Monitoring mAb proteoforms in mouse plasma using an automated immunocapture combined with top-down and middle-down mass spectrometry

Jonathan Dhenin[1,2,3,*], Valérie Lafont[3], Mathieu Dupré[3], Alain Krick[3], Christine Mauriac[3], Julia Chamot-Rooke[1,*]

[1]Institut Pasteur, Université Paris Cité, CNRS UAR2024, Mass Spectrometry for Biology, Paris, 75015, France
[2]Université Paris Cité, Sorbonne Paris Cité, 75013 Paris, France
[3]DMPK, Sanofi R&D, 91380 Chilly-Mazarin, France

*To whom correspondence should be addressed

Corresponding authors:

Jonathan Dhenin – DMPK, Sanofi R&D, 91380 Chilly-Mazarin, France. Email: jonathan.dhenin@sanofi.com
Julia Chamot-Rooke – Institut Pasteur, Université Paris Cité, CNRS UAR2024, Mass Spectrometry for Biology, Paris, 75015, France. Email: julia.chamot-rooke@pasteur.fr

Keywords: immunocapture, middle-down mass spectrometry, monoclonal antibody, proteoforms, top-down mass spectrometry

Total number of words:

ABSTRACT: Monoclonal antibodies (mAbs) have established themselves as the leading biopharmaceutical therapeutic modality. Once the developability of a mAb drug candidate has been assessed, an important step is to check its *in vivo* stability through pharmacokinetics (PK) studies. The gold standard is ligand-binding assay (LBA) and LC–MS (Liquid Chromatography- Mass Spectrometry) performed at the peptide level (bottom-up approach). However, these analytical techniques do not allow to address the different mAb proteoforms that can arise from biotransformation. In recent years, top-down and middle-down mass spectrometry approaches have gained popularity to characterize proteins at the proteoform level but are not yet widely used for PK studies. We propose here a workflow based on an automated immunocapture followed by top-down and middle-down LC-MS/MS approaches to characterize mAb proteoforms spiked in mouse plasma. We demonstrate the applicability of our workflow on a large concentration range using pembrolizumab as a model. We also compare the performance of two state-of-the-art Orbitrap platforms (Tribrid Eclipse and Exploris 480) for these studies. The added value of our workflow for an accurate and sensitive characterization of mAb proteoforms in mouse plasma is highlighted.

STATEMENT OF SIGNIFICANCE:

1. Introduction

In the last 25 years, monoclonal antibodies (mAbs) have become the major modalities in the biopharmaceutical industry and are now largely approved against previously "undruggable" targets in several therapeutic areas[1–3]. In most recent years, biotherapeutics in research pipelines have evolved towards a variety of scaffolds including antibody-drug conjugates (ADCs), fusion proteins, antibody fragments such as NANOBODY® VHHs (Nbs), antigen-binding fragments (Fabs) or single-chain variable fragments (scFv)[4–6]. With a growing interest in multispecifics, even more complex and diverse formats are now being designed such as CODV-Ig (Cross-over dual variable-Ig like), mAb-scFv or tandem-Nbs. Since the complexity of these new biotherapeutic constructs potentially makes them more prone to *in vivo* biotransformation[7–9], there is nowadays a crucial need to precisely characterize and quantify biotransformation products. Biotransformation of a therapeutic protein includes proteolytic cleavages and post-translational modifications (PTMs) such as deamidation, oxidation or glycation among others. Biotransformation can therefore greatly impact pharmacokinetics/pharmacodynamics (PK/PD) profiles, efficacy and in some extreme cases immunogenicity and safety[10,11].

Ligand-binding assay (LBA) and LC–MS (Liquid Chromatography-Mass Spectrometry) are the most used techniques for the *in vivo* analysis of mAb-based drugs in PK/PD studies. Traditionally, quantitation of large biotherapeutics in biological matrices is performed by LBA, due to its high sensitivity and high throughput[12]. However, LBA can be subject to potential cross-reactivity and does not allow to achieve structural information including biotransformation. In last years, improvements in instrumentation and methods have made LC-MS approaches essential in PK studies of biotherapeutics. Conventionally, protein-based drugs are digested by trypsin and resulting peptides are analyzed by LC-MS/MS. This approach allows to get both sequence information including localization of PTMs[13–16] and quantitation at the peptide level. The monitoring of a set of reporter peptide ions (distributed in the different functional domains of the biotherapeutic drug) by targeted MS-based approach, such as multiple-reaction monitoring (MRM), proved to be a highly sensitive strategy with detection up to several weeks after administration[17–19]. The overlay of all selected peptides can be used to evaluate the *in vivo* stability of the administrated product. However, this approach does not allow to address the different proteoforms present in a sample that can arise from biotransformation. The structure of the biotransformation products that circulate in the body is lost after trypsin digestion. Alternative strategies have recently emerged to overcome this limitation. They are either based on the analysis of proteins at the intact level (intact mass analysis and top-down MS/MS approaches) or at the subunit level (middle-down analysis). These subunits can easily be obtained using structure-specific enzymes and/or by reduction of the disulfide

bonds[20–24]. Both top-down and middle-down approaches have gained popularity in academic and industry laboratories and are developed on a variety of high-resolution instruments including mainly Q-TOF and Orbitrap mass spectrometers.

At the intact level, top-down LC-MS approaches allow to achieve the analysis of mAbs proteoforms in both denaturing and native conditions[25–32]. However, the large molecular weight of intact mAb-based drugs still represents a major challenge for MS/MS sequencing [33–37]. This can be substantially improved when fragmenting subunits[32,38–43]. For instance, the complete sequencing of Complementarity Determining Regions (CDRs) has been described on above-hinge subunits with multiple activation techniques[32,44–47]. A recent interlaboratory study highlights the benefit and pitfalls of both approaches for the characterization of intact antibodies[48]. Nonetheless, it should be noted that almost all the work published so far is based on the use of a large amount of starting material (a few micrograms), which is not sustainable for the analysis of *in vivo* biotransformation products. Improvements in stationary phases[49–55], low-flow chromatography and alternative front-end separations[56–58] have recently opened the way to higher sensitivity studies. For instance, both the qualitative and quantitative top-down and middle-down analysis of biotherapeutics extracted from plasma has been described in the last years[59–63]. However, the lower sensitivity of these protein-centric approaches, compared to the peptide-centric ones, still represents a challenge to their routine use in preclinical/clinical studies. This is particularly the case for *in vivo* studies dealing with multiple biological matrices (plasma, tissues, tumor samples), very limited volumes (microsampling) and very low concentrations of circulating biotherapeutics.

To address the challenge of sensitivity, an attractive possibility is to use an immunoaffinity enrichment step before the MS analysis[64–68]. Multiple immunocapture platforms are commercially available (plate-, bead-, tip- or cartridge-based) and can be used in an automated fashion to simplify sample preparation and establish robust workflows. Initially used for the analysis of mAbs, these workflows have recently been adapted to the challenging structural diversity of next-generation biotherapeutics, which can also go with an unexpected PK behavior.

We introduce here a new workflow based on the combination of an automated immunocapture with top-down and middle-down analysis of pembrolizumab spiked in mouse plasma. The most recent Orbitrap platforms (Orbitrap Exploris 480 and Tribrid Eclipse) were used and compared. Pembrolizumab was chosen because it belongs to the growing class of immune checkpoint inhibitors of high interest for cancer immunotherapy. We also took advantage of the presence of multiple proteoforms in our pembrolizumab stock solution to mimic

potential biotransformation products. These proteoforms arise from partial glycation and C-terminal glycine clipping. We illustrate the complementarity of top-down and middle-down approaches to characterize pembrolizumab proteoforms across a concentration range compatible with *in vivo* PK studies. The method was developed starting from only 10 µL of plasma.

2. Materials and methods

2.1. Pembrolizumab stock solution

Pembrolizumab was supplied as a stock solution in DPBS buffer pH 7.4 (Dulbecco's phosphate buffer saline) at 10.1 mg/mL. The purity of the mAb stock solution was assessed in a preliminary LC-MS experiment after deglycosylation with PNGase F (New England Biolabs). This deglycosylation step is mandatory to evaluate the extent of glycation since this modification adds 162 Da, which also corresponds to the Δmass between the major G0F and G1F glycans. Figure S1 gathers the total ion chromatogram (TIC) and deconvolved mass spectrum of pembrolizumab stock solution analyzed on the Orbitrap Exploris 480 (250 ng on column).

2.2. Sample preparation: immunocapture and enzymatic digestion

Pembrolizumab was either analyzed intact, after digestion with IdeS or after digestion with IdeS and reduction. All sample preparation steps were performed on an AssayMAP Bravo robotic platform (Agilent Technologies, CA). Streptavidin cartridges (SA-W, Agilent Technologies, CA) were first primed with 100 µL of 1% formic acid (FA) in water and then equilibrated with 100 µL of DPBS buffer pH 7.4. 1.48 µg of human secreted programmed cell death (PD-1)-Fc fusion protein (BPS Bioscience), 5.4 µg of anti-human IgG Fc (Jackson Immunoresearch) or 5.4 µg of anti-human IgG $F(ab')_2$ (Jackson Immunoresearch) were used as capture reagent and immobilized onto the cartridges at a flow rate of 5 µL/min. Capture reagent-to-mAb ratio was common to all experiments and fixed to 5:1 (molar ratio). For middle-down approaches only (PD-1)-Fc fusion protein and anti-human IgG $F(ab')_2$ were used as capture reagents. Cartridges were then washed twice with 50 µL of DPBS buffer. Affinity purification was performed by loading 200 µL of the diluted plasma samples (10 µL of plasma diluted 20 times in DPBS buffer) at 5 µL/min. Plasma samples were prepared by spiking blank mouse plasma with pembrolizumab across a wide concentration range (0.5 – 100 µg/mL). The cartridges were then washed twice with 50 µL of DPBS buffer. Deglycosylation was performed by aspiring 500 units of PNGase F through the cartridges over 2 hours at 45°C. Cartridges were washed with 50 µL of DPBS buffer and 50 µL of 10% acetonitrile (ACN) in water. For middle-down approaches, cartridges were re-equilibrated with 50 µL of DPBS and 10 units of IdeS enzyme (Promega) were aspirated through the cartridge over 1 hour at 45°C. Reaction was stopped by loading 15 µL of water as chase buffer. The chase buffer was collected in the elution plate. Cartridges were then washed with 50 µL of water and 50 µL of 10% ACN in water. For all approaches, the intact mAb and

the still-bound subunits were eluted from the cartridges with 15 µL of 1% formic acid in water at 5 µL/min. From these 15 µL (intact) and 30 µL (middle-down), only respectively 2.5 µL or 5 µL ($1/6^{th}$) were used for each LC-MS injection. For middle-down MS/MS approach only, $F(ab')_2$ and Fc/2 subunits were reduced into Lc, Fd and Fc/2 subunits with 50 mM of TCEP at 45°C for 30 min under vigorous agitation.

2.3. LC-MS & LC-MS/MS analysis

Immuno-enriched samples were analyzed by LC-MS using a Vanquish Horizon UHPLC system (Thermo Scientific, San Jose, CA) coupled to an Orbitrap Tribrid Eclipse (Thermo Scientific, San Jose, CA) or an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Bremen, Germany) both fitted with a H-ESI source. Proteins were separated on MAbPac™ RP columns (2.1 mm i.d. x 50 or 100 mm, 4 µm particle size, 1500 Å pore size, Thermo Scientific, San Jose, CA) heated at 85°C with a gradient of 0.1% FA in ACN at 250 µL/min: from 10 to 60% in 2 min for intact mAb, from 20 to 60% in 3.27 min for $F(ab')_2$ and Fc/2 and from 25 to 35% in 5 min for Lc, Fd and Fc/2 subunits. For all experiments spray voltage was set to 3.8 kV, sheath gas settings was 25, auxiliary gas settings was 5, vaporizer temperature was 150°C and ion transfer tube temperature was 325°C. MS spectra were acquired at 7,500 resolving power (at m/z 200) with a scan range set to m/z 500-5,000, 5 microscans (µscans) per MS scan, an automatic gain control (AGC) target value of $4x10^5$ (Orbitrap Tribrid Eclipse) or $3x10^6$ (Orbitrap Exploris 480) and a maximum injection time mode set to Dynamic with at least 10 points across the peak (expected peak width 6 s). RF values were set to 150% for intact mAb and 60% for all other subunits. Source fragmentation energy on the Orbitrap Tribrid Eclipse was 60V for intact mAb and $F(ab')_2$ and 10V for Fc/2, Lc and Fd subunits. Source fragmentation energy on the Orbitrap Exploris 480 was 80V for intact mAb and $F(ab')_2$ and 10V for Fc/2, Lc and Fd subunits. Fragmentation data in the middle-down approach were recorded using targeted LC-MS/MS experiments at 120,000 resolving power (at m/z 200) with EThcD and HCD activations on the Orbitrap Tribrid Eclipse and HCD on the Orbitrap Exploris 480. Four precursor charge states were multiplexed for each targeted subunit. More details about the MS/MS parameters can be found in Table S1. All experiments were conducted using the Intact Protein mode with a pressure in the ion-routing multipole (IRM) set to standard (8 mTorr for Orbitrap Tribrid Eclipse; 8.25 mTorr for Orbitrap Exploris 480) or low (1 mTorr for Orbitrap Tribrid Eclipse; 2.47 mTorr for Orbitrap Exploris 480).

2.4. Data analysis

MS spectra were deconvolved with Genedata Expressionist® software using a time-resolved deconvolution and the Maximum Entropy (MaxEnt) algorithm. Minimum and maximum masses were respectively set to 10 and 150 kDa and a 0.2 Da mass step was used. MS/MS spectra were averaged across the appropriate subunit elution windows and then deconvolved using the embedded Xtract algorithm in FreeStyle™ (v.1.6.75.20) with a signal-

to-noise ratio threshold of 3, a fit factor of 80%, a remainder threshold of 25% and maximum charge set to the precursor charge state. Lists of decharged and deisotoped monoisotopic masses were imported into ProSight Lite (v. 1.4) and used for fragment assignments with a 5 ppm mass tolerance. Only b- and y-ions were considered for HCD while b-, c-, y- and z-ions were searched for EThcD.

3. Results and discussion

Our work focused on the development of multi-level LC-MS strategies coupled to immuno-affinity purification (Figure 1) which satisfy as many desirable criteria as possible: automated sample preparation, robustness and ease of implementation, speed of analysis, repeatability, and data quality. First, a combination of top-down MS with immunocapture was evaluated with multiple capture reagents on two state-of-the-art Orbitrap mass spectrometers. Second, a middle-down approach based on digestion with IdeS was implemented in an automated fashion and compared to the intact approach. Finally, targeted MS/MS acquisitions (middle-down MS/MS approach) were conducted to benchmark the sequence coverage of mAb subunits that can be expected on both systems after extraction from mouse plasma.

3.1. Top-down MS coupled to immunocapture

Intact mass analysis remains the most straightforward strategy to analyze biotherapeutics at high throughput. In our work pembrolizumab was spiked in mouse plasma across a wide concentration range (0.5 – 100 µg/mL) often observed in PK studies and only 10 µL of plasma were used as starting material to develop a workflow that could fit requirements of both serial (microsampling) and non-serial (destructive) studies. A very short gradient was set up on a Vanquish Horizon UHPLC system to ensure a robust and high-throughput analysis of immunocaptured mAbs from plasma samples. Our workflow leverages therefore the use of a robotic immunocapture and a fast LC-MS analysis (less than 7.5 min including extensive column washing and equilibration) to analyze more than 120 plasma samples per night. Figure 2 gathers the total ion chromatograms (TIC) and the deconvolved spectra of the plasma samples immuno-enriched by the PD-1 antigen and analyzed on both mass spectrometers. A sharp chromatographic peak was obtained for the immunocaptured intact mAb with a full width half maximum (FWHM) of around 3 seconds. The combination of a sharp elution profile and a very fast MS acquisition (Orbitrap resolution set at 7.5k) leads to a sensitive detection of the intact mAb even at the lowest concentration. Indeed, signal-to-noise ratios (S/N) of the most abundant charge state are ranging between respectively 27 and 441 (Exploris 480) or 17 and 383 (Tribrid Eclipse) for the 0.5 µg/mL and 100 µg/mL samples (Figures S2-S3). High-quality zero-charge deconvolved spectra were generated with the Genedata Expressionist software suite (Figure 2, insets B-C). Three proteoforms with decreased intensities could be identified: unmodified pembrolizumab, pembrolizumab with one glycation (+ 162 Da) and with two

glycations (+ 324 Da). The doubly modified proteoform, which was of very low abundance, could only be detected with an acceptable mass error (< 20 ppm) down to 1 µg/mL. This simple mass profiling shows that qualitative data of high interest can be achieved in a short time frame, which can be useful when studying biotransformation products. A semi-quantitative evaluation was also carried out for the three tested antigens on both LC-MS systems. The sum of the deconvolved intensities of the three proteoforms was plotted against the spiked mAb concentration for all tested antigens (Figure 3). Excellent fits were obtained with r^2 between 0.993 and 0.999. The higher signal response observed with the human PD-1 is in line with the higher specificity of this capture reagent compared to more generic anti-$F(ab')_2$ and anti-Fc antigens. Nevertheless, these generic antigens can be useful to develop a generic immunocapture for any humanized mAb-based drug. Beside the evaluation of a possible linearity of the response, method precision (coefficient of variance) and accuracy (bias) were calculated for three quality control (QC) samples at concentrations 2, 15 and 75 µg/mL spiked into plasma in three replicates. The limits of detection (LOD) and quantitation (LOQ) were considered respectively as the lowest concentration for which the unmodified proteoform was still detected and the lowest QC concentration with acceptable accuracy and precision (within ± 25% according to established guidelines[69]). All results are gathered in Table S2 for both Orbitrap mass spectrometers and each antigen. LODs were found identical, i.e. 0.5 µg/mL, for every tested antigen on both mass spectrometers and in accordance with the hybrid assays described in the literature for a similar sample input[64–66,68]. LOQs of 2 µg/mL were achieved except for the anti-Fc (on the Orbitrap Exploris), probably due to an issue during the affinity purification step. Note that the calculated LOD/LOQ are global but that our data also allow individual values to be calculated for each specific proteoform. Although valuable data can be obtained from intact mAb analysis, the low Orbitrap resolution that is used to maximize sensitivity can complicate the analysis if proteoforms of very similar masses (less than 100 Da difference) are generated. This is illustrated on Figure S4 where the experimental deconvolved mass spectrum of the intact pembrolizumab was compared to a simulated one. An enlargement on the left side of the peak is observed on the experimental trace (Figure S4, inset A), which could be due to the presence of a clipped proteoform at the C-terminal glycine of one heavy chain (- 57.03 Da). The presence of this lighter proteoform is confirmed by the data obtained at 15k Orbitrap resolution (Figure S4, inset B). To solve this issue, one can either increase resolution, but in that case drastically lose sensitivity or resort to middle-down experiments to decrease the size of proteoforms. This strategy can be of particular interest when analyzing next-generation biotherapeutics (CODV-Ig, mAb-Nb hybrids, …), which can have molecular masses much higher than mAbs.

3.2. Middle-down MS coupled to immunocapture

We chose a middle-down approach based on a digestion with IdeS that we incorporated into our automated immunocapture workflow. IdeS leads to $F(ab')_2$ and Fc/2 subunits of respectively 100 and 25 kDa or only 25 kDa when combined with a S-S bond reduction. The LC gradient was adapted to maintain a high throughput for the analysis (almost 100 samples per night) with a baseline chromatographic separation of $F(ab')_2$ and Fc/2 subunits. For the MS method, we used time-dependent in-source energy settings to achieve the best possible sensitivity for both subunits (10V for Fc/2 and 60-80V for the $F(ab')_2$). The data obtained for pembrolizumab spiked in mouse plasma and immuno-enriched by PD-1 are depicted in Figure 4. After deconvolution, two glycated proteoforms could be detected for $F(ab')_2$, as obtained for the intact mAb. For Fc/2, the spectrum is much more complex with the presence of at least six different proteoforms among which four could be assigned to: C_{ter} glycine clipped Fc/2 (proteoform 1), unmodified expected Fc/2 (proteoform 2), Fc/2 with an unprocessed C_{ter} lysine (proteoform 5) as well as a glycated Fc/2 (proteoform 6). The table S3 gathers the theoretical and experimental molecular masses of all proteoforms. The overall sensitivity of the analysis was not affected by the additional on-cartridge digestion step since the two most intense proteoforms for each subunit can still be detected down to 0.5 µg/mL of spiked mAb. As for the intact approach, LODs and LOQs were respectively calculated at 0.5 and 2 µg/mL. Method precision and accuracy also meet the recommended guidelines (Figure S5, Table S4). The obtained data also allow us to evaluate the abundance of the clipped mAb, which cannot be obtained from the top-down MS results. To do so, we took the fractional abundance of the clipped Fc/2 proteoforms, that we divided by two to account for the equal probability of both heavy chains to be clipped (symmetric mAb). This leads to a relative intensity of around 8.3% for the clipped mAb. This value is confirmed by the simulation performed at 15k resolution where a very good fitting of the left part of the peak is observed between experimental and theoretical distributions (Figure S6). For the right part of the peak, the discrepancy may be explained by the presence of unknown heavier proteoforms (containing the Fc/2 proteoforms #3-5). These results show that the middle-down approach clearly brings additional information compared to the top-down MS one, with no cost on sensitivity. We also demonstrate that this approach can be performed in a fully automated fashion, which can be of high interest for PK studies.

3.3. Middle-down MS/MS coupled to immunocapture

A last but important step in our workflow is the confirmation of proteoform structures by MS/MS. Although the clipping of C-terminal glycine is a well reported modification, unexpected ones have to be validated. In addition, MS/MS could also provide information on glycated proteoforms. A reduction of the disulfide bonds with TCEP was therefore added as a final step to generate only 25 kDa subunits (Lc, Fd and Fc/2). These subunits can, in theory, be easily separated by LC and fragmented with high efficiency to achieve excellent

sequence coverages. Figure S7 and table S3 report the MS data obtained and highlight the presence of seven assignable proteoforms. We selected the four most abundant ones for fragmentation, for each spiked concentration: unmodified Lc, unmodified Fd, deglycosylated Fc/2 as well as the C_{ter} glycine-clipped Fc/2. EThcD and HCD fragmentations were used on the Tribrid Eclipse and HCD only on the Exploris 480 using multiplex precursor selection for all. Since Exploris platforms are spreading faster than Tribrid, in particular in biopharma laboratories, it was of interest to evaluate how this instrument, which is largely used for bottom-up experiments, could be used on intact proteins. All results are depicted in Figure 5 (two fragmentation experiments for each subunit are merged: one EThcD spectrum and one HCD spectrum for the Eclipse; two HCD spectra for the Exploris 480). As expected, the sequence coverage decreases with the concentration in plasma. The Eclipse starts with a much higher sequence coverage than the Exploris (62% vs 28%), thanks to the EThcD contribution, but leads to exploitable results only down to 5 mg/mL. HCD on the Exploris seems less affected by the concentration. We can also note that different sequence coverage are obtained for the various subunits, in line with their concentration in the sample. Fragmentation maps achieved at various spiked concentrations are reported in Tables S5-S6 for each subunit. For the clipped Fc/2, the Eclipse fragmentation map confirms the removal of the glycine (and subsequent amidation) at the C-terminus even in plasma samples with only 15 µg/mL of spiked pembrolizumab. For the Exploris, enough diagnostic C-terminal fragments are still detected at 5 µg/mL. These concentrations are of interest for PK studies where circulating concentrations between 1 and 100 µg/mL are often observed. Considering that only 10 mL of plasma were used for each analysis, 5 µg/mL corresponds to only 50 ng of protein, among which only 8.3 ng were used for each injection. For comparison, a very recent *in vivo* study combining immunoaffinity and middle-down MS/MS[70], indicates a sequence coverage of around 30% for the fragmentation of the Lc of a therapeutic mAb by ECD for 1 mg injected on column. In our case, this sequence coverage is obtained for only 42 ng on column, showing that our workflow is much more sensitive.

4. Concluding remarks

In this work we set up a fully automated workflow for the top-down and middle-down LC-MS/MS analysis of a mAb spiked in mouse plasma. This workflow is based on an immunoaffinity purification and the use of state-of-the-art instrumentation (Orbitrap Tribrid Eclipse and Exploris 480). Our results show that both approaches yield complementary information. The intact mass analysis provides a first glimpse on some specific proteoforms present in the sample (here glycated ones) but does not allow to capture the full picture. The middle-down approach, based on an IdeS digestion offers a more complete analysis by analyzing smaller subunits. For pembrolizumab, additional low-abundance proteoforms corresponding to C-terminal clipping

could be revealed and confirmed by MS/MS experiments. The combination of an automated sample preparation and customized MS methods ensures a sensitive detection of both the intact mAb and its subunits in plasma down to 0.5 μg/mL. Moreover, the precision and accuracy of our approaches meet the guidelines published for such studies. Our work is a first step toward a more general use of top-down and middle-down Orbitrap mass spectrometry approaches for the characterization and monitoring of mAbs in PK studies.

ACKNOWLEDGMENTS: This work was supported by the European Union's Horizon 2020 Research and Innovation Program under the grant num-bers 829157 (TopSpec) and 823839 (EPIC-XS). J.D acknowl-edges the Association Nationale de la Recherche et de la Tech-nologie for funding his PhD under agreement CIFRE2020/0483. J.D. and J.C.R acknowledge the Region Ile de France (DIM 1HEALTH) and the LABEX IBEID (grant n°ANR-10-LABX-62-IBEID from the Programme d'Investissements d'Avenir) for funding the Orbitrap Tribrid Eclipse. The authors would like to thank Paul Ferrari's team (Biologics research France, E-Biology, Sanofi R&D, France) for the production of pembrolizumab.

ASSOCIATED DATA: Data are available via ProteomeXchange with identifier PXD040896.

CONFLICT OF INTEREST: The authors have declared no conflict of interest.

REFERENCES:

[1] Kaplon, H., Chenoweth, A., Crescioli, S., Reichert, J.M., Antibodies to watch in 2022. *mAbs* 2022, 14, 2014296.
[2] Walsh, G., Biopharmaceutical benchmarks 2018. *Nat Biotechnol* 2018, 36, 1136–1145.
[3] Lyu, X., Zhao, Q., Hui, J., Wang, T., et al., The global landscape of approved antibody therapies. *Antib Ther* 2022, 5, 233–257.
[4] Carter, P.J., Lazar, G.A., Next generation antibody drugs: pursuit of the "high-hanging fruit." *Nat Rev Drug Discov* 2018, 17, 197–223.
[5] Labrijn, A.F., Janmaat, M.L., Reichert, J.M., Parren, P.W.H.I., Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discov* 2019, 18, 585–608.
[6] Arbabi-Ghahroudi, M., Camelid Single-Domain Antibodies: Promises and Challenges as Lifesaving Treatments. *Int J Mol Sci* 2022, 23, 5009.
[7] Hall, M.P., Biotransformation and In Vivo Stability of Protein Biotherapeutics: Impact on Candidate Selection and Pharmacokinetic Profiling. *Drug Metab Dispos* 2014, 42, 1873–1880.
[8] Walles, M., Berna, M., Jian, W., Hauri, S., et al., A Cross Company Perspective on the Assessment of Therapeutic Protein Biotransformation. *Drug Metab Dispos* 2022, 50, 846–857.
[9] Ball, K., Bruin, G., Escandón, E., Funk, C., et al., Characterizing the Pharmacokinetics and Biodistribution of Therapeutic Proteins: An Industry White Paper. *Drug Metab Dispos* 2022, 50, 858–866.
[10] Zhu, L., Glick, J., Flarakos, J., Bioanalytical Challenges in Support of Complex Modalities of Antibody-Based Therapeutics. *AAPS J* 2020, 22, 130.
[11] Tang, Y., Cao, Y., Modeling Pharmacokinetics and Pharmacodynamics of Therapeutic Antibodies: Progress, Challenges, and Future Directions. *Pharmaceutics* 2021, 13, 422.
[12] Todoroki, K., Mizuno, H., Sugiyama, E., Toyo'oka, T., Bioanalytical methods for therapeutic monoclonal antibodies and antibody–drug conjugates: A review of recent advances and future perspectives. *Journal of Pharmaceutical and Biomedical Analysis* 2020, 179, 112991.
[13] Millán-Martín, S., Jakes, C., Carillo, S., Buchanan, T., et al., Inter-laboratory study of an optimised peptide mapping workflow using automated trypsin digestion for monitoring monoclonal antibody product quality attributes. *Anal Bioanal Chem* 2020, 412, 6833–6848.
[14] Liu, T., Li, Y., Xu, J., Guo, Q., et al., Mass spectrometry-based multi-attribute method for mutation analysis in the early development of therapeutic proteins. *J Pharm Biomed Anal* 2022, 220, 115018.
[15] Jakes, C., Millán-Martín, S., Carillo, S., Scheffler, K., et al., Tracking the Behavior of Monoclonal Antibody Product Quality Attributes Using a Multi-Attribute Method Workflow. *J. Am. Soc. Mass Spectrom.* 2021, 32, 1998–2012.
[16] Camperi, J., Dahotre, S., Guillarme, D., Stella, C., Monitoring multiple quality attributes of a complex Fc-fusion protein during cell culture production processes by mD-LC-MS peptide mapping. *Talanta* 2022, 246, 123519.

[17] Jong, K.A. de, Breugel, S.J. van, Hillebrand, M.J., Rosing, H., et al., Bottom-up sample preparation for the LC–MS/MS quantification of anti-cancer monoclonal antibodies in bio matrices. *Bioanalysis* 2020.
[18] Falck, D., Thomann, M., Lechmann, M., Koeleman, C.A.M., et al., Glycoform-resolved pharmacokinetic studies in a rat model employing glycoengineered variants of a therapeutic monoclonal antibody. *mAbs* 2021, 13, 1865596.
[19] Li, F., Weng, Y., Zhang, G., Han, X., et al., Characterization and Quantification of an Fc-FGF21 Fusion Protein in Rat Serum Using Immunoaffinity LC-MS. *AAPS J* 2019, 21, 84.
[20] Sjögren, J., Olsson, F., Beck, A., Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. *Analyst* 2016, 141, 3114–3125.
[21] Johansson, B.P., Shannon, O., Björck, L., IdeS: a bacterial proteolytic enzyme with therapeutic potential. *PLoS One* 2008, 3, e1692.
[22] von Pawel-Rammingen, U., Johansson, B.P., Björck, L., IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *The EMBO Journal* 2002, 21, 1607–1615.
[23] Moelleken, J., Endesfelder, M., Gassner, C., Lingke, S., et al., GingisKHAN™ protease cleavage allows a high-throughput antibody to Fab conversion enabling direct functional assessment during lead identification of human monoclonal and bispecific IgG1 antibodies. *MAbs* 2017, 9, 1076–1087.
[24] Čaval, T., Hecht, E.S., Tang, W., Uy-Gomez, M., et al., The lysosomal endopeptidases Cathepsin D and L are selective and effective proteases for the middle-down characterization of antibodies. *FEBS J* 2021, 288, 5389–5405.
[25] Deslignière, E., Ehkirch, A., Duivelshof, B.L., Toftevall, H., et al., State-of-the-Art Native Mass Spectrometry and Ion Mobility Methods to Monitor Homogeneous Site-Specific Antibody-Drug Conjugates Synthesis. *Pharmaceuticals* 2021, 14, 498.
[26] Füssl, F., Strasser, L., Carillo, S., Bones, J., Native LC–MS for capturing quality attributes of biopharmaceuticals on the intact protein level. *Current Opinion in Biotechnology* 2021, 71, 32–40.
[27] Ehkirch, A., D'Atri, V., Rouviere, F., Hernandez-Alba, O., et al., An Online Four-Dimensional HIC×SEC-IM×MS Methodology for Proof-of-Concept Characterization of Antibody Drug Conjugates. *Anal Chem* 2018, 90, 1578–1586.
[28] Ehkirch, A., Goyon, A., Hernandez-Alba, O., Rouviere, F., et al., A Novel Online Four-Dimensional SEC×SEC-IM×MS Methodology for Characterization of Monoclonal Antibody Size Variants. *Anal. Chem.* 2018, 90, 13929–13937.
[29] Ehkirch, A., Hernandez-Alba, O., Colas, O., Beck, A., et al., Hyphenation of size exclusion chromatography to native ion mobility mass spectrometry for the analytical characterization of therapeutic antibodies and related products. *Journal of Chromatography B* 2018, 1086, 176–183.
[30] Farsang, E., Guillarme, D., Veuthey, J.-L., Beck, A., et al., Coupling non-denaturing chromatography to mass spectrometry for the characterization of monoclonal antibodies and related products. *Journal of Pharmaceutical and Biomedical Analysis* 2020, 185, 113207.
[31] Carillo, S., Criscuolo, A., Füssl, F., Cook, K., Bones, J., Intact multi-attribute method (iMAM): A flexible tool for the analysis of monoclonal antibodies. *Eur J Pharm Biopharm* 2022, 177, 241–248.
[32] Fornelli, L., Srzentić, K., Huguet, R., Mullen, C., et al., Accurate Sequence Analysis of a Monoclonal Antibody by Top-Down and Middle-Down Orbitrap Mass Spectrometry Applying Multiple Ion Activation Techniques. *Anal. Chem.* 2018, 90, 8421–8429.
[33] Fornelli, L., Ayoub, D., Aizikov, K., Liu, X., et al., Top-down analysis of immunoglobulin G isotypes 1 and 2 with electron transfer dissociation on a high-field Orbitrap mass spectrometer. *Journal of Proteomics* 2017, 159, 67–76.
[34] Fornelli, L., Damoc, E., Thomas, P.M., Kelleher, N.L., et al., Analysis of intact monoclonal antibody IgG1 by electron transfer dissociation Orbitrap FTMS. *Mol Cell Proteomics* 2012, 11, 1758–1767.
[35] Mao, Y., Valeja, S.G., Rouse, J.C., Hendrickson, C.L., Marshall, A.G., Top-Down Structural Analysis of an Intact Monoclonal Antibody by Electron Capture Dissociation-Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry. *Anal. Chem.* 2013, 85, 4239–4246.
[36] Tsybin, Y.O., Fornelli, L., Stoermer, C., Luebeck, M., et al., Structural Analysis of Intact Monoclonal Antibodies by Electron Transfer Dissociation Mass Spectrometry. *Anal. Chem.* 2011, 83, 8919–8927.
[37] Shaw, J.B., Liu, W., Vasil'ev, Y.V., Bracken, C.C., et al., Direct Determination of Antibody Chain Pairing by Top-down and Middle-down Mass Spectrometry Using Electron Capture Dissociation and Ultraviolet Photodissociation. *Anal. Chem.* 2020, 92, 766–773.
[38] Fornelli, L., Ayoub, D., Aizikov, K., Beck, A., Tsybin, Y.O., Middle-Down Analysis of Monoclonal Antibodies with Electron Transfer Dissociation Orbitrap Fourier Transform Mass Spectrometry. *Anal. Chem.* 2014, 86, 3005–3012.

[39] Cotham, V.C., Brodbelt, J.S., Characterization of Therapeutic Monoclonal Antibodies at the Subunit-Level using Middle-Down 193 nm Ultraviolet Photodissociation. *Anal. Chem.* 2016, 88, 4004–4013.
[40] Hernandez-Alba, O., Houel, S., Hessmann, S., Erb, S., et al., A Case Study to Identify the Drug Conjugation Site of a Site-Specific Antibody-Drug-Conjugate Using Middle-Down Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2019, 30, 2419–2429.
[41] Martelet, A., Garrigue, V., Zhang, Z., Genet, B., Guttman, A., Multi-attribute method based characterization of antibody drug conjugates (ADC) at the intact and subunit levels. *Journal of Pharmaceutical and Biomedical Analysis* 2021, 201, 114094.
[42] Srzentić, K., Nagornov, K.O., Fornelli, L., Lobas, A.A., et al., Multiplexed Middle-Down Mass Spectrometry as a Method for Revealing Light and Heavy Chain Connectivity in a Monoclonal Antibody. *Anal. Chem.* 2018, 90, 12527–12535.
[43] Gstöttner, C., Reusch, D., Haberger, M., Dragan, I., et al., Monitoring glycation levels of a bispecific monoclonal antibody at subunit level by ultrahigh-resolution MALDI FT-ICR mass spectrometry. *mAbs* 2020, 12, 1682403.
[44] Greisch, J.-F., den Boer, M.A., Beurskens, F., Schuurman, J., et al., Generating Informative Sequence Tags from Antigen-Binding Regions of Heavily Glycosylated IgA1 Antibodies by Native Top-Down Electron Capture Dissociation. *J Am Soc Mass Spectrom* 2021, 32, 1326–1335.
[45] Greisch, J.-F., den Boer, M.A., Lai, S.-H., Gallagher, K., et al., Extending Native Top-Down Electron Capture Dissociation to MDa Immunoglobulin Complexes Provides Useful Sequence Tags Covering Their Critical Variable Complementarity-Determining Regions. *Anal. Chem.* 2021, 93, 16068–16075.
[46] Bondt, A., Hoek, M., Tamara, S., de Graaf, B., et al., Human plasma IgG1 repertoires are simple, unique, and dynamic. *Cell Systems* 2021, 12, 1131-1143.e5.
[47] He, L., Anderson, L.C., Barnidge, D.R., Murray, D.L., et al., Analysis of Monoclonal Antibodies in Human Serum as a Model for Clinical Monoclonal Gammopathy by Use of 21 Tesla FT-ICR Top-Down and Middle-Down MS/MS. *J. Am. Soc. Mass Spectrom.* 2017, 28, 827–838.
[48] Srzentić, K., Fornelli, L., Tsybin, Y.O., Loo, J.A., et al., Interlaboratory Study for Characterizing Monoclonal Antibodies by Top-Down and Middle-Down Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2020, 31, 1783–1802.
[49] Fekete, S., Murisier, A., Beck, A., Lawhorn, J., et al., New wide-pore superficially porous stationary phases with low hydrophobicity applied for the analysis of monoclonal antibodies. *Journal of Chromatography A* 2021, 1642, 462050.
[50] Fekete, S., Bobály, B., Nguyen, J.M., Beck, A., et al., Use of Ultrashort Columns for Therapeutic Protein Separations. Part 1: Theoretical Considerations and Proof of Concept. *Anal. Chem.* 2021, 93, 1277–1284.
[51] Fekete, S., Murisier, A., Nguyen, J.M., Bolton, M.J., et al., Use of Ultra-short Columns for Therapeutic Protein Separations, Part 2: Designing the Optimal Column Dimension for Reversed-Phase Liquid Chromatography. *Anal. Chem.* 2021, 93, 1285–1293.
[52] Nguyen, J.M., Liu, X., DeLoffi, M., Murisier, A., et al., Aptamer-based immunoaffinity LC-MS using an ultra-short column for rapid attomole level quantitation of intact mAbs. *Journal of Chromatography B* 2021, 1173, 122694.
[53] Bobály, B., Veuthey, J.-L., Guillarme, D., Fekete, S., New developments and possibilities of wide-pore superficially porous particle technology applied for the liquid chromatographic analysis of therapeutic proteins. *Journal of Pharmaceutical and Biomedical Analysis* 2018, 158, 225–235.
[54] Bobály, B., Lauber, M., Beck, A., Guillarme, D., Fekete, S., Utility of a high coverage phenyl-bonding and wide-pore superficially porous particle for the analysis of monoclonal antibodies and related products. *Journal of Chromatography A* 2018, 1549, 63–76.
[55] Murisier, A., D'Atri, V., Pirner, S., Larraillet, V., et al., Ultra-Fast Middle-Up Reversed Phase Liquid Chromatography Analysis of Complex Bispecific Antibodies Obtained in Less Than One Minute. *Pharmaceutics* 2022, 14, 2315.
[56] Park, H.-M., Winton, V.J., Drader, J.J., Manalili Wheeler, S., et al., Novel Interface for High-Throughput Analysis of Biotherapeutics by Electrospray Mass Spectrometry. *Anal. Chem.* 2020, 92, 2186–2193.
[57] Campuzano, I.D.G., Pelegri-O'Day, E.M., Srinivasan, N., Lippens, J.L., et al., Part 1. High-Throughput Mass Spectrometry for Biopharma: A Universal Modality and Target Independent Analytical Method for Accurate Biomolecule Characterization. *J. Am. Soc. Mass Spectrom.* 2022.
[58] Sawyer, W.S., Srikumar, N., Carver, J., Chu, P.Y., et al., High-throughput antibody screening from complex matrices using intact protein electrospray mass spectrometry. *Proc Natl Acad Sci USA* 2020, 117, 9851–9856.

[59] Kang, L., Camacho, R.C., Li, W., D'Aquino, K., et al., Simultaneous Catabolite Identification and Quantitation of Large Therapeutic Protein at the Intact Level by Immunoaffinity Capture Liquid Chromatography–High-Resolution Mass Spectrometry. *Anal. Chem.* 2017, 89, 6065–6075.
[60] Kang, L., Xu, S., Pang, Y., Kirchner, T., et al., Application of middle-down approach in quantitation and catabolite identification of protein by LC–high-resolution mass spectrometry. *Bioanalysis* 2021, 13, 465–479.
[61] Li, W., Srikumar, N., Forrest, W.F., Ellerman, D., et al., Characterizing and Quantitating Therapeutic Tethered Multimeric Antibody Degradation Using Affinity Capture Mass Spectrometry. *Anal. Chem.* 2020, 92, 6839–6843.
[62] Kotapati, S., Passmore, D., Yamazoe, S., Sanku, R.K.K., et al., Universal Affinity Capture Liquid Chromatography-Mass Spectrometry Assay for Evaluation of Biotransformation of Site-Specific Antibody Drug Conjugates in Preclinical Studies. *Anal. Chem.* 2020, 92, 2065–2073.
[63] Xu, K., Liu, L., Saad, O.M., Baudys, J., et al., Characterization of intact antibody–drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography–mass spectrometry. *Analytical Biochemistry* 2011, 412, 56–66.
[64] Kellie, J.F., Alelyunas, Y.W., Albert, J., Schneck, N.A., et al., Intact mAb LC–MS for drug concentration from pre-clinical studies: bioanalytical method performance and in-life samples. *Bioanalysis* 2020, 12, 1389–1403.
[65] Kellie, J.F., Kehler, J.R., Mencken, T.J., Snell, R.J., Hottenstein, C.S., A whole-molecule immunocapture LC–MS approach for the *in vivo* quantitation of biotherapeutics. *Bioanalysis* 2016, 8, 2103–2114.
[66] Kellie, J.F., Tran, J.C., Jian, W., Jones, B., et al., Intact Protein Mass Spectrometry for Therapeutic Protein Quantitation, Pharmacokinetics, and Biotransformation in Preclinical and Clinical Studies: An Industry Perspective. *J. Am. Soc. Mass Spectrom.* 2021, 32, 1886–1900.
[67] Jashnani, A., Kotapati, S., Deshpande, M., Yamazoe, S., et al., Automated and Faster Affinity Capture Method for Biotransformation Assessment of Site-Specific Antibody Drug Conjugates. *Anal. Chem.* 2021, 93, 5371–5376.
[68] Vasicek, L.A., Zhu, X., Spellman, D.S., Bateman, K.P., Direct quantitation of therapeutic antibodies for pharmacokinetic studies using immuno-purification and intact mass analysis. *Bioanalysis* 2019, 11, 203–213.
[69] Jenkins, R., Duggan, J.X., Aubry, A.-F., Zeng, J., et al., Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. *AAPS J* 2015, 17, 1–16.
[70] Kellie, J.F., Schneck, N.A., Causon, J.C., Baba, T., et al., Top-Down Characterization and Intact Mass Quantitation of a Monoclonal Antibody Drug from Serum by Use of a Quadrupole TOF MS System Equipped with Electron-Activated Dissociation. *J. Am. Soc. Mass Spectrom.* 2023, 34, 17–26.

FIGURE LEGENDS:

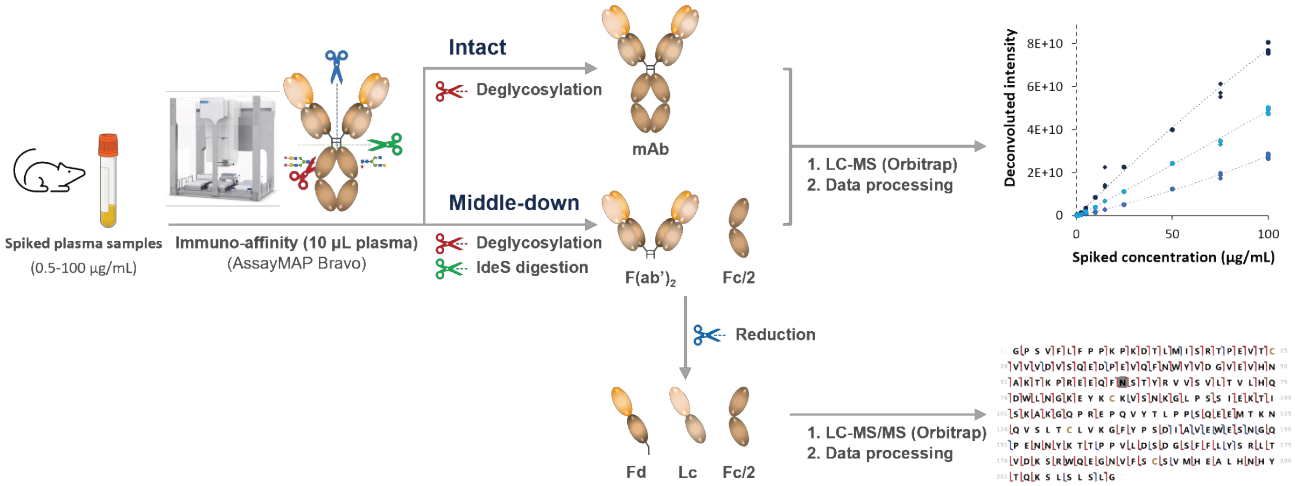

Figure 1: Overview of the developed automated workflow on the AssayMAP Bravo system combining immunocapture, top-down and middle-down LC-MS.

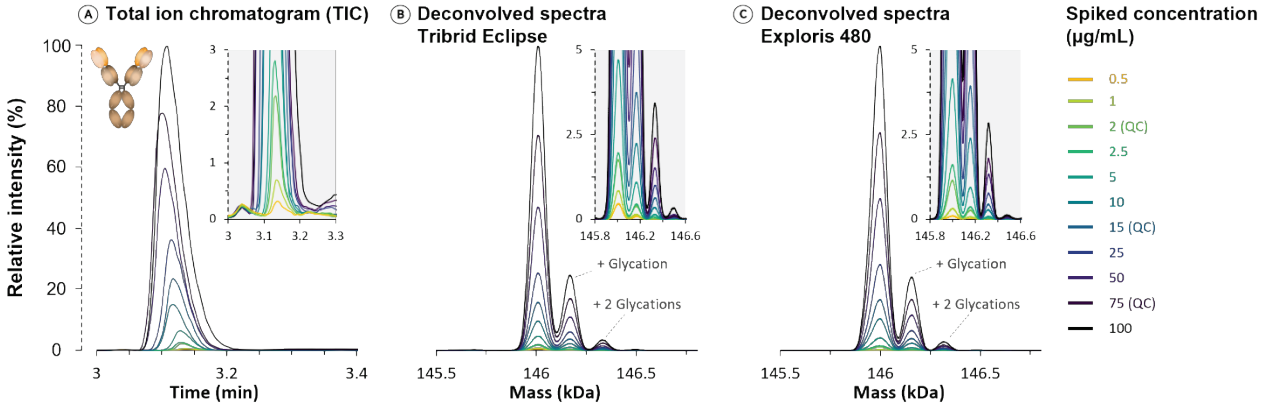

Figure 2: Total ion chromatogram (A) and deconvolved spectra (B – C) of intact pembrolizumab immunocaptured by PD-1 from mouse plasma (0.5 – 100 µg/mL) and deglycosylated. QC: quality control.

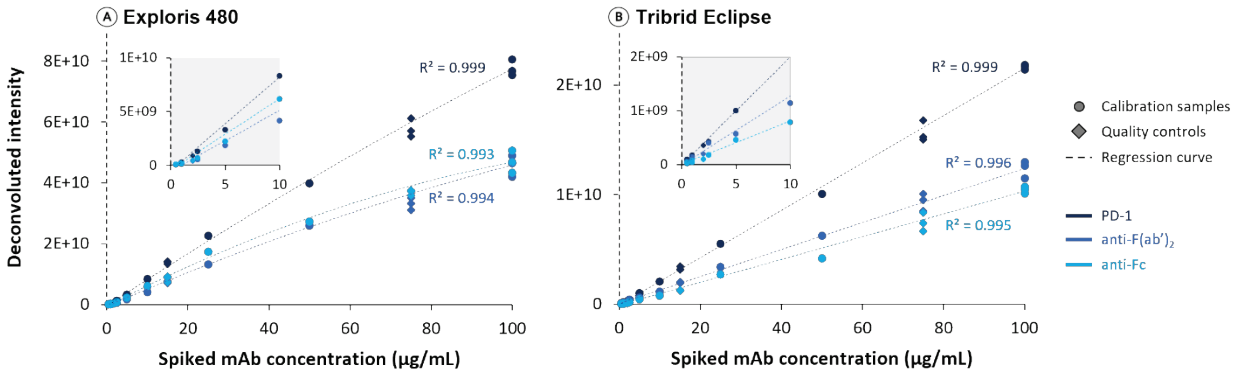

Figure 3: Calibration curves on the Orbitrap Exploris 480 (A) and Tribrid Eclipse (B) for the total intact mAb semi-quantitation after immunocapture by PD-1 (dark blue), anti-F(ab')$_2$ (blue) and anti-Fc (light blue) antigens.

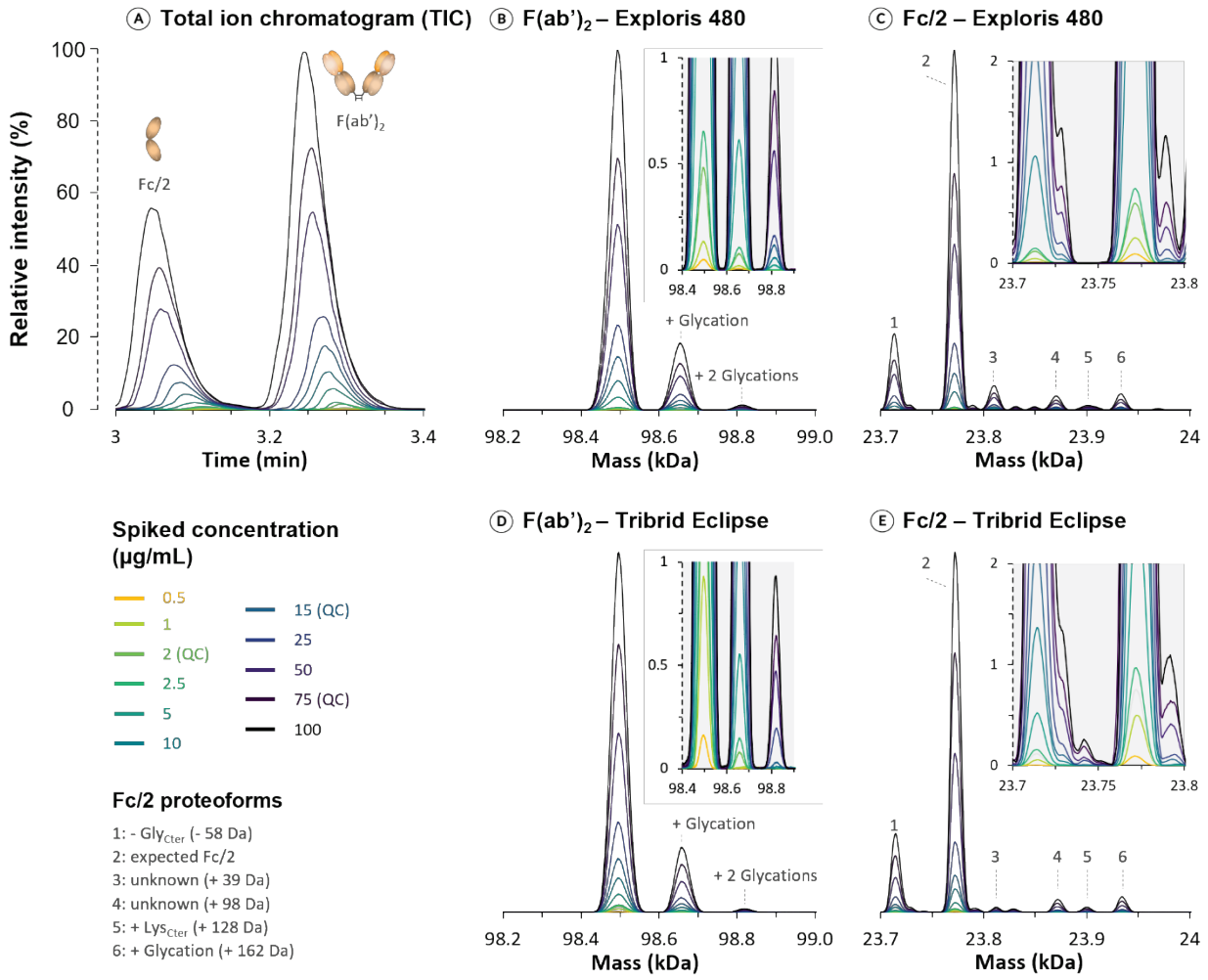

Figure 4: Total ion chromatogram (A) and deconvolved spectra (B – E) of pembrolizumab immunocaptured by PD-1 from mouse plasma (0.5 – 100 µg/mL), deglycosylated and digested with IdeS. QC: quality control.

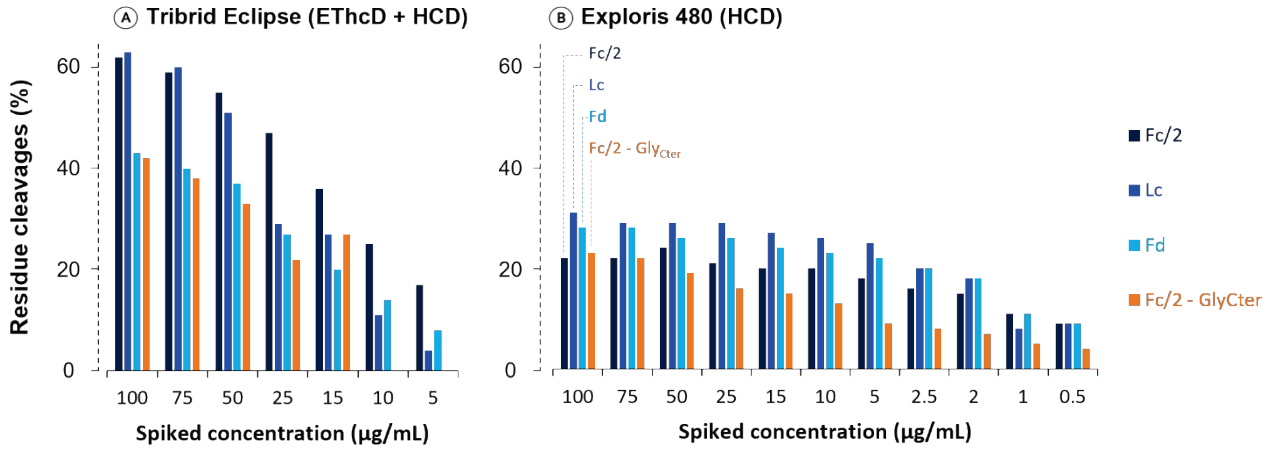

Figure 5: Sequence coverage in middle-down LC-MS/MS experiments for the four targeted mAb subunits on the Orbitrap Tribrid Eclipse (A) and Exploris 480 (B).