V.

#### 3.3. Identification of IgG subclasses using automated classification methods

#### 3.3.1. Description of workflows used to create classification schemes

In order to clusterize mAbs from the same subclass regardless of intraclass variations, the classification module of CIUSuite 2 was used to build automated categorization methods at intact and middle levels<sup>287,403</sup>. In the following example, adalimumab (IgG1), panitumumab (IgG2) and reslizumab (IgG4) are considered as reference mAbs for F(ab')<sub>2</sub> subunits, triplicate fingerprints serving as inputs to create our in-house classification (step 1, Figure 8A).

First, the centroid of each ATD needs to be standardized at trap CV = 0 V to ensure comparison solely on unfolding patterns, irrespective of drift time variations due to different masses. Then, the UFS plot provides an overview of diagnostic regions that could be exploited for IgG identification. Although a wide range of CVs are available in this example, energies chosen for subclass determination can be fine-tuned by using cross-validation (step 2, Figure 8B). This step consists of performing a "leave one out" analysis, where each reference replicate takes a turn being the tested data to see how accurate the classification is. Cross-validation helps to assess the accuracy of categorization at each possible

number of included voltages (Figure 8B). Voltages are added to the scheme in decreasing order of scores from UFS graph: means and standard deviations are considered in our method, which implies that points with high variability will eventually score poorly even with high -log<sub>10</sub>(p-value) values. In this example, choosing only the highest scoring feature (125 V on UFS plot) would be insufficient to provide an accurate IgG identification (cross-validation accuracy = 65%) (Figure 8B). Our scheme could use two to eight voltages for optimized classification (accuracy = 100%). At equal accuracy values, the highest number of features will be favored to have a wider diagnostic CVs window that still takes into account slight intraclass variations. These eight best CVs are highlighted in grey on the UFS plot (Figure 8B). CIUSuite 2 then performs a linear discriminant analysis (LDA) based on voltages selected by the user, allowing to visualize the separation of each mAb subclass (step 3, Figure 8C). Here, the three reference mAbs are well clusterized, each represented by a specific region (IgG1 in blue, IgG2 in orange and IgG4 in pink). Poor classification parameters would result in an overlap from data across all groups. Finally, additional samples can be automatically categorized using the defined scheme based on CIU similarities. Classification results obtained from each CV for the three replicates are depicted on the LDA plot (grey, Figure 8C). In this example, the F(ab')<sub>2</sub> subdomain of trastuzumab is accurately recognized as an IgG1 with a score of  $95.3 \pm 0.7\%$ .



**Figure 8.** Workflow used to create a classification scheme. **(A)** Reference mAbs' fingerprints chosen for  $F(ab')_2$  subunits (z = 21+). **(B)** The UFS plot identifies discriminating regions between fingerprints of mAb isotypes. Best CVs for classification (in grey) are selected using cross validation. **(C)** LDA helps to better visualize how well each group clusterizes (lgG1 blue, lgG2 orange, lgG4 pink). The classification method accurately categorizes trastuzumab.

# 3.3.2. Evaluation of categorization schemes to determine IgG subclasses: the eculizumab case study

Our strategy was successfully applied to several mAbs from different subclasses (see publication 5 in appendix), among which two are presented thereafter. For intact natalizumab, results obtained still evidence similarities between IgGs 1 and 4, with 36.8% of IgG1-like behavior, even if the mAb is mostly categorized as an IgG4 (56.6  $\pm$  5.1% of IgG4, Figure 9A). Classification scores increase after Ides digestion, up to 80.3  $\pm$  3.8% for the F(ab')<sub>2</sub> subunit, ensuring unambiguous IgG4 identification. Automated classification at the middle level proved to be particularly relevant for precise characterization of hybrid mAb formats, as exemplified by eculizumab (IgG2/4). Although intact-level analysis failed to reveal the hybridicity of the mAb, the F(ab')<sub>2</sub> subdomain was identified as an IgG2 (96.9  $\pm$  0.6%) while the Fc region was found to be IgG4-like (75.8  $\pm$  0.7%) (Figure 9B). Despite very similar fingerprints obtained for Fc subunits, especially for IgGs 1 and 4, our classification method was able to differentiate between all isotypes to correctly assign the subclass of eculizumab.

Altogether, our categorization schemes offer clear-cut characterization of IgGs, with benefits of middle-level CIU further evidenced in the case of engineered hybrid mAbs. Integration of CIU into biopharmaceutical pipelines would be of utmost interest to screen mAb isotypes, allowing to complement more classically-used reversed-phase LC (rpLC-MS) or capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) that sometimes cannot fully address the complexity of last-generation mAb products<sup>149</sup>.



**Figure 9.** Classification of **(A)** natalizumab (IgG4) and **(B)** eculizumab (IgG2/4). Categorization was performed at the intact level (z = 22+), for the F(ab')2 subunit (z = 21+), and Fc subdomains (z = 12+).

#### Scientific communication

These developments have been included in a peer-reviewed article:

Botzanowski, T.; Hernandez-Alba, O.; Malissard, M.; Wagner-Rousset, E.; Deslignière, E.; Colas, O.; Haeuw, J.-F.; Beck, A.; Cianférani, S., Middle level IM-MS and CIU experiments for improved therapeutic immunoglobulin subclass fingerprinting. *Anal Chem* **2020**, *92* (13), 8827-8835, DOI: 10.1021/acs.analchem.0c00293.

# 4. Development of CIU approaches to monitor mAb conjugation process: Application to the site-specific T-GlyCLICK-DM1 ADC

# 4.1. Linear TWIMS fails to detect conformational changes upon mAb conjugation

ADCs consist of a tumor-targeting mAb covalently attached to cytotoxic drugs *via* a chemical linker. nIMS-MS has been employed to characterize second-generation<sup>152,153</sup> and site-specific<sup>17</sup> ADCs. In these studies, CCS values predicted from masses indicate that minor differences that fall within the mass error of the linear TWIMS measurement (~2%) might be observed between DAR populations<sup>263</sup>. Only slight <sup>TW</sup>CCS<sub>N2</sub> variations were indeed observed, and reported <sup>TW</sup>CCS<sub>N2</sub> measurements suggested that increases in <sup>TW</sup>CCS<sub>N2</sub> were solely related to mass increments resulting from drug binding. Again, these experiments highlight limitations of low resolution TWIMS for the separation of species with very close conformations. This was further exemplified by mAb-biotin conjugates, which exhibit only very minor CCS differences (< 2%) compared to unconjugated mAbs<sup>397</sup>. As no significant information can be deduced from <sup>TW</sup>CCS<sub>N2</sub> measurements, CIU fingerprinting represents an attractive tool to monitor conformational changes as a result of the conjugation process<sup>17,397</sup>.

#### 4.2. Synthesis of site-specific T-GlyCLICK-DM1

Early-generation ADCs rely on conjugation through cysteine or lysine residues, resulting in highly heterogeneous populations<sup>144</sup>. In order to ensure controlled position and stoichiometry of drugs, new strategies have been developed to produce more homogeneous site-specific ADCs. Among available conjugation strategies, anchoring drugs to Fc glycans located at the Asn297 residue appears as a valuable technique to synthesize homogeneous ADCs<sup>404</sup>. In this context, the GlyCLICK<sup>®</sup> technology from Genovis, based on glycan-remodeling and click chemistry, was used to generate a custom-made DAR2 ADC<sup>405</sup>. The synthesis includes three steps: deglycosylation, azide activation and click reaction (Figure 10A). N-glycans are first trimmed after the innermost N-acetylglucosamine (GlcNAc) glycan moiety. Then, the azide activation involves the addition of a N-azidoacetylgalactosamine (GalNAz) on remaining glycans to provide a specific reactive site for copper-free click reaction, with any alkyne containing payload of choice<sup>405</sup>. As the conjugation occurs on the Fc subdomain, the Fab region responsible for antigen binding is preserved, and so the immunoreactivity of the product is not impaired. Here, the cytotoxic drug conjugated to trastuzumab is a microtubulin polymerization inhibitor, mertansine, a maytansine derivative known as DM1. First, online SEC-nMS experiments carried out on each reaction products allowed to ensure the successful completion of the conjugation. Results confirm the expected avDAR of 2.0 for T-GlyCLICK-DM1, with a single homogeneous population detected (Figure 10B, see publication 3 for further information). In order to gain further insight into the bioconjugation process, we next aimed at monitoring conformational landscapes of all reaction products (from T0 to T-GlyCLICK-DM1), to explore the influence of each conjugation step on the mAb conformation. As reported for other ADCs, nIMS-MS measurements did not yield significant conformational information, as only minor mass-related <sup>TW</sup>CCS<sub>N2</sub> variations were detected (see publication 3). We thus turned to CIU approaches with the aim of observing different unfolding behaviors resulting from the conjugation process.



**Figure 10. (A)** Synthesis of T-GlyCLICK-DM1 using the GlyCLICK technology. N-glycans of trastuzumab T0 are trimmed, leading to deglycosylated trastuzumab T1, which is further azide-activated (T2) to provide specific site for final DM1 conjugation (T-GlyCLICK-DM1). **(B)** Zoom on SEC-nMS spectra of the different reaction products;  $\bullet$  = a-fucosylation (-146 Da), **\*** = glycation (+162 Da).

# 4.3. CIU to monitor gas-phase stability along the conjugation process

In order to tackle small conformational differences between compounds along the synthesis, CIU fingerprints were generated for charge states 23+ and 24+. For the 24+ charge state, the CIU pattern of glycosylated trastuzumab T0 exhibits three transitions (Figure 11A). Upon deglycosylation of trastuzumab, four conformational features are still detected for the T1 intermediate, except CIU50 values differ (Figure 11B). Although CIU50 values of the first transition remain identical for T0 and T1 (32.7 V), the second unfolding transition happens at lower energies for T1 (57.1 V) than for glycosylated T0 (66.6 V), and the third one occurs at 177.8 V for T1 but only at 192.6 V for T0. These results corroborate previous observations which highlighted that glycan trimming reduces resistance towards gas-phase unfolding compared to glycosylated samples<sup>16,398,406</sup>. HDX-MS studies pinpoint higher global deuterium uptake after endoS2 treatment<sup>407</sup>, indicating that solvent exposure increases without the

steric hindrance of intact glycans, which leads to deglycosylated mAbs being more prone to unfolding<sup>406</sup>. After the azide activation step, slightly higher energies are required to reach the first (37.7 V) and second unfolding transitions (72.7 V) for the T2 intermediate (Figure 11C). Automated CIU50 analysis fails to detect the third conformational transition related to the apparition of state 3, as only the most intense feature (state 2) is detected. Shifts in transition voltages suggest that azide-activated T2 retains a better resistance to unfolding than T0 and T1. Lastly, the conjugation of two DM1 payloads on T2 provides increased resistance towards collisional activation to the end product T-GlyCLICK-DM1, with only two transitions occurring at higher CIU50 values (42.7 and 82.4 V) than initial and intermediate products, suggesting that the click chemistry step mostly contributes to the gas-phase stabilization of T-GlyCLICK-DM1 (Figure 11D). Stabilization resulting from drug fixation is also observed on CIU fingerprints of the 23+ charge state (see supplementary data of publication 3).



**Figure 11.** CIU fingerprints acquired at the intact level (z = 24+) for **(A)** glycosylated trastuzumab T0, **(B)** deglycosylated trastuzumab T1, **(C)** azide-activated T2, and **(D)** T-GlyCLICK-DM1. CIU50 analysis (lower panel) helps to compare relative gas-phase stabilities along the synthesis.

Overall, CIU approaches offer definite advantages over standard CCS measurements, allowing to tackle small conformational variations that cannot be detected based on the sole use of IMS-MS. CIU represents a useful tool to monitor the gas-phase stability of products along ADC development. We have shown here that drug conjugation confers a better resistance to gas-phase unfolding to the mAb for the GlyCLICK synthesis. Similar conclusions were obtained for a DAR4 site-specific ADC<sup>17</sup>. It should be noted that drug binding does not necessarily stabilize the mAb, as exemplified on a model biotin-antibody conjugate<sup>397</sup>.

#### Scientific communication

This project has been published, and presented through poster communications.

# Peer-reviewed article (selected for the June 2021 cover issue)

Deslignière, E.; Ehkirch, A.; Duivelshof, B. L.; Toftevall, H.; Sjögren, J.; Guillarme, D.; D'Atri, V.; Beck, A.; Hernandez-Alba, O.; Cianférani, S., State-of-the-Art Native Mass Spectrometry and Ion Mobility 119 Methods to Monitor Homogeneous Site-Specific Antibody-Drug Conjugates Synthesis. *Pharmaceuticals* **2021**, *14* (6), 498, DOI: 10.3390/ph14060498.

#### Poster communications

<u>Deslignière, E.;</u> Hernandez-Alba, O.; Ehkirch, A.; Beck, A.; Toftevall<sup>,</sup> H.; Nordgren, M.; Sjögren<sup>,</sup> J.; Cianférani, S., In-depth Characterization of a Site-Specific Antibody-Drug Conjugate Generated Through Enzymatic Remodeling and Click Chemistry.

 Spectrométrie de Masse et Analyse Protéomique (SMAP), September 16–19<sup>th</sup> 2019, Strasbourg (France).

o Festival of Biologics, October 15–17<sup>th</sup> 2019, Basel (Switzerland).

# 5. Influence of cysteine conjugation and reduction of structuring disulfide bonds on ADC gas-phase stability

In order to get a broader overview of the effect of drug conjugation on ADCs, the next step consisted of studying additional ADCs obtained through different conjugation strategies. In particular, the focus was put on cysteine-based ADCs, for which structuring interchain disulfide bonds are reduced.

# 5.1. Trastuzumab deruxtecan (Cys-ADC)

#### 5.1.1. Cysteine-based conjugation strategy

In 2019, the U.S. FDA granted approval to trastuzumab deruxtecan (T-DXd, Enhertu<sup>®</sup>, Daiichi Sankyo/Astrazeneca) for the treatment of HER2-positive breast cancers<sup>408</sup>. Conjugation occurs on cysteine residues of trastuzumab after disulfide reduction. Interchain disulfide bonds are more likely to be reduced than intrachain ones, allowing for a controlled reduction of the four interchain disulfide bridges. Up to eight reactive thiol groups are then available for conjugation. The DXd (derivative of exatecan DX-8951) cytotoxic agent is a topoisomerase I inhibitor, bound to the mAb *via* an enzymatically cleavable maleimide GGFG peptide linker<sup>409,410</sup> (Figure 12A). Contrary to classical cysteine-based conjugation which generates heterogeneous populations with even numbers of DARs from 0 to 8, a homogeneous drug distribution was achieved with an avDAR of 8.0 (Figure 12B, C)<sup>410</sup>. Of note, T-DXd was found to be highly stable in plasma, with low levels of clearance, contradicting the wildly spread principle that high DARs are detrimental to pharmacokinetics due to faster clearance and increased aggregation<sup>410-412</sup>.

As previously described, gas-phase unfolding behaviors are mainly driven by interchain disulfide bridges<sup>16</sup>. Here, disulfide bonds between heavy chains and heavy/light chains have all been reduced and interchain interactions are entirely noncovalent, which could affect CIU patterns.



**Figure 12. (A)** Structure of trastuzumab deruxtecan T-DXd. **(B)** SEC-nMS spectrum of intact deglycosylated T-DXd (Vc = 120 V; O71171). **(C)** Deconvoluted SEC-nMS spectrum generated with UniDec v4.1.0.

#### 5.1.2. CIU experiments at intact and middle levels

We intended to assess the influence of complete interchain Cys-conjugation on CIU fingerprints by comparing deglycosylated trastuzumab and T-DXd. At the intact level, for the 22+ charge state, two conformational transitions are detected for T-DXd (47.5 and 117.2 V), while a single transition at 42.6 V is observed for trastuzumab (Figure 13A). This suggests that the absence of covalent disulfide bonds between the different chains alters the global resistance to unfolding of T-DXd compared to its parent mAb. The same destabilization effect was observed for the 21+ charge state (data not shown). In order to determine whether this shift in gas-phase stability was indeed due to modifications in the F(ab')<sub>2</sub> region upon conjugation, we moved to middle-level fingerprinting, after IdeS digestion. As all species under investigation are noncovalent, interactions between light and heavy chains are more easily broken and fragmentation occurs when increasing voltages, which makes the generation of CIU fingerprints particularly challenging for  $F(ab')_2$  subunits. Indeed, between 150 – 175 V, only the 19+ charge state of the  $F(ab')_2$  subdomain was still available (Figure 13B). For this charge state, the first transition happens at similar energies for both compounds (~37.5 V). However, the most unfolded state of T-DXd becomes the major feature at 137.6 V, while it barely starts appearing for trastuzumab (> 175 V). These results further evidence a gas-phase destabilization after disulfide bonds reduction. Conversely, for the 12+ charge state of Fc subdomains, transitions are highly similar for the ADC and its parent mAb, which corroborates the fact that decreased gas-phase resistance of T-DXd is mostly related to the absence of interchain disulfide bridges in the  $F(ab')_2$  region (Figure 13C).


**Figure 13.** CIU fingerprints and CIU50 values of deglycosylated trastuzumab *versus* deglycosylated T-DXd. Experiments were performed **(A)** at the intact level (z = 22+), **(B)** on F(ab')<sub>2</sub> subunits (z = 19+), and **(C)** on Fc subunits (z = 12+).

### 5.2. Brentuximab vedotin (Cys-ADC)

In order to strengthen our hypothesis regarding the influence of cysteine conjugation on gas-phase behaviors, we next aimed at analyzing a second cysteine-linked ADC (BV).

#### 5.2.1. Cysteine-based conjugation strategy

Brentuximab vedotin (BV, Adcetris<sup>®</sup>, Seattle Genetics) was approved in 2011 by the U.S. FDA for the treatment of Hodgkin lymphomas and systemic anaplastic large cell lymphomas<sup>413</sup>. Conjugation occurs on thiol groups of cysteine residues after reduction of the four interchain disulfide bonds. The cytotoxic inhibitor of microtubulin polymerization, monomethyl auristatin E (MMAE), is attached to the anti-CD30 lgG1 *via* a cleavable maleimide valine-citrulline dipeptide linker<sup>144</sup> (Figure 14A). BV has an avDAR of  $4.0^{152}$ , which is commonly targeted to achieve optimum efficiency. Different populations are generated, with even numbers of conjugated drugs (n = 0, 2, 4, 6, 8) resulting in a heterogeneous mixture of covalent and noncovalent species (Figure 14B-D). BV thus offers the opportunity to monitor the gas-phase stability as a function of DAR, contrary to T-DXd for which only one population can be studied.





#### 5.2.2. CIU at middle level

As the conjugation occurs on the F(ab')<sub>2</sub> region, and because F(ab')<sub>2</sub> CIU traces are more informative than intact ones, we focused on CIU fingerprints at the middle level after IdeS digestion, for different DAR populations. Again, not all DARs were available for fingerprinting because of low initial intensities and/or too easy fragmentation of subunits at high CVs due to fewer covalent interactions, especially for D6 and D8 species. Hence, fingerprints were generated for D0, D2 and D4 populations. For the 18+ charge state, D0 exhibits one transition at 42.6 V. All interchain disulfide bonds remain intact, which stabilizes the F(ab')<sub>2</sub> subunit (Figure 15A). For D2 species, the first transition occurs at 47.5 V. A second one, although very subtle, was detected at 112.6 V after fine-tuning of feature detection parameters (Figure 15B). Positional isomers cannot be resolved in linear TWIMS<sup>152</sup>, and so the obtained CIU pattern results from a combination of both covalent and noncovalent species (Figure 14B), which can explain why the effect of DAR conjugation on gas-phase behaviors is not obvious. For D4 populations, all

species are noncovalent. A new transition is observed at high voltages (172.4 V), suggesting that the reduction of additional interchain disulfide bonds has a destabilizing effect on the ADC (Figure 15C).



**Figure 15.** CIU experiments performed on the F(ab')<sub>2</sub> subdomain of deglycosylated BV. **(A-C)** CIU fingerprints and their associated CIU50 values were obtained for species corresponding to D0, D2 and D4.

Altogether, the gas-phase stability of the product is impaired as the number of payloads increases. These experiments confirm the fact that cysteine conjugation decreases the resistance to unfolding of the ADC, because of the absence of covalent disulfide bridges that are crucial to maintain the structure and stability of mAb products. These results are in good agreement with the destabilization seen for T-DXd.

#### 5.3. Trastuzumab emtansine (Lys-ADC)

In order to definitely confirm our observations related to cysteine-conjugation, we next focused on the characterization of a lysine-conjugate, which serves as a "control" sample, considering that all inter- and intrachain disulfide bonds remain intact.

#### 5.3.1. Lysine-based conjugation strategy

Trastuzumab emtansine (T-DM1, Kadcyla<sup>®</sup>, Genentech) was approved in 2013 by the U.S. FDA to treat patients with HER2-positive breasts cancers. Lysine conjugation proceeds through the formation of amide bonds between the reactive amine side chains of lysine residues and activated esters<sup>144,414</sup>. DM1 payloads are conjugated onto the lysine residues of trastuzumab *via* a noncleavable linker containing an N-hydroxysuccinimide ester (SMCC)<sup>414</sup> (Figure 16A). IgG1s contain ~90 possible sites for lysine-conjugation of T-DM1, among which 40 are most susceptible to be conjugated due to higher solvent accessibility<sup>415</sup>. Of all the ADCs studied in this chapter, T-DM1 is the most heterogeneous one, with covalent populations ranging from DAR 0 to 8, for a theoretical avDAR of 3.5 <sup>153</sup> (Figure 16B).



Figure 16. (A) Structure of T-DM1. (B) SEC-nMS spectrum of intact deglycosylated T-DM1 (Vc = 180 V; O62365); \* = linker adducts (+220 Da). (C) Deconvoluted SEC-nMS spectrum.

#### 5.3.2. CIU at intact and middle levels

CIU patterns were first obtained at the intact level for DAR species from D1 to D5. Higher DARs were not available for fingerprinting due to low intensities. As D0 populations also exhibit weak signal intensities, CIU50 values were obtained directly from the deglycosylated parent mAb trastuzumab. CIU fingerprints presented thereafter were generated without quadrupole selection to ensure straightforward comparison with previous results from T-GlyCLICK-DM1.

For the 24+ charge state, all intact species display the same number of conformational states, with three transitions occurring at identical CIU50 values ( $\Delta$ CIU50 < 5 V are not considered to be significant) (Figure 17A). These comparisons suggest that the conjugation does not stabilize nor destabilize the mAb in the gas phase, contrary to T-GlyCLICK-DM1. At the middle level, IdeS-digested F(ab')<sub>2</sub> subdomains are also highly similar between the different DAR populations. Two transitions are observed at ~33 V and ~164 V for the 20+ charge state (Figure 17B). This is in agreement with conclusions drawn for intact species, which means that lysine-conjugation in the case of T-DM1 does not influence the gas-phase stability of the mAb.

These experiments first validate the hypothesis that the destabilization seen for cysteineconjugates is indeed related to the absence of covalent interchain disulfide bonds, as no such effect was noted for lysine- (T-DM1) or glycan-based- (T-GlyCLICK-DM1) conjugation. In addition, these results illustrate that gas-phase behaviors highly depend on the conjugation strategy.



**Figure 17.** CIU experiments of deglycosylated T-DM1. **(A)** Fingerprints obtained at the intact level (z = 24+) for the parent mAb and D5 species of T-DM1. CIU50 values are given as a function of DAR. **(B)** Fingerprints were also acquired for F(ab')<sub>2</sub> subdomains (z = 20+).

#### 6. Conclusions and Perspectives

Benefits of CIU approaches to circumvent low resolution CCS measurements have been illustrated for different mAb formats. First, CIU fingerprints provide a signature pattern for each IgG isotype, especially for  $F(ab')_2$  subdomains, which allowed us to develop automated classification methods to achieve a precise identification of IgG subclasses. CIU experiments also proved to be useful to monitor mAb gas-phase behavior along the synthesis of a site-specific ADC, highlighting in this case a stabilization upon conjugation. In addition, CIU fingerprinting was of interest to evaluate the influence of drug fixation and conjugation strategies on the gas-phase stability of mAbs, helping to investigate small conformational changes when IMS-MS failed to. In particular, CIU strongly suggested that cysteine-conjugates are destabilized with increasing number of drug payloads, as a consequence of the reduction of structuring interchain disulfide bridges. However, we cannot rule out the possibility that variations in CIU patterns may be due to interferences with species of higher DARs after drug deconjugation. Similar to the charge stripping phenomenon, D(n) species resulting from D(>n)deconjugation could overlap with the actual D(n) CIU trace. In addition, though fingerprints could be obtained for most of the DARs, it would be worth recording experiments with quadrupole selection as nMS spectra of second-generation ADCs are complex, which would probably allow to access higher DAR patterns.

Other mAb-derived formats are on the rise, among which domain antibodies (nanobodies), multispecific mAbs, and antibody-protein fusion biologics that could open up new possibilities<sup>141</sup>. Indepth characterization of mAb products remains challenging, given their size, structural variations, along with multiple potential PTMs. To support the ever-growing portfolio of biotherapeutics, rapid and sensitive screening techniques are required. In this context, CIU has the potential to be part of the analytical toolbox of biopharmaceutical companies to complement more classical screening approaches. CIU data have already been shown to correlate well with unfolding patterns obtained through DSC, albeit CIU affords improved sensitivity and selectivity, appearing as a method of choice to acquire conjugation-dependent stability shift information for biotherapeutics<sup>149,397,416</sup>. Although recent software developments for easier data processing could usher in the integration of CIU into drug discovery pipelines, the true expansion of CIU into a high-throughput screening method will require the introduction of automated data acquisition techniques.

# Publication 3

State-of-the-Art Native Mass Spectrometry and Ion Mobility Methods to Monitor Homogeneous Site-Specific Antibody-Drug Conjugates Synthesis

Deslignière, E.; Ehkirch, A.; Duivelshof, B. L.; Toftevall, H.; Sjögren, J.; Guillarme, D.; D'Atri, V.; Beck, A.; Hernandez-Alba, O.; Cianférani, S.

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# Native Mass Spectrometry and Ion Mobility Methods for Site-Specific Antibody-Drug Conjugates Analysis

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## State-of-the-Art Native Mass Spectrometry and Ion Mobility Methods to Monitor Homogeneous Site-Specific Antibody-Drug Conjugates Synthesis

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Abstract: Antibody-drug conjugates (ADCs) are biotherapeutics consisting of a tumor-targeting monoclonal antibody (mAb) linked covalently to a cytotoxic drug. Early generation ADCs were predominantly obtained through non-selective conjugation methods based on lysine and cysteine residues, resulting in heterogeneous populations with varying drug-to-antibody ratios (DAR). Sitespecific conjugation is one of the current challenges in ADC development, allowing for controlled conjugation and production of homogeneous ADCs. We report here the characterization of a sitespecific DAR2 ADC generated with the GlyCLICK three-step process, which involves glycan-based enzymatic remodeling and click chemistry, using state-of-the-art native mass spectrometry (nMS) methods. The conjugation process was monitored with size exclusion chromatography coupled to nMS (SEC-nMS), which offered a straightforward identification and quantification of all reaction products, providing a direct snapshot of the ADC homogeneity. Benefits of SEC-nMS were further demonstrated for forced degradation studies, for which fragments generated upon thermal stress were clearly identified, with no deconjugation of the drug linker observed for the T-GlyGLICK-DM1 ADC. Lastly, innovative ion mobility-based collision-induced unfolding (CIU) approaches were used to assess the gas-phase behavior of compounds along the conjugation process, highlighting an increased resistance of the mAb against gas-phase unfolding upon drug conjugation. Altogether, these state-of-the-art nMS methods represent innovative approaches to investigate drug loading and distribution of last generation ADCs, their evolution during the bioconjugation process and their impact on gas-phase stabilities. We envision nMS and CIU methods to improve the conformational characterization of next generation-empowered mAb-derived products such as engineered nanobodies, bispecific ADCs or immunocytokines.

**Keywords:** native mass spectrometry; size-exclusion chromatography (SEC); ion mobility-mass spectrometry (IM-MS); collision-induced unfolding (CIU); antibody-drug conjugate (ADC); site-specific conjugation

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#### 1. Introduction

In the last decade, antibody-drug conjugates (ADC) have evolved into promising and efficient therapeutic agents for targeted chemotherapy, with 9 ADCs currently approved by the Food and Drug Administration (FDA), and more than 80 in clinical studies [1]. ADCs are generated through the conjugation of monoclonal antibodies (mAbs) which specifically target the tumor cell, with highly potent cytotoxic drug payloads. Both elements are covalently bound via a cleavable or non-cleavable chemical linker. First-generation ADCs suffered from insufficient potency of the payload or toxicity due to the instability of the ADC, leading to premature drug release [2]. Extensive development efforts led to second-generation ADCs, with more potent payloads, improved linker stability and lower levels of unconjugated mAbs [3]. Bestselling second-generation ADCs include brentuximab vedotin (BV, Adcetris® from Seattle Genetics) and trastuzumab emtansine (T-DM1, Kadcyla<sup>®</sup> from Roche) [4]. However, challenges remain for these ADCs, most notably related to product heterogeneity. Drug conjugation typically occurs through primary amines of lysine side-chains (T-DM1) or cysteine thiol groups after reduction of the interchain disulfide bonds (BV). The conjugation process results in a heterogeneous mixture of species ranging from 0 to 8 payload molecules per antibody, with average drug-to-antibody ratios (avDAR) of 3-4. Drawbacks of the second-generation ADCs include competition with unconjugated mAbs, but also fast clearance and possible aggregation of high DAR species [5,6].

Building on lessons learned from past-generations products, several strategies to produce more homogeneous site-specific ADCs with improved pharmacokinetics have been developed [3,7,8], including the addition of engineered cysteine residues at specific sites [7–11], the use of microbial transglutaminases to attach amine-containing payloads to glutamine residues in the antibody backbone, thus connecting the drug to the antibody via a stable amide linkage [12–14], and the introduction of unnatural amino acids to provide a chemical handle on their conjugation [15,16]. As an alternative, recent development of new heterobifunctional reagents for maleimide conjugations were also described to produce homogeneous site-specific ADCs [17,18]. Among the different approaches that can be used to generate homogeneous ADCs, glycan-mediated conjugation based on the Asn297 residue appears as an appealing alternative [19]. The glycan moiety contained in the Fc region of mAbs can be modified through different engineering strategies to accommodate cargo molecules and produce homogeneous site-specific ADCs [19,20]. We used this technology developed by van Geel et al. to generate a custom-made DAR2 ADC with two drugs per antibody [19]. The conjugation uses a three-step procedure, consisting of deglycosylation, azide activation and click reaction (Figure 1). Specifically, the deglycosylation step allows the glycans to be trimmed after the innermost N-acetylglucosamine (GlcNAc) glycan moiety. Then, the addition of an N-azidoacetylgalactosamine (GalNAz) is performed through the azide activation step. As result, the azido-modified glycans become site-specifically reactive for copper-free click reaction with any alkyne containing payload of choice. By applying this strategy, the drug stoichiometry is controlled in a sitespecific manner and localized on the Fc region of the mAb, while the antibody-binding region (Fab) is preserved, and thus, minimal influence on the immunoreactivity is expected.



**Figure 1.** Schematic overview of the bioconjugation protocol. N-glycans remodeling of trastuzumab (T0) through deglycosylation, azide activation and click-chemistry, generating deglycosylated trastuzumab (T1), azide-activated trastuzumab (T2) and T-GlyCLICK-DM1, respectively.

The development and optimization of ADCs involve in-depth analytical and bioanalytical characterization along the production process, to monitor several critical quality attributes, such as the drug load distribution (DLD), the amount of unconjugated antibody (D0), the avDAR ratio and the presence of size variants [21,22]. State-of-the-art approaches for ADC analysis comprise chromatographic, electrophoretic and mass spectrometric techniques [21,23]. Among them, native mass spectrometry (nMS), which retains noncovalent assemblies, has now entered into R&D laboratories. Valliere-Douglass et al. first highlighted the benefits of nMS for intact mass measurement and relative distribution of drug-loaded species in the case of cysteinyl-linked ADCs [24]. Chen et al. described successful use of nanoESI instead of conventional ESI for cysteine-linked ADCs after proteolytic drug removal [25]. Online coupling of size exclusion chromatography (SEC) to nMS was then implemented by different groups for the analysis of mAbs and ADCs [26–30], paving the way for routine integration of nMS in high throughput analytical workflows of biopharmaceutical companies. An additional level of separation can be achieved through ion mobility spectrometry coupled to nMS (nIM-MS), which provides conformational characterization in the gas phase. nIM-MS was employed for the direct determination of distribution profiles and avDAR values of second-generation ADCs, BV and T-DM1 [31,32]. Although the DAR calculation based on nIM-MS results is not as straightforward as from nMS data, the overall drift time of ADC species obtained from nIM-MS analysis allows DAR comparison in a rapid manner. Drug binding can also be assessed by measuring collision cross sections (CCS), which correspond to the momentum transfer between ion and gas particles, and represent the effective area of ions interacting with the buffer gas [30-32]. However, nIM-MS sometimes fails to separate co-drifting species with closely related conformations due to its low resolution, as exemplified by mAb-biotin conjugates, which exhibit only very minor CCS differences (<2%) compared to unconjugated mAbs [33]. IM-based collision-induced unfolding (CIU) approaches have proved to be efficient to circumvent poor linear travelling-wave IM (TWIMS) resolution, offering further insight into gas-phase behavior upon ion activation in the instrument trap cell [34]. Destabilization of biotinylated model ADCs was detected with CIU even for low amounts

of conjugated biotin, highlighting the potential of CIU to tackle small conformational changes between the ADC and its parent mAb [33]. Another study performed on a site-specific DAR4 ADC evidenced increased resistance to gas-phase unfolding of the ADC compared to its unconjugated counterpart mAb [30]. Few papers have reported the characterization of ADCs using CIU, most likely because of the heterogeneity of early-generation ADCs, yet this technique can provide valuable information to evaluate the gas-phase stabilization or destabilization along the conjugation process.

We highlight in this study the potential of last generation cutting edge nMS and IM methodologies for the characterization a customized DAR2 trastuzumab-GlyCLICK-DM1 (T-GlyCLICK-DM1) generated through glycan-based enzymatic remodeling and click chemistry. SEC-nMS allows thorough identification and quantification of the different species involved either during the synthesis or in the context of forced degradation studies. Innovative IM-based CIU approaches were used to monitor the modifications in the unfolding pattern of the different conjugational intermediates isolated during T-GlyCLICK-DM1 formation. The combination of SEC-nMS and gas-phase CIU experiments provided better characterization of ADCs, affording new techniques to monitor the binding, gas-phase and conformational stabilities of the different intermediates during the conjugation process (Figure 2). IM-based CIU experiments presented in this work allow to broaden the scope of analytical information available for ADCs physicochemical characterization, from the basic assessment of the number of payloads and the drug-load distribution (SEC-nMS) to gas-phase conformational behavior. We propose here SEC-nMS and IM-based CIU methods as innovative analytical techniques, complementary to more classical biophysical techniques already implemented in most R&D laboratories, to improve the conformational characterization of next-generation empowered ADCs.



Figure 2. Analytical workflow used to monitor the conjugation of T-GlyCLICK-DM1.

#### 2. Results and Discussion

#### 2.1. Online SEC-nMS to Monitor the Conjugation Process

We first investigated the initial (T0), intermediate (T1 and T2) and end (T-GlyCLICK-DM1) products using SEC-nMS, a methodology particularly well-suited not only for fast desalting of mAbs products, but also for size variant identification and quantification [27,35] (Figure 3, Table 1).

For the initial T0 compound, monomeric trastuzumab (>99.5% based on the SEC-UV chromatogram) was detected as the main compound with its glycoforms by SEC-nMS, along with the presence of very low amounts of high-molecular weight species (HMWS, peak I, Figure 3A), in agreement with previously published trastuzumab SEC-nMS analyses (Figure 3) [27]. The first step led to the formation of a main product T1 corresponding to deglycosylated trastuzumab, bearing hallmarks of the deglycosylation process through core fucose and GlcNac residues (+349 Da on each HC). Minor species corresponding to T1, with one of its heavy chain (HC) having only one GlcNac moiety attached (+203 Da), and to glycation of the T1 intermediate (+162 Da) were also detected (Figure 3B). The azide

activation leads to the conversion of the triplet peak into another triplet (T2), with a mass increase of +244 Da on each HC (Figure 3B). Several additional low-molecular weight species (LMWS) were detected by SEC-UV, suggesting that the azide activation step slightly affects the stability of the mAb and forms higher amounts of LMWS, including Fc-Fab (peak III) or LC, Fd and Fab fragments (peak IV) (Figure 3A, Table 1). Finally, drug conjugation was monitored in the last step, ending up with a homogeneous peak with a mass of 148,957 ± 1 Da, corresponding to the binding of two DM1 molecules (one on each HC). T-GlyCLICK-DM1 exhibits a single avDAR2 population in agreement with the site-specific glycan-based conjugation, resulting in a straightforward SEC-nMS spectrum contrary to the highly heterogeneous and complex T-DM1 spectrum with species from D0 to D8 (Figure S1A,B). Of note, lower amounts of LMWS were obtained for the final T-GlyCLICK-DM1 product compared to azide-activated T2, with LC, Fd or Fab fragments that were not observed on the chromatogram, but still detected by nMS which has a higher sensitivity than SEC-UV.

Altogether, the mass accuracy of nMS combined with SEC separation allowed to unambiguously identify and quantify all products, highlighting the versatility of SEC-nMS for ADC analysis.



**Figure 3.** Online SEC-nMS analysis of initial (T0), intermediate (T1 and T2) and end (T-GlyCLICK-DM1) conjugation products. (**A**) Zoom on SEC-UV chromatograms at 280 nm; I = HMW dimers, II = main product, III = Fc-Fab fragments and IV = LC, Fd and Fab fragments. (**B**) Zoom on SEC-nMS spectra obtained for the different main products; • = a-fucosylation (-146 Da), **\*** = glycation (+162 Da).

**Table 1.** Masses of species detected with SEC-nMS. Relative quantification of the different species was assessed based on SEC-UV signals. \* ND = mass not determined because of very low intensities on the nMS spectrum.

	Τ0	T1	T2		T-GlyCLICK-DM1			
	99.6%	99.7%	96.6%		98.5%			
-	G0F/G0 147,930 ± 4 Da	_						
Main	(G0F)2 148,067 ± 4 Da	_	146,372 ± 2 Da			148,957 ± 1 Da		
Product	G1F/G0F 148,228 ± 2 Da	145,875 ± 1 Da						
_	(G1F)2 148,387 ± 2 Da							
	G2F/G1F 148,548 ± 1 Da							
HMW	0.4%	0.3%	0.5%		0.2%			
Dimers	296,828 ± 25 Da	291,719 ± 25 Da		292,911 ± 23 Da		ND *		
LMWS			2.7%	Fc-Fab	99,319 ± 6 Da	1.3%	Fc-Fab	101,910 ± 8 Da
				LC	23,473 ± 2 Da		LC	23,474 ± 3 Da
	-	-	0.2%	Fd	23,618 ± 4 Da	<0.1%	Fd	23,615 ± 3 Da
				Fab	47,129 ± 9 Da		Fab	47,091 ± 8 Da

#### 2.2. Forced Degradation Studies

To evaluate the stability of the T-GlyCLICK-DM1 product, we performed forced degradation studies at high-temperature (50 °C) for 15 days followed by SEC-nMS analysis [36].

Forced degradation studies of the final T-GlyCLICK-DM1 product revealed four main peaks on the SEC chromatogram (Figure 4A). Two main species were observed on the MS spectrum of peak II. The intact T-GlyCLICK-DM1 degraded upon thermal stress, resulting in two species with masses of 148,415 ± 10 Da (–545 Da compared to the intact product) and 147,882 ± 9 Da (–1078 Da) (Figure 4B). As no mass shifts were observed for T0, T1 and T2 after thermal stress, these two degradation products most likely correspond to the loss of maytansinol after ester hydrolysis within the DM1 drug (–548 Da) [37]. Similarly, losses of –560 and –1111 Da were detected on Fc-Fab fragments (peak III, Figure 4B). No deconjugation was observed on T-GlyCLICK-DM1, as minor species still correspond to DAR2.

Previous thermal stress studies performed on mAbs have evidenced the formation of LMWS, which result mainly from fragmentation in the hinge region, and formation of HMW aggregates [36,38]. While the aggregation and hinge-fragmentation of therapeutic mAbs have been extensively studied [39–41], only few papers have dealt with stressed ADCs, focusing mainly on their aggregation, but lacking a detailed characterization of LMWS [42,43]. Wakankar et al. showed, using SEC analysis, that T-DM1 was more prone to aggregation than unconjugated trastuzumab, which was further emphasized after storage at 40 °C for 70 days [44]. Temperature-induced aggregation as a function of increasing DAR was also examined for a cysteine-linked ADC, highlighting that high DAR species were far more likely to form aggregates under stressed conditions [45].

For the GlyCLICK conjugation process, higher amounts of HMWS and LMWS are generated for the initial, intermediate and final reaction products upon thermal stress. Additional LMWS (peak IV) corresponding to LC, Fab and Fd fragments that were not observed on the SEC-UV chromatograms of non-stressed samples (expect for T2, Figure 3) were detected (Figures 4A and S2). In particular, for T-GlyCLICK-DM1 (Figure 4), an increased amount of HMWS corresponding to dimers was detected for the thermally-stressed sample compared to the non-stressed one (peak I, 4.6 vs. 0.2%, respectively). Regarding LMWS, the fraction of Fc-Fab species (peak III) significantly increased upon thermal stress (+9.1%), and a substantial amount of Fab, LC and Fd fragments could be observed (peak IV, 4.6%).

Of note, different species were identified as Fab fragments, with a ladder of cleavage sites on the HC upper hinge sequence C<sup>223</sup>/D/K/T/H/T/C<sup>229</sup>, as already reported for IgG1 mAbs [40,41]. These Fab fragments have been described as a result of direct hydrolysis of peptide bonds, or radical transfer between the aforementioned residues [46–48]. Other LMW species detected within peak IV include a Asp<sup>1</sup>-Glu<sup>213</sup> LC fragment and a Glu<sup>1</sup>-Ser<sup>222</sup> Fd fragment, generated after cleavage of the HC-LC disulfide bond. The scission of the Cys<sup>223</sup>-Cys<sup>214</sup> bond can occur either via  $\beta$ -elimination [48] or via a radical reaction mechanism [47]. The presence of sulfurized cysteines following the disruption of the Cys<sup>223</sup>-Cys<sup>214</sup> bond was also previously demonstrated (+32 Da, Figure 4B) [47]. These different cleavage products were observed for all products of the GlyCLICK reaction (Figure S2). The amount of LMW cleavage products was significantly enhanced under thermal stress; however, some species were also detected for non-stressed T2 (Table 1). Interestingly, no deconjugation of the drug linker was detected, as DAR2 species are mostly detected on intact T-GlyCLICK-DM1.

Overall, SEC-nMS allows to monitor the formation of HMW aggregates and LMW hinge-related species for all our reaction compounds subjected to thermal stress conditions. Upon thermal stress, the final T-GlyCLICK-DM1 produces higher amounts of HMWS (+4.4% compared to the non-stressed sample) and LMWS (+13.6%) than the initial product T0 (+1.2% for HMWS and +9.3% for LMWS). T-DM1 exhibits higher resistance to thermally induced fragmentation (+2.4% of LMWS) compared to T-GlyCLICK-DM1 and

T0, but is more prone to aggregation (+15.0%), in agreement with conclusions published on unconjugated trastuzumab vs. T-DM1 using SEC-UV analysis [44,49] (Figure S1A). However, SEC as a standalone technique does not provide sufficient information on the nature of the degradation products. Our results show a clear benefit of the SEC-nMS coupling, which offers both quantification and identification of fragments in a straightforward way, within a single run.



**Figure 4.** Online SEC-nMS analysis of thermally-stressed T-GlyCLICK-DM1. (**A**) Overlaid SEC chromatograms of stressed (solid line) and non-stressed (dotted line) samples. Relative amounts of HMWS and LMWS are given for the stressed sample; I = HMW dimers, II = main product, III = Fc-Fab fragments and IV = LC, Fd and Fab fragments. (**B**) SEC-nMS spectra of species generated upon thermal stress.  $\diamond$  = sulfurized Cys<sup>214</sup> (+32 Da compared to LC Asp<sup>1</sup>-Cys<sup>214</sup>).

#### 2.3. nIM-MS to Monitor the Conformational Landscape during the Conjugation Process

We next used IM-based methodologies to investigate conformational changes upon the drug conjugation process.

We first performed a <sup>TW</sup>CCS<sub>N2</sub> calculation on both intact and IdeS-digested conjugation compounds (Table S1). Based on mass-derived CCS predictions of intact products, only very slight differences (<1.3%) that fall within the mass error of the IM measurement (2%) might be observed between all species under investigation. Indeed, at the intact level, differences in <sup>TW</sup>CCS<sub>N2</sub> were between 0.3 and 1.2% for the 23+ charge state. Middle-up level measurements provide slightly higher <sup>TW</sup>CCS<sub>N2</sub> variations for the Fc fragment (between 0.6 and 4.2%), which correspond to mass-related differences. <sup>TW</sup>CCS<sub>N2</sub> values obtained for the F(ab')<sub>2</sub> subdomain was similar for all products, as conjugation sites are located on Fc fragments. Altogether, these results suggest that the chemical conjugation process does not drastically affect the overall global conformation of the mAb. However, drawing clear-cut conclusions solely from nIM-MS measurement for mAbs with very close conformations remains challenging at both intact and middle-up levels due to the low resolution of linear TWIMS [50]. The preliminary CCS measurements are the rationale for performing further CIU experiments, as an alternative to tackle small conformational variations that would result in differences in CIU patterns.

CIU experiments were then performed on two different charge states (24+ and 23+) of the reaction products obtained along the drug conjugation process, with the aim to end up with different unfolding patterns. CIU patterns of T0 to T-GlyCLICK-DM1 are represented in Figure 5. For the 24+ charge state, the CIU fingerprint of glycosylated trastuzumab T0 reveals three unfolding transitions (four conformational states) in the 0-200 V range (Figure 5A). After the first deglycosylation step, three transitions are still detected for the T1 intermediate (Figure 5B). While the first one occurs at the same voltage for T0 and T1 (32.7 V), the second transition exhibits lower CIU50 values for T1 (57.1 V) than for glycosylated T0 (66.6 V) and the third transition happens at 177.8 V for T1, but only at 192.6 V for T0. As previously reported using CIU experiments [51,52], these results indicate that deglycosylated trastuzumab T1 is more prone to unfolding than its glycosylated counterpart, also in agreement with hydrogen-deuterium exchange (HDX) data showing increased deuterium uptake after EndoS2 deglycosylation [52]. Upon azide activation, the CIU fingerprint still looks very similar, but with slightly higher CIU50 values for the first and second transitions (37.7 and 72.7 V, respectively) (Figure 5C). The third transition (at high voltages) is not detected for T2 using automated CIU50 analysis, as the most unfolded state only starts appearing, with state 2 remaining the most intense feature until 200 V. CIU50 values suggest that the conformational states of azide-activated T2 are more resistant towards unfolding than T0 and T1, in favor of a gas-phase stabilization just before the click chemistry reaction. Finally, the conjugation of the DM1 drug on T2 confers a better gas-phase resistance to unfolding to the end product T-GlyCLICK-DM1, with two conformational transitions occurring at higher CIU50 values (42.7 and 82.4 V) than the other reaction compounds, suggesting that the click chemistry step mostly contributes to the increased resistance to unfolding of T-GlyCLICK-DM1 (Figure 5D). Similarly, CIU fingerprints of the 23+ charge state illustrate the improved stability towards unfolding of the final product compared to T0, T1 and T2 (Figure S3).

Altogether, these results highlight that drug conjugation reinforces the overall stability of the mAb towards gas-phase unfolding, as already reported for a DAR4 site-specific ADC [30].



**Figure 5.** CIU experiments at the intact level for the 24+ charge state. CIU fingerprints (upper panel) and CIU50 analysis (lower panel) were acquired to compare the resistance to gas-phase unfolding of the reaction compounds (**A**) T0, (**B**) T1, (**C**) T2 and (**D**) T-GlyCLICK-DM1.

#### 3. Materials and Methods

#### 3.1. Sample Preparation

T-DM1 was N-deglycosylated by incubating one unit of IgGZERO (Genovis, Lund, Sweden) per microgram of ADC for 30 min at 37 °C. For middle-up nIM-MS level experiments, IdeS digestion was performed by incubating one unit of FabRICATOR enzyme (Genovis, Lund, Sweden) per microgram of mAb or ADC for 60 min at 37 °C.

#### 3.2. Manual Buffer Exchange

Prior to nIM-MS, products T0, T1, T2 and T-GlyCLICK-DM1 were desalted against 100 mM ammonium acetate (pH 6.9), using eight cycles of centrifugal concentrator with 10 and 50 kDa cutoffs for IdeS-digested and intact mAbs, respectively (Vivaspin, Sartorius, Göttingen, Germany). Protein concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Graffenstaden, France). Each solution was diluted in 100 mM ammonium acetate at pH 6.9 to 10  $\mu$ M prior to nIM-MS and CIU acquisitions.

#### 3.3. Online SEC-nMS

An Acquity UPLC H-class system (Waters, Wilmslow, UK) composed of 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 coupled to a Synapt G2 HDMS mass spectrometer (Waters, Wilmslow, UK) for online SEC-nMS experiments. The SEC column used was an Acquity BEH SEC 200 Å, 1.7  $\mu$ m, 4.6 × 150 mm (Waters). The separation was carried out in isocratic mode with a 100 mM AcONH<sub>4</sub> mobile phase at pH 6.9. The Synapt G2 was operated in positive ionization mode with a capillary voltage of 3 kV and a sample cone voltage of 180 V. The backing pressure of the Z-Spray source was set to 6 mbar. Acquisitions were performed in the 1000–10,000 *m*/*z* range. External calibration was performed using singly charged ions produced by a 2 g/L solution of cesium iodide in 2-propanol/water (50/50 *v*/*v*). SEC-nMS data interpretations were performed using MassLynx v4.1 (Waters, Manchester, UK).

#### 3.4. nIM-MS and CIU Experiments

The Synapt G2 HDMS was coupled to the automated chip-based nanoESI device (TriVersa NanoMate, Advion, Ithaca, USA). The cone voltage of the Synapt G2 was fixed to 80 V to avoid in-source ion activation while ensuring ion transmission. The backing pressure was 6 mbar. The argon flow rate was set to 5 mL/min. Ions were focused in the helium cell (120 mL/min), prior to IM separation. The N<sub>2</sub> flow rate in the IM cell was 60 mL/min. The wave height and velocity were fixed to 40 V and 850 m/s, respectively. Drift times were converted into CCS values using avidin (for middle-up level data), concanavalin A, alcohol dehydrogenase and pyruvate kinase (for intact-level data) as external calibrants [53,54]. ATDs were extracted using MassLynx v4.1.

CIU experiments were carried out by increasing the collision voltage in the trap cell from 0 to 200 V using steps of 5 V. CIU data were processed using the CIUSuite 2 v2.2 software [55]. ATDs were smoothed using a Savitsky-Golay algorithm with a window length of 5 and a polynomial order of 2. CIU acquisitions were performed in triplicate to generate averaged CIU fingerprints with their associated RMSD using the 'Basic Analysis' module of the CIUSuite 2 software. RMSDs under 15% between technical replicates account for a good reproducibility of CIU data (Table S2). CIU50 values, which allow to quantitatively assess unfolding transitions, were determined with the 'Stability Analysis' module.

#### 4. Conclusions

This study clearly highlights the benefits of using innovative nMS and IM methodologies for the analytical characterization of ADCproducts. In the present work, a customized homogeneous site-specific ADC generated through glycan-based enzymatic remodeling and click chemistry was used as a case study.

First, the combination of SEC with nMS was found to be particularly well suited to monitor the ADC conjugation process. Indeed, thanks to an excellent mass accuracy and sensitivity, the characterization and quantification of the different reaction products (intermediates) obtained during the drug conjugation process were easily assessed. SEC-nMS was also found to be relevant in forced degradation studies, for the simultaneous identification and quantification of LMWS and HMWS within the same run. Indeed, upon thermal stress, several HMWS and LMWS were produced and clearly identified with SEC-nMS. With the site-specific ADC product investigated in this work, no deconjugation of the drug linker was detected. The SEC-nMS data emphasize the importance of the technique to accurately characterize the drug form and bioconjugation intermediates prior to moving on to in vivo studies. Based on its noticeable advantages, SEC-nMS is expected to soon become a standard in R&D biopharmaceutical laboratories [27].

Next, IM-based methodologies were used to investigate conformational changes upon the drug conjugation process. Even if CCS measurements are not highly informative on intact ADCs nor subunits obtained after protease treatment, results suggest showed that the chemical conjugation process does not drastically affect the overall global conformation of the mAb. However, drawing clear conclusions solely from CCS values was difficult due to the low resolution of linear TWIMS. Therefore, advanced innovative CIU experiments were performed to compare the resistance to gas-phase unfolding of the different intermediates observed during the conjugation process. Based on the unfolding patterns, it was possible to conclude that the drug conjugation improves the overall stability of the mAb against gas-phase unfolding, allowing to circumvent limitations of CCS measurements for mAb-based products. These results demonstrate that CIU approaches offer clear benefits over standard nIM-MS experiments to detect subtle conformational differences that translate into different CIU patterns. In addition, CIU data have been reported to correlate with unfolding patterns observed using differential scanning calorimetry (DSC), suggesting a solution-phase memory effect of mAbs products in the gas phase [33,50]. CIU offers significant benefits over DSC, with improved sensitivity and selectivity, and thus, appears as an appealing approach to acquire conjugation-dependent gas-phase stability shift information for biotherapeutics.

**Supplementary Materials:** The following are available online at www.mdpi.com/1424-8247/14/6/498/s1, Figure S1: Online SEC-nMS analysis of T-DM1, Figure S2: Online SEC-nMS analysis of T0, T1 and T2 after thermal stress, Table S1: TWCCSN2 measurements of intact and IdeS-digested reaction products, Figure S3: CIU experiments at the intact level for the 23+ charge state, Table S2: RMSDs between triplicates for CIU fingerprints at the intact level for 23+ and 24+ charge states.

**Author Contributions:** Conceptualization and methodology, S.C., D.G., J.S. and A.B.; formal analysis and investigation, E.D., A.E. and O.H.-A.; resources, H.T. and J.S.; writing—original draft preparation, E.D. and S.C.; writing—review and editing, E.D., S.C., O.H.-A., A.B., D.G., V.D., B.L.D. and J.S.; supervision, S.C.; funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript.

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### SUPPLEMENTARY INFORMATION

# State-of-the-Art Native Mass Spectrometry and Ion Mobility Methods to Monitor Homogeneous Site-Specific Antibody-Drug Conjugates Synthesis.

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- **Table S2:** RMSDs between technical triplicates for CIU fingerprints at the intact level for 23+ and 24+ charge states.



**Figure S1.** Online SEC-nMS analysis of T-DM1. (A) Overlaid SEC chromatograms of stressed (solid line) and nonstressed (dotted line, grey) samples. (B) SEC-nMS spectrum of intact non-stressed T-DM1 with corresponding UV chromatogram (280 nm) depicted in inset; **\*** = linker adducts (+220 Da). (C) SEC-nMS spectrum of thermally-stressed T-DM1.





**Table S1.** <sup>TW</sup>CCS<sub>N2</sub> measurements of intact and IdeS-digested reaction products. <sup>¶</sup> Mass-based estimation of CCS, CCS =  $2.435 \times MW^{2/3}$  according to Ruotolo *et al.* (*Nat Protoc* **2008**, 3(7), 1139-1152).

		™CCS <sub>N2</sub> (nm²)					
		то	T1	T2	T-GlyCLICK-DM1		
	Predicted <sup>¶</sup>	68.1	67.5	67.6	68.4		
Intact	23+	73.8 ± 0.2	73.4 ± 0.2	73.6 ± 0.2	74.3 ± 0.2		
	24+	75.3 ± 0.2	74.9 ± 0.1	75.1 ± 0.1	75.9 ± 0.1		
Fa	Predicted <sup>¶</sup>	33.3	32.3	32.5	33.6		
fragment	12+	$34.1 \pm 0.1$	33.2 ± 0.1	$33.4 \pm 0.1$	34.6 ± 0.2		
nuginent	13+	35.2 ± 0.1	$34.4 \pm 0.1$	34.7 ± 0.1	$35.9 \pm 0.1$		
F(ab')	Predicted <sup>¶</sup>	51.6	51.6	51.6	51.6		
fragment	20+	56.8 ± 0.1	56.8 ± 0.1	56.6 ± 0.2	57.0 ± 0.1		
oment	21+	58.2 ± 0.1	58.2 ± 0.1	58.0 ± 0.2	58.2 ± 0.1		



**Figure S3.** CIU experiments at the intact level for the 23+ charge state. CIU fingerprints (upper panel) and CIU50 analysis (lower panel) were acquired to compare the resistance to gas-phase unfolding of the reaction compounds (A) T0, (B) T1, (C) T2 and (D) T-GlyCLICK-DM1.

Table S2. RMSDs between technical triplicates for CIU fingerprints at the intact level for 23+ and 24+ charge states.

	RMSD between technical replicates (n = 3)						
	то	T1	T2	T-GlyCLICK-DM1			
23+	13.3	7.3	11.2	8.4			
24+	9.7	5.1	11.2	7.9			

## Chapter 3 – Coupling of SEC to CIU

#### 1. Analytical context

Although many biopharmaceutical companies have now integrated nMS into their discovery pipelines, the introduction of CIU into R&D laboratories remains scarce<sup>417</sup>, mostly because of the level of expertise required for nIMS-MS and CIU experiments, but also because of a lack of automation of the overall procedure. Indeed, while recent software developments have significantly helped to ease and automate data interpretation<sup>418</sup>, buffer exchange and CIU data acquisition still lack in automation, hampering the routine use of CIU in an industrial environment.

CVs can be ramped either manually or in a semi-automatic way, using a pre-programmed sample list for sequential acquisition, resulting in a laborious and error-prone process. In addition, CIU experiments are carried out through direct injection in nanoESI mode, using either automated chipbased devices<sup>17,148,149,171</sup> or glass capillary nanoemitters<sup>15,159,168,174</sup>. However, the nanoelectrospray may suffer from instabilities along the CV ramp due to nozzle or capillary clogging<sup>26</sup>, leading to a tedious process for the operator. Altogether, the classical CIU workflow remains highly time-consuming, as it requires approximately three hours for one sample, ranging from sample preparation (manual desalting) to data acquisition (from 0 to 200 V, in triplicate, with 10 V steps).

Very few solutions have been proposed to increase CIU throughput. ORIGAMI<sup>MS</sup>, an open-source software dedicated to TWIMS instruments from Waters, offers automated data acquisition, albeit manual buffer exchange is still needed<sup>285</sup>. Another strategy suggested by Vallejo *et al.* consists of focusing only on median voltages of CIU transitions and features for mAb classification purposes, although complete fingerprints have to be established first to target CVs of interest<sup>164</sup>.

In this context, the hyphenation of SEC to nIMS-MS is highly appealing to automate the CIU pipeline and promote its wide adoption, as it affords a fast and efficient desalting, leading to a significant reduction of the overall time process<sup>289</sup>. As online SEC coupling provides a controlled continuous flowrate and maintains a stable electrospray, CIU acquisition can be synchronized with the sample elution.

#### 2. Objectives

The aim of this work is to automate CIU approaches from sample preparation to data interpretation using the coupling of SEC to nIMS-MS (SEC-CIU). The first objective was to evaluate the feasibility of this new coupling by comparing the obtained fingerprints to those generated with regular nanoESI-CIU experiments. Then, in order to fully exploit the potential of SEC-CIU, high-throughput strategies were developed for mAb classification at intact and middle levels.

#### 3. Development of SEC-CIU

#### 3.1. Optimization of SEC and CIU parameters

Since data acquisition is performed along the sample elution, the SEC flowrate, which is generally comprised between 0.100 - 0.250 mL/min for therapeutic mAbs<sup>289</sup>, was slowed down to broaden chromatographic peaks and maximize the number of CV steps contained within a run. The flowrate was set to 0.035 mL/min, as it affords the best compromise between nIMS-MS signal intensities, S/N ratio, and CIU parameters (number of scans and steps, scan time) (Figure 18A). Then, the selection of the SEC column represents a critical step to implement high-throughput SEC-CIU experiments. As the use of short columns does not impair the resolution of fingerprints despite a reduced number of CIU scans compared to longer SEC columns (Acquity BEH 200 Å, 4.6 x 300 mm, Waters), shorter columns with reduced pore size (125 Å, 4.6 x 30 mm) were preferred for fast mAb desalting<sup>289</sup> (< 5 min) (Figure 18B). An additional benefit of 30 mm columns lies in the fact that mAbs all exhibit the same retention time, leading to a unique SEC-CIU acquisition method which facilitates rapid mAb screening.

Next, CIU acquisition slots, referred to as nIMS-MS functions, were aligned with the sample elution (~2 min with enough nIMS-MS intensity for intact mAbs) for automated data acquisition using MassLynx software (Figure 18A). Several key points should be considered for the optimization of SEC-CIU methods:

- First, at least two or three chromatographic runs are required to generate a complete fingerprint (0 - 200 V), as the number of functions within each file is limited. SEC-CIU were carried out using 10 V increments, which provided well-resolved fingerprints for mAb classification while avoiding the multiplication of SEC runs. A method based on three runs (seven CV steps for each run, Figure 18A) was ultimately favored to guarantee a higher number of scans for improved definition of CIU fingerprints compared to those obtained with two runs. If needed, an additional run with specific CVs can help to target and better resolve features appearing only in a narrow voltage range or highly populated regions.

- CVs are generally ramped with increasing values, although it could be argued that extra activation needed to remove salts adducts that may be present in the peak tail might distort the CIU plots. The comparison of CIU fingerprints obtained by stepping the voltages either up or down resulted in RMSD < 6 %, highlighting the robustness of the SEC-CIU approach (Figure 18C).</p>

Altogether, the combination of efficient high-throughput desalting and automated data collection offered by SEC-CIU substantially shortens the overall time process of CIU experiments, saving around two hours (depending on the desalting device used) compared to nanoESI-CIU experiments with manual buffer exchange. In addition, the SEC-CIU setup yields RMSDs < 10% between technical replicates (n = 3) of a fingerprint, in line with U.S FDA recommendations<sup>288</sup>, and similar to values obtained with nanoESI-CIU.



**Figure 18. (A)** Acquisition of SEC-CIU data for intact mAbs. In this case, a fingerprint replicate is acquired in three runs. Datasets are combined to generate an averaged (n = 3) fingerprint, as depicted for the 27+ charge state of nivolumab. **(B)** Fingerprint generated on a 300 mm SEC column, and in comparison with 30 mm SEC column results. **(C)** Fingerprint obtained with decreasing CVs, compared to one acquired with an increasing CV ramp. Both datasets were acquired on a 30 mm column.

#### 3.2. Proof-of-concept through comparison with nanoESI- and ESI-CIU fingerprints

A preliminary study consisted of ensuring that the coupling of SEC to CIU maintains key features of CIU unfolding patterns, that is, number of transitions and their associated conformational states. The analysis of intact adalimumab (IgG1), serves as a proof-of-concept to validate the SEC-CIU setup.

Among SEC-ESI-CIU parameters that could influence unfolding plots, the temperature of the desolvation gas within the ESI Z-spray source was first evaluated. Since mAbs are highly stable proteins, SEC-nMS analyses can be performed under quite harsh desolvation temperatures to achieve more efficient desolvation, with subsequent better mass accuracy<sup>17,289</sup>. However, great care must be taken to guarantee that additional heating does not result in ion pre-activation. As depicted in Figure 19A, SEC-ESI-CIU fingerprints generated at desolvation gas temperature of 150°C *versus* 450°C present strong similarities in terms of conformational states and transitions, with RMSD ~7% for the 28+ charge state of intact adalimumab. These observations were confirmed on different mAbs from distinct IgG subclasses (see supplementary data of publication 4). These first experiments demonstrate that high desolvation gas temperatures do not affect SEC-ESI-CIU plots, as only relative intensities between the different conformational states may vary (Figure 19B).

SEC-ESI-CIU fingerprints were then compared to those obtained with nanoESI-CIU experiments. As the desolvation gas is not heated in nanoESI-CIU experiments when using the TriVersa NanoMate robot, a first comparison of ground states corresponding to CV = 0 V allowed to confirm that initial conformations are very close ( $\Delta^{TW}CCS_{N2} < 1\%$ ): the main conformer for the 26+ charge state of

adalimumab exhibits a <sup>TW</sup>CCS<sub>N2</sub> value of 77.3  $\pm$  0.1 nm<sup>2</sup> in nanoESI mode *versus* 77.5  $\pm$  0.1 nm<sup>2</sup> with SEC-ESI. In addition, both fingerprints display the same activated conformational states and number of unfolding transitions (Figure 19C, D). Slight differences in terms of relative intensities between co-existing features were observed, leading to an apparent translation towards lower CVs for SEC-ESI-CIU (RMSD ~15%, Figure 19C). For example, the most unfolded state (state 4) becomes the main feature at 150 V for SEC-CIU *versus* 190 V for nanoESI-CIU. In order to determine whether these minor variations could be related to the SEC column, an additional fingerprint was recorded using direct ESI injection under the same conditions as SEC-ESI-CIU, except for the flowrate. Both SEC-ESI and ESI conditions result in highly similar CIU fingerprints (RMSD ~ 7%), which corroborates the fact that the coupling of SEC to CIU does not strongly alter mAb CIU plots (Figure 19C, D).



**Figure 19. (A)** SEC-CIU fingerprints and differential plot of intact adalimumab (z = 28+) at desolvation gas temperatures of 450 and 150 °C. **(B)** ATDs (grey dots) of the 28+ charge state were extracted at CV = 0 V for both temperatures. Gaussian fitting performed with CIUSuite 2 shows three conformers C1, C2 and C3. **(C)** SEC-CIU fingerprints and differential plots obtained for intact adalimumab (z = 26+) with different infusion modes. **(D)** Extracted ATDs for nanoESI (pink), SEC-ESI (blue) and ESI (black).

#### 4. SEC-CIU for fast mAb classification

#### 4.1. IgG fingerprinting and subclass classification at intact and middle levels

Based on the work presented in the previous chapter to identify mAb subclasses at both intact and middle levels using nanoESI-CIU<sup>149</sup>, the SEC-CIU strategy was next employed for the differentiation of three mAbs belonging to distinct subclasses: adalimumab (IgG1), panitumumab (IgG2) and reslizumab (IgG4).

At the intact level, fingerprints of the 27+ charge state provide a straightforward differentiation of the IgG1, as it is the sole subclass with three unfolding transitions (Figure 20A). While two transitions are observed for both IgG2 and IgG4, the two mAbs could still be clearly distinguished using CIU50 values (36 and 166 V for IgG2 *versus* 30 and 117 V for IgG4). In particular, the UFS plot pinpointed one diagnostic region between 90 – 125 V, which was subsequently used to build a subclass classification method, considering the three previous mAbs as references (Figure 20A). Trastuzumab and ofatumumab were successfully recognized as IgG1s, with scores of 94.9  $\pm$  1.2% and 86.0  $\pm$  4.3% respectively, while nivolumab was identified as an IgG4 (75.0  $\pm$  4.4%) (Figure 20B). Of note, although nivolumab is not a wild-type IgG4 like reslizumab, but an hinge-stabilized IgG4 (i.e. with improved resistance to gas-phase unfolding conferred by a mutation in its hinge sequence to avoid Fab arm exchange<sup>148</sup>), it could still be unequivocally classified, demonstrating that inter-subclasses differences are greater than intra-subclasses ones. Accurate classifications were also obtained from 26 and 28+ charges states (see supplementary data of publication 4).

The categorization of mAbs was further applied at the middle level after IdeS digestion, which is more informative than the intact level<sup>149</sup>. Since F(ab')<sub>2</sub> and Fc subdomains do not coelute on short columns and thus cannot be activated simultaneously, the focus was put on  $F(ab')_2$  fragments, which possess more pronounced differences between unfolding patterns of subclasses<sup>149</sup>. Indeed, for the 21+ charge state of the  $F(ab')_2$  subunit, all three subclasses exhibit distinct number of conformational transitions, offering a clear-cut IgG identification (Figure 20C). The UFS plot evidenced discriminating CV values in the 85 – 120 V range. It is worth noting that differentiation scores are lower than those achieved in nanoESI-CIU using the same reference mAbs and charge states. This can be explained by the fact that less time is spent on each scan in SEC-CIU due to chromatographic limitations (2 scans x 3 s) compared to nanoESI-CIU (11 scans x 4 s), which slightly reduces the resolution of SEC-CIU features. However, categorization capabilities of our SEC-CIU setup are not impaired. Indeed, the classification built based on the diagnostic region resulted in accurate characterization of trastuzumab and ofatumumab as IgG1s (95.6  $\pm$  1.2% and 92.8  $\pm$  2.7%, respectively), and nivolumab as IgG4 (92.2  $\pm$  2.5%) (Figure 20D). As expected from Botzanowski et al.<sup>149</sup>, the categorization of IgGs after IdeS digestion yields higher scores than at the intact level, with lower standard deviations. In addition, scores were similar to those obtained with nanoESI-CIU fingerprints (> 90%)<sup>149</sup>, highlighting the potential of SEC-CIU to guarantee a rapid and accurate identification of IgG subclasses.



**Figure 20.** (A) SEC-CIU fingerprints of intact reference mAbs (z = 27+), and associated UFS plot obtained after interpolation by a factor 2. The diagnostic region for classification is shown in red. (B) Subclass categorization of intact trastuzumab, ofatumumab and nivolumab obtained with an in-house classification method. (C) SEC-CIU fingerprints of F(ab')<sub>2</sub> subunits of reference mAbs (z = 21+) with corresponding UFS plot. The diagnostic region is shown in red. (D) Subclass categorization of trastuzumab, ofatumumab and nivolumab function of trastuzumab.

#### 4.2. Increasing SEC-CIU classification throughput

The SEC-CIU method previously described allows to generate full fingerprints (0 – 200 V) with precise classification of IgG subclasses in ~45 min, reducing by threefold the overall CIU acquisition time compared to nanoESI-CIU. Several strategies were next developed to further enhance the throughput of SEC-CIU experiments.

#### 4.2.1. Targeted-scheduled SEC-CIU

Once complete reference fingerprints were established and classification parameters optimized, the aim was to implement a targeted-scheduled IgG screening relying solely on the most diagnostic CVs. Hence, a single-run SEC-CIU method based on seven diagnostic CVs was applied, using the same CIU scan parameters as Figure 18, but with 5 V steps to match CVs identified with interpolated UFS plots (Figure 20). Targeted-scheduled SEC-CIU offers high-confidence classification with scores as conclusive as those obtained in three SEC runs (> 85%) at both intacst and middle levels, as exemplified by ofatumumab, trastuzumab and nivolumab (Figure 21). This strategy allows to generate clear-cut categorization of IgGs while drastically reducing the data collection time (~15 min for each triplicate).



**Figure 21.** Targeted-scheduled SEC-CIU applied to the classification of **(A)** of atumumab, **(B)** trastuzumab, and **(C)** nivolumab.

#### 4.2.2. Multiplexed SEC-CIU

Since intact mAbs co-elute on short SEC columns, simultaneous activation of multiple mAbs can be performed in the trap cell, allowing to generate several fingerprints at once, as long as species possess distinct *m/z* ratio to avoid overlapping CIU plots. Characterizing mAbs mixtures is of main interest, as coformulated mAbs with synergic effects appear as promising therapeutic entities<sup>419</sup>. A mixture of three mAbs from different subclasses (trastuzumab - IgG1, ofatumumab - IgG1, nivolumab - IgG4) was thus analyzed to assess the potential of multiplexed SEC-CIU for IgGs identification in a single experiment.

In order to guarantee minimal signal interferences between CIU fingerprints of two consecutive MS peaks, mAbs with a mass difference > 450 Da (based on the resolution R ~310 for 27+ charge states) were mixed. As online SEC desalting ensures enough resolution of MS peaks, it was possible to extract well-defined ATDs which still provide unequivocal IgG differentiation (Figure 22A). Nonetheless, an additional point that needs to be checked for multiplexed experiments are potential interferences between species entering the trap cell. Since several populations are activated simultaneously, ion-ion interactions might occur and influence unfolding patterns<sup>164,176</sup>. Comparisons of ATDs acquired either with single-mAb analysis or multiplexed SEC-CIU show that unfolding profiles are highly similar, demonstrating that CIU patterns are not altered even if other mAbs are present in the sample matrix (Figure 22B-D). Hence, classification results remain accurate even in multiplexing conditions (Figure 22E-G). Of note, F(ab')<sub>2</sub> fragments also co-elute on short SEC columns, and so the multiplexed SEC-CIU strategy could be generalized to the middle-level (see supplementary data of publication 4).

Overall, mAb-multiplexed SEC-CIU offers considerable improvements over standard CIU approaches, as it maximizes the information content (plots of multiple proteins and their charge states) gathered in a single experiment, with increased mAb-fingerprinting throughput.


**Figure 22.** Multiplexed SEC-CIU experiments at the intact level. **(A)** Native mass spectrum of an equimolar (15  $\mu$ M) mAb mixture (trap CV = 90 V). SEC-CIU fingerprints were generated for the three intact mAbs, ofatumumab, panitumumab and nivolumab. **(B-D)** Extracted ATDs afford a direct comparison of unfolding profiles obtained with mAb-multiplexed (solid lines) and single-mAb experiments (dotted lines). **(E-G)** Categorization of the three intact multiplexed mAbs for the 27+ charge state.

# 4.2.3. Targeted-scheduled multiplexed SEC-CIU

Lastly, the combination of targeted and multiplexed SEC-CIU strategies allows to further increase the throughput of SEC-CIU for classification purposes. Data collection focuses on the most diagnostic CVs, with categorization of multiple mAbs at once. In order to replicate experimental conditions for the two previous paragraphs, the following example is based on the acquisition of seven CVs for a mixture of intact ofatumumab, trastuzumab and nivolumab. High scores (> 75%) are achieved (Figure 23), providing precise classifications similar to those obtained either for each separated mAb in targeted mode (Figure 21), or in multiplexed mode (Figure 22). Altogether, this method offers a straightforward identification of different IgGs at once in ~15 min. In manual nanoESI-CIU experiments, ~5 hours would be required to categorize three mAbs. Targetedscheduled multiplexed SEC-CIU thus appears as an attractive strategy for high-throughput screening of complex mAbs mixtures.



Figure 23. Targeted-scheduled multiplexed SEC-CIU applied to the classification of (A) ofatumumab, (B) trastuzumab, and (C) nivolumab.

# Scientific communication

These results have been published and presented through oral and poster communications.

# Peer-reviewed article

Deslignière, E.; Ehkirch, A.; Botzanowski, T.; Beck, A.; Hernandez-Alba, O.; Cianférani, S., Toward Automation of Collision-Induced Unfolding Experiments through Online Size Exclusion Chromatography Coupled to Native Mass Spectrometry. *Anal Chem* **2020**, *92* (19), 12900-12908, DOI: 10.1021/acs.analchem.0c01426.

# Oral presentation

<u>Deslignière, E.</u>; Ehkirch, A.; Botzanowski, T.; Beck A.; Hernandez-Alba, O.; Cianférani, S., Increased Collision-Induced Unfolding Experiments Throughput using Online Size Exclusion Chromatography Coupled to Native MS. Journées Françaises de Spectrométrie de Masse (JFSM), June 14-24<sup>th</sup> 2021 (Online meeting).

# Poster communications

<u>Deslignière, E.</u>; Ehkirch, A.; Botzanowski, T.; Beck A.; Hernandez-Alba, O.; Cianférani, S., Towards automation of Collision Induced Unfolding experiments through online Size Exclusion Chromatography coupled to native Mass Spectrometry.

- Spectrométrie de Masse et Analyse Protéomique (SMAP), September 16-19<sup>th</sup> 2019, Strasbourg (France).
- Premier Congrès Français de Biologie Structurale Intégrative, October 7-11<sup>th</sup> 2019, Toulouse (France).
- 68<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, June 1-12<sup>th</sup> 2020 (On-line meeting).

# 5. SEC-CIU to differentiate gas-phase unfolding behaviors of tsAb conformers

The SEC-CIU setup was next applied to a more challenging and complex engineered tsAb. This work is a follow-up to the results presented in part III (chapter 3) regarding the differentiation of tsAb isomers based on their IMS gas-phase behaviors. Standalone cIMS-MS provided a clear separation of both conformers at the middle level after enzymatic digestion above the hinge region (FabALACTICA<sup>®</sup>, Genovis), which was not the case at the intact level. These variations were attributed to proline isomerization on a YPP motif contained in the Fab domain, yielding *cis* and *trans* isomers<sup>385</sup>. Owing to an additional dimension of separation, SEC-IMS-MS experiments carried out on the linear TWIMS-MS instrument revealed small conformational differences between intact tsAb conformers, with  $\Delta^{TW}CCS_{N2} = 0.4 \text{ nm}^2$  and 0.6 nm<sup>2</sup> for charge states 28+ and 29+, respectively (Figure 24A). In order to strengthen these observations, we performed SEC-CIU on the two separated chromatographic peaks to determine whether tsAb isomers exhibit distinct gas-phase unfolding patterns at intact level.

The extraction and superposition of ATDs allows for direct comparison of gas-phase behaviors upon activation (Figure 24B). For the 29+ charge state, both conformers start unfolding at the same voltage (CV = 30 V). At 40 V, the conformer C1, which is initially more compact than C2, unfolds and becomes more extended than C2. At 60 V, the two species have adopted their final unfolded conformation, which exhibit identical drift times (20.8 ms), suggesting that both conformers ultimately rearrange into the same activated conformation. Overall, a single transition is detected for the two species in the 0 – 200 V range, which can be further visualized on CIU plots (Figure 24C). Nonetheless, when conformational differences are subtle, CIU fingerprints may be difficult to interpret for non-experts, especially for a precise comparison of unfolding patterns as traces cannot be superimposed. We thus used another representation, which is better adapted to illustrate variations between the two conformers. Figure 24D, generated with Benthesikyme<sup>286</sup>, represents the intensity weighted mean of each ATD (IWMATD), and helps to capture small changes in average drift times and peak width. This graph highlights a steep unfolding slope for C1 starting at 20 V, which indicates a transition towards a more extended conformation. Conversely, the slope remains shallower for C2 at low voltages (< 40 V), with a breaking point at 40 V reflecting a clear conformational shift. Interestingly, C2 reaches its final extended conformation at 70V, before C1, which was not detected on extracted ATDs. These differences in transitions could be further evaluated through CIU50 values, which confirm that unfolding occurs at lower CV values for C1 (38.7 V) compared to C2 (48.9 V) (Figure 24E). In conclusion, although small conformational differences are observed considering that the highest -log<sub>10</sub>(p-value) does not exceed 0.3 (35 V, Figure 24F), we still consider these differences to be significant. Indeed,  $\Delta$ CIU50 are repeatable on all three technical replicates of the 29+ charge state, but also for charge states 30+ and 31+. In addition, RMSDs obtained when comparing fingerprints from C1 and C2 (RMSD = 9%) are higher than RMSDs between technical replicates (4%), meaning that inter-group variations are greater than intra-group ones (data not shown).

Altogether, these experiments illustrate the potential of SEC-CIU to successfully analyze more tricky mAb formats and to achieve a more complete characterization of these products.



**Figure 24.** SEC-CIU experiments for the 29+ charge state of tsAb conformers. (A) SEC-UV chromatogram with the two identified C1 (blue) and C2 (red) tsAb conformers. <sup>TW</sup>CCS<sub>N2</sub> values are given at CV = 0 V. (B) Extracted ATDs (z = 29+). Above 100 V, identical ATDs were obtained. (C) CIU fingerprints. (D) Intensity weighted mean of ATDs represented as a function of CVs for both conformers. (E) Evaluation of CIU50 values. (F) UFS plot.

# Scientific communication

These data will be included in an article currently in preparation.

# 6. Conclusions

In this chapter, the capabilities of the online coupling of SEC to nIMS-MS for the automation of CIU experiments were explored. SEC-CIU offers several benefits over classical nanoESI-CIU, among which an improved and fast online desalting, and a significant reduction of the overall data acquisition time (from ~3 hours to 15 min with targeted-scheduled SEC-CIU). The SEC-CIU coupling preserves the quality and key features of nanoESI-CIU fingerprints, allowing to generate specific signatures of each IgG subclass for rapid and accurate categorization at both intact and middle levels. We next aimed at developing different methods with the aim of further increasing the SEC-CIU throughput, either using targeted-scheduled SEC-CIU based solely on diagnostic CVs, or through mAb multiplexing. These 141

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strategies help to tackle more complex samples while broadening the scope of information comprised within a single run. SEC-CIU thus appears as an appealing tool to rapidly investigate protein gas-phase unfolding. Indeed, our setup proved to be efficient to differentiate gas-phase behaviors of two tsAb conformers, showing the potential of SEC-CIU to address concrete challenges encountered along the development of new engineered products in biopharmaceutical companies. The automation of CIU experiments can pave the way to widen its use and applications, and ease the integration of this approach in biopharmaceutical industries and R&D laboratories. In addition, the SEC dimension could be replaced by IEX or HIC to provide conformational information on charge variants or on the different Dn species of ADCs.

# Publication 4

Toward Automation of Collision-Induced Unfolding Experiments through Online Size Exclusion Chromatography Coupled to Native Mass Spectrometry

Deslignière, E.; Ehkirch, A.; Botzanowski, T.; Beck, A.; Hernandez-Alba, O.; Cianférani, S.



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Article

# **Toward Automation of Collision-Induced Unfolding Experiments** through Online Size Exclusion Chromatography Coupled to Native Mass Spectrometry

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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 IM mass spectrometry (SEC-CIU). Online automated SEC-CIU experiments offer



several benefits over nanoESI–CIU, among which are (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; and (iii) maintaining the number of unfolding transitions. We then evaluate the potential of SEC-CIU to distinguish monoclonal antibody (mAb) 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 mAb-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-ligand interactions.<sup>1</sup> In the last decade, ion mobility (IM) combined with MS has grown into a valuable asset for the study of proteins and noncovalent complexes<sup>2,3</sup> mainly because of the implementation of IM, notably travelling wave IM spectrometry<sup>4</sup> (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 (CCSs), the latter being related to the global conformation of the ion.<sup>5</sup> However, IM fails to differentiate codrifting species with closely related conformations because of 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 separation.<sup>6–8</sup> 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 buildup of internal energy in the ions.<sup>6</sup> 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 because of the unfolding or compaction of the protein.<sup>9</sup> These conformational changes are reflected through ATD variations. Thereby, CIU experiments generate multidimensional datasets, with ATDs being acquired at each CV.

Early studies on collisional activation were carried out by Shelimov et al. in 1997 and include the observation of apomyoglobin conformations<sup>10</sup> and the different gas-phase unfolding of cytochrome c versus bovine pancreatic trypsin

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inhibitor which differ in their number of disulfide bridges (none and three, respectively).<sup>11</sup> Following the commercialization of quadrupole/TWIMS/time-of-flight mass spectrometers,<sup>4</sup> modern technological developments have further extended the range of CIU examples. Current applications of CIU encompass probing protein–protein complexes,<sup>12,13</sup> protein–ligand interactions,<sup>9,14–18</sup> and binding of lipids to membrane proteins.<sup>19,20</sup> CIU also plays a key role to investigate therapeutically relevant proteins, allowing more conformational insights into monoclonal antibodies (mAbs),<sup>21–24</sup> biosimilars,<sup>25</sup> and antibody–drug conjugates.<sup>26,27</sup> In particular, Tian et al.<sup>7</sup> 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 clearcut categorization than the intact level.<sup>28</sup>

Even if native MS approaches have entered R&D laboratories of biopharma companies, the use of CIU approaches is still scarce mostly because of 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.<sup>9,29–33</sup> CIUSuite, CIUSuite 2, PULSAR, and ORIGAMI<sup>ANALYSE</sup> 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 online data acquisition. Increase in CV is usually carried out either manually or in a semiautomatic way using sequential data acquisition through a preprogrammed sample list. Altogether, the classical CIU data acquisition pipeline results in a tedious and time-consuming process which hampers its routine use. Classical "semiautomated" experiments (manual desalting followed by automated sequential acquisition) require around 3 h ranging from sample preparation to data acquisition. Vallejo et al.<sup>3</sup> suggested focusing only on median voltages of CIU features and transitions for faster mAb differentiation, once full fingerprints have been established. Only one open-source software (ORIGAMI<sup>MS</sup>) allows automated acquisition of CIU data but prior manual desalting is still necessary.<sup>31</sup>

In this context, the use of size exclusion chromatography (SEC) is of main interest for fast online desalting. Indeed, SEC has been successfully coupled 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 industry.<sup>35</sup> We thus explored the feasibility of SEC coupled to native IM-MS for CIU analyses.

We here present 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. Article

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 mAb subclasses at both intact and middle levels, while overall diminishing threefold the acquisition time necessary to record CIU experiments. Finally, 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 the IdeS enzyme (FabRICA-TOR, Genovis) was added per microgram of mAb prior deglycosylation. The mixture was incubated at 37 °C for 60 min.

**Manual Buffer Exchange.** For electrospray (ESI)–CIU and nanoESI–CIU experiments, samples were desalted against 100 mM ammonium acetate at pH 7.0, using eight cycles of a centrifugal concentrator (Vivaspin, 30 kDa cutoff, Sartorius, Göttingen, Germany). The 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  $\mu$ M prior to native ESI–CIU 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 coupled 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 200 Å, 1.7  $\mu$ m, 4.6  $\times$  300 mm and an Acquity BEH SEC 125 Å, 1.7  $\mu$ m, 4.6  $\times$  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 increased 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 increased to 0.250 mL/min for 0.4 min. A total of 20 and 12  $\mu$ g were injected for intact- and middle-level analyses, respectively.

The Synapt G2 was operated in the sensitivity mode and positive polarity with a capillary voltage of 3.0 kV. Desolvation and source temperatures were set to 450 and 100 °C, respectively. Desolvation and cone gas flow rates were 750 and 60 L/h, 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<sub>2</sub> flow rate was 60 mL/min. The IM wave velocity and height were 800 m/s and 40 V, respectively. Data were



Figure 1. Schematic representation of the acquisition workflow of SEC-CIU data (0-60 V range) for intact mAbs. For each CV, one IM-MS function (gray scale bar) is implemented in the MassLynx MS file along the sample elution. Scan parameters were set to 5 scans of 3 s 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.

acquired in the 1000–10,000 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). CVs 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, and 140-200 V). For each voltage step of intact mAb experiments, the number of scans and scan time were set to 5 and 3 s, respectively. These 15 s acquisition slots, that is, 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 an ESI source using a syringe infusion pump (KD Scientific, Holliston, MA, USA) using 250  $\mu$ L syringe (Hamilton, Bonaduz, Switzerland) at an infusion rate of 3.5  $\mu$ L/min. Desolvation and source temperatures were set to 450 °C and 100 °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 an 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 °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.

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Data Treatment. Chromatographic, IM, and MS data were analyzed using MassLynx v4.1. CIU data were processed using the CIUSuite 2 v2.1 software.<sup>33</sup> Data sets were collected in triplicate (with three SEC runs for each replicate) to generate averaged CIU fingerprints. ATDs were smoothed using a Savitzky-Golay algorithm with a window length of 5 and a polynomial order of 2. Interpolation by a factor of 2 allowed us to double the number of steps along the CV axis. Averaged and differential plots with their associated rmsd's were generated using the "Basic Analysis" module. rmsd's 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: the 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 the maximum value for each CV; and transition region padding = 15 V. When features coexist at a 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<sup>ANALYSE</sup> v1.2.1.4 software.<sup>31</sup>

For mAb subclass categorization, the centroid of the ATDs was standardized at CV = 0 V to ensure comparison solely on unfolding patterns, irrespective of drift time variations because of different masses. Adalimumab (IgG1), panitumumab (IgG2), and reslizumab (IgG4) were chosen as reference mAbs to build classification methods at intact and middle



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.

levels using the "Classification" module of the CIUSuite 2 software.<sup>33,36</sup> Univariate feature selection (UFS) plots were used to select the most diagnostic voltages, that is, 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 CVs from 0 to 200 V prior to IM separation. CVs are ramped either manually or sequentially using a sample list, resulting in an error-prone and highly time-consuming process (~75 min for each sample, in triplicate, with 10 V steps). Previous manual CIU studies have been performed in the nanoESI mode using either glass capillary nanoemitters<sup>9,15–17</sup> or automated chip-based nanoESI devices.<sup>18,21,27,28</sup> Both techniques hardly maintain spray stability throughout the whole CV ramp because of clogging,<sup>37,38</sup> leading to a laborious process for the MS operator. Hence, coupling of SEC to native IM-MS is of major interest to automate CIU experiments while substantially reducing the overall time process. Because online SEC coupling ensures a continuous controlled flow rate with a stable ESI, CIU data can be recorded automatically during the sample elution.

**Optimization of the 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 BEH 200 Å 4.6 × 300 mm and an Acquity BEH 125 Å, 4.6 × 30 mm (Waters), requiring runs of 18 and 5 min, respectively. Although the longer elution on the 300 mm column affords an increased number of scans and improved desalting efficiencies,<sup>35</sup> no significant difference was observed on CIU fingerprints (rmsd < 6%, Figure S1). Because 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 the SEC Flow Rate.** Automated SEC– native MS experiments for therapeutic proteins are usually performed at flow rates comprised between 0.10 and 0.25 mL/ min.<sup>35</sup> 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 a 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 the number of steps, scan parameters, and IM-MS signal, leading to CIU data acquired for 2 min with enough MS intensity.

Optimization of CIU Parameters. CIU parameters, that is, 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 15 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 mAb subclasses. After data processing in CIUSuite 2 (see Materials and Methods), at least 8 specific diagnostic CVs were available for mAb 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 s) to improve the S/N ratio. Once the run has started, CVs are automatically increased following the user's method. Of note, CVs can also



**Figure 3.** Intact- and middle-level 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')<sub>2</sub> fragments corresponding to our three reference mAbs. (d) Middle-level subclass classification (z = 21+) of ofatumumab (IgG1), trastuzumab (IgG1), and nivolumab (IgG4).

be ramped in the decreasing order without altering CIU fingerprints (Figure S3). For each of the three runs (0–60 V, 70–130 V, and 140–200 V), CIU data are stored within a single MassLynx.raw file compatible with the CIUSuite 2 software<sup>33</sup> for data analysis. Because 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 fast online desalting<sup>35</sup> compared to classical nanoESI-CIU experiments which require manual buffer exchange, and the possibility to automate data acquisition to further reduce the data collection time, saving 2 h 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.<sup>7,21-23,28</sup> 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 ones<sup>28</sup> in order to assess whether CIU key features are retained with our SEC-CIU setup. As ESI generates larger droplets with higher charge states than nanoESI,<sup>37</sup> only one charge state (26+) was available for comparison between nanoESI-CIU and SEC-ESI-CIU (Figure 2a). Both methods provided the same number of unfolding transitions (Figure 2b,c,e), and identical conformational states are obtained in both fingerprints (Figure S4c-e), but relative intensities between coexisting states slightly differ, which results in an apparent translation toward 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 versus 30 V for nanoESI–CIU and SEC–CIU, respectively (Figure 2e). Although 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 under SEC–ESI and ESI conditions (rmsd < 8%, Figure S4b,c), as depicted for the 26+charge state of adalimumab at the intact level (Figure 2c–e), which demonstrates that the coupling of SEC to CIU does not significantly affect mAb CIU patterns.

mAbs being very stable molecules, we usually perform our native MS analyses under quite harsh temperature desolvation conditions, either in SEC-native MS<sup>35</sup> or nanoESI-CIU,<sup>21,28</sup> 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-CIU and SEC-CIU fingerprints. No significant difference (rmsd < 9% for the 28+ charge state) was observed between SEC-ESI-CIU fingerprints generated at 150 or 450 °C desolvation temperature (the 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 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



**Figure 4.** mAb-multiplexed SEC-CIU experiments. (a) Native SEC-CIU mass spectrum of an equimolar  $(15 \ \mu\text{M})$  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-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 mAb-multiplexed SEC-CIU (solid lines) experiments at the intact level.

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-CIU or ESI-CIU.

SEC-CIU for mAb Classification. Previous studies have demonstrated that mAb subclasses, which, namely, differ in their number and bridging of disulfide bonds, could be classified based on their unfolding patterns.<sup>7,28</sup> 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 the native structure, signal intensity, and CIU information content, it will be used thereafter for classification purposes (additional charge states are shown in the Supporting Information, 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, mAb subclasses could unambiguously be distinguished based on their specific ATD 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. observations.<sup>7</sup> 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 and 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, <sup>21,28</sup> although unfolding patterns between mAbs from the same subclass are not strictly identical probably because of the contribution of the variable domains of full fragment antigen-binding [F(ab')<sub>2</sub>] 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 S9b). 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 mAb subclass identification, whereas fragment crystallizable (Fc) subdomains do not allow clear-cut classification because of similar unfolding patterns.<sup>28</sup> Because F(ab')<sub>2</sub> and Fc fragments do not coelute on the 30 mm column and thus could 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 because of 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 CVs in the 85–120 V range (Figure S9c). Our middle-level classification based on the three reference mAbs (adalimumab, panitumumab, and reslizumab) unequivocally categorizes of atummab 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 fingerprints.<sup>28</sup> 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.<sup>28</sup>

Once full CIU fingerprints (0-200 V) of reference mAbs have been generated and classification parameters were optimized, we developed a targeted scheduled SEC-CIU based on the most diagnostic CVs extracted from UFS plots.<sup>33</sup> This allowed us to propose a single-run SEC-CIU strategy for rapid IgG classification (15 min for triplicate CIU maps) by recording only 7 diagnostic CVs (in 5 V steps, 90 to 120 V for z = 27 + at the intact level, or 85 and 95 to 120 V for z = 21+at the 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 mAb categorization and also affords a drastic reduction in the overall acquisition time.

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

**SEC–CIU for mAb-Multiplexed Analyses.** An additional benefit of short SEC columns stems from the coelution 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 us 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 a distinct m/z ratio to avoid overlapping CIU fingerprints. The characterization of mAb mixtures is of key interest, as coformulated mAbs with synergic effects represent promising therapeutic entities.<sup>40,41</sup>

In order to fully exploit the potential of SEC-CIU for fast mAb classification, we subsequently aimed at analyzing the three mAb subclasses in a single experiment. We thus evaluated our SEC-CIU strategy on a mixture containing intact of atumumab (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 us to extract ATDs at m/z ranges specific to each mAb, resulting in well-defined CIU fingerprints which still afford an unambiguous subclass classification (Figure 4b). An issue of quadrupole nonselected CIU lies in possible interferences between species

activated in the trap cell which might influence unfolding transitions.<sup>20,34</sup> 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 mAb mixture analysis provided that enough MS resolution is available.

Altogether, mAb-multiplexed experiments provide significant improvements over standard CIU approaches. Our method offers a wide range of information, that is, fingerprints of multiple proteins and their different charge states, while reducing data acquisition time (the same time for one to three mAb analyses). As  $F(ab')_2$  fragments also coelute on the 30 mm SEC column, our multiplexing strategy can be further expanded to the middle level for mAb subclass classifications (Figure S13).

#### CONCLUSIONS

We have demonstrated here the potential of online coupling 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 h to 15 min 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 subclass 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 sample 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 biotherapeutic 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 https://pubs.acs.org/doi/10.1021/acs.analchem.0c01426.

rmsd's of technical SEC–CIU replicates; SEC–CIU fingerprints on 300 vs 30 mm SEC columns; SEC–CIU fingerprints with 2 vs 3 runs; SEC–CIU fingerprints with increasing vs decreasing CVs; comparisons between nanoESI–CIU/SEC–ESI–CIU; influence of ESI desolvation temperature on SEC–CIU fingerprints; influence of source temperature on nanoESI–CIU fingerprints; additional SEC–CIU fingerprints and classifications; ATDs of  $F(ab')_2$  for reference mAbs; targeted scheduled SEC–CIU classifications; and middle-level mAb-multiplexed SEC–CIU (PDF)

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#### 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

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# SUPPORTING INFORMATION

# Toward Automation of Collision-Induced Unfolding Experiments through Online Size Exclusion Chromatography Coupled to Native Mass Spectrometry

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- **Table S1**: RMSDs of technical SEC-CIU replicates at intact and middle levels.
- **Figure S1**: SEC-CIU fingerprints of the 27+ charge state of intact reslizumab obtained on (a) an Acquity BEH SEC 200Å, 1.7 μm, 4.6 x 300 mm and (b) an Acquity BEH SEC 125Å, 1.7 μm, 4.6 x 30 mm.
- **Figure S2**: Comparison of SEC-CIU fingerprints obtained with three-runs and two-runs methods for the 27+ charge state of intact (a) adalimumab and (b) nivolumab.
- **Figure S3**: SEC-CIU fingerprints obtained with increasing or decreasing CV ramps for (a) nivolumab at intact level (z = 27+) and (c) the F(ab')<sub>2</sub> fragment of trastuzumab (z = 21+).
- Figure S4: Comparisons between nanoESI- and SEC-ESI-/ESI- CIU for intact adalimumab.
- Figure S5: Influence of ESI desolvation temperature on SEC-CIU fingerprints.
- Figure S6: Influence of source temperature (40, 60, 100 °C) on nanoESI-CIU fingerprints of intact adalimumab.
- **Figure S7**: SEC-CIU experiments and classification at intact level for the 26+ charge state.
- Figure S8: SEC-CIU experiments and classification at intact level for the 28+ charge state.
- Figure S9: SEC-CIU experiments at intact (z = 27+) and middle (z = 21+) levels.
- **Figure S10**: SEC-CIU experiments and classification at middle level for the 22+ charge state of F(ab')<sub>2</sub> fragments.
- Figure S11: ATDs of F(ab')<sub>2</sub> fragments for reference mAbs (z = 21+).
- Figure S12: Targeted scheduled SEC-CIU subclass classifications.
- Figure S13: Middle-level mAbs-multiplexed SEC-CIU experiments.

		RMSDs (%) of technical replicates (n = 3)					
	z+	adalimumab	panitumumab	reslizumab	ofatumumab	trastuzumab	nivolumab
Intact	26	4.16	6.87	7.20	4.10	6.53	-
	27	3.25	6.84	8.90	4.78	8.03	3.87
	28	2.90	4.29	3.96	4.01	4.89	3.82
F(ab')2	21	8.77	7.75	6.65	5.52	7.24	6.90
	22	7.56	5.87	6.89	6.22	5.57	7.54

**Table S1:** RMSDs of technical SEC-CIU replicates at intact and middle levels. RMSDs are given for fingerprints acquired at desolvation and source temperatures of 450 °C and 100 °C, respectively.



**Figure S1:** SEC-CIU fingerprints of the 27+ charge state of intact reslizumab obtained on (a) an Acquity BEH SEC 200Å, 1.7  $\mu$ m, 4.6 x 300 mm and (b) an Acquity BEH SEC 125Å, 1.7  $\mu$ m, 4.6 x 30 mm. (c) The differential plot between the two fingerprints pinpoints highly similar fingerprints. (d) ATDs were extracted from 0 to 200 V in 20 V steps for both 300 mm (dark blue) and 30 mm (light blue) columns.



**Figure S2:** Comparison of SEC-CIU fingerprints obtained with three-runs and two-runs methods for the 27+ charge state of intact (a) adalimumab and (b) nivolumab. Fingerprints acquired in three and two runs are depicted in the upper and lower panels, respectively.



**Figure S3:** SEC-CIU fingerprints obtained with increasing or decreasing CV ramps for (a) nivolumab at intact level (z = 27+) and (c) the F(ab')<sub>2</sub> fragment of trastuzumab (z = 21+). Differential plots between both ramps show RMSDs < 6 % for (b) nivolumab and (d) trastuzumab.



**Figure S4:** Comparisons between nanoESI- and SEC-ESI-/ESI- CIU for intact adalimumab. Differential plots (z = 26 +) for (a) SEC-ESI- versus nanoESI- CIU and (b) SEC-ESI- versus ESI- CIU. (c) Table summarizing median drift times of each feature for the different ionization modes. (d) CCS obtained at trap CV = 0 for nanoESI (red) and SEC-ESI (blue). (e) ATDs obtained at CV = 0 for nanoESI (red) and SEC-ESI (blue). (e) ATDs obtained at CV = 0 for nanoESI (red) and SEC-ESI (blue).

Avidin, concanavalin A, pyruvate kinase and alcohol dehydrogenase were used as external CCS calibrants as reported by Bush et al. (*Anal Chem* 2010, 82, 22, 9557–9565, doi: 10.1021/ac1022953). IM drift times were determined with the 'Gaussian Fitting' module of CIUSuite 2, and converted into CCS as described elsewhere (Ruotolo *et al., Nat Protoc* 2008, 3, 7, 1139–1152, doi: 10.1038/nprot.2008.78).



**Figure S5:** Influence of ESI desolvation temperature on SEC-CIU fingerprints. (a) SEC-CIU fingerprints obtained at 150 and 450 °C for intact adalimumab, ofatumumab, panitumumab and reslizumab (z = 28 +). (b) Differential plots between 150 and 450 °C fingerprints. (c) Tables summarizing mAbs CCS values calculated for 150 and 450 °C at trap CV = 0 V. ATDs were extracted for z = 26 + for both desolvation temperatures (right panel).

# Source Temperature



**Figure S6:** Influence of source temperature (40, 60 and 100 °C) on nanoESI-CIU fingerprints of intact adalimumab. (a) 23+ charge state (most intense z+) nanoESI-CIU fingerprints and table with associated median drift times. (b) 26+ charge state (used for nanoESI versus SEC-ESI-CIU comparisons) nanoESI-CIU fingerprints and table with corresponding median drift times.



**Figure S7:** SEC-CIU experiments and classification at intact level for the 26+ charge state. (a) The UFS plot pinpoints the most diagnostic CVs (red box) for IgGs classification. (b) Fingerprints of reference mAbs, adalimumab, panitumumab and reslizumab. (c) Fingerprints of clusterized mAbs, ofatumumab and trastuzumab. (d) Subclass classification obtained on clusterized mAbs.



**Figure S8:** SEC-CIU experiments and classification at intact level for the 28+ charge state. (a) The UFS plot pinpoints the most diagnostic CVs (red boxes) for IgGs classification. (b) Fingerprints of reference mAbs, adalimumab, panitumumab and reslizumab. (c) Fingerprints of clusterized mAbs, ofatumumab, trastuzumab and nivolumab. (d) Subclass classification obtained on clusterized mAbs.



**Figure S9:** SEC-CIU experiments at intact (z = 27+) and middle (z = 21+) levels. The UFS plot allows to select the most diagnostic CVs (red boxes) for IgGs classification at (a) intact and (c) middle levels. (b, d) SEC-CIU fingerprints of clusterized mAbs.



**Figure S10:** SEC-CIU experiments and classification at middle level for the 22+ charge state of F(ab')<sub>2</sub> fragments. (a) The UFS plot pinpoints the most diagnostic CVs (red boxes) for IgGs classification. (b) Fingerprints of reference mAbs, adalimumab, panitumumab and reslizumab. (c) Fingerprints of clusterized mAbs, ofatumumab, trastuzumab and nivolumab. (d) Subclass classification obtained on clusterized mAbs.



**Figure S11:** ATDs of  $F(ab')_2$  fragments for reference mAbs (z = 21+). ATDs were extracted at CV = 60, 100 and 170 V for adalimumab (blue), panitumumab (green), and reslizumab (grey).



**Figure S12:** Targeted scheduled SEC-CIU subclass classifications (single-run method) for (a) trastuzumab and (b) nivolumab. Single-run fingerprints are acquired using the 7 most diagnostic voltages of intact (z = 27+, 90 to 120 V, 5 V steps) or middle (z = 21+, 85 and 95 to 120 V, 5 V steps) levels.



**Figure S13:** Middle-level mAbs-multiplexed SEC-CIU experiments. (a) Native SEC-CIU mass spectrum of  $F(ab')_2$  fragments of an equimolar (7 µM) mAb mixture containing three IdeS-digested mAbs, ofatumumab, panitumumab and nivolumab (trap CV = 70 V). Masses are given for the  $F(ab')_2$  domains. (b) 21+ charge state SEC-CIU fingerprints of each  $F(ab')_2$  fragment obtained from multiplexing experiments. (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 middle level.

# Chapter 4 – Exploring the Capabilities of the High-Resolution cIMS-MS Instrument to Characterize mAbs' Unfolding Patterns

# 1. Analytical context

As described in part III, chapter 3 for large and heterogeneous mAbs, broad ATDs generated in cIMS-MS reflect the presence of multiple conformational families, in agreement with the flexible and dynamic nature of mAbs<sup>98,155</sup>. As proteins in their native states are composed of a continuum of conformers, increases in IMS resolving power alone cannot resolve multiple overlapping features. The combination of "classical" CIU approaches and high-resolution cIMS-MS has not been reported in the literature, however both methods could benefit from each other, with improved resolution to enhance subtle differences detected through CIU. In addition, tandem IMS is particularly useful to overcome limitations of IMS<sup>1</sup> analyses, especially if ions are activated between IMS cycles<sup>89</sup>. For a long time, the use of tandem IMS was restricted to specialized research groups which had the knowledge and skills to construct such instruments in-house. The cIMS-MS instrument is currently the only commercialized platform offering IMS<sup>n</sup> capabilities, with activation occurring upon reinjection from the prestore into the cIMS cell of the selected population<sup>14</sup> (see paragraph 3.2). This approach has been applied to monitor the conversion between the different conformational intermediates of cytochrome c, allowing to propose a sequence of gas-phase unfolding events<sup>19,20</sup>. Interconversion was also exemplified by human amylin, which exhibits two different native conformations whose ATDs become superimposable upon activation<sup>19</sup>. High-resolution cIMS-MS combined to CIU approaches appears as a promising method to probe folding and dynamics of specific features contained within broad conformational ensembles, using CIU approaches.

## 2. Objectives

This chapter aims at evaluating the potential of the high-resolution cIMS-MS instrument for CIU approaches, in order to:

- Achieve more accurate CIU fingerprinting and classification of the different IgG subclasses;

- Provide further insights into unfolding behaviors of IgG2 disulfide variants, using new (CIU)-IMS-CIU-IMS capabilities of the cIMS-MS device.

This work is a follow-up of results presented in part III, chapter 3, and was conducted in collaboration with Dr. Hélène Rogniaux and Simon Ollivier (INRAE, Nantes, France).

# 3. Performing CIU experiments on the cIMS-MS instrument

# 3.1. Activation in the trap cell (CIU-cIMS)

As for linear TWIMS-MS instruments, CIU experiments can be performed by raising CVs in the trap cell prior to the cIMS cell (Figure 25A). While the trap CV is limited to 200 V on the Synapt G2 HDMS, values can be increased up to 240 V on the cIMS-MS device, which could be of interest to observe new features at high voltages. After their activation in the trap cell, ions are separated in the cIMS cell, either with a single pass, or multiple passes (steps 2–4, Figure 25B). These experiments will be further referred to as "CIU-cIMS".



**Figure 25.** CIU-cIMS experiment. **(A)** CIU occurs in the trap cell, located prior the cIMS cell. **(B)** Activated ions are separated in IMS, either with a single pass or multiple passes.

# 3.2. Activation upon reinjection in the cIMS cell

# 3.2.1. Without pre-activation in the trap cell (IMS-CIU-IMS)

The cIMS-MS device offers the possibility to perform IMS<sup>n</sup> experiments, where a specific population can be selected after IMS separation, and stored to the prestore while remaining populations are ejected towards the ToF detector (steps 1–5, Figure 26A). The selected population can then be activated upon reinjection into the cIMS cell (step 6, Figure 26A). To active the ions on re-entry to the array, the overall potential of the prestore is increased with respect to the array<sup>14</sup> (Figure 26B). More precisely, prearray gradient and bias are raised while the array offset is kept constant, thus forming a gradient that increases the kinetic energy of the ions. Ions will collide with the background gas from

the cIMS region (N<sub>2</sub>), which eventually leads to the generation of newly-activated conformations. As activation occurs through sequential increase of prestore voltages, the x-axis of fingerprints corresponds to the voltage increase compared to the initial prearray values (if default parameters = gradient 95 V/bias 85 V, then +100 = gradient 195 V/bias 185 V) (Figure 26C). Lastly, excited ions are separated during the IMS<sup>2</sup> stage (steps 7–8 on Figure 26A). In theory, this can be done a limitless number of times due to the geometry of the instrument, however the signal intensity will drop with both the number of IMS<sup>n</sup> cycles and the CV increase. These experiments will be further referred to as "IMS-CIU-IMS".

Selection of a native conformational population followed by its activation allows to tackle even minor populations, with improved resolution and detection of new features<sup>14</sup>.



**Figure 26.** IMS-CIU-IMS experiments. **(A)** Sequence used on the cIMS device. (1-5) lons are separated in the IMS<sup>1</sup> stage. A population of interest is selected and ejected to the prestore. Other features are ejected to the ToF. (6) The selected feature is activated upon reinjection into the cIMS cell. (7-8) Newly-activated species are separated in the IMS<sup>2</sup> stage. **(B)** Schematic representation of ATDs resulting from each IMS stage. **(C)** Potential energy diagram showing the increase of voltage required for activation upon reinjection to the array. Adapted from Giles *et al.*, 2019 (ref. 14). **(D)** IMS-CIU-IMS fingerprint. The collision voltage axis starts at the initial prearray value.

# 3.2.2. With pre-activation in the trap cell (CIU-IMS-CIU-IMS)

An extension of the IMS-CIU-IMS mode consists of pre-activating ions in the trap cell. This methodology combines the two activation modes presented above, and can thus be referred to as "CIU-IMS-CIU-IMS". The sequence used in these CIU-IMS-CIU-IMS experiments remains almost identical to Figure 26, except that the selected feature has been activated upstream of the cIMS cell (= step 1 from Figure 25 followed by the complete sequence from Figure 26). This mode of acquisition allows to monitor a specific activated state, which provides information on unfolding events/mechanisms occurring between the different conformational states<sup>19,20</sup>.

# 4. Classical CIU approaches combined to high resolution cIMS (CIU-cIMS)

### 4.1. High-resolution CIU for improved IgG fingerprinting and subclass categorization

# 4.1.1. Benefits of high-resolution CIU-cIMS for better IgG fingerprinting

A first study consisted of combining high-resolution cIMS-MS to CIU approaches for the differentiation of IgG subclasses. Fingerprints generated with the cIMS-MS instrument were first compared to those obtained on a linear TWIMS-MS device. In the following experiments, ions were activated solely in the trap cell prior to the IMS cell (10 V steps). It should be noted that direct comparisons of transition voltages between both platforms are not relevant considering that these instruments possess different geometries, which induces variations in CIU50 values. In addition, the background gas for collisions is Ar on the linear instrument, which is more activating than N<sub>2</sub> used on the cIMS-MS platform, because of a higher polarizability<sup>252,254</sup>.

As unfolding patterns of the most native charge states (i.e. lower charge states) are generally more informative<sup>16</sup>, we focused on the 27+ charge state of intact mAbs. For intact trastuzumab (lgG1), six features are detected with CIU-cIMS, with minor newly-resolved states (Figure 27A). Indeed, states 1 and 2 ( $\Delta t_D = 2.6 \text{ ms}$ ), which are present only on a small CV range, are well defined contrary to the linear CIU for which both conformers are comprised within a single broad ATD. CIU-cIMS also highlights an equilibrium between states 3 and 4, with a better estimation of the relative intensities of the two coexisting conformers due to an increase of +11% in valley separation (Figure 27B). Benefits of high-resolution CIU-cIMS are even more evident for lgG4s. For intact nivolumab, six conformational states are detected upon activation using cIMS-MS, while only four features are clearly observed on the linear CIU plot (Figure 27C). Features 3 and 4 are separated on the cIMS-MS device (19% valley separation), whereas the linear TWIMS-MS does not differentiate the two populations because of poor IMS resolution (Figure 27D).

Lastly, for the IgG2 subclass, CIU plots are very similar on cIMS-MS and linear TWIMS-MS instruments for intact panitumumab (Figure 27E). Unfolding patterns are generally less crowded than those of IgG1s and IgG4s, with fewer transitions due to increased stability conferred by additional disulfide bridges in the IgG2 hinge region. Because IgG2 patterns are less complex (sequential unfolding) than other subclasses for which different features co-exist and need to be better separated, IgG2 fingerprints generated on the linear TWIMS-MS instrument are already well-defined.

We have seen in part III (chapter 3) that two disulfide-related conformers were detected in cIMS for native profiles of IgG2s. However, the co-existence of disulfide variants at CV = 0 V is not obvious on CIU-cIMS representations because the two conformers are not well-resolved, resulting in a broad CIU trace. The distinction of disulfide variants is lost upon activation (Figure 27F), which either means that isoforms have completely identical unfolding behaviors, or that the resolution is not sufficient to distinguish activated species that could arise from slightly different disulfide patterns.

Altogether, CIU-cIMS offers clear improvements of fingerprints' graphic resolution as reflected by % valley separations, allowing for better fingerprinting of IgG subclasses with additional conformational states uncovered. Of note, RMSD values between technical replicates were lower in CIU-cIMS (~4%) compared to linear TWIMS CIU (~7%) for all mAbs under investigation. CIU-cIMS also proved to be a robust approach, with RMSD values ~7% compliant with U.S. FDA recommendations<sup>288</sup>.



**Figure 27.** CIU-cIMS experiments ( $T^{\circ}_{source} = 50^{\circ}$ C,  $T^{\circ}_{desolvation gas} = 250^{\circ}$ C, Vc = 80 V). (**A**, **C**, **E**) Comparisons of fingerprints acquired on the cIMS-MS device after one pass or the linear TWIMS-MS instrument, for three intact deglycosylated mAbs. Features detected on the cIMS but not on the linear TWIMS are represented in red. (**B**, **D**, **F**) Extracted ATDs at trap = 80 V for 27+ charge states, with associated % valley separation calculated between the two main features (B – trastuzumab, D – nivolumab, F – panitumumab).

# 4.1.2. UFS plots show better differentiation for CIU-cIMS than for linear TWIMS CIU

As CIU-cIMS provides an enhanced "spectral definition" of CIU fingerprints for the different IgG subclasses, we next assessed whether these improvements would influence UFS plots. Better separation of conformational states does not necessarily guarantee higher differentiation scores, as IgG1s and IgG4s now exhibit very similar unfolding patterns, with the same number of features contrary to linear TWIMS CIU. Yet, for the 27+ charge state of intact mAbs, the UFS plot generated based on trastuzumab (IgG1), panitumumab (IgG2) and nivolumab (IgG4) clearly evidences advantages of CIU-cIMS over linear TWIMS CIU (Figure 28A). While the maximum –log<sub>10</sub>(p-value) value achieved on the linear TWIMS instrument is 0.86, CIU-cIMS affords differentiation scores up to 1.33. cIMS also yields lower standard deviations than linear TWIMS CIU, especially between 50 – 100 V, in agreement 149

with previous observations on RMSD values between technical replicates. These results are further demonstrated for the 28+ charge state, which definitely illustrates how increased IMS resolution contributes to an easier differentiation of IgG subclasses (Figure 28B). The most discriminating region identified for CIU-cIMS ranges from 100 to 140 V, with nine CVs whose  $-log_{10}$ (p-value) values are all greater than the best score that could be reached with linear TWIMS (0.74).

In conclusion, a wider range of diagnostic CVs with higher differentiation scores is available in CIUcIMS, leading to more accurate categorization of IgG subclasses, as exemplified by the 27+ charge state of intact IgG1 elotuzumab (Figure 28C). For this comparison, classification methods were both built based on the seven best diagnostic CVs (either for cIMS or linear TWIMS). On the linear TWIMS, elotuzumab is recognized as an IgG1 (59.7  $\pm$  2.7%), even if the IgG4 isotype cannot be unambiguously ruled out (34.3  $\pm$  2.4%), showing once again how the distinction of IgG1 *versus* 4 can be challenging at the intact level with a low IMS resolution. Conversely, scores of 87.3  $\pm$  1.5% are obtained using cIMS, allowing for unambiguous IgG1 identification.



**Figure 28.** UFS plots obtained with CIU-cIMS and linear TWIMS CIU for **(A)** 27+ charge states and **(B)** 28+ charge states. Classifications are based on intact deglycosylated trastuzumab (lgG1), panitumumab (lgG2) and nivolumab (lgG4). **(C)** Categorization obtained for elotuzumab (lgG1).

# 4.1.3. Benchmarking of classification strategies

# 4.1.3.1. Categorization after generation of complete CIU-cIMS fingerprints

Our classification method for the 27+ charge state was further applied to several intact mAbs belonging to different IgG subclasses (Figure 29). Ofatumumab and natalizumab were successfully identified as IgG1 (90.6  $\pm$  3.1%) and IgG4 (84.8  $\pm$  5.1%), respectively (Figure 29A, C). In addition, denosumab was recognized as an IgG2 with an excellent score (99.4  $\pm$  0.7%) despite having an "atypical" CIU fingerprint, with one additional conformational transition between 75 – 150 V compared to the reference IgG2 used to create the classification scheme (Figure 29B). These results corroborate the fact that IgG2s exhibit a distinct behavior compared to other subclasses, with an unequivocal categorization.

These experiments demonstrate that the use of high-resolution cIMS-MS combined to automated classification tools offers a clear-cut and straightforward classification of all mAbs, with scores > 85%, without the need to perform prior IdeS digestion (except for ambiguous hybrid formats). On the other

hand, the categorization at intact level on linear TWIMS instruments is difficult as it requires a very careful optimization of classification methods, which is far from being evident for unexperienced users.



**Figure 29.** CIU-cIMS fingerprints and classification scores obtained for the 27+ charge state of intact deglycosylated **(A)** of atumumab, **(B)** denosumab, and **(C)** natalizumab.

# 4.1.3.2. Targeted-scheduled multiplexed CIU-cIMS

Lastly, we performed targeted-scheduled multiplexed CIU-cIMS, which reduces the data collection time along with drastic reduction of acquisition time (see part IV, chapter 3). Based on UFS plots, only the seven most discriminating CVs (125 to 155 V for the 27+ charge state) were recorded, on a mixture of two different mAbs, elotuzumab (IgG1) and natalizumab (IgG4) (Figure 30A). High classification scores of  $85.9 \pm 2.6\%$  IgG1 for elotuzumab and  $85.7 \pm 4.9\%$  IgG4 for natalizumab were obtained (Figure 30B, C). Those values are extremely close to those achieved after the generation of complete CIU plots (87.3 and 84.8% for elotuzumab and natalizumab, respectively), meaning that accurate categorization is retained, albeit with less time spent on recording CIU fingerprints (from ~45 to 15 min).





# 4.2. CIU-multipass cIMS to further enhance the definition of fingerprints

In order to further explore the potential of high-resolution IMS for CIU fingerprinting, CIU plots were generated either after a single pass or multiple passes (two passes) on the cIMS-MS instrument.
Fingerprints after one or two passes for the 27+ charge state of intact trastuzumab (IgG1) exhibit the same number of conformational states (six features, Figure 31A). Although no new features are uncovered, co-existing activated states are better separated as the resolving power increases. First, features are distributed over a larger arrival time range after two passes, with  $\Delta t_{D (0-200V)} = 32$  ms between the most folded state and the most unfolded one, while  $\Delta t_{D (0-200V)} = 16$  ms at one pass. The % valley separation also improves with more passes. At trap CV = 80 V, co-existing conformers are separated at 57% valley after two passes, which represents an increase of +17% compared to one pass (Figure 31B). Similarly, at high CVs (180 V), a gain of +19% valley separation is obtained between the main features.

Overall, benefits of multipass CIU-cIMS for enhanced graphic resolution of CIU fingerprint are well illustrated in the case of intact trastuzumab. Yet, it is important to note that recording multipass CIU remains challenging. Indeed, the ATD window for data acquisition, which is tuned based on ATDs at CV = 0 V, is kept constant along the whole CV ramp. However, upon activation, unfolded species with lower mobilities are generated, which means than the most extended conformations at high voltages could eventually be excluded from that fixed ATD window. More specifically, folded states would be recorded at *n* passes, while unfolded ones would not have completed pass *n-1*. As the number of passes increases, this effect will be stronger, in addition to possible wrap-around which also limits possibilities for multipass CIU.

In order to ease data collection and treatment, CIU fingerprints can be acquired at one pass, which is already enough to achieve significant improvements in terms of IgG classifications compared to CIU experiments carried out on linear TWIMS-MS instruments.



**Figure 31.** CIU-cIMS experiments at one *versus* two passes. **(A)** Fingerprints obtained for the 27+ charge state intact deglycosylated trastuzumab. **(B)** ATDs were extracted at different trap CVs at one (black) and two (red) passes, with associated % valley separation calculated between the two main features.

## 5. Exploiting (CIU)-IMS-CIU-IMS capabilities of the cIMS-MS instrument to characterize IgG2 disulfide variants

As a reminder, we have seen in part III (chapter 3) that high-resolution cIMS-MS is able to separate (at least) two conformers for native IgG2s, which are most probably related to disulfide isoforms (Figure 32). As gas-phase behaviors of IgG2 disulfide variants have not been extensively documented, we next intended to gain more information on these isoforms and their unfolding patterns by using IMS<sup>n</sup> capabilities of the cIMS-MS instrument combined to CIU approaches.



**Figure 32.** cIMS-MS reveals the presence at least two conformers in IgG2s, as exemplified by **(A)** panitumumab and **(B)** denosumab. A slight interconversion towards a more extended form is observed for selection I.

## 5.1. Selection of co-existing excited states to differentiate their gas-phase behaviors

A point that caught our attention was the presence of two co-existing conformational states between 75 – 150 V for the 27+ charge state of intact denosumab, considering that our reference IgG2 panitumumab displays a single feature in this CV region (Figure 33A). As isoforms for denosumab are initially separated contrary to panitumumab for which one conformer is seen as a shoulder, a hypothesis could be that differences between disulfide variants of denosumab are more pronounced, and eventually led to separation into two clearly distinguishable states upon activation. Consequently, our first idea was to perform CIU-IMS-CIU-IMS, with a pre-activation in trap at 130 V, followed by selection at two passes (IMS<sup>1</sup>) and further activation of each population during IMS<sup>2</sup> (states 2 and 3, Figure 33A, B). These experiments could help (i) to see whether the two activated features have different unfolding behaviors upon reinjection into the cIMS cell, and (ii) to determine if state 4 arises from rearrangement of the conformational state 2 or 3.

The first step to conduct CIU-IMS-CIU-IMS experiments is to ensure that the selection and isolation of each population is efficient, i.e. that no interconversion occurs between states 2 and 3. Indeed, if both states immediately interconvert, performing CIU-IMS-CIU-IMS would be pointless as CIU fingerprints resulting would be the same for both selected features.

We thus performed CIU-IMS<sup>2</sup> experiments to check for possible interconversion (Figure 33B). Unfortunately, after selection of each conformational population, strictly identical ATDs were obtained. Interconversion between states 2, 3, and 4 is observed (IMS<sup>2</sup>, Figure 33B). Although we could not pursue on CIU-IMS-CIU-IMS, these results suggest that the co-existence of activated features for denosumab is not related to disulfide variants, as spontaneous interconversion between disulfide isoforms in the gas-phase is unlikely<sup>378</sup>. This conclusion is perhaps not so surprising as IgG4s also display the same equilibrium between two features in the 75 – 150 V range (Figure 27B, 29C). The differentiation of IgG2s in IMS is a real challenge and this aspect should be investigated in greater detail with further experiments.



**Figure 33. (A)** CIU-cIMS fingerprints of intact panitumumab *versus* denosumab after one pass. Extracted ATDs at trap CV = 130 V. **(B)** CIU-IMS<sup>2</sup>. After activation in the trap at CV = 130 V, each conformer can be selected (IMS<sup>1</sup>) and ejected to the prestore, before being reinjected for separation (IMS<sup>2</sup>). States 2 and 3 were successively selected. Resulting extracted ATDs show an interconversion between features 2, 3 and 4.

## 5.2. IMS-CIU-IMS to differentiate IgG2 disulfide variants

### 5.2.1. Results

Again, the aim is to see if both conformational populations separated for denosumab display different CIU fingerprints. We have seen that the separation of disulfide variants is lost after activation in the trap cell (CIU-cIMS), which prevents the monitoring of each conformer's evolution in the gas phase through classical CIU-cIMS approach. Thus, we decided to work directly on the two conformers identified in native conditions. Here, IMS-CIU-IMS experiments are more adapted, with activation occurring only after selection of each native state. In order to achieve a better separation of both states, selection was performed after four passes for the 27+ charge state of intact denosumab (Figure 34A). The IMS<sup>2</sup> stage consists of a single pass analysis. This experiment can be thus summarized as follows: IMS<sup>1</sup>(4 passes)  $\rightarrow$  CIU  $\rightarrow$  IMS<sup>2</sup>(1 pass).

IMS-CIU-IMS fingerprints for the two selected features are highly similar, with unfolding starting at +70 V (Figure 34B). While differences are clear for native states (RMSD = 43%), RMSD values decrease to 20% during unfolding. Broader CIU traces are obtained for window I, which is logical considering that we had previously pinpointed a slight interconversion of this feature towards a more extended conformer of low intensity (Figure 32B; refer to part III, chapter 3 for more details). As this minor conformer would not be detected using CIU50 analysis, we moved to waterfall representations and

Gaussian fitting to better visualize subtle differences that could arise between the two selections (Figure 34C, D). Additional populations in the 75 – 90 V range are detected for slice I (five to six features), suggesting a wider conformational space upon activation than slice II (four features). Even if minor variations could be observed, this region is difficult to interpret as continuous unfolding occurs, without clear separation of conformers.

Drawing definite conclusions on different gas-phase unfolding for the two selected windows thus remains elusive. Further investigation in reducing conditions could help to clarify behaviors of disulfide isoforms.



**Figure 34.** IMS-CIU-IMS experiments on the 27+ charge state of intact deglycosylated denosumab. **(A)** IMS selection of windows I/II at four passes. **(B)** CIU occurs upon reinjection from the prestore. Generated CIU fingerprints were compared through a differential plot. **(C)** Extracted ATDs for each selection. **(D)** Gaussian fitting was performed for ATDs extracted at +85V, allowing to highlight differences between the two selected species.

## 5.2.2. Limitations of IMS-CIU-IMS for intact native mAbs

Prestore CIU experiments are limited as the prearray gradient starts at 95 V and cannot exceed 200 V. In addition, activation performed on re-entry to the array is less energetic that CIU in the trap cell. While only 25 V in the trap cell are needed to reach the first transition (Figure 33A), the transition starts after an increase of +80 V in the prestore (Figure 34B), most probably as a result of higher pression in the cIMS cell (1.75 mbar) compared to the trap cell (10<sup>-2</sup> mbar).

In the end, the interest of IMS-CIU-IMS, which provides only a "mild" activation, seems limited for intact mAbs. These large molecules possess many degrees of freedom, and are able to accommodate for more energy before ultimately unfolding. Hence, kinetic energies conferred to ions are not enough to reach more unfolded states which could be more informative.

## 6. Conclusions

The combination of CIU to high-resolution cIMS-MS provides further insights into gas-phase behaviors of biotherapeutics. Additional features are uncovered upon activation, allowing to refine IgG classification as a result from more accurate graphical definition of CIU fingerprints. CIU-cIMS experiments offer decisive advantages over linear TWIMS CIU and even high-resolution IMS-MS measurements.

IMS<sup>n</sup> experiments open up new possibilities to explore gas-phase unfolding of (minor) selected features. These multifunction capabilities could help to move towards a detailed understanding of unfolding pathways, and structural identification/modelling of conformational states through computational MD approaches. Although IMS-CIU-IMS does not seem to be suitable for intact mAbs, IdeS digestion could be an attractive alternative as the activation of smaller subunits requires less energy because of fewer degrees of freedom.

### Scientific communication

A scientific publication reporting on these results is currently in preparation.

#### **Oral presentation**

<u>Deslignière, E.</u>; Ollivier, S.; Beck, A.; Giles, K.; Richardson, K.; Hernandez-Alba, O.; Ropartz, D.; Rogniaux, H.; Cianférani, S., High-resolution cyclic ion mobility-mass spectrometry for improved conformational characterization and subclass differentiation of native intact therapeutic monoclonal antibodies. 69<sup>th</sup> ASMS Conference on Mass Spectrometry and Allied Topics, October 31-November 4<sup>th</sup> 2021 (Philadelphia, PA, USA).

## **Part IV – Conclusions**

In this part, benefits of CIU approaches for in-depth characterization of different therapeutic mAbderived formats have been outlined. CIU offers the potential to produce significantly richer structural datasets for biotherapeutics compared to IMS-MS measurements.

• First, I showed that CIU is a versatile tool for biotherapeutics:

- Through the evaluation of new bioinformatics tools, I was able to develop classification methods to achieve a more quantitative and accurate IgG isotype categorization. IgG subclasses present distinct unfolding patterns, in particular for  $F(ab')_2$  regions. Identification of isotypes at the middle level is especially interesting for engineered hybrid formats.

- I next illustrated the structuring role of disulfide bonds in the case of cysteine-based ADCs, for which disulfide reduction impairs the resistance of conjugates against gas-phase unfolding.

- CIU was also employed to detect subtle conformational differences along the synthesis of a sitespecific DAR2 ADC, helping to monitor conjugation-dependent gas-phase stability.

• Next, I tackled the **challenging issue of CIU automation**. For this, I developed an online SEC-CIU coupling. SEC-CIU affords a fast and efficient desalting and preserves key features of CIU fingerprints, while allowing for drastic reduction of the overall time process. The coupling thus holds great potential to meet high-throughput biopharma requirements. As SEC-nMS is amenable to rapid analysis of multiple next-generation formats<sup>289</sup>, including nanobodies, antibody-protein fusion biologics, and bsAbs that currently account for 20% of the clinical antibody pipeline<sup>141</sup>, our SEC-CIU setup could push forward the routine use of CIU in R&D laboratories.

• Lastly, I evaluated the potential of **high-resolution IMS-MS for CIU experiments**. The cIMS-MS instrument used in this thesis offers an improved definition of CIU fingerprints with increased separation of co-existing conformational states, resulting in even more precise classification of IgG isotypes than for linear TWIMS. Multipass and IMS<sup>n</sup> modes available on the cIMS-MS platform open up new possibilities for CIU experiments, allowing to focus on the extensive characterization of selected features with higher resolution<sup>14</sup>. As of today, very few studies have exploited these capabilities for native proteins<sup>19,20</sup>, and so there is still a huge amount of work ahead to better understand unfolding mechanisms of therapeutic mAbs and protein complexes.

As a final note on CIU, I would like to mention the development of a fast CIU workflow recently described by the group of Ruotolo<sup>420</sup>. This technique relies on microfluidic-based sample introduction. Nanoliter droplets separated by a perfluorodecalin carrier phase are generated from a multiwell plate into a tubing connected to a syringe pump. Droplets are then transferred to a nanoESI tip using a homebuilt polydimethylsiloxane union for direct injection into a Synapt instrument. This technique allows

to analyze 96 compounds in  $\sim$ 60 min (10 s per sample in triplicate, including wash steps between samples), which makes it attractive for protein/ligand screening.

# General Conclusions and Perspectives

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## **General Conclusions and Perspectives**

My PhD work was centered on technical developments in nMS and IMS-MS to better address problems encountered either by structural biologists or by biopharma companies. In this context, my thesis has focused on different methodological developments aiming at:

• Improving the sample preparation step for noncovalent assemblies analyses: At the beginning of my PhD work, the SEC-nMS coupling was used routinely for mAbs in the lab. I demonstrated that the SEC-nMS coupling is amenable to the analysis of a broader range of protein complexes (protein/DNA and high molecular weight species), offering a fast online buffer exchange tool, and avoiding in-solution complex destabilization that can occur with manual desalting.

• Integrating nMS and nIMS-MS data into structural biology programs: Through my involvement in a structural biology program centered on RuvBL helicases and their associated partners, I was able to show the potential of nMS, nIMS-MS and newly-developed mass photometry techniques to complement conventional biophysical approaches. In particular, this project highlighted that nIMS-MS data were in excellent agreement with SEC experiments and CCS predicted from crystal structures, proving that native conformations are retained in the gas-phase. This brought me to the conclusion that nMS and nIMS-MS are valuable tools to tackle macromolecules, and their online coupling with SEC will surely be of benefit for the structural biology community.

• Evaluating benefits of high-resolution IMS-MS for the characterization of therapeutic proteins: For this, I focused on one high-resolution IMS-MS instrument, the cyclic IMS geometry developed by Waters. After describing the architecture of the cIMS cell, along with its way of operation and advantages, its multifunction capabilities (isolation, IMS<sup>n</sup>) were presented and applied to a series of problems related to the conformational characterization of mAbs. A first study consisted of highlighting the advantages of high-resolution IMS-MS for the determination of disulfide pairings on a tryptic digest of an IgG4 bearing one additional Cys residue in the CDR3 region of its heavy chain. This project highlighted benefits of cIMS-MS to resolve new conformational features and enhance the separation of disulfide-bridged peptide isomers compared to linear TWIMS-MS, leading to clear-cut identification of disulfide pairings from the ATD profiles of a single IMS-MS measurement. I next had the opportunity to illustrate the potential of the high-resolution cIMS-MS instrument for more accurate IgG subclasses categorization based solely on ATD profiles. Although specific bimodal profiles were unveiled for IgG2s, differences were less obvious for IgG1s versus IgG4s, first results however hinting at wider conformational spaces for IgG4s. Finally, benefits of cIMS-MS were pivotal for the characterization of a more complex engineered multispecific antibody. High-resolution cIMS-MS provided definitive proof of existence of two conformational isomers that could only be clearly resolved at the middle level using cIMS-MS, whereas linear TWIMS-MS provided a limited separation of the two species. These are the first applications of cIMS-MS to the conformational characterization

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of intact native biotherapeutics. Overall, results obtained on the cIMS-MS platform illustrate the definite advantages of high resolution second-generation IMS-MS instruments over first-generation ones, and will, with no doubt, be of interest for in-depth analysis of mAb-based products and more challenging biological complexes.

Automating CIU approaches and developing high-resolution CIU-MS approaches: After describing the influence of several MS parameters on CIU unfolding patterns to show how important it is to carefully tune temperature/pressure to avoid preemptive unfolding, I applied CIU approaches to a series of projects of increasing complexity related to mAb characterization. Through the evaluation of new bioinformatics tools to improve the exploitation of CIU fingerprints, I was able to propose a data treatment workflow that pinpoints diagnostic CVs regions for the differentiation of IgG subclasses at intact and middle levels. In addition, CIU was of utmost interest not only to monitor the conjugation of a next-generation site-specific ADC, but also to highlight the key role of disulfide bonds in the maintain of cysteine-linked ADCs. A central part of this PhD work was the direct hyphenation of SEC to CIU, which allowed to design a fully automated CIU pipeline. Thanks to the fast online SEC desalting, the SEC-CIU coupling significantly reduces data collection time. Even higher throughput could be achieved with targeted-scheduled and/or multiplexed strategies for IgG classification. This represents the first setup integrating a nondenaturing LC dimension with CIU experiments. Finally, I developed high-resolution CIU approaches on the cIMS-MS instrument, demonstrating benefits of higher resolving powers for more straightforward CIU feature resolution/detection and subsequent IgG categorization than on first-generation IMS instruments. Multifunction capabilities of the cIMS-MS platform were also investigated to generate preliminary results for the differentiation of IgG2 disulfide isoforms' gas-phase unfolding behaviors. Altogether, CIU has the potential to become a highthroughput screening tool for the development of biotherapeutics, even for unusual antibody architectures.

Even if structural MS techniques are now well-established in the field of structural biology, and have started to enter biopharmaceutical R&D laboratories, nMS and nIMS-MS still face many challenges that will have to be overcome in the next years.

• Analytical challenges related to high MW analysis by nMS: We have most likely reached the upper limit of what can be measured through conventional ESI-nMS, i.e. by deducing masses from a charge state distribution. This is the approach used in commercial instruments. Snijder *et al.* suggested that complexes up to 20 MDa could be studied by nMS (authors used a modified Q-ToF 1 instrument)<sup>8</sup>. Yet, for heterogeneous populations, the limit is much smaller<sup>421</sup>.

A first way of dealing with high molecular weight species in a straightforward manner is to use mass photometry. This in-solution technique is well adapted to rapidly assess the heterogeneity (in terms of binding stoichiometries) of macromolecular assemblies up to 5 MDa<sup>196,198</sup>. Measurements are

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extremely fast (< 1 min). This technique requires smaller quantities (< pmole) and less concentrated samples (100  $\mu$ M – 100 nM) than nMS, which makes mass photometry particularly attractive for structural biology programs where protein purification yields low amounts of material<sup>197</sup>. The major drawbacks of this technique are the lack of detailed structural/conformational information, and the limited concentration range which will most likely be lifted in the near future with instrumental improvements<sup>197</sup>. Mass photometry could undoubtedly become an essential routine tool for structural biology projects, and will be of utmost interest for X-ray crystallography and cryo-EM communities.

To move one step forward into the gigadalton regime, single-molecule MS methods that have emerged in the last decade appear promising<sup>422</sup>.

Nanomechanical resonators represent an attractive alternative to study large macromolecular assemblies, even in the gigadalton range<sup>423</sup>. Here, individual particles are deposited on a nanoresonator, whose frequency is highly dependent on the particle mass. Shifts in frequencies are monitored as molecules accrete on the resonator, which allows to deduce the mass of each particle (independently of the charge) in real time<sup>424</sup>. This technology is amenable to analyzing not only ions but also neutral particles<sup>425</sup>. Hentz and coworkers successfully characterized a 108.4 MDa DNA-filled bacteriophage T5 capsid<sup>426</sup>. Current limitations lie in the fact that experiments are time-consuming (hours), with also low resolutions in some cases. Nanomechanical systems could open new applications in the field of structural biology, by achieving the performance required to tackle supramolecular assemblies, and in particular large viruses used for gene therapy delivery or cell therapy transduction.

Among single-molecule techniques, charge detection MS (CDMS) seems to be the most promising approach for the analysis of mega- and gigadaltons species. In CDMS, individual ions pass through a conducting cylinder. The charge can be determined from the amplitude of the current induced when the ion travels through the detector tube, while the m/z ratio is calculated from the flight time and kinetic energy of each ion<sup>427</sup>. Deconvolution of an m/z spectrum thus becomes unnecessary, avoiding the need for resolved charge states that would be required in traditional nMS. The implementation of CDMS on Orbitrap instruments has afforded increased sensitivity and resolution, enabling the analysis of even more complicated mixtures of proteoforms<sup>428,429</sup>. A recent example of CDMS is the characterization of the heavily glycosylated trimeric SARS-CoV-2 spike protein (560 kDa), which represents a challenge due to glycan heterogeneity<sup>430</sup>. CDMS has also been largely used for viruses, among which genome-filled adeno-associated viruses (4.9 MDa)<sup>431</sup>, intact Flock House virus (9.4 MDa)<sup>432</sup>, and live-attenuated rotavirus (RotaTeq vaccine, 61.1 MDa)<sup>433</sup>. CDMS experiments remain lengthy because ions are weighed individually, but Harper et al. have demonstrated that up to 13 ions could be monitored simultaneously to considerably reduce analysis time<sup>434</sup>. It is thus conceivable that with further instrumental developments, CDMS will become compatible with prior nondenaturing LC separation, easing the characterization of high molecular weight species<sup>353</sup>. In addition, the groups of Jarrold and Clemmer are currently working on the coupling of high-resolution IMS to CDMS, to provide additional conformational information that would complement data generated from CDMS. IMS-CDMS has the potential to grow into a powerful approach to conquer the nanoworld.

• Towards more user-friendly nIMS-MS workflows (calibration, data interpretation, etc.): Multiple challenges also need to be addressed to promote the use of nIMS-MS not only for structural biology projects, but also for detailed characterization of biotherapeutics.

Over the past five years, technological developments have made high-resolution IMS accessible to a wider audience, with the release of TIMS, SLIM and cIMS instruments. Studies of protein complexes on these platforms are still at their beginnings, and so it is too early to comment on TIMS and SLIM performances regarding native assemblies. On the other hand, in light of results obtained during this thesis, cIMS-MS appears to be an efficient qualitative tool to distinguish overlapping conformational families. Yet, because samples frequently contain multiple conformers, the interpretation of rich IMS-MS data is difficult, even with high-resolution instruments that cannot fully resolve co-existing species. Deconvolution into Gaussian contributions is helpful, but can be criticized as the selection of appropriate width for Gaussian curves and subsequent number of detected conformers are often subjective. Recent software developments have facilitated IMS-MS analyses, but there is still room to improve the treatment of high-resolution IMS-MS data.

A crucial step to push forward more quantitative high-resolution IMS-MS approaches will require the generation of higher accuracy CCS standards for calibration purposes<sup>14,269</sup>. Current uncertainties in available reference CCS values used for TWIMS calibration are between 1 and 2%. The new calibration approach and associated software package proposed by Richardson *et al.* improve calibration accuracies, and the procedure seems adapted not only for linear TWIMS-MS, but also for cIMS-MS<sup>269</sup>. However, the usefulness of calibrated high-resolution IMS-MS measurements is still limited<sup>435</sup>. Reporting values below those of reference ones would be meaningless considering systematic errors within CCS databases. Building an adapted CCS library, with higher accuracy CCS standards, thus calls for strong commitment of the IMS community.

• **Towards more in-depth interpretation of IMS-MS based datasets:** Another key point to fully exploit nIMS-MS data relies on linking IMS-MS to solution behavior and to be able to model unfolding events that happen in CIU experiments. Those latest objectives mostly rely on progress in the field of MD. To this day, gas-phase structural changes remain hard to predict. Several issues have to be dealt with to advance gas-phase MD simulations<sup>436</sup>:

- A first requirement is to aim at a better understanding of structural changes occurring upon desolvation, and to assess the extent to which gas-phase structures can be predicted from solution ones. Electrostatic interactions should be taken into account over longer distances when working in vacuum, which tremendously slows down calculations and limits simulation time scales. In addition,

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force fields commonly used for MD are adapted for in-solution species and may deviate from what really happens in the gas phase<sup>437</sup>.

- Secondly, because charges play an important role in structural dynamics, it is essential to determine the location of charges on proteins to provide accurate MD simulations<sup>438</sup>. Charge placement is a real challenge considering the number of possible charge isomers that need to be sampled. This area of research is still in its infancy, with ongoing MD studies in the group of Konermann: very recently, they conducted CIU simulations using different MD models, either with static or mobile H<sup>+</sup>, and showed that unrealistic CIU outcomes are obtained if H<sup>+</sup> migration and its effect on salt bridges are not considered<sup>439</sup>.

It is possible that these hurdles will be overcome in a not too distant future, allowing to propose macromolecular structures with contributions from nIMS-MS data. More importantly, such computational approaches could bridge the knowledge gap that remains for CIU experiments. Indeed, a detailed understanding of unfolding pathways with structural identification of intermediate conformational states is lacking, which avoids the full exploitation of CIU fingerprints. A complete model able to predict unfolding patterns based on the structure of the protein still needs to be found.

• Push the development of nTD-MS: Lastly, another promising track that I would like to discuss is the rise of TD-MS approaches, even in native conditions. Several fragmentation techniques (HCD, ETD, ECD, UVPD, etc.) are used for protein dissociation. These techniques allow for the identification and location of proteoforms (PTMs). So far, TD-MS has been performed mainly in denaturing conditions. In native conditions, inducing dissociation in the protein backbone is far more difficult, especially as protein size and complexity increase. nTD-MS gives information on exposed protein regions in a given conformation. nTD-MS results could possibly be linked to conformational data generated from IMS-MS, in order to assess the influence of proteoforms on a protein's global conformation along with its gas-phase stability. nTD-MS could help to decipher unfolding intermediates seen on CIU fingerprints, by pinpointing sequence parts exposed upon activation. These data could be integrated into MD simulations. This approach is particularly interesting for the characterization of mAb-related products (CQA monitoring, conjugation sites, position of fusion proteins, etc.), but is not exclusive to biotherapeutics, and could ultimately be extended to more challenging samples, such as membrane proteins or crude extracts.

To conclude, given the increasingly sophisticated questions that structural MS is expected to answer, we could imagine an integrative workflow for in-depth multi-level characterization of native proteins, as follows (Figure 1):

- Upstream of the mass spectrometer, a first online separative step consisting of SEC or IEX would afford online desalting and separation of size/charge variants. HIC is another option for ADC analysis. Capillary electrophoresis can also be used.

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- After chromatographic separation, conformational information would be recorded using IMS measurements or CIU experiments.

- Finally, each separated species would be fragmented prior to MS detection, allowing to directly connect proteoforms and conformation. MS<sup>n</sup> can even be considered at this stage.



**Figure 1.** Schematic representation of a nondenaturing LC-IMS-TD-MS coupling. The influence of proteoforms on a protein's conformation could be assessed (top). Unfolding intermediates and CIU pathways could also be investigated (bottom).

To this day, the most advanced commercial instruments on which this would be feasible are the cIMS-MS from Waters, especially since the latest integration of the ExD cell, and the SLIM-MS platform from MOBILion (CID fragmentation, along with the possibility to add an ExD cell<sup>440</sup>). Orbitrap platforms released on the market (Fusion Lumos Tribrid and Eclipse Tribrid from Thermo Fisher Scientific) are the most attractive for TD approaches, but are not equipped with an IMS cell able to perform CCS measurements.

Obviously, there is still plenty of work to achieve this advanced LC-IMS/CIU-TD-MS coupling. For the time being, connecting "offline" IMS-MS and TD-MS approaches, in denaturing and then native conditions, would already pave the way towards a better understanding of structure-function relationships, impacting on our knowledge of biological processes as well.

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## Experimental Section

**Experimental Section** 

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## **Experimental Section**

The experimental section summarizes the sample preparation, instrumentation used, and sequences of proteins analyzed along this PhD work.

### 1. Manual desalting

Manual buffer exchange prior to nMS and nIMS-MS experiments was carried out by using two types of desalting devices:

- **Gel filtration**: This technique is based on SEC separation, meaning that low molecular weight salts will enter the bead pores from the stationary resin phase, whereas high molecular weight analytes of interest (proteins) will be not be retained within pores. Proteins thus separate from the original buffer salts, and exchange into the column buffer. Zeba columns (Thermo Fisher Scientific) with a cutoff of 7 kDa were used during this PhD thesis.

- Ultrafiltration: This technique separates species based on their sizes using centrifugation. The sample is deposited on a semipermeable membrane, whose pore size determines which proteins will be able to pass through. Centrifugation is applied to force solvent through the membrane: species whose MWs are lower that the cutoff will traverse the membrane, while larger analytes will be retained. Several dilution/concentration cycles are required to ensure an efficient desalting of proteins. Vivaspin ultrafiltration columns (Sartorius), composed of a polyethersulfone membrane, were used during this work, with cutoffs of 10, 30, and 50 kDa.

After the manual buffer exchange step, the sample concentration is measured based on UV absorbance at 280 nm with a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). The molecular mass and extinction coefficient are determined based on the theoretical sequence using ProtParam Expasy, allowing to access to the sample concentration *via* the Beer-Lambert law.

### 2. Instrumentation

## 2.1. ToF instrument: LCT

Figure 1 represents the LCT from Micromass (U.K.), which was upgraded for high *m/z* values by MS Vision. The LCT was coupled to the automated chip-based nanoESI TriVersa NanoMate device (Advion, U.S.).

#### 2.2. Orbitrap instrument: Exactive Plus EMR

Figure 2 represents the Exactive Plus EMR Orbitrap from Thermo Fisher Scientific (Germany), coupled to the TriVersa NanoMate device.

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**Figure 1.** Schematic representation of the LCT ToF. The TriVersa NanoMate was coupled to the LCT for experiments performed in part II, chapters 1 (ERR) and 2 (R1R2).



**Figure 2.** Schematic representation of the Exactive Plus EMR Orbitrap. The TriVersa NanoMate was coupled to the EMR for experiments performed in part II, chapter 2 (DPCD, R1R2 complex, R2D complex).

#### 2.3. SEC-nMS coupling

The SEC-nMS coupling uses an Acquity H-Class chromatographic system (Waters). The Acquity H-Class is composed of a quaternary pump (QSM), a sampler manager (FTN), a column oven, set at ambient temperature during my experiments, and a UV detector. The system is controlled by MassLynx v4.1. Figure 3 presents the coupling on the Synapt G2 HDMS.



Figure 3. Photograph of the Acquity H-Class coupled to the Synapt G2 HDMS.

### 3. Protein sequences

The following tables provide the sequence and molecular mass of proteins analyzed during this PhD work. The desalting method is also indicated.

3.1.	Part II -	<b>Chapter</b>	<b>1.</b> '	Versatility	of	SEC-nMS
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Protein	Molecular mass (Da)	Desalting	Sequence
ERRα	46 389.5	150 mM AcONH₄, pH 6.9 Zeba (2 cycles) or Acquity SEC column BEH200 4.6 x 150 mm, 1.7 μm	MGSSQVVGIEPLYIKAEPASPDSPKGSSETETEPPVALAPGPAPTRCLPG HKEEEDGEGAGPGEQGGGKLVLSSLPKRLCLVCGDVASGYHYGVASCE ACKAFFKRTIQGSIEYSCPASNECEITKRRKKACQACRFTKCLRVGMLKE GVRLDRVRGGRQKYKRRPEVDPLPFPGPFPAGPLAVAGGPRKTAAPVN ALVSHLLVVEPEKLYAMPDPAGPDGHLPAVATLCDLFDREIVVTISWAKS IPGFSSLSLSDQMSVLQSVWMEVLVLGVAQRSLPLQDELAFAEDLVLDE EGARAAGLGELGAALLQLVRRLQALRLEREEYVLLKALALANSDSVHIED AEAVEQLREALHEALLEYEAGRAGPGGGAERRRAGRLLLTLPLLRQTAG KVLAHFYGVKLEGKVPMHKLFLEMLEAMMDHHHHHH
DNA WC26	15 941.30	150 mM AcONH₄, pH 6.9 Zeba (2 cycles)	ATGTCAAGGTCACCGTGACCTTTACG TCGTAAAGGTCACGGTGACCTTGACA
Yeast Rvb1	50 744.40	150 mM AcONH₄, pH 7.5 Zeba (2 cycles) or Acquity SEC column BEH450 4.6 x 150 mm, 2.5 μm	GPHMVAISEVKENPGVNSSNSGAVTRTAAHTHIKGLGLDESGVAKRVE GGFVGQIEAREACGVIVDLIKAKKMSGRAILLAGGPSTGKTALALAISQEL GPKVPFCPLVGSELYSVEVKKTETLMENFRRAIGLRIKETKEVYEGEVTEL TPEDAENPLGGYGKTISHVIVGLKSAKGTKTLRLDPTIYESIQREKVSIGDV IYIEANTGAVKRVGRSDAYATEFDLETEEYVPLPKGEVHKKKEIVQDVTLH DLDVANARPQGQDVISMMGQLLKPKKTEITEKLRQEVNKVVAKYIDQ GVAELIPGVLFIDEVNMLDIEIFTYLNKALESNIAPVVVLASNRGMTTVRG TEDVISPHGVPPDLIDRLLIVRTLPYDKDEIRTIIERRATVERLQVESSALDL LATMGTETSLRYALQLLAPCGILAQTSNRKEIVVNDVNEAKLLFLDAKRS TKILETSANYL
Yeast Rvb2	51 611.6	150 mM AcONH₄, pH 7.5 Zeba (2 cycles) or Acquity SEC column BEH450 4.6 x 150 mm, 2.5 μm	MSIQTSDPNETSDLKSLSLIAAHSHITGLGLDENLQPRPTSEGMVGQLQ ARRAAGVILKMVQNGTIAGRAVLVAGPPSTGKTALAMGVSQSLGKDVP FTAIAGSEIFSLELSKTEALTQAFRKSIGIKIKEETELIEGEVVEIQIDRSITGG HKQGKLTIKTTDMETIYELGNKMIDGLTKEKVLAGDVISIDKASGKITKLG RSFARSRDYDAMGADTRFVQCPEGELQKRKTVVHTVSLHEIDVINSRTQ GFLALFTGDTGEIRSEVRDQINTKVAEWKEEGKAEIVPGVLFIDEVHMLD IECFSFINRALEDEFAPIVMMATNRGVSKTRGTNYKSPHGLPLDLLDRSIII TTKSYNEQEIKTILSIRAQEEEVELSSDALDLLTKTGVETSLRYSSNLISVAQ QIAMKRKNNTVEVEDVKRAYLLFLDSARSVKYVQENESQYIDDQGNVQI SIAKSADPDAMDTTE

Protein	Molecular mass (Da)	Desalting	Sequence
Human R1 FL	51 759.7		MVHHHHHHLVPRGSKIEEVKSTTKTQRIASHSHVKGLGLDESGLAKQAAS GLVGQENAREACGVIVELIKSKKMAGRAVLLAGPPGTGKTALALAIAQELG SKVPFCPMVGSEVYSTEIKKTEVLMENFRRAIGLRIKETKEVYEGEVTELTPC ETENPMGGYGKTISHVIIGLKTAKGTKQLKLDPSIFESLQKERVEAGDVIYIE ANSGAVKRQGRCDTYATEFDLEAEEYVPLPKGDVHKKKEIIQDVTLHDLDV ANARPQGGQDILSMMGQLMKPKKTEITDKLRGEINKVVNKYIDQGIAELV PGVLFVDEVHMLDIECFTYLHRALESSIAPIVIFASNRGNCVIRGTEDITSPH GIPLDLLDRVMIIRTMLYTPQEMKQIIKIRAQTEGINISEEALNHLGEIGTKTT LRYSVQLLTPANLLAKINGKDSIEKEHVEEISELFYDAKSSAKILADQQDKYM K
Human R2 FL	51 886.4	200 mM AcONH₄, pH 7.5 Zeba (2 cycles)	MATVTATTKVPEIRDVTRIERIGAHSHIRGLGLDDALEPRQASQGMVGQL AARRAAGVVLEMIREGKIAGRAVLIAGQPGTGKTAIAMGMAQALGPDTP FTAIAGSEIFSLEMSKTEALTQAFRRSIGVRIKEETEIIEGEVVEIQIDRPATGT GSKVGKLTLKTTEMETIYDLGTKMIESLTKDKVQAGDVITIDKATGKISKLGR SFTRARDYDAMGSQTKFVQCPDGELQKRKEVVHTVSLHEIDVINSRTQGFL ALFSGDTGEIKSEVREQINAKVAEWREEGKAEIIPGVLFIDEVHMLDIESFSF LNRALESDMAPVLIMATNRGITRIRGTSYQSPHGIPIDLLDRLLIVSTTPYSEK DTKQILRIRCEEEDVEMSEDAYTVLTRIGLETSLRYAIQLITAASLVCRKRKGT EVQVDDIKRVYSLFLDESRSTQYMKEYQDAFLFNELKGETMDTSLEVLFQ
Human R1 ΔDII	40 322.8		MVHHHHHHLLVPRGSKIEEVKSTTKTQRIASHSHVKGLGLDESGLAKQAAS GLVGQENAREACGVIVELIKSKKMAGRAVLLAGPPGTGKTALALAIAQELG SKVPFCPMVGSEVYSTEIKKTEVLMENFRRAIGLRIKEGPPGIIQDVTLHDLD VANARPQGGQDILSMMGQLMKPKKTEITDKLRGEINKVVNKYIDQGIAEL VPGVLFVDEVHMLDIECFTYLHRALESSIAPIVIFASNRGNCVIRGTEDITSPH GIPLDLLDRVMIIRTMLYTPQEMKQIIKIRAQTEGINISEEALNHLGEIGTKTT LRYSVQLLTPANLLAKINGKDSIEKEHVEEISELFYDAKSSAKILADQQDKYM K
Human R2 ΔDII	40 743.7		MATVTATTKVPEIRDVTRIERIGAHSHIRGLGLDDALEPRQASQGMVGQL AARRAAGVVLEMIREGKIAGRAVLIAGQPGTGKTAIAMGMAQALGPDTP FTAIAGSEIFSLEMSKTEALTQAFRRSIGVRIKEGPPGVVHTVSLHEIDVINSR TQGFLALFSGDTGEIKSEVREQINAKVAEWREEGKAEIIPGVLFIDEVHMLD IESFSFLNRALESDMAPVLIMATNRGITRIRGTSYQSPHGIPIDLLDRLLIVSTT PYSEKDTKQILRIRCEEEDVEMSEDAYTVLTRIGLETSLRYAIQLITAASLVCR KRKGTEVQVDDIKRVYSLFLDESRSTQYMKEYQDAFLFNELKGETMDTSLE VLFQ
DPCD	23 505.0	For R2D reconstitution, or in preformed R2D 200 mM AcONH₄, pH 7.5 Zeba (2 cycles)	GPHMAVTGWLESLRTAQKTALLQDGRRKVHYLFPDGKEMAEEYDEKTSE LLVRKWRVKSALGAMGQWQLEVGDPAPLGAGNLGPELIKESNANPIFMR KDTKMSFQWRIRNLPYPKDVYSVSVDQKERCIIVRTTNKKYYKKFSIPDLDR HQLPLDDASLSFAHANCTLIISYQKPKEVVVAESELQKELKKVKTAHSNDGD CKTQ
RPAP3 396-665	31 830.1	Preformed R2T'P' complex 200 mM AcONH4, pH 7.5	MDYKDDDDKASELIEKGHWDDVFLDSTQRQNVVKPIDNPPHPGSTKPLK KVIIEETGNLIQTIDVPDSTTAAAPENNPINLANVIAATGTTSKKNSSQDDLF PTSDTPRAKVLKIEEVSDTSSLQPQASLKQDVCQSYSEKMPIEIEQKPAQFA TTVLPPIPANSFQLESDFRQLKSSPDMLYQYLKQIEPSLYPKLFQKNLDPDVF NQIVKILHDFYIEKEKPLLIFEILQRLSELKRFDMAVMFMSETEKKIARALFNH IDKSGLKDSSVEELKKRYGG
PIH1D1 199-290	11 273.1	zeba (z cycles)	MVGRAESGPEKPHLNLWLEAPDLLLAEIDLPKLDGALGLSLEIGENRLVMG GPQQLYHLDAYIPLQINSHESKAAFHRKRKQLMVAMPLLPVPSGSLEVLFQ
<b>SPAG</b> 622-926 (± <u>Flag</u> )	34 907.2 <u>36 060.4</u>	For R2S'P reconstitution, on in preformed R2S'P' 200 mM AcONH4, pH 7.5 Zeba (2 cycles)	MTFKALKEEGNQCVNDKNYKDALSKYSECLKINNKECAIYTNRALCYLKLCQ FEEAKQDCDQALQLADGNVKAFYRRALAHKGLKNYQKSLIDLNKVILLDPSI IEAKMELEEVTRLLNLKDKTAPFNKEKERRKIEIQEVNEGKEEPGRPAGEVS MGCLASEKGGKSSRSPEDPEKLPIAKPNNAYEFGQIINALSTRKDKEACAHL LAITAPKDLPMFLSNKLEGDTFLLLIQSLKNNLIEKDPSLVYQHLLYLSKAERF KMMLTLISKGQKELIEQLFEDLSDTPNNHFTLEDIQALKRQYEL <u>ASDYKDDD</u> <u>DK</u>
PIH1D2 225-315	12 144.3	Preformed R2S'P' 200 mM AcONH₄, pH 7.5 Zeba (2 cycles)	MVKMPAYELKIVHDHSEKPLKIELKVELPGINSVSLCDLSVSEDDLLIEVSEKY RLHLNLPKLIDTEMTTAKFIKEKSTLIITMPLVGSLEVLFQ
PIH1D2 FL	36 248.1	For R2S'P reconstitution 200 mM AcONH₄, pH 7.5 Zeba (2 cycles)	GPHMETSSKGLLTQVTQFWNLLDDLAQSDPEGYEKFIQQQLKEGKQLCAA PEPQLCLQTRILKPKEKILFINLCQWTRIPAPQSTTHPVPLTVGKPEDTTEISD AYTVIDVAYNPDVLHAAEKDQVKKNQLIQMAMKCIEEKFQFTLSHSYHITK FRIKGSIQRMKQNLMGIQTDSIDLREKMRRELTLGQIRSSTMSNPDHFPQL LLPKDQVSGKAVCLIEEISSTEIQVEMKMPAYELKIVHDHSEKPLKIELKVELP GINSVSLCDLSVSEDDLLIEVSEKYRLHLNLPKLIDTEMTTAKFIKEKSTLIITM PLV

## 3.2. Part II – Chapter 2. RuvBL1/2 Complexes

## 3.3. Part III (Chapter 3) and Part IV (All Chapters)

Protein	Molecular	Desalting	Sequence
	mass (Da)		Light chain
adalimumab (deglycosylated)	145 890		DIQMTQSPSSLSASVGDRVTITCRASQGIRNYLAWYQQKPGKAPKLLIY AASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDVATYYCQRYNRAPYTFG QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC Heavy chain EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEW VSAITWNSGHIDYADSVEGRFTISRDNAKNSLYLQMNSLRAEDTAVYYC AKVSYLSTASSLDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAAL GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK
brentuximab B (deglycosylated) Parent mAb of BV	146 010	100 mM AcONH₄ pH 6.9 Vivaspin 50 kDa for intact mAbs Vivaspin 10/30 kDa for IdeS-digested mAbs (6 – 8 cycles) or	DIVETQSPASLAVSLGQIRATISCKASQSVDFDGDSYMINWYQQRPGQPP KVLIYAASNLESGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQSNEDP WTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYAC EVTHQGLSSPVTKSFNRGEC Heavy chain QIQLQQSGPEVVKPGASVKISCKASGYTFTDYYITWVKQKPGQGLEWIG WIYPGSGNTKYNEKFKGKATLTVDTSSSTAFMQLSSLTSEDTAVYFCANY GNYWFAYWGQGTQVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT YICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPG
denosumab (deglycosylated)	145 161	Acquity SEC column BEH200 4.6 x 300 mm, 1.7 μm Acquity SEC column BEH200 4.6 x 150 mm, 1.7 μm Acquity SEC column BEH125 4.6 x 30 mm, 1.7 μm	Light chain EIVLTQSPGTLSLSPGERATLSCRASQSVRGRYLAWYQQKPGQAPRLLIY GASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVFYCQQYGSSPRTFG QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC Heavy chain EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEW VSGITGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA KDPGTTVIMSWFDPWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTA ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPS SSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK
eculizumab (deglycosylated)	145 679		Light chain DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIY GATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFG QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC Heavy chain VQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEW MGEILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCA RYFFGSSPNWYFDVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS NFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLPP PKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR EEQFBSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQ KSLSLSLG

elotuzumab (deglycosylated)	145 896		Light chain DIQMTQSPSSLSASVGDRVTITCKASQDVGIAVAWYQQKPGKVPKLLIY WASTRHTGVPDRFSGSGSGTDFTLTISSLQPEDVATYYCQQYSSYPYTFG QGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC Heavy chain EVQLVESGGGLVQPGGSLRLSCAASGFDFSRYWMSWVRQAPGKGLE WIGEINPDSSTINYAPSLKDKFIISRDNAKNSLYLQMNSLRAEDTAVYYCA RPDGNYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF PPKKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGK
natalizumab (deglycosylated)	146 619	100 mM AcONH₄ pH 6.9 Vivaspin 50 kDa for intact mAbs Vivaspin 10/30 kDa for IdeS-digested mAbs (6 – 8 cycles)	Light chain DIQMTQSPSSLSASVGDRVTITCKTSQDINKYMAWYQQTPGKAPRLLIH YTSALQPGIPSRFSGSGSGRDYTFTISSLQPEDIATYYCLQYDNLWTFGQ GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC Heavy chain QVQLVQSGAEVKKPGASVKVSCKASGFNIKDTYIHWVRQAPGQRLEW MGRIDPANGYTKYDPKFQGRVTITADTSASTAYMELSSLRSEDTAVYYC AREGYYGNYGVYAMDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSES TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV PSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPAPEFLGGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKA KGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP ENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNH YTQKSLSLSLGK
nivolumab (deglycosylated)	144 030	Acquity SEC column BEH200 4.6 x 300 mm, 1.7 μm Acquity SEC column BEH200 4.6 x 150 mm, 1.7 μm Acquity SEC column BEH125 4.6 x 30 mm, 1.7 μm	Light chain EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDA SNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQSSNWPRTFGQG TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC Heavy chain QVQLVESGGGVVQPGRSLRLDCKASGITFSNSGMHWVRQAPGKGLE WVAVIWYDGSKRYYADSVKGRFTISRDNSKNTLFLQMNSLRAEDTAVY YCATNDDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVK DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTKT YTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFBS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLG
ofatumumab (deglycosylated)	146 505		Light chain EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDA SNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPITFGQG TRLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNR Heavy chain not available
panitumumab (deglycosylated)	144 733		Light chain DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIY DASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYFCQHFDHLPLAFGG GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQ GLSSPVTKSFNRGEC Heavy chain VQLQESGPGLVKPSETLSLTCTVSGGSVSSGDYYWTWIRQSPGKGLEWI GHIYYSGNTNYNPSLKSRLTISIDTSKTQFSLKLSSVTAADTAIYYCVRDRV TGAFDIWGQGTMVTVSSASTKGPSVFPLAPCSR5TSESTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYT CNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPKPKDTLM ISRTPEVTCVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTF RVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVY TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPML DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTOKSLSI SPG

			Light chain
			DIQMTQSPSSLSASVGDRVTITCLASEGISSYLAWYQQKPGKAPKLLIYG
			ANSLQTGVPSRFSGSGSATDYTLTISSLQPEDFATYYCQQSYKFPNTFGQ
		100 mM AcONH <sub>4</sub> pH 6.9	GTKVEVKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK
			VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH
		Vivaspin 50 kDa	QGLSSPVTKSFNRGEC
		for intact mAbs	Heavy chain
reslizumab	144 260		EVQLVESGGGLVQPGGSLRLSCAVSGLSLTSNSVNWIRQAPGKGLEWV
(deglycosylated)	144 260	Vivaspin 10/30 kDa for IdeS-digested mAbs	GLIWSNGDTDYNSAIKSRFTISRDTSKSTVYLQMNSLRAEDTAVYYCARE
			YYGYFDYWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDY
			FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYT
			CNVDHKPSNTKVDKRVESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTL
		(6 – 8 cycles)	MISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS
		or	TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQV
			YTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
			LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLG
		Acquity SEC column	Light chain
	)	BEH200.4.6 x 200 mm	
			SASELYSGVPSRESGSRSGTDETLTISSLQPEDEATYYCQQHYTTPPTFGQ
		1.7 μm	
trastuzumah			
(deglugesulated)		Acquity SEC column	Heavy chain
(degrycosylated)		BEH200 4.6 x 150 mm.	EVOLVESGGGI VOPGGSI BI SCAASGENIKDTYIHWVROAPGKGI EWV
	145 866	1 7 um	
Parent mAb of T-	140 000	1.7 μπ	
DM1 and T-			GCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLOSSGLYSLSSVVTVPSSS
GIVCLICK-DM1		Acquity SEC column	LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL
GIVELER DIVIT		BEH125 4.6 x 30 mm,	FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
		1.7 um	PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
		p	GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
			NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY
			TQKSLSLSPGK

*Note:* The sequence of the tsAb antibody is confidential.

### 4. IMS parameters

Proteins	IMS parameters			
R1R2, R2D, R2TP, R2SP complexes	WV = 550 m/s WH = 40 V He flowrate = 120 mL/min N <sub>2</sub> flowrate = 60 mL/min			
DPCD	$WV = 1250 \text{ m/s}$ $WH = 40 \text{ V}$ He flowrate = 150 mL/min $N_2 \text{ flowrate} = 25 \text{ mL/min}$			
mAbs	Linear TWIMS (Intact & Middle) WV = 800 m/s WH = 40 V He flowrate = 120 mL/min N <sub>2</sub> flowrate = 60 mL/min	<b>Cyclic TWIMS</b> (Intact) WV = 900 m/s WH = 45 V He flowrate = 150 mL/min N <sub>2</sub> flowrate = 45 mL/min		
tsAb	Linear TWIMS (Intact/Middle) WV = 923/1200 m/s WH = 40/35 V He flowrate = 130/150 mL/min N <sub>2</sub> flowrate = 45/45 mL/min	<b>Cyclic TWIMS</b> (Intact/Middle) WV = 900/650 m/s WH = 45/32 V He flowrate = 150/150 mL/min N <sub>2</sub> flowrate = 45/45 mL/min		
ADCs	Linear TWIMS (Intact & Middle) WV = 850 m/s WH = 40 V He flowrate = 120 mL/min N <sub>2</sub> flowrate = 60 mL/min			

Experimental Section

# Annex 1

## Publication 5

Middle Level IM–MS and CIU Experiments for Improved Therapeutic Immunoglobulin Subclass Fingerprinting

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Annexes



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#### Article

## Middle Level IM–MS and CIU Experiments for Improved Therapeutic Immunoglobulin Subclass Fingerprinting

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connections. Different analytical techniques have been reported to assess intact IgGs subclasses, with recently special interest in native ion mobility (IM) and collision induced unfolding (CIU) mass spectrometry (MS). However, these two techniques exhibit significant limitations to differentiate mAb subclasses at the intact level. In the present work, we aimed at developing a unique IM–MS-based approach for the characterization of mAb subclasses at the middle level. Upon IdeS-digestion, the unfolding patterns of the  $F(ab')_2$  and Fc domains were simultaneously analyzed in a single run to provide deeper structural

insights of the mAb scaffold. The unfolding patterns associated with the  $F(ab')_2$  domains are completely different in terms of unfolding energies and number of transitions. Thereby,  $F(ab')_2$  regions are the diagnostic domain to provide specific unfolding signatures to differentiate IgG subclasses and provide more confident subclass categorization than CIU on intact mAbs. In addition, the potential of middle-level CIU was evaluated through the characterization of eculizumab, a hybrid therapeutic IgG2/4 mAb. The unfolding signatures of both domains were allowed to corroborate, within a single run, the hybrid nature of eculizumab as well as specific subclass domain assignments to the  $F(ab')_2$  and Fc regions. Altogether, our results confirm the suitability of middle-level CIU of  $F(ab')_2$  domains for subclass categorization of canonical and more complex new generation engineered antibodies and related products.

D uring the last 20 years, monoclonal antibody (mAb) development and engineering have significantly evolved due to their therapeutic efficiency against many diseases, such as cancer and autoimmune diseases.<sup>1</sup> More than 80 antibody-based products are currently approved by regulatory agencies (FDA and EMA), while ~600 others are in clinical studies, including more than 60 in phase III clinical trials.<sup>2</sup>

Among the different post-translational modifications (PTMs) that can occur within the primary sequence of mAbs, glycosylation and interchain disulfide linkages strongly contribute to the stabilization of the tertiary structure of these proteins. Indeed, therapeutic mAbs can be classified into four subclasses (IgG1, IgG2, IgG3, and IgG4) with different inter- and intrachain disulfide connectivities. While human IgG3 subclass is usually not considered for therapeutic mAbs engineering and production due to its limited potential associated with its shorter half-life,<sup>1</sup> human IgG1, IgG2, and IgG4 mAb subclasses represent the main classes of mAb-based therapeutics. One of the main structural differences between the three therapeutic subclasses (IgG1, IgG2, and IgG4) is the number (four for IgG1 and IgG4 and six for IgG2) and the connectivities of interchain disulfide bridges<sup>3</sup> (Figure 1). The heavy and light chains of all



Figure 1. Schematic representation of interchain disulfide bridges (yellow bars) characteristic of the IgG1 (a), IgG2 (b), IgG4 (c), and hybrid IgG2/4 (d) mAb subclasses.

subclasses are linked by one disulfide bond, while the two heavychains can be linked either by two (for IgG1 and IgG4) or four (for IgG2) disulfide bonds located in the hinge region of the

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antibody.<sup>3</sup> In addition, mAbs global structure is also maintained with 12 intrachain disulfide bridges that connect two cysteines that belong to the same domain. The interchain disulfide bridges network, which is characteristic of each individual subclass, has an impact on different mAb properties (structure, stability, surface hydrophobicity, isoelectric point, etc.)<sup>4</sup> and modulate their higher-order structure.<sup>5–8</sup> Thereby, mAbs from different subclasses will differ in their secondary immune functions.<sup>9,10</sup> In terms of mAb developability, there is a continuous interest for improvement of new analytical techniques to characterize the impact of the different interchain disulfide patterns on therapeutic mAb structures and structure–function relationships.

Ion mobility coupled to mass spectrometry (IM-MS), and its collision induced unfolding (CIU) variant, have been used to characterize the structure and dynamics of proteins<sup>11-14</sup> and protein complexes<sup>15,16</sup> in the gas-phase. During the last 5 years, CIU has been increasingly used in structural biology to characterize a wide range of biological systems and has integrated the analytical portfolio of international regulatory agencies.<sup>17</sup> Although the CIU approach still remains as a laborious and time-consuming process, significant efforts have been made to improve data acquisition<sup>18</sup> and interpretation.<sup>19,20</sup> CIU experiments allowed us in some cases to circumvent the limitations associated with IM resolution to separate and differentiate mAbs with very similar global structure. Thereby, CIU afforded structural insights that led to the differentiation of human nontherapeutic mAb subclasses,<sup>21,22</sup> ADCs' characterization,<sup>23,24</sup> and stabilized vs wild-type therapeutic IgG4 mAbs among others.<sup>25</sup> More particularly, the group of Ruotolo reported that glycan moieties contribute to the stabilization of the mAb scaffold in the gas-phase, thus glycosylated mAbs require around 15% more energy to undergo unfolding compared to their nonglycosylated counterparts.<sup>22</sup> The same study also highlighted the role of interchain disulfide linkage in mAb gas-phase stability, the latter being proposed as responsible for differences observed on CIU patterns from four human mAb subclasses.<sup>22</sup> However, in some cases, the CIU-based categorization/characterization of intact mAb subclasses remains challenging due to the very subtle differences observed in their intact CIU fingerprints.

In the present work, we aimed at improving IM-MS and CIU workflows to better differentiate the subclasses of therapeutic mAbs (IgG1, IgG2, and IgG4), including engineered hybrid mAbs. For this purpose, we developed middle-level IM-MS and CIU approaches where the mAb scaffold is IdeS-digested<sup>26</sup> prior to IM-MS or CIU analysis. In this case, a thorough characterization of therapeutic mAbs scaffold is performed based on the individual analysis of the  $F(ab')_2$  and Fc subdomains. The global structure along with the gas-phase dynamics associated with each subunit highlighted the structural similarities/differences induced by the interchain connectivities of each therapeutic subclass and provided more evidence to improve mAb subclass differentiation. Finally, the middle-level CIU allowed to unravel the subclass of a hybrid engineered mAb, pinpointing its suitability to characterize and differentiate the subclass of canonical and hybrid therapeutic mAbs.

#### MATERIALS AND METHODS

Sample Preparation. Eculizumab (Soliris, Alexion Pharmaceuticals Inc.), panitumumab (Vectibix, Amgen) natalizumab (Tysabri, Biogen), adalimumab (Humira, Abbvie), trastuzumab (Herceptin, Roche), ipilimumab (Yervoy, BMS),

and reslizumab (Cinqair, Teva Pharmaceuticals) were sourced from their respective manufacturers as EMA-approved drug products. N-Glycans were enzymatically removed to obtain more homogeneous native mass spectra. Thereby mAbs were Ndeglycosylated during 30 min at 37 °C with IgGZERO (Genovis). In the case of middle-level analysis, the deglycosylated mAbs were degraded with IdeS enzyme (immunoglobulindegrading enzyme of Streptococcus pyogenes, FabRICATOR, Genovis). A 1  $\mu$ g/unit ratio was used to achieve an efficient digestion and subsequently, the mixture was incubated during 60 min at 37 °C. After deglycosylation and/or IdeS digestion, therapeutic mAbs were then desalted against 100 mM ammonium acetate at pH 7.0 prior to native MS analysis, using about six to eight cycles of centrifugal concentrator (Vivaspin, 30 kDa cutoff, Sartorious, Göttingen, Germany). The concentration of each individual solution after desalting process was measured by UV absorbance using a nanodrop spectrophotometer (Thermo Fisher Scientific, France). Prior to native MS analysis, each sample was diluted in 100 mM ammonium acetate at pH 7.0 to a final concentration of 5  $\mu$ M.

**Native MS Analysis.** Native mass spectra were acquired on an Orbitrap Exactive Plus EMR (Thermo Fisher Scientific, Bremen, Germany) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion, Ithaca, U.S.A.) operating in the positive ion mode. The capillary voltage and the pressure of the nebulizer gas were set at 1.7-1.9 kV and 0.15-0.20 psi, respectively. The source parameters were tuned to obtain the best mass accuracy for native MS experiments as follows: briefly, the in-source voltage was set to 150 eV, the HCD cell voltage was fixed to 50 eV. The pressure of the backing region and the HCD cell were fixed to 2 mbar and  $10^{-5}$  mbar, respectively. Native MS data interpretations were performed using Xcalibur software v4.0 (Thermo Fisher Scientific, Bremen, Germany).

Native IM-MS and CIU Experiments. Ion mobility and CIU experiments were performed on a hybrid Q-IM-TOF mass spectrometer (Synapt G2, Waters, Manchester, U.K.). The cone voltage was fixed at 80 V to improve the ion transmission and avoid in source ion activation. The backing pressure of the Zspray source was set to 6 mbar and the argon flow rate was 5 mL/ min. Ions were focused with a helium flow rate of 120 ml/min and separated in the IM cell with a  $N_2$  flow rate of 60 mL/min. Ion mobility parameters were tuned to improve ion separation and prevent ion heating as described in Hernandez et al.<sup>25</sup> Briefly, the wave velocity and height were fixed to 800 m/s and 40 V, respectively. IM drift times of each mAb were converted in collision cross sections using three charge states of concanavalin A, pyruvate kinase, and alcohol deshydrogenase as external calibrants as reported elsewhere.<sup>27</sup> The drift times of the reference proteins and the analytes ions were measured under the same experimental conditions. MassLynx software (Waters, Manchester, U.K.) was used to generate arrival time distributions.

Collision induced unfolding experiments were performed by increasing the collision voltage of the trap by 5 V steps from 0 to 200 V prior to IM separation. All data were acquired consecutively on the same subclass mAb batch with strictly identical instrumental parameters to reduce fluctuations in the backing, trap, and IM pressures. Individual IM data were gathered to generate CIU fingerprint using the last version of CIUSuite2 software (version 2.1) and in particular the CIUSuite2\_BasicAnalysis and the CIUSuite2\_StabilityAnalysis modules to obtain average and differential plots, and then to



**Figure 2.** Intact level CIU experiments. CIU experiments of the 22+ charge state of adalimumab (IgG1) (a), panitumumab (IgG2) (b), natalizumab (IgG4) (c), and hybrid IgG2/4 eculizumab (d). CIU fingerprints are depicted in the upper panels. ATDs extracted at 150 V corresponding to the three therapeutic mAbs are depicted in the lower panels (e, f, g, and h). Table summarizing the IM drift times of the observed unfolding states (i).

determine CIU50 values to assess the stability of each transition directly from the CIU data. Each plot corresponds to the average of the three analysis replicates with a root-mean-square deviation lower than 10% showing a good reproducibility of the experiment. ATD intensities were normalized to a maximum value of 1 and classical smoothing parameters were used (Savitzky-Golay algorithm with a window length of 3 and a polynomial order of 2) as that in the previous version of the software. Prior to subclass classification and CIU comparison, the centroid of the ATDs at zero volts were standardized (same initial IM centroid) to provide a straightforward comparison of the unfolding patterns. Adalimumab, panitumumab, and natalizumab were chosen as the IgG1, IgG2, and IgG4 reference mAbs, respectively, to create the in-house classification methods at both intact and middle levels using the CIUSuite2 algorithm. Four additional therapeutic mAbs (eculizumab, trastuzumab, ipilimumab, and reslizumab) from different mAb subclasses were included in the present study to evaluate the reliability of our in-house classification methods. Three CIU replicates of all mAbs were used to generate the whole data set. Collision voltages with the highest score in the univariate feature selection plot (UFS) were specifically selected<sup>19,28</sup> to provide an adequate classification of clusterized mAbs (eculizumab, trastuzumab, ipilimumab, and reslizumab).

**RPLC Analysis.** Separation of the different IgG1, IgG2, IgG2/4, and IgG4 subclasses were performed in a Zorbax RRHD column (2.1 mm × 50 mm, 1.8  $\mu$ m, 300 Å) from Agilent Technologies (Wilmington, DE, U.S.A.). The column was loaded with 1  $\mu$ L of the intact mAbs solution at 5 mg/mL final concentration (5  $\mu$ g). Mobile phase A was composed of 0.1% TFA, 2% isopropyl alcohol (IPA) in water, and mobile phase B was 0.1% TFA, 25% acetonitrile, in IPA. Samples were eluted with a constant flow rate of 250  $\mu$ L/min and using a chromatographic gradient from 10% to 25% B over 9 min,

followed by a shallow gradient up to 27.8% B over 7 min. Then, the gradient increased up to 29.8% B over 1 min, followed by 29.8-50% B over 2 min.

nrCE-SDS Analysis. IgGs were analyzed in nonreduced condition using a Maurice system (Protein Simple) equipped with the Compass software. Chemicals were provided from the Maurice CE-SDS application kit from the provider. Samples were diluted in  $1 \times$  sample buffer to a final concentration of 1 mg/mL, from which  $50-\mu$ L-aliquot samples were made. Then 2  $\mu$ L of internal standard was added to each sample. 2.5  $\mu$ L of a 250-mM stock solution of the alkylating agent IAM was added to each 50- $\mu$ L sample to block disulfide scrambling or exchange. They were denatured at 70 °C for 10 min, cooled on ice for 5 min and mixed by vortex. Each sample was then transferred to a 96-well plate and spun down in a centrifuge for 10 min at 1000g. All samples were electrokinetically injected into the cartridge capillary by applying 4600 V for 20 s before separation by electrophoresis at 5750 V during 35 min. Electropherograms were analyzed with the Empower data software.

**Differential Scanning Calorimetry (DSC).** DSC experiments were performed on a MicroCal VP-Capillary DSC instrument (Malvern Instruments). Samples were buffer exchanged into PBS Dulbecco pH 7.4 buffer or 25 mM His/ His-HCl, 150 mM NaCl, pH 6.5 and diluted to 1 mg/mL in according buffer. 400  $\mu$ L of the protein solution as well as 400  $\mu$ L of the according buffer were dispensed in 96-well plates, loaded to the capillary sample cell while the reference cell contained the corresponding buffer. The chamber was pressurized to 3 atm, and the temperature ramped from 40 to 100 °C at 1 °C/min heating rate. The recorded DSC thermograms were baseline subtracted and subjected to a multicomponent Gaussian fitting in the MicroCal VP-Capillary DSC software 2.0 (Malvern Instruments).

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The temperatures for three major transitions were extracted from the fitted Gaussian models, relating to the unfolding of  $CH_2$ , Fab, and  $CH_3$  domains. For each sample, 3 independent experiments were carried out allowing us to use a value of 1 °C as the cutoff limit for evaluating the significance of the differences observed in melt temperatures.

#### RESULTS AND DISCUSSION

Intact Level IM-MS and CIU Experiments for mAb Subclass Classification. Since mAb glycosylation induces additional heterogeneity on mass spectra without affecting subclass determination by intact-CIU fingerprints,<sup>22</sup> we first analyzed three deglycosylated mAbs-adalimumab (IgG1), panitumumab (IgG2), and natalizumab (IgG4)-at the intact level by native MS (Figure S1 of the Supporting Information, SI) and IM-MS (Figure S2). Overall, the charge state distributions observed on the native mass spectra are centered either on the 24+ or 23+ charge states. The same therapeutic mAbs were next analyzed by native IM-MS at the intact level to provide more insights into their global conformation. For all charge states, IM–MS provides very similar <sup>TW</sup>CCS<sub>N2</sub> values within the error of the CCS measurement, avoiding classification of subclasses on the sole basis of CCS measurements. The coelution of all reference therapeutic mAbs upon IM separation leads to the conclusion that current TWIMS resolution cannot afford an efficient differentiation of the three subclasses as previously reported on nontherapeutic mAbs $^{22,25}$  (Figure S2a). Of note, even though the centroid of the collision cross section distribution (CCSD) profiles are very similar, the FWHM of the IgG2 CCSD is slightly broader (1.65 nm<sup>2</sup> versus 1.10 nm<sup>2</sup>, and 1.15 nm<sup>2</sup> for IgG1 and IgG4, respectively), suggesting potential coexistence of several isomers, in agreement with a more flexible IgG2 scaffold. The high similarity in terms of primary sequence between these mAbs leads to the analysis of quasi-isobaric (<2% mass difference) and quasi-iso-cross sectional (<1% CCS difference) (Figure S2b,c) proteins for which classical native IM-MS instrumentation can only provide limited information.

As differences in CIU fingerprints of different mAb subclasses from human serum were mainly related to differences in the number and connectivities of interchain disulfide bridges contained within the structure of mAbs,<sup>20,22</sup> we next performed and compared CIU experiments on the three therapeutic mAbs at the intact level (Figure 2). Overall, the CIU patterns of the three mAb subclasses look very similar to two unfolding transitions present on the three CIU fingerprints.

While the three canonical mAb subclasses (adalimumab, panitumumab, and natalizumab) exhibit the same IM migration times at the ground state, some subtle differences can be observed upon ion activation (Figure 2a–c). One diagnostic CIU region is comprised between the 100 and 200 V range, where ATDs from the three subclasses exhibit different distributions (Figure 2e–g), allowing the discrimination of the therapeutic mAb subclasses at the intact level, as previously reported.<sup>20,22,25</sup> Although mAb subclasses can be differentiated when these structures populate excited unfolding states upon activation with the background gas, CIU fingerprints at the intact level only provide very limited and subtle differences, hindering a clear-cut classification of therapeutic mAb subclasses.

Middle-Level CIU Analysis Affords Easier Mab Subclass Categorization than Intact CIU. In order to circumvent intact level IM–MS and CIU limitations, we next performed



**Figure 3.** Middle-level IM–MS analysis of  $F(ab')_2$  domains. CCSDs of the 21+ charge state of the  $F(ab')_2$  domains of eculizumab, adalimumab, panitumumab, and natalizumab (a). Evolution of the  $F(ab')_2$ <sup>TW</sup>CCS<sub>N2</sub> as a function of the charge state (b). Table summarizing the measured <sup>TW</sup>CCS<sub>N2</sub> of the  $F(ab')_2$  domains (c).

native IM–MS and CIU experiments (Figures 3, 4, and 5) at the middle level to further characterize the global conformation and the gas-phase stability of the  $F(ab')_2$  and Fc domains of IdeS-digested adalimumab, panitumumab, and natalizumab (see Materials and Methods section).

Regarding IM-MS results, the measured <sup>TW</sup>CCS<sub>N2</sub> of the  $(Fab')_2$  domains (20+ and 21+ charge states) are very similar (average differences in CCS comprised between 0.66 and 2.62%), which avoids clear assessment of mAb subclass based solely on <sup>TW</sup>CCS<sub>N2</sub> measurements. While broad CCSD exhibiting two distributions were observed for the  $F(ab')_2$ domain of IgG1 and IgG4 mAbs (the second feature being most likely due to gas phase activation), only one narrow CCS distribution is observed for the IgG2 panitumumab (Figure 3). For the 12+ charge state of the Fc region, the measured  $^{TW}CCS_{N2}$  are 33.1 ± 0.1 nm<sup>2</sup>, 33.2 ± 0.1 nm<sup>2</sup>, and 33.1 ± 0.1 nm<sup>2</sup> for IgG1, IgG2, and IgG4 references, respectively (Figure S3), showing that the categorization/characterization of IgG1, IgG2, and IgG4 mAb subclasses cannot be performed based on the  ${}^{TW}CCS_{\rm N2}$  measurements of the Fc domains. These results  $(^{TW}CCS_{N2}$  measurements along with CCSDs) prompted us to suggest that the only potential information that might be deduced directly from IM-MS data is that IgG2 subclass might be differentiated from IgG4/IgG1 with the  $F(ab')_2$  regions.

We next compared CIU experiments on both  $F(ab')_2$  and Fc subunits of adalimumab, panitumumab, and natalizumab after IdeS digestion (Figures 4 and Figure 5, respectively). Overall, the differentiation of the mAb subclasses upon collisional activation of the  $F(ab')_2$  domains is clearly evidenced based not only on the number of unfolding transitions observed in the CIU fingerprints, but also due to the different collision energies associated with each transition (Figure 4). While only two unfolding transitions are observed in the CIU fingerprint of the IgG2  $F(ab')_2$  domain (17.9 and 37.6 V, respectively) (Figure 4b), three transitions are observed in the case of the IgG1  $F(ab')_2$  domain (23.6, 67.3, and 123.5 V, respectively) (Figure 4a), and five transitions in the IgG4  $F(ab')_2$  CIU fingerprint (18.5, 72.5, 113.0, 147.6, and 162.4 V) (Figure 4c). The most unfolded state of the IgG2  $(Fab')_2$  domain is populated at lower voltages compared to the IgG1 and IgG4 subclasses. However,

#### **Analytical Chemistry** pubs.acs.org/ac Article d) eculizumab a) adalimumab b) panitumumab c) natalizumab IgG1 F(ab')<sub>2</sub> IgG2 F(ab'), IgG4 F(ab'), IgG2/4 F(ab') 22. 20.0 SE 17.5 JI 12. CIU50: 23.6, r2=: 0.99 CIU50: 67.3, r2=: 0.97 CIU50: 123.5, r2=: 0.97 CIU50: 17.9, r2=: 1.00 CIU50: 37.6, r2=: 0.91 CIU50: 19.3, r2=: 0.99 CIU50: 42.3, r2=: 0.92 22. 20.0 E 17.5 E 15.0 E 12 CIU50: 50 75 100 125 150 Collision Voltage (V) 50 75 100 125 150 Collision Voltage (V) 100 125 150 175 on Voltage (V) 75 100 125 150 Collision Voltage (V)

**Figure 4.** Middle-level CIU experiments on  $F(ab')_2$  domains. CIU fingerprints (top panel) and stability analysis "CIU50" of 21+ charge state of  $F(ab')_2$  domain of IgG1 (a), IgG2 (b), IgG4 (c), and IgG2/4 (d) from 0 to 200 V trap collision voltage. Gaussian fitting and collision voltages associated with the unfolding transitions are depicted in the lower panels.



Figure 5. Middle-level CIU experiments on Fc domains. CIU fingerprint of 12+ charge state of Fc domains corresponding to adalimumab (IgG1) (a), panitumumab (IgG2) (b), natalizumab (IgG4) (c), and eculizumab (IgG2/4) (d). The CIU fingerprints and the corresponding Gaussian fitting are depicted in upper and lower panels, respectively.

this final state is kinetically stabilized from 40 to 200 V, whereas the different unfolded states of IgG1, and IgG4 subclasses are only kinetically stabilized during shorter voltage ranges. The gasphase stability of the  $F(ab')_2$  domain of the IgG2 subclass stems more likely from the higher number of disulfide bridges in the hinge region (four interchain S-S) that prevents the unfolding process of the domain upon ion heating. Our data thus suggest that middle-level CIU patterns of  $F(ab')_2$  domains allow easier and more confident subclass determination than intact level CIU fingerprints. This is also supported by the UFS plots obtained from CIUSuite2 comparing the classification methods for intact mAbs and the  $F(ab')_2$  subunits (Figure S4). While all the UFS scores of the intact-CIU method are lower than 1.5, suggesting relatively similar subclasses, the  $F(ab')_2$  UFS plot shows a highly diagnostic region between 60 and 140 V with higher scores (-log p-values >1.5), pinpointing higher differences in  $F(ab')_2$  CIU

fingerprints than on intact CIU plots, leading to more reliable classification.

For the Fc domains, CIU fingerprints of the three reference mAbs (adalimumab, panitumumab, and natalizumab) exhibit very similar unfolding patterns with two unfolding transitions that lead to an increase of the collision cross section, and a final transition around 120 V where the global conformation of the Fc domain is compacted (Figure 5). Therefore, relatively low UFS scores were obtained for Fc domain-based subclass categorization (Figure S4c), revealing that clusterization of therapeutic mAbs based on CIU fingerprints of Fc domains might lead to inaccurate subclass differentiation. This result is consistent with the high similarity (~95%) of the Fc sequence between the three subclasses (Table S1) leading to very similar gas-phase stabilities and dynamics. However, the collision energy associated with the unfolding transitions observed on the IgG1 Fc fingerprint (41.5, 86.4, and 131.5 V) are slightly higher compared to those

observed in the IgG2 (25.0, 67.5, and 122.5 V) and IgG4 (27.7 V, 67.5 V, 117.4 V) Fc domains suggesting a slightly higher gasphase stabilization of the IgG1 Fc domain. This observation might be related to the influence of the noncovalent interactions that contribute to the stabilization and dimerization of the mAb Fc domain.<sup>29–33</sup> Indeed, the strongest CH<sub>3</sub>–CH<sub>3</sub> interaction was found in the IgG1 structure (up to 10<sup>6</sup>-fold) in comparison to the other subclasses,<sup>29</sup> which is in good agreement with the gas-phase stability observed in the Fc CIU fingerprints. To strengthen this hypothesis, stability of the constant regions was also investigated using differential scanning calorimetry (DSC) (Table S2).<sup>23</sup> In this case, the melting temperatures corresponding to the denaturation of the CH<sub>2</sub> and CH<sub>3</sub> domains of the different mAbs subclasses evidenced a higher thermal stability for the IgG1 heavy chain constant domains, in agreement with results obtained by middle-level CIU experiments.

Altogether, our middle-level CIU results on three model mAbs depict that middle level CIU patterns of  $F(ab')_2$  domains generated after IdeS digestion enable more easy and confident subclass classification than intact level CIU data.

Validation/Benchmarking of Middle-Level F(ab')<sub>2</sub> CIU Pattern for Better mAb Subclass Assessment. To strengthen our claims, we performed additional middle-level CIU experiments on  $F(ab')_2$  domains of three additional therapeutic mAbs: two IgG1 (trastuzumab and ipilimumab) and one IgG4 (reslizumab), no additional IgG2 being available for inclusion in this validation phase. This series of different therapeutic mAbs was subjected to our in-house mAb classification method based on the three reference mAbs previously detailed (adalimumab, panitumumab, and natalizumab) (Figure S5). After selection of the most diagnostic collision voltages of the  $F(ab')_2$  reference fingerprints (from 60 to 140 V), our middle-level CIU classification method led to the unambiguous identification of trastuzumab and ipilimumab as IgG1 (with 77.0  $\pm$  14.2% and 90.8  $\pm$  8.7%, respectively) and reslizumab as IgG4 (97.1  $\pm$  0.2% of confidence) (Figure S5g-i). Even though the CIU fingerprints of the different mAbs from the same subclass do not exhibit strictly the same unfolding features, most likely due to the contribution of the specific variable domains of the  $F(ab')_2$  regions<sup>25</sup> of each mAb, middle-level CIU of F(ab')2 domains afforded clear-cut subclass identification and categorization of therapeutic mAbs.

Middle-Level CIU Strategies Provide Accurate Characterization of Eculizumab, a Hybrid IgG2/4. Finally, to challenge our middle-level CIU method based on (Fab')2 subclassification, we analyzed eculizumab as a model for hybrid mAb constructs. Eculizumab is a humanized hybrid IgG2/4 mAb directed against the complement protein C52 and indicated to treat the rare hemolytic disease paroxysmal nocturnal hemoglobinuria.<sup>34</sup> The heavy-chain constant region of the parental antibody was repaved with components of both human IgG2 and IgG4 constant regions. The heavy chain of the hybrid mAb includes the CH1 and hinge regions of human IgG2 fused to the CH<sub>2</sub> and CH<sub>3</sub> regions of human IgG4. To avoid the generation of an antigenic site during the fusion, a restriction endonuclease cleavage site common to both IgG2 and IgG4 was used to join the two constant regions (31 amino acids flanking the fusion site are identical between IgG2 and IgG4).<sup>35</sup> The unique combination of an IgG2/4 constant region makes this molecule fail to bind to Fc receptors (IgG2) and does not activate complement cascade (IgG4), which reduces the proinflammatory potential of the antibody.<sup>36</sup> Due to its inherent

hybrid constitution, classical analytical techniques used for intact-mAb subclass categorization applied to eculizumab characterization provide a series of unclear/contradictory results. For example, nonreduced capillary electrophoresissodium dodecyl sulfate (nrCE-SDS) eculizumab analysis presents a single peak which is rather in agreement with an IgG4 than with an IgG2 nrCE-SDS behavior for which doublet peaks are expected (Figure S6).<sup>37</sup> Conversely, reversed-phase high performance liquid chromatography (rpHPLC) analysis of eculizumab clearly presents an IgG2-like behavior with three peaks reflecting IgG2 structural isoforms A, B, and A/B (Figure S7).<sup>38,39</sup> As classical analytical methods for intact mAb characterization are not adapted to depict and dissect the complex structural scaffold of hybrid mAb formats, there is a need for complementary analytical techniques able to tackle this issue. We thus applied our CIU workflows for eculizumab characterization.

Regarding the <sup>TW</sup>CCS<sub>N2</sub> measurements and as expected from our results on reference IgGs, hybrid eculizumab cannot be differentiated from reference therapeutic mAbs at the intactlevel owing to their similar global conformations (overall difference in CCS < 2%, Figure S2). Interestingly, a broad CCSD (fwhm =  $1.42 \text{ nm}^2$ ) is observed for eculizumab. In addition, a small shoulder is also depicted on the right side of its CCSD, which might be related to the presence of different ion populations. At the middle-level, independently of the charge state, the  $^{TW}CCS_{N2}$  of the F(ab')<sub>2</sub> region of eculizumab is closer to those of the IgG2 reference (panitumumab), which might be considered as a first hint toward eculizumab  $F(ab')_2$  region behaving as an IgG2 (Figure 3b and c). As expected, no conclusions can be drawn from middle-level native IM-MS  $^{\rm TW}{\rm CCS}_{\rm N2}$  measurements regarding the Fc part of eculizumab owing to the high primary sequence similarity ( $\sim$ 95%) with the three mAbs (Table S1, and Figure S3). Altogether IM-MS investigation provides very limited information toward the characterization of the "hybridicity" of eculizumab.

We thus moved to CIU experiments. Intact-level CIU fingerprint of eculizumab (hybrid IgG2/IgG4) was compared to those of adalimumab, panitumumab, and natalizumab (references IgG1, IgG2, and IgG4, respectively) previously described (Figure 2d). Overall, the eculizumab CIU fingerprint shows four unfolding states and three transitions, revealing one additional unfolding transition compared to the reference IgG2 or IgG4 subclasses. In more detail, the first transition occurs at 36.6 V, the second at ~145 V, and the last one at 168 V. Automated subclass classification using the CIUSuite2 module<sup>40</sup> mainly recognizes eculizumab as an IgG2 (Figure S8d). However, the classification algorithm is not well adapted to characterize the hybrid structure of eculizumab since there is not a significant correlation between eculizumab's fingerprint and a secondary mAb subclass (either IgG1, or IgG4). Indeed, the IgG1 and IgG2 subclasses equally contribute to the unfolding pattern of the hybrid mAb ( $8.5 \pm 2.7$  and  $11.8 \pm 3.0\%$ ), which impedes assessment of the second most prevailing mAb subclass in the eculizumab structure. Interestingly, a closer manual data interpretation allowed highlighting that the first CIU transition of eculizumab (37 V) is similar to the first transition of IgG2 or IgG4, while the two other eculizumab transitions correspond to the second transition of reference IgG4 (145 V for eculizumab versus 147 V) or IgG2 (168 V), respectively, which might suggest that eculizumab CIU fingerprint could result from a composite/hybrid of the two IgG4 and IgG2 CIU patterns. This first evidence can be strengthened based on the centroid IM drift

times of each unfolding state. The IM drift time of the second state of eculizumab is similar to the drift time of the second state of natalizumab (blue values in Figure 2i) while the drift time of the third state of eculizumab is close to the second unfolding state drift time of panitumumab (red values in Figure 2i). These data suggest that eculizumab gas-phase unfolding behavior is a hybrid between reference IgG2 and IgG4 mAbs. However, even if the intact-level CIU allows for concluding that the eculizumab CIU fingerprint is clearly different from reference IgG2/IgG4 ones, it does not allow us to draw any conclusion about the origin of this difference related to its inherent "hybridicity".

We finally performed CIU experiments on the  $F(ab')_2$  and Fc subunits of eculizumab obtained upon IdeS digestion (Figures 4 and 5). Overall, the CIU fingerprint of the  $F(ab')_2$  subdomain of eculizumab exhibits a very similar CIU pattern (same number of unfolding transitions at very similar collision energies) with the reference IgG2  $F(ab')_2$  (Figure 4b and d), suggesting an IgG2-like gas-phase unfolding of eculizumab  $F(ab')_2$ . Automatic subclass classification algorithm included in the open source CIUSuite2 software<sup>19</sup> (Figure S8h) assessed the  $F(ab')_2$  subdomain of eculizumab  $F(ab')_2$  as an IgG2-type CIU pattern (79.3  $\pm$  11.3% of IgG2). These results corroborate middle-IM–MS CCS measurements, and clearly show that the (Fab')<sub>2</sub> of eculizumab can be unambiguously associated with an IgG2 subclass.

For the Fc subdomains, as expected, very similar CIU patterns were observed for all mAbs (Figure 5). Automatic subclass detection of the CIUSuite2 software<sup>19</sup> revealed that eculizumab Fc unfolding pattern was slightly closer to the Fc subdomain of the IgG4 reference (natalizumab) rather than the IgG1 or IgG2 ones (Figures 5 and S8). These subtle but significant differences stem from the very close collision energies associated with each individual unfolding transitions observed in the eculizumab and IgG4 Fc fingerprints (27.7, 67.5 and 117.8 V for eculizumab compared to 27.7, 67.5, and 117.4 V for the reference IgG4). Conversely, all voltages associated with IgG1 unfolding pattern were significantly higher when compared to eculizumab, while only one voltage associated with the third unfolding event allows distinguishing eculizumab (117.8 V) from IgG2 (122.5 V) (Figures 5 and S8). Thereby, the resulting RMSD upon comparison of the eculizumab Fc CIU fingerprint with the three references, IgG1, IgG2, and IgG4, were 28.08%, 8.84%, and 4.50%, respectively, suggesting that the unfolding behavior of the Fc part of eculizumab resembles an IgG4-like subclass. These results were also corroborated after using the classification algorithm. Upon selection of the most significant collision voltages (45 and 135 V), the Fc fingerprint of eculizumab is recognized as an IgG4 at  $(84,2 \pm 2.8\%)$ .

Altogether, our results clearly demonstrate that middle-level CIU probed the duality/hybridicity of engineered mAbs formats. In our case, among all tested analytical techniques (nrCE-SDS, rpHPLC–UV, native IM–MS, and CIU-IM–MS at the intact level), middle-level CIU experiments was able to provide structural evidence of eculizumab hybrid format and to assess the subclass of each of its domains, all in one single run. The energy associated with the unfolding transitions along with the number of unfolding events present on  $F(ab')_2$  and Fc CIU fingerprints afforded an accurate and straightforward identification of eculizumab hybrid construction.

#### CONCLUSIONS

This work presents an alternative and complementary IM–MSbased approach for mAb subclass distinction. Indeed, as all mAb subclasses have signature peptides, subclass confirmation usually consists of peptide mass fingerprinting after enzymatic digestion.<sup>41,42</sup> To investigate mAb subclass on intact entities, a panel of classical analytical techniques like capillary electrophoresis and liquid chromatography are available. However, ambiguous or even controversial results can be obtained, especially when new formats like hybrid mAbs are concerned. As mAb subclass identification can also be addressed by IM–MS based approaches,<sup>20,43</sup> this study presents a middle-level (after IdeS digestion of mAbs) IM–MS strategy based on CIU experiments to tackle mAb subclassification. We demonstrate here that middle-level CIU affords better distinction of mAb subclasses than similar analyses performed at the intact level.

Considering IM-MS and CCS measurements at both intact and middle levels, mAb subclasses usually present "co-drifting"/ overlapping ATDs when using commercially available IM-MS instruments due to a lack of IM resolution, which prevents subclass classification through a simple CCS measurement. Slight differences in ATD profiles and/or ATDs fwhm might eventually suggest rough trends.

In this study, we demonstrate how the analysis of large mAb fragments (50-100 kDa) can be used to compliment native protein CIU data sets. Indeed, stronger conclusions for mAb subclass determination were obtained from middle-level CIU experiments, especially from  $F(ab')_2$  CIU pattern interpretation (100 kDa). As the vibrational energy redistribution is more efficient upon collision of the smaller  $F(ab')_2$  and Fc domains with the background gas, the ions in the gas-phase can populate additional excited unfolding states, which provide clear-cut specific signature characteristics of mAb subclasses. Conversely to CIU fingerprints of therapeutic mAbs recorded at the intact level that only present subtle differences in the 100-200 V region,  $F(ab')_2$  CIU fingerprints exhibit significantly different unfolding features both in terms of number and associated energies of unfolding transitions along the whole voltage range (from 0 to 200 V). As a consequence, the  $F(ab')_2$  CIU fingerprints can be considered as the most diagnostic region to differentiate mAb subclasses since the number of unfolding transitions and their associated energies are clearly different for IgG1, IgG2, and IgG4 mAbs. The unfolding behavior of the  $F(ab')_2$  domains is mainly related to subclass specific interchain disulfide connectivities that drive specific structures, leading to diagnostic CIU features. The influence of the variable domains in the CIU patterns of the  $F(ab')_2$  subunits is also observed when comparing CIU fingerprints from the same mAb subclass (IgG1 or IgG4), however, similar unfolding features among each mAb subclass are detected, allowing their classification.

Although CIU unfolding patterns of Fc domains (50 kDa) are overall very similar owing to the absence of covalent connectivities in Fc domains (no interchain S–S bridges that connect Fc noncovalent dimers) and the high primary sequence similarities of Fc regions (>93% in our study), minor differences can also be detected between middle-level CIU fingerprints of Fc domains. Indeed, a careful and detailed data interpretation of transition energies related to noncovalent dimeric Fc domains also affords distinguishing: (i) IgG1 from IgG2 or IgG4, with overall higher unfolding energies for all transition states for IgG1 and (ii) IgG2 from IgG4 on the basis of one unique transition (the more energetic at 117.4 V for IgG4 versus 122.5 V for IgG2). Ranking of gas-phase stabilities and resistance to unfolding of Fc noncovalent dimers (IgG1 > IgG2 > IgG4) were directly correlated to strength of noncovalent CH<sub>3</sub>-CH<sub>3</sub> interactions. Our results thus show that middle CIU fingerprints

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are not only sensitive to covalent connectivities (disulfide bridges) differences that drive mAb structure and rigidity  $(F(ab')_2 \text{ domains})$  but also to noncovalent interactions (Fc domains).

Benefits of middle IM-MS and CIU approaches are illustrated for the characterization of hybrid mAb-formats like the IgG2/IgG4 eculizumab. While classical analytical techniques used for intact mAb subclass validation such as nrCE-SDS or rpHPLC-MS led to controversial results and failed in identifying the hybridicity of eculizumab, intact-level CIU approach provided a first strong hint toward a composite IgG2/ IgG4 CIU pattern. The precise hybrid IgG2/IgG4 character of eculizumab was definitely, more clearly, and accurately assessed by middle-level CIU. Analysis of the middle-level CIU fingerprints of eculizumab pointed out that the  $F(ab')_2$ unfolding pattern corresponds to an IgG2-like mAb reference, confirming the trends identified in middle IM-MS analysis, while the Fc domain behaves as an IgG4-like subclass. For eculizumab, middle-level CIU experiments allowed us to face the challenge of hybrid mAb-format characterization, allowing us within one single CIU experiment to identify specific structural subclass features but also to attribute subclass to its corresponding mAb subdomain.

Altogether, our results highlight the suitability of middle-level CIU experiments to differentiate and classify the subclass of large mAb fragments, including complex new generation hybrid formats. Middle-level CIU provides more informative insights that enable a further characterization of therapeutic proteins to overcome the limitation of classical analytical techniques used for intact or large mAb fragments. We believe that the analysis of large mAb fragments by middle-level CIU provides unique information content that can not only assist in the analysis of new mAb hybrids but also beneficially expand the current IM– MS and CIU method toolbox.

#### ASSOCIATED CONTENT

#### **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00293.

Table S1, Sequence homology of the  $F(ab')_2$  and Fc domains of IgG1, IgG2, IgG2/4, and IgG4 mAbs; Table S2, melting temperatures of  $CH_2$  and  $CH_3$  domains obtained from the thermograms of different mAbs isotypes; Figure S1, native mass spectra of adalimumab, panitumumab, natalizumab, and eculizumab; Figure S2, CCSDs of 22+ charge state of IgG1, IgG2, IgG4, and IgG2/4; Figure S3, CCSDs of the 12+ charge state of the Fc subunits; Figure S4, univariate feature selection plots of the classification methods of IgG1, IgG2, and IgG4 subclasses; Figure S5, CIU comparison of the 21+ charge state of the  $F(ab')_2$  CIU fingerprints of reference mAbs and the classified mAbs; Figure S6, electropherograms of IgG2, IgG2/4, and IgG4; Figure S7, RPLC chromatogram of adalimumab, panitumumab, eculizumab, and natalizumab; and Figure S8, differential CIU fingerprint between eculizumab and the three reference mAbs are available free of charge (PDF)

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#### Notes

The authors declare no competing financial interest.

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# Annex 2

Summary of multifunctional modes on the cIM-MS instrument

Annexes





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Développement d'approches de spectrométrie de masse native et de mobilité ionique de nouvelle génération pour la caractérisation de complexes multiprotéiques et protéines thérapeutiques

## Résumé

Ce travail de thèse porte sur des développements méthodologiques en spectrométrie de masse native (nMS) et mobilité ionique (IMS) afin de mieux répondre aux problématiques rencontrées par les biologistes ou les entreprises biopharmaceutiques. Le couplage de la chromatographie d'exclusion stérique (SEC) à la nMS a d'abord été étendu à une large variété de complexes biologiques. L'intérêt des méthodes de nMS et nIMS-MS pour des études intégratives de biologie structurale a ensuite été montré dans le cas de complexes multiprotéiques de hauts poids moléculaires. D'autre part, l'apport du nouvel instrument cyclique de haute résolution IMS-MS pour la caractérisation d'anticorps monoclonaux (mAbs) par rapport aux instruments IMS-MS de première génération a été évalué. Enfin, les approches de *collision-induced unfolding* (CIU) ont été automatisées en développant un nouveau couplage de la SEC à la CIU. L'intérêt de la haute résolution CIU pour l'étude des mAbs a également été illustré.

<u>Mots-clés :</u> spectrométrie de masse native, mobilité ionique, collision-induced unfolding, anticorps monoclonaux, complexes multiprotéiques.

## Résumé en anglais

This PhD work focuses on methodological developments in native MS (nMS) and ion mobility spectrometry (IMS) to better address problems encountered either by structural biologists or by biopharmaceutical companies. First, the coupling of size exclusion chromatography (SEC) to nMS was extended to a large variety of noncovalent biological complexes. The interest of nMS and nIMS-MS methods for integrative structural biology studies was demonstrated through the analysis of high molecular weight multiprotein complexes. Then, benefits of the new high-resolution cyclic IMS-MS platform and its multifunction capabilities over first-generation IMS-MS instruments were illustrated for the characterization of therapeutic monoclonal antibody (mAb) products. Next, a fully automated collision-induced unfolding (CIU) workflow was developed by coupling SEC to CIU, affording high-throughput CIU of mAbs. Lastly, potentialities of high-resolution CIU approaches were evaluated for in-depth mAb analysis.

<u>Keywords:</u> native mass spectrometry, ion mobility, collision-induced unfolding, monoclonal antibodies, multiprotein complexes.