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Bispecific antibody characterization by a combination of intact and site-specific/chain-specific LC/MS techniques

Bastiaan L. Duivelshof [a,b], Alain Beck [c], Davy Guillarme [a,b], Valentina D'Atri [a,b,*]

[a] Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO), University of Geneva, CMU-Rue Michel Servet 1, 1211, Geneva 4, Switzerland
[b] School of Pharmaceutical Sciences, University of Geneva, CMU-Rue Michel Servet 1, 1211, Geneva 4, Switzerland
[c] IRPF - Centre d'Immunologie Pierre-Fabre (CIPF), 5 Avenue Napoléon III, BP 60497, Saint-Julien-en-Genevois, France

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ABSTRACT

Bispecific antibodies (bsAbs) are considered as an important class of biopharmaceutical drugs, with about 160 products in clinical trials. From an analytical point of view, the correct chain-association is one of the most critical challenge to monitor during bsAbs development and production. In the present study, a full analytical workflow has been developed based on the use of various chromatographic modes: size exclusion chromatography (SEC), ion exchange chromatography (IEX), reversed phase liquid chromatography (RPLC), and hydrophilic interaction chromatography (HILIC), all combined with high resolution mass spectrometry (MS). This analytical strategy was applied to Hemlibra® (emicizumab), which is certainly the most successful commercial bsAb to date. Using this strategy, it was possible to monitor the presence of mispaired bsAb species and detect and identify additional post-translational modifications (PTMs).

1. Introduction

With recent successes of therapeutic monoclonal antibodies (mAbs) for treatment of diseases such as arthritis, cancer, diabetes and cardiovascular diseases, the field of protein biopharmaceuticals has become one of the fastest growing and most innovative class of human therapeutics. Fueled by this success, many new mAb-based formats have been developed (*e.g.*, fusion proteins, antibody drug conjugates and nanobodies) to fulfill the increasing demand for clinical efficacy and lower adverse effects in the next generation of antibody therapeutics [1]. Among these new formats bispecific antibodies (bsAbs) combine the antigen recognition sites of two or more antibodies in a single protein construct and therefore allow to target two (or more) different epitopes either on the same or on different antigens [2]. As recently reported by Mullard, bsAbs might be considered as the rising star of the antibody field, representing nearly 20% of the clinical antibody pipeline, with almost 160 bispecific and multispecific agents currently in clinical trials [1].

Indeed, the therapeutic potential of bsAbs is extremely appealing, especially for immuno-oncology applications, since they are potentially able to selectively bind to malignant cells, while concurrently recruiting immune cells for enabling targeted cell depletion. Besides, applications of bsAbs for non-cancer indications are also present in the clinical development pipeline (although to a lesser extent) and include different disease areas, such as HIV-1 infections, immune-mediate and autoimmune disorders, diabetes, asthma, and rheumatoid arthritis, among others [3].

However, the vast number of therapeutic applications results in a vast diversity within the bsAb family, literally referred as a zoo by Brinkmann & Kontermann, including more than 100 different combination of antigen-binding moieties and (homo/hetero) dimerization modules, classified based on their format (fragment-based, symmetric, and asymmetric) and their valency (number of binding sites, generally 1 + 1, 1 + 2, or 2 + 2) [2,3].

This molecular (complex) variability arises from the need to develop bsAbs with desired specificity and functionality to serve diverse therapeutic applications, but also from the need to meet developability criteria for fitting production and upstream/downstream processing [2]. This aspect has led to incredible innovations in the field of antibody engineering and antibody biology, and also led to the establishment of more than 20 different commercialized technology platforms for bsAb creation and development [3,4].

Considering the plethora of conceptual and technical innovations developed in this field and the number of molecules currently in clinical

* Corresponding author. Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO), School of Pharmaceutical Sciences, University of Geneva, CMU - Rue Michel Servet 1, 1211, Geneva 4, Switzerland.
E-mail address: valentina.datri@unige.ch (V. D'Atri).

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studies, a large number of bsAbs are expected to reach the market in the upcoming years, even though only four bsAb have been approved by the FDA to date. This includes catumaxomab (2009, Removab®, Trion, withdrawn in 2017 for commercial reasons), blinatumomab (2015, Blincyto®, Amgen Inc.), emicizumab (2017, Hemlibra®, Genentech), and amivantamab (2021, Rybrevant®, Johnson & Johnson). Whereas blinatumomab and amivantamab are used for hematological and solid malignancies, respectively, emicizumab is used as cofactor mimetic for the routine prophylaxis of patients with hemophilia A who are missing the plasma clotting factor VIII (FVIII) [5]. Indeed, emicizumab is able to mimic the function of FVIII by recognizing the activated coagulation factor IX (FIXa) and coagulation factor X (FX) for placing them into spatially appropriate position to allow FIXa to activate FX, without having any homology to the native FVIII [6,7].

From a structural point of view, emicizumab is an IgG4 based bsAb, having 1 + 1 asymmetric format with heterodimerizing heavy chains (HC), one for each target (namely, FIXa and FX), and common light chain (cLC), as represented in Fig. 1. This particular format is also generally referred as biclonic [2]. In addition, emicizumab represents a perfect example of asymmetric reengineering technology immunoglobulin (ART-Ig), containing commonly used IgG4 mutations, improving stabilization and downstream processing (S228P/K196Q/R409K/H435R), and reduction of C-terminal (and charge) heterogeneity (L445P/G446del/K447del), in addition to a specific p*I* engineering (K196Q/F296Y), and mutations inducing the heterodimerization of the heavy chains (E356K x K438E) [3,4,7]. This latter strategy is specifically used for enforcing the correct coupling of heavy chains coming from two different parent antibodies and consists in switching the charged residues polarity of specific amino acids (E356K x K438E), resulting in a stable ionic interaction for the heterodimer versus a repulsive interaction in the case of homodimerization [8]. Detailed information about the emicizumab sequence and its mutations have been reported in Fig. S1.

Indeed, the chain-association issue is one of the most critical analytical challenge to monitor during bsAbs development and production. Several engineered technologies have been developed to ensure correctly paired bsAb, mainly focus to prevent the formation of HC homodimers and LC mispairing, in the case of IgG-like formats [2,3,8,9]. To cite some examples, engineering of the Fc-region (specifically, the CH3 domain) have been implemented to prevent the formation of HC homodimers through the inclusion of specific amino acid mutations that promote HC heterodimerization through steric hindrance (Knob-into--Hole, KiH), electrostatic steering effects or hydrophobic mutations, strand-exchange engineered domains (SEED technology) or addition of an heterodimerization-driving domain, such as a leucine zipper in the C-terminus of the CH3 domain (LUZ-Y technology) [9]. Similarly, LC mispairing can be prevented by generating bsAbs with common light chains (cLC) or through more sophisticated approaches such as the application of CrossMab and DuetMab technologies, among others [9].

Despite the efforts in preventing mispaired formats, the formation of correctly associated bsAb cannot be completely ensured and therefore precise and sensitive analytical approaches are applied during the engineering and development process for monitoring the presence of mispaired byproducts that might be assembled simultaneously with the desired bsAb. In addition, common mAb-related chemical and enzymatic post-translational modifications (PTMs, *e.g.*, deamidation, oxidation, *etc.*) also need to be monitored in reason of their potential impact on the stability, safety, and efficacy of the final bsAb [9,10]. In this scenario, the characterization of bsAbs requires a wide panel of analytical tools and liquid chromatography (LC) coupled to mass spectrometry (MS) based analysis represents the ultimate approach for detection, identification, and quantification of mispaired bsAb species, eventually bearing additional PTMs.

Here, multiple LC-MS strategies in a multi-level approach were set out to characterize the bsAb emicizumab. At intact protein level, size-exclusion chromatography coupled to MS (SEC-MS) was used to monitor the presence of protein size variants caused by aggregate formation and product fragments eventually resulting from incorrect LC and HC binding [11–14]. These size variants are often associated with adverse immunogenic effects and loss of efficacy and therefore need to be well characterized [15].

Other PTMs such as, *e.g.*, oxidation, deamidation and glycation, result in the formation of charge variants and are considered critical

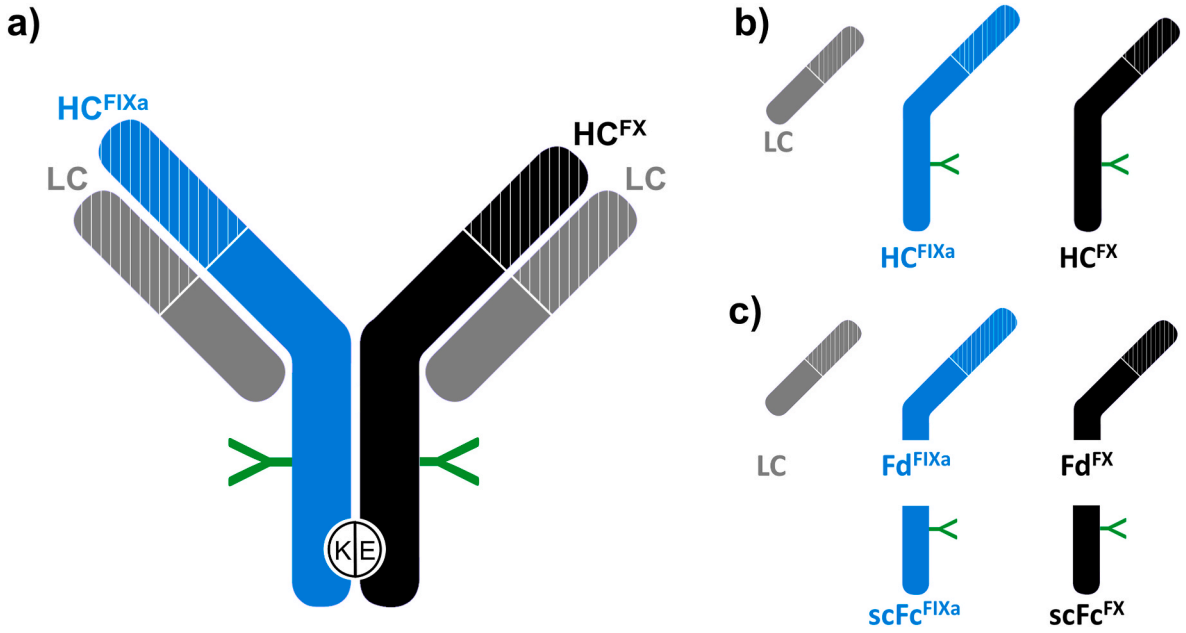

Fig. 1. Emicizumab structure: IgG4 bsAb consisting of two identical light chains (LC), reported in grey, and two different heavy chains (HC) of different length, namely HC^{FIXa} and HC^{FX} reported in blue and black, respectively. (a) The HC and LC variable domains have been highlighted by a pattern with lines, while the glycosylation sites have been reported in green. The white circle identifies the strategy used for enforcing correct heavy chain heterodimerization, consisting in switching the charged residues polarity of specific amino acids (E356K x K438E) to obtain a stable ionic interaction for the heterodimer. Complete amino acid sequence and list of mutations are reported in Fig. S1. Emicizumab subunits obtained after (b) DTT reduction and (c) *IdeS* digestion combined with DTT reduction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

quality attributes (CQAs) due to their influence on the pharmacokinetics and stability of the protein. Therefore, intact level analysis of emicizumab was performed using cation exchange chromatography coupled to MS (CEX-MS) to detect undesired product variants.

However, a major downside of intact protein analysis is the lack of site-specific information when analyzing asymmetric BsAbs, such as emicizumab consisting of two different HCs.

Fortunately, middle-up level analysis provides an interesting approach to obtain site-specific information on BsAbs. The middle-up level refers to the analysis of protein subunits (25–50 kDa) that are generated after protease digestion (*e.g.*, *IdeS*) and/or chemical reduction of the disulphide bonds and enable more accurate peak identification. Recently, this approach was used to analyze a glycan-mediated antibody-drug conjugate (ADC) and determine the correct Fc-drug conjugation in a site-specific manner using RPLC- and HILIC-MS methods [16]. For bsAbs, a similar middle-up level approach provides highly informative fragments that can be used for the localization of chain specific PTMs and glycosylation profile (Fig. 1).

In this study, we therefore present a comprehensive characterization of the bispecific commercial protein emicizumab by using a wide variety of orthogonal chromatographic methods coupled to MS.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (AcN), methanol (MeOH) and water were LC-MS grade (Optima®) and obtained from Fisher Chemical (Reinach, Switzerland). For SEC and IEX experiments, ultrapure water was obtained from a MilliQ purification system from Millipore (Bedford, MA, USA). Isopropanol (ULC-MS grade), formic acid (ULC-MS, FA, 99%) and trifluoroacetic acid (ULC-MS, TFA, >99.0%) were obtained from Biosolve BV (Valkenswaard, the Netherlands). DL-dithiothreitol (DTT, >99%) was obtained from Axon Lab AG (Baden, Switzerland). Ammonium acetate solution (5 M) and Trizma® hydrochloride (>99.0%) were obtained from Sigma-Aldrich (Buchs, Switzerland). *IdeS* enzyme (FabRICATOR®) was purchased from Genovis AB (Lund, Sweden). IonHance CX-MS pH concentrate A (10X) and IonHance CX-MS pH concentrate B (10X) were obtained from Waters (Waters, Milford, MA, USA). Emicizumab (30 mg/mL) was obtained as European Union pharmaceutical-grade drug product from its manufacturer.

2.2. Sample preparation

For intact analysis in IEX-MS, the emicizumab stock solution of 30 mg/mL was placed into polypropylene HPLC vials prior to analysis. For intact analysis in SEC-MS, the stock solution was diluted to 10 mg/mL. For emicizumab middle-up analyses, subunits were generated using the following procedures: reduction of the interchain disulphide bridges was achieved by the addition of 100 mM of DTT solution to 2 mg/mL of protein material and incubating for 30 min at 45 °C. Digestion and reduction of emicizumab was performed by reconstituting 270 units of *IdeS* enzyme with 120 μg of mAb in a solution of 10 mM Tris buffer (pH 7.3). The final concentration of 2 mg/mL was incubated for 30 min at 45 C. Consequently, 100 mM of DTT was added to the digested material and incubated for 30 min at 45 °C. All sample materials were stored at 4 °C until analysis.

2.3. SEC-MS analyses: instrumentation and experimental conditions

An UHPLC system (ACQUITY UPLC H-Class, Waters), equipped with a quaternary solvent delivery pump, an auto-sampler including a 15 μL flow-through-needle injector, and a TUV detector operating at 280 nm, was coupled to an ESI-TWIMS-Q-TOF mass spectrometer (Vion, Waters, Milford, USA) to perform the SEC-MS analyses. The Vion was operated in the sensitivity mode and positive polarity to acquire continuum data in the range of 1000–16000 *m/z* with a scan time of 2 s. Capillary voltage was set at 3.0 kV, cone voltage at 150 V, and source offset at 80 V. Source temperature was set at 150 °C, desolvation temperature at 500 °C, and desolvation gas flow at 750 L/h. The system was calibrated by using a 200 pg/μL sodium iodide solution diluted in a mixture of water/isopropanol 50/50 (v/v) with 0.1% FA. UNIFI v1.9.4 was used for data acquisition, while protein mass spectra data treatment was performed with MassLynx software (Waters). The deconvoluted spectra were generated after manual identification of the multiply charged species after which the spectra were transformed on to a molecular mass axis. The transformation was performed using a predefined resolution of 0.1 Da between the data points and by selection of the lowest and highest molecular mass to include in the algorithm. The considered mass ranges were: 147000–150000 Da for intact proteins, 23000–25000 Da for light chains (LC), 24000–26000 for single chains of the crystallisable fragment (scFc) and 25000–27000 for the antigen binding fragments of the heavy chains (Fd').

SEC-MS analysis were performed with a prototype PEEK coated 4.6 × 300 mm column (YMC-SEC MAB, 3 μm, 250 Å) kept at room temperature. The separation was carried out in isocratic mode by using 100 mM ammonium acetate as mobile phase, a flow-rate of 0.15 mL/min and injection volume of 10 μL. A split of the LC flow was realized with a PEEK T-junction so that the flow rate entering to the MS was of 0.05 mL/min.

2.4. CEX-, RPLC-, and HILIC-MS experiments: instrumentation and chromatographic conditions

CEX-, RPLC-, and HILIC-MS experiments were performed using an ACQUITY UPLC I-Class system coupled to a TUV detector for UV detection (280 nm) and to a high-resolution BioAccord ToF mass spectrometer from Waters. For CEX-MS experiments, the mass spectrometer was operated in the ESI+ mode with 1.5 kV capillary voltage, 350 °C desolvation temperature and a sampling cone voltage of 150 V. Full scan acquisition was performed with Intelligent Data Capture (IDC) on in the range of 400–7000 *m/z* and a scan rate of 2 Hz. Charge variants analysis was performed by using the BioResolve SCX mAb (2.1 mm × 50 mm, 3 μm) column and the IonHance CX-MS pH concentrate A and B as mobile phases. The concentrates were diluted 10x with MilliQ water to obtain a concentration of 50 mM ammonium acetate in 2% acetonitrile (pH 5.0) and 160 mM ammonium acetate in with 2% acetonitrile (pH 8.5), to prepare mobile phase A and B, respectively. The gradient applied consisted of an isocratic step at 50%B for 1 min followed by 50%–98%B in 16.5 min. 98%B was kept for 1.5 min to wash the column and followed by a column re-equilibration step at 50%B for 7 min. The flow rate was 0.1 mL/min, column temperature 30 °C and an injection volume of 1 μL (30 mg/mL sample material) was used.

For RPLC-MS and HILIC-MS the mass spectrometer was operated in the ESI+ mode with a capillary voltage of 1.5 kV, desolvation temperature of 550 °C and a cone voltage 120 V for HILIC-MS and 70 V for RPLC-MS. Full scan acquisition was performed with IDC on and a mass range of 400–7000 *m/z* with a scan time of 2 Hz. The separation in HILIC and RPLC was performed by using 0.1% TFA water and 0.1% TFA in AcN as mobile phases A and B, respectively. In RPLC mode, the gradient applied was 29%–38%B in 12 min. This was followed by an isocratic step at 70%B (1 min) for column washing and 5-min re-equilibration step at 29%B. For HILIC, a gradient of 85% to 73%B in 0.2 min, followed by 73% to 65%B in 12 min was applied. Then, an isocratic step at 15%B for 1 min was used to wash the column, followed by 9 min column re-equilibration at 85%B. In both separation modes, the flow rate was 0.4 mL/min, column temperature 80 °C and injection volume 0.5 μL (2 mg/mL sample material). The Waters Acquity UPLC Protein BEH C4 (1.7 μm, 150 mm × 2.1 mm, 300 Å) and Waters Acquity UPLC Glyco-Protein Amide (1.7 μm, 150 mm × 2.1 mm, 300 Å) columns were used for separations in RPLC and HILIC modes, respectively.

3. Results and discussion

3.1. CEX-MS

One of the main goal of the CEX-MS analysis was to confirm the correct hetero-dimerization of the heavy chains (LC-HC^{FIXa}-HC^{FX}-LC format, having p*I* 6.9) and the absence of homo-dimers traces (LC- HC^{FX}-HC^{FX}-LC, having p*I* 6.1, or LC-HC^{FIXa}- HC^{FIXa}-LC, having p*I* 8.3) [7]. In parallel, the identification of other PTMs eventually related to the formation of charge variants was performed. In this context, as reported by Du et al., modifications that may induce acidic species include *e.g.* the presence of deamidation, glycation, and glycans containing sialic acid or high mannose, while modifications that may induce basic species include C-terminal Lys, isomerization of Asp, succinamide, Met oxidation, and aglycosylation among others [17]. In addition, both acidic and basic species can be related to the formation of pyroglutamate (pE), depending on the precursor of the cyclization process. In fact, the formation of pE from the N-terminal Glu (E) or Gln (Q) may respectively result in a basic or an acidic variant, with the charge variant containing the pE being less basic than the one containing the Q, and less acidic than the one containing the E [17,18]. The CEX-MS analysis was performed with a narrow bore column and MS-compatible mobile phases. 1 μL of 30 mg/mL sample was injected onto the column and elution was performed in pH gradient mode. The LC gradient was adapted to allow the proper separation of the minor species from the main peak, UV detection (280 nm) was used for quantification purposes, and MS source conditions were optimized to allow the detection and MS deconvolution of all the species, with a special attention devoted to minor charge variants.

The emicizumab CEX profile obtained under optimized method conditions was reported in Fig. 2 and consists of a main peak (M), one acidic variant (A1), and two basic variants (B1 – B2). Detailed charge variant retention times and mass assignments are reported in Table S1. The UV detection was used for quantifying the amount of acidic/basic species (Fig. 2a), while the MS detection was used for identification purposes. The total ion chromatogram (TIC, Fig. 2b) was consistent with the UV trace, and each variant was easily identified after mass deconvolution (Fig. 2c). This allowed to confirm the absence of homo-dimers traces together with the presence of several PTMs. Specifically, the main peak (M) was showing homogenous N-terminal modifications (two Q/pE conversions) and several protein glycoforms mostly related to complex and fucosylated species, with G0F/G0F, G0F/G1F, G1F/G1F as the major forms. Interestingly, the acidic species (A1) was characterized by high-mannose and afucosylated glycoforms (namely, M5/M5, G0/G0, and G0/G0F), with a homogenous presence of pE as N-terminal modification (Q/pE), while the first basic variant (B1) was characterized by complex and fucosylated glycoforms with 2 QpE conversions at the N-terminal end. Finally, the second basic variant (B2) was characterized by

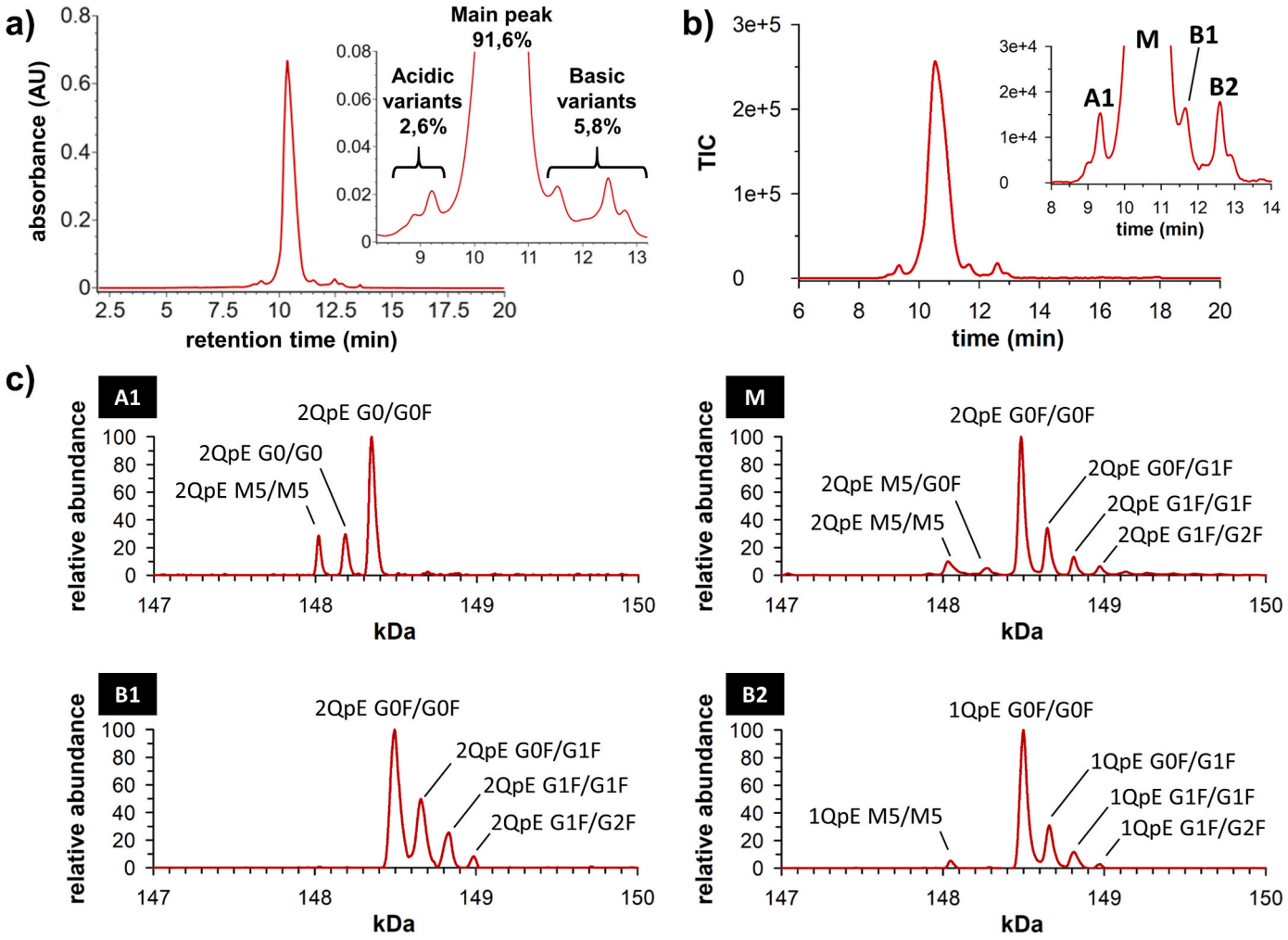

Fig. 2. Emicizumab CEX-MS analysis: identification and peak assignment of charge variants. (a) CEX-UV chromatogram at 280 nm and zoom highlighting the relative amounts of the main peak and the acidic/basic variants. (b) Total ion chromatogram (TIC) and zoom highlighting the minor variants. (c) Mass spectra deconvolution and glycoform annotation of peaks A1, M, B1, and B2, respectively. QpE refers to the cyclization of the N-terminal Q in pyroE. Complete list of peak assignments is reported in Table S1.

complex and fucosylated glycoforms bearing only one QpE conversion as N-terminal modification. In fact, the proteoforms containing the combination Q-pE resulted as basic species in comparison to the proteoforms having the pE-pE combinations (detected in the other charge variants), since the Q is more basic than the pE.

3.2. SEC-MS

SEC-MS was used to perform size variants characterization. The separation was performed with a prototype PEEK coated column to minimize any secondary interaction between the bsAb and the column hardware when using MS-compatible mobile phases [19]. 10 μL of 10 mg/mL sample was injected onto the column and elution was performed in isocratic mode. As reported in Fig. 3, emicizumab shows an almost perfect profile in terms of size variants. Indeed, the UV profile (Fig. 3a) shows an incredible homogeneous sample, with no low-molecular weight species (LMWS) and a negligible amount of high-molecular weight species (HMWS, corresponding to only 0.2%), as highlighted in the inset reporting the relative quantification based on the areas of the chromatographic peaks. Mass spectra of the two detected peaks (dimer and monomer) and the deconvoluted spectrum of the main peak have been reported in Fig. 3b and c, respectively. Thanks to the direct coupling to MS, the identity of each chromatographic peak was easily unveiled and the complete glycoforms assignment was performed for the main species corresponding to the monomer (Fig. 3c). In addition to the different glycoforms, the cyclization of the N-terminal Q in pyroE was

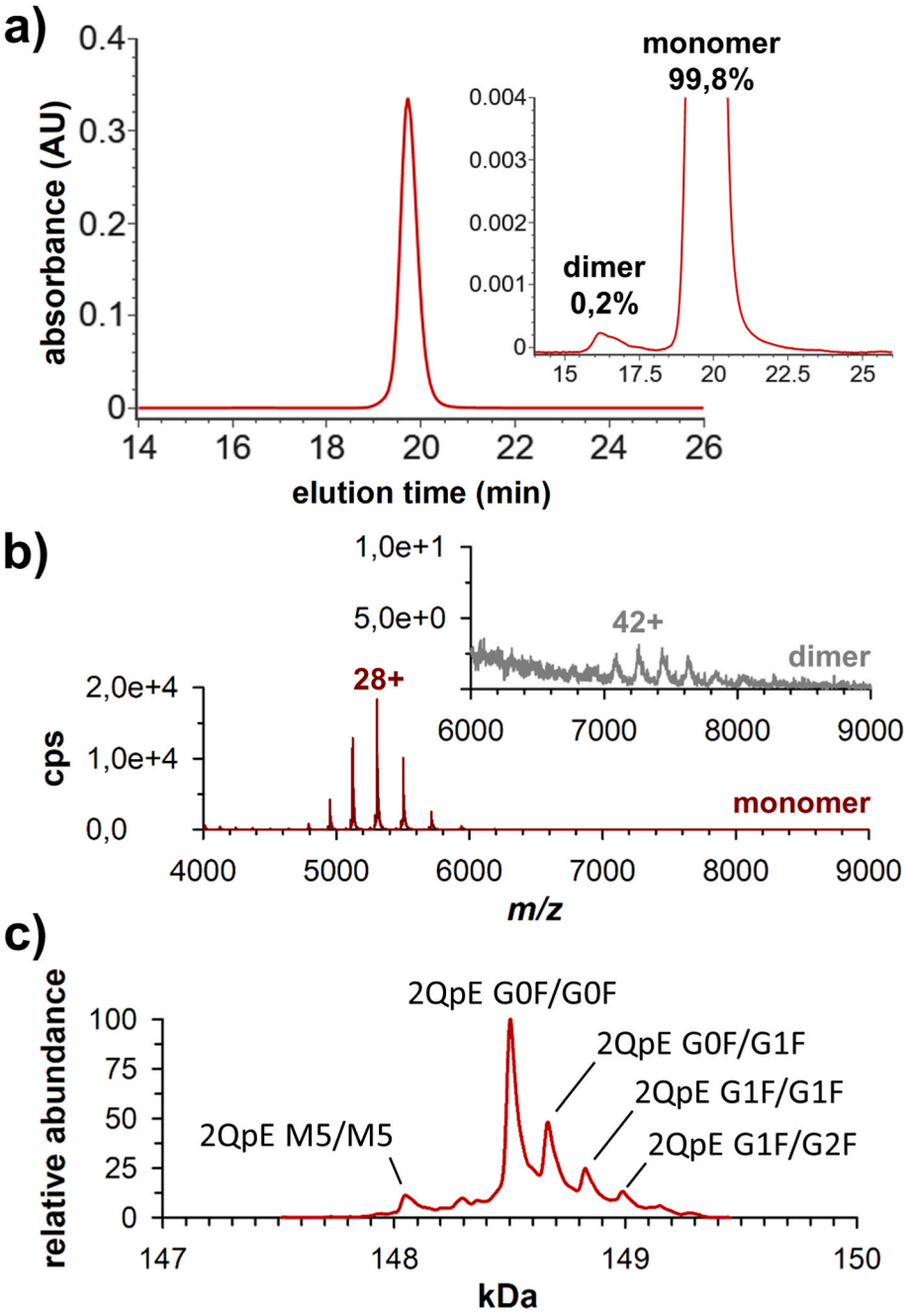

Fig. 3. Emicizumab SEC-MS analysis: identification and peak assignment of size variants. (a) SEC-UV chromatogram at 280 nm and zoom highlighting the main peak (monomer) and the HMWS variant (dimer) with their relative amounts. (b) Mass spectra of monomer and dimer peaks and (c) deconvolution and glycoform annotation of the monomeric species. QpE refers to the cyclization of the N-terminal Q in pyroE. Complete list of peak assignments is reported in Table S2.

also identified as major PTM (Fig. 3c and Table S2). Overall, the results were in line with the MS assignment performed by IEX-MS at intact level and the glycan characterization performed by middle-up HILIC-/RPLC-MS (see section 3.3). In addition, beyond the accurate size variants analysis, SEC-MS also allowed to confirm the exceptional homogeneity of the sample consisting in the correct hetero-dimerization of the heavy chains (LC-HC^{FIXa}-HC^{FX}-LC format) and the absence of homo-dimers traces (no LC- HC^{FX}-HC^{FX}-LC or LC-HC^{FIXa}- HC^{FIXa}-LC detected).

3.3. RPLC- and HILIC-MS/denaturing chromatographic conditions

Intact analysis of emicizumab was first performed using RPLC and HILIC coupled to MS detection. The coupling of these denaturing techniques to MS is widely used in routine environments and commonly used for intact mass determination. For intact emicizumab, it was observed that a better peak shape was obtained in RPLC vs. HILIC mode, but in both techniques, no variants of emicizumab were chromatographically separated (Fig. S2). This is related to the limited chromatographic selectivity obtained at the intact protein level and can be improved by reducing the analyte size [20]. Mass deconvolution of both peaks demonstrated the presence of intact emicizumab with twice the formation of N-terminal pyroglutamic acid and the presence of G0F/G0F as major glycoforms (Table S3). Furthermore, no species were observed with masses corresponding to the incorrect heterodimerization of the heavy chains. However, limited information on PTMs was obtained at the intact protein level due to the broad isotopic distribution for large proteins in MS detection that hampers the accurate detection of small mass differences. Moreover, intact level analysis does not provide site-specific information on the detected PTMs, which is of particular interest when analysing bispecific antibodies such as emicizumab. Therefore, to improve the chromatographic performance and increase the MS sensitivity a middle-up approach was used.

3.3.1. Middle-up RPLC-MS

To get more informative fragments, intact emicizumab was enzymatically digested and chemically reduced using *IdeS* digestion and DTT reduction. Protein subunits of approximately 25–50 kDa were generated and subsequently analysed first using RPLC-MS.

Fig. S3a illustrates the RPLC separation of emicizumab after DTT reduction of the disulphide bridges to generate the common LC and the HC corresponding to FIXa and FX. It was observed that the LC eluted first and was followed by multiple peaks corresponding to the HC subunits. The presence of multiple peaks corresponding to the HC subunits could indicate the presence of isomeric variants of the FX and FIXa chains. This was previously observed for a site-specific ADC product after analysis using both RPLC-MS and HILIC-MS on middle-up level [16]. Moreover, two species for a single HC fragment of a bispecific antibody were also observed by Verscheure et al. after RPLC-MS analysis [21]. Another potential explanation is the presence of partially and fully reduced variants. The incomplete reduction of intramolecular disulphide bridges could create a mixture of subunit variants with different reduction levels that can be resolved chromatographically or by using capillary electrophoresis [21,22]. Here, mass deconvolution demonstrated minor differences between the first and second eluting peak corresponding to the HC of FX and HC of FIXa, respectively (Table S4). However, the accurate assignment of the approx. 2 Da difference resulting from an intact disulphide bridge is hampered by the limited mass accuracy of the MS instrument at the subunit level.

To further increase the chromatographic resolution, *IdeS* digested and DTT reduced emicizumab was analysed using RPLC-MS. Fig. 4a illustrates the obtained RPLC separation for the common LC and the Fd' and scFc corresponding to both the FIXa and FX chains. It was observed that based on the sequence differences between the FIXa and FX antigen binding regions, the Fd' subunits could be well resolved and the Fd^{FX} subunit was less retained on the column compared to the Fd^{FIXa}. Mass deconvolution of both Fd' peaks confirmed the formation of N-terminal pyroglutamic acid (QpE) in all cases (Table S5). In addition, adduct formation was observed for both Fd' subunits and is considered an experimental artefact related to the use of TFA in the mobile phase prior to the ESI-MS. This was confirmed by the observation of identical adducts on the Fd subunits of other mAb products in a separate analysis using identical experimental conditions (Fig. S4).

Furthermore, chromatographic separation of the $scFc^{FIXa}$ and $scFc^{FX}$

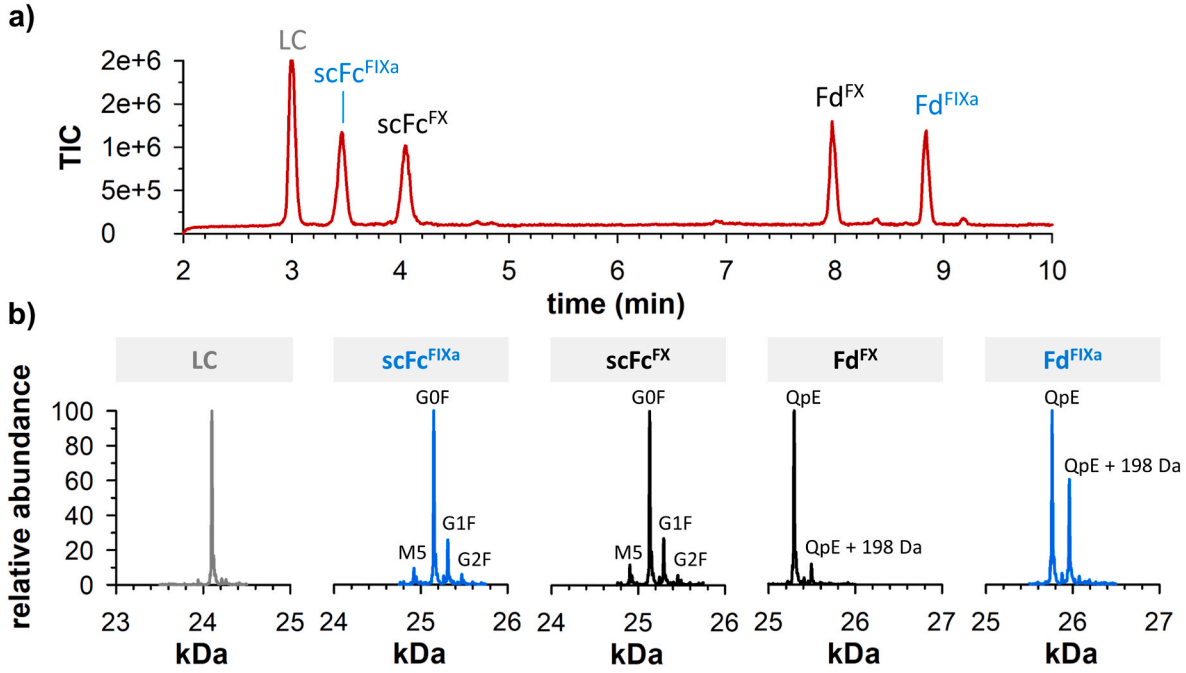

Fig. 4. Middle-up RPLC-MS analysis: identification and peak assignment of subunits. (a) Total ion chromatogram (TIC) of *IdeS* digested and DTT reduced emicizumab and (b) mass spectra deconvolution of each peak reporting the corresponding glycoform/PTM annotation. QpE refers to the cyclization of the N-terminal Q in pyroE. Complete list of peak assignments is reported in Table S5.

was observed. This demonstrates the strong efficiency of the RPLC separation since the sequence differences between the two scFc chains was limited to 3 amino acid residues (Fig. S1). Moreover, it was observed that the $scFc^{FIXa}$ eluted before the $scFc^{FX}$ which is contrary to the elution order observed for the Fd' subunits. Deconvolution of the mass spectra corresponding to both scFc peaks showed the M5, G0F, G1F and G2F as main glycan species on both FIXa and FX crystallisable fragments (Fig. 4b). In addition, the deconvoluted mass spectra of the scFc subunits showed no differences in glycan abundance levels, therefore indicating a symmetric glycosylation profile between the FIXa and FX chains.

3.3.2. *Middle-up HILIC-MS*

Middle-up HILIC-MS analysis was performed to obtain complementary information on the subunits of emicizumab. Since HILIC analysis is driven by hydrophilic interaction with the stationary phase, it enables the chromatographic separation of the subunit glycan profile based on their differences in hydrophilicity. For bispecific antibodies, this could provide the possibility to characterize the glycosylation based on chain specific chromatographically resolved glycan profiles. Therefore, emicizumab subunits of approximately 25–50 kDa were generated and subsequently analysed using HILIC-MS.

The HILIC analysis of the emicizumab subunits after DTT reduction of the disulphide bridges provides less information compared to the RPLC analysis. Fig. S3b illustrates the obtained HILIC-MS profile of reduced emicizumab, in which, poorly resolved peaks were observed for the HC^{FIXa} and HC^{FX} (Table S6). This co-eluting effect could be related to the minor differences in glycosylation between the FIXa and FX HCs, leading to minimal differences in hydrophilicity. Moreover, the minimal differences in the amino acid sequence and large size further reduce the separation efficiency of the HILIC analysis.

Further fragmentation of emicizumab into smaller fragments of approx. 25 kDa using the combined *IdeS* digestion and DTT reduction allowed to greatly improve the chromatographic resolution. Fig. 5a illustrates the separation of the common LC and the Fd' and scFc corresponding to both the FIXa and FX chain of emicizumab. Contrary to RPLC, it was observed that the Fd' fragments eluted first, followed by the LC and both the scFc variants with multiple resolved glycoforms (Table S7). More specifically, the elution order for the two Fd' subunits was opposite to the order observed in RPLC with the Fd' of FIXa eluting before the Fd' of FX. This confirms a more hydrophobic character of the Fd' of FIXa when compared to the Fd' of FX.

For the scFc subunits, multiple glycoforms could be resolved. However, due to the minimal differences in the amino acid sequence between the $scFc^{FIXa}$ and $scFc^{FX}$ and the small differences in retentivity caused by the various glycoforms, multiple peaks were found closely eluting. To characterize the glycosylation profiles of each scFc separately, the extracted ion chromatograms (EIC) corresponding to the masses of subunits carrying various glycoforms were used. Fig. 5b shows an overlay of the EICs and demonstrates the separation of the G0F, M5, G1F and G2F glycans of each chain. Moreover, it was shown that coelution between the chains was present for the $scFc^{FX}$ subunit carrying the G0F glycan and the $scFc^{FIXa}$ subunit carrying the M5 glycan. More interestingly, it was observed that each glycoform of the FIXa subunit elutes prior to the respective FX subunit with the same glycan attached. Therefore, the elution order of the scFc subunits was found identical between the RPLC and HILIC analysis despite their orthogonality in retention mechanism. At last, identical glycoforms were observed on both chains and based on the EIC profiles no differences were found in glycan abundance levels. This is in good correlation with the RPLC-MS characterization and confirms the identification of a symmetric glycosylation profile between FIXa and FX.

4. Conclusions

This paper describes a comprehensive LC/MS workflow for the intact and site-specific characterization of bispecific antibody, namely Emicizumab. For this purpose, various chromatographic methods (IEX, SEC, RPLC and HILIC) were combined with high resolution MS.

The IEX-MS analysis confirms the absence of homo-dimers traces and the presence of several PTMs (high mannose glycan species, complex and fucosylated glycoforms, presence of N-terminal modification). SEC-MS shows an incredible homogeneous sample with no LMWS and a negligible amount of HMWS (corresponding to only 0.2%) and confirms the excellent homogeneity of the sample consisting in the correct hetero-

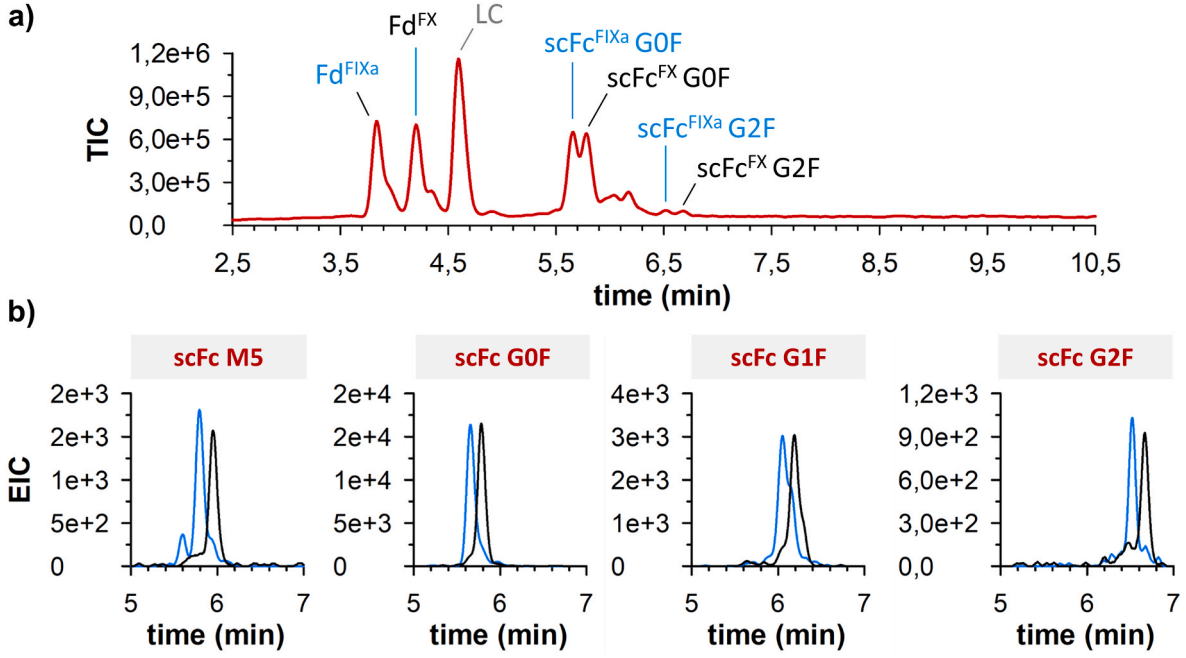

Fig. 5. Middle-up HILIC-MS analysis: identification and peak assignment of subunits. (a) Total ion chromatogram (TIC) of *IdeS* digested and DTT reduced emicizumab and (b) extracted ion chromatograms (EIC) of the scFc peaks reporting the corresponding glycoform/PTM annotation. QpE refers to the cyclization of the N-terminal Q in pyroE. Complete list of peak assignments is reported in Table S7.

dimerization of the heavy chains and the absence of homo-dimers traces. The formation of N-terminal pyroglutamic acid was confirmed by RPLC-MS at the middle-up level of analysis. In HILIC-MS, multiple glycoforms could be resolved and no differences were found between both chains of the bsAb (FIXa and FX) in terms of glycan abundance levels (symmetric glycosylation). Obviously, the analytical workflow developed here can be applied to any other bispecific antibody.

CRediT author contribution statement

Bastiaan L. Duivelshof: Investigation, Data curation, Visualization, Writing - original draft; **Alain Beck:** Conceptualization, Writing - review & editing; **Davy Guillarme:** Conceptualization, Supervision, Writing - original draft; Writing - review & editing; **Valentina D'Atri:** Project administration, Data curation, Visualization, Writing - original draft; Writing - review & editing.

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.

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Appendix A. Supplementary data

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References

[1] A. Mullard, FDA approves 100th monoclonal antibody product, Nat. Rev. Drug Discov. 20 (2021) 491–495, https://doi.org/10.1038/d41573-021-00079-7.
[2] U. Brinkmann, R. Kontermann, The making of bispecific antibodies, mAbs 9 (2017), https://doi.org/10.1080/19420862.2016.1268307.
[3] A.F. Labrijn, M.L. Janmaat, J.M. Reichert, P.W.H.I. Parren, Bispecific antibodies: a mechanistic review of the pipeline, Nat. Rev. Drug Discov. (2019), https://doi.org/10.1038/s41573-019-0028-1.
[4] C. Dumet, J. Pottier, V. Gouilleux-Gruart, H. Watier, Insights into the IgG heavy chain engineering patent landscape as applied to IgG4 antibody development, mAbs 11 (2019) 1341–1350, https://doi.org/10.1080/19420862.2019.1664365.
[5] L.J. Scott, E.S. Kim, Emicizumab-kxwh: first global approval, Drugs 78 (2018) 269–274, https://doi.org/10.1007/s40265-018-0861-2.
[6] Z. Sampei, T. Igawa, T. Soeda, M. Funaki, K. Yoshihashi, T. Kitazawa, A. Muto, T. Kojima, S. Nakamura, K. Hattori, Non–antigen-contacting region of an asymmetric bispecific antibody to factors IXa/X significantly affects factor VIII-mimetic activity, mAbs 7 (2015) 120–128, https://doi.org/10.4161/19420862.2015.989028.
[7] Z. Sampei, T. Igawa, T. Soeda, Y. Okuyama-Nishida, C. Moriyama, T. Wakabayashi, E. Tanaka, A. Muto, T. Kojima, T. Kitazawa, K. Yoshihashi, A. Harada, M. Funaki, K. Haraya, T. Tachibana, S. Suzuki, K. Esaki, Y. Nabuchi, K. Hattori, Identification and multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor Activity, PloS One 8 (2013), e57479, https://doi.org/10.1371/journal.pone.0057479.
[8] C. Klein, C. Sustmann, M. Thomas, K. Stubenrauch, R. Croasdale, J. Schanzer, U. Brinkmann, H. Kettenberger, J.T. Regula, W. Schaefer, Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies, mAbs 4 (2012) 653–663, https://doi.org/10.4161/mabs.21379.
[9] M. Amaral, S. Hölper, C. Lange, J. Jung, H. Sjuts, S. Weil, M. Fischer, K. Radoevic, E. Rao, Engineered technologies and bioanalysis of multispecific antibody formats, J. Appl. Bioanal. 6 (2020) 26–51, https://doi.org/10.17145/jab.20.005.
[10] A.R. Evans, A.S. Hebert, J. Mulholland, M.J. Lewis, P. Hu, ID–MAM: a validated identity and multi-attribute monitoring method for commercial release and stability testing of a bispecific antibody, Anal. Chem. 93 (2021) 9166–9173, https://doi.org/10.1021/acs.analchem.1c01029.
[11] A. Goyon, V. D'Atri, O. Colas, S. Fekete, A. Beck, D. Guillarme, Characterization of 30 therapeutic antibodies and related products by size exclusion chromatography: feasibility assessment for future mass spectrometry hyphenation, J. Chromatogr. B 1065–1066 (2017) 35–43, https://doi.org/10.1016/j.jchromb.2017.09.027.
[12] A. Ehkirch, A. Goyon, O. Hernandez-Alba, F. Rouviere, V. D'Atri, C. Dreyfus, J.-F. Haeuw, H. Diemer, A. Beck, S. Heinisch, D. Guillarme, S. Cianferani, A novel online four-dimensional SEC×SEC-IM×MS methodology for characterization of monoclonal antibody size variants, Anal. Chem. 90 (2018) 13929–13937, https://doi.org/10.1021/acs.analchem.8b03333.
[13] A. Ehkirch, O. Hernandez-Alba, O. Colas, A. Beck, D. Guillarme, S. Cianférani, Hyphenation of size exclusion chromatography to native ion mobility mass spectrometry for the analytical characterization of therapeutic antibodies and related products, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1086 (2018) 176–183, https://doi.org/10.1016/j.jchromb.2018.04.010.
[14] E. Deslignière, M. Ley, M. Bourguet, A. Ehkirch, T. Botzanowski, S. Erb, O. Hernandez-Alba, S. Cianférani, Pushing the limits of native MS: online SEC-native MS for structural biology applications, Int. J. Mass Spectrom. 461 (2021) 116502, https://doi.org/10.1016/j.ijms.2020.116502.
[15] M. Haberger, M. Leiss, A.-K. Heidenreich, O. Pester, G. Hafenmair, M. Hook, L. Bonnington, H. Wegele, M. Haindl, D. Reusch, P. Bulau, Rapid characterization of biotherapeutic proteins by size-exclusion chromatography coupled to native mass spectrometry, mAbs 8 (2016) 331–339, https://doi.org/10.1080/19420862.2015.1122150.
[16] B.L. Duivelshof, E. Deslignière, O. Hernandez-Alba, A. Ehkirch, H. Toftevall, J. Sjögren, S. Cianferani, A. Beck, D. Guillarme, V. D'Atri, 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. 92 (2020) 8170–8177, https://doi.org/10.1021/acs.analchem.0c00282.
[17] Y. Du, A. Walsh, R. Ehrick, W. Xu, K. May, H. Liu, Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies, mAbs 4 (2012) 578–585, https://doi.org/10.4161/mabs.21328.
[18] A. Goyon, M. Excoffier, M.C. Janin Bussat, B. Bobaly, S. Fekete, D. Guillarme, A. Beck, Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies, J. Chromatogr. B 1066 (2017) 119–128, https://doi.org/10.1016/j.jchromb.2017.09.033.
[19] A. Murisier, S. Fekete, D. Guillarme, V. D'Atri, The importance of being metal-free: the critical choice of column hardware for size exclusion chromatography coupled to high resolution mass spectrometry, Submitt. to Anal. Chim. Acta. (2021).
[20] S. Fekete, A. Beck, J.-L. Veuthey, D. Guillarme, Proof of concept to achieve infinite selectivity for the chromatographic separation of therapeutic proteins, Anal. Chem. 91 (2019) 12954–12961, https://doi.org/10.1021/acs.analchem.9b03005.
[21] L. Verscheure, M. Oosterlynck, A. Cerdobbel, P. Sandra, F. Lynen, K. Sandra, Middle-up characterization of monoclonal antibodies by online reduction liquid chromatography-mass spectrometry, J. Chromatogr. A 1637 (2021) 461808, https://doi.org/10.1016/j.chroma.2020.461808.
[22] C. Gstöttner, S. Nicolardi, M. Haberger, D. Reusch, M. Wuhrer, E. Domínguez-Vega, Intact and subunit-specific analysis of bispecific antibodies by sheathless CE-MS, Anal. Chim. Acta 1134 (2020) 18–27, https://doi.org/10.1016/j.aca.2020.07.069.