



HAL
open science

SEC-MS in denaturing conditions (dSEC-MS) for in-depth analysis of rebridged monoclonal antibody-based formats

Rania Benazza, Ilias Koutsopetras, Valentine Vaur, Guilhem Chaubet, Oscar Hernandez Alba, Sarah Cianferani

► To cite this version:

Rania Benazza, Ilias Koutsopetras, Valentine Vaur, Guilhem Chaubet, Oscar Hernandez Alba, et al.. SEC-MS in denaturing conditions (dSEC-MS) for in-depth analysis of rebridged monoclonal antibody-based formats. *Talanta*, 2024, 272, pp.125727. 10.1016/j.talanta.2024.125727 . hal-04547655

HAL Id: hal-04547655

<https://hal.science/hal-04547655>

Submitted on 15 Apr 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



SEC-MS in denaturing conditions (dSEC-MS) for in-depth analysis of rebridged monoclonal antibody-based formats

Rania Benazza^{a,b}, Ilias Koutsopetras^c, Valentine Vaur^c, Guilhem Chaubet^c, Oscar Hernandez-Alba^{a,b}, Sarah Cianféroni^{a,b,*}

^a Laboratoire de Spectrométrie de Masse BioOrganique, IPHC UMR 7178, Université de Strasbourg, CNRS, 67087 Strasbourg, France

^b Infrastructure Nationale de Protéomique ProFI—FR2048, 67087 Strasbourg, France

^c Bio-Functional Chemistry (UMR 7199), Institut du Médicament de Strasbourg, University of Strasbourg, 74 Route du Rhin, 67400 Illkirch-Graffenstaden, France

ARTICLE INFO

Handling Editor: Qun Fang

Keywords:

Mass spectrometry

Size-exclusion chromatography

ABSTRACT

Disulfide rebridging methods are emerging recently as new ways to specifically modify antibody-based entities and produce future conjugates. Briefly, the solvent-accessible disulfide bonds of antibodies or antigen-binding fragments (Fab) thereof are reduced under controlled conditions and further covalently attached with a rebridging agent allowing the incorporation of one payload per disulfide bond. There are many examples of successful rebridging cases providing homogeneous conjugates due to the use of symmetrical reagents, such as dibromomaleimides. However, partial rebridging due to the use of unsymmetrical ones, containing functional groups with different reactivity, usually leads to the development of heterogeneous species that cannot be identified by a simple sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) due to its lack of sensitivity, resolution and low mass accuracy. Mass spectrometry coupled to liquid chromatography (LC-MS) approaches have already been demonstrated as highly promising alternatives for the characterization of newly developed antibody-drug-conjugate (ADC) and monoclonal antibody (mAb)-based formats. We report here the in-depth characterization of covalently rebridged antibodies and Fab fragments in-development, using size-exclusion chromatography hyphenated to mass spectrometry in denaturing conditions (denaturing SEC-MS, dSEC-MS). DSEC-MS was used to monitor closely the rebridging reaction of a conjugated trastuzumab, in addition to conjugated Fab fragments, which allowed an unambiguous identification of the covalently rebridged products along with the unbound species. This all-in-one approach allowed a straightforward analysis of the studied samples with precise mass measurement; critical quality attributes (CQAs) assessment along with rebridging efficiency determination.

1. Introduction

Since the approval of the first antibody-drug conjugate (ADC), Mylotarg® (gemtuzumab ozogamicin), for the treatment of acute myeloid leukemia (AML) [1], ADCs have emerged as a very promising class of therapeutics for targeted cancer therapy. By August 2023, 12 ADCs have been approved by the Food and Drug Administration (FDA) and/or the European Medicine Agency (EMA), among which 6 target hematological indications and 7 target solid-tumours [2]. In addition, more than 140 ADCs are currently in clinical trials, among which 11 are in late-stage studies [2–7]. ADCs are constructed by conjugating highly cytotoxic payloads via a linker to a receptor-targeting monoclonal antibody (mAb) delivering the payload directly to tumor cells to reduce

systemic toxicity and increase the selectivity of the drug [8–10]. Each of these components plays an important role in determining the toxicity and the stability of the ADC [6]. Innovative chemical strategies have been developed to conjugate the linker-drug payload to the mAb, either through stochastic approaches, on lysine (Lys) or reduced cysteine (Cys) sites, or via site-selective strategies, using the introduction of engineered reactive cysteine residues, unnatural amino acids, glycol-conjugation or by disulfide rebridging [3,11–14]. The latter strategy remains an appealing method for the generation of highly homogeneous and stable site-selective ADCs with less cost and time of development. Rebridging strategies usually consist of a first interchain disulfide reduction step followed by a rebridging step through a linker bearing the cytotoxic payload [15] (Fig. 1a and b). Recently developed mAb rebridging

* Corresponding author. Laboratoire de Spectrométrie de Masse BioOrganique, IPHC UMR 7178, Université de Strasbourg, CNRS, 67087 Strasbourg, France.

E-mail address: sarah.cianferani@unistra.fr (S. Cianféroni).

<https://doi.org/10.1016/j.talanta.2024.125727>

Received 18 July 2023; Received in revised form 4 December 2023; Accepted 27 January 2024

Available online 29 January 2024

0039-9140/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

technologies include pyridazinedione reagents (PDs) [16], divinyl pyrimidines (DVPs) [17], isobutylene [18] and next-generation malimides (NGMs), with the latter being widely used in industry [13,14]. As all chemical modifications of ADCs can affect the final efficacy and safety of an ADC [19], powerful and robust analytical techniques are required for in-depth monitoring of development and optimization phases of this new class of drugs.

To monitor and characterize mAb-based formats, analytical methods have been developed concomitantly to new mAb formats developments [20–30]. Mass spectrometry (MS)-based methods, coupled to liquid chromatography (LC) or electrophoretic methods play a central role in the characterization of mAb formats. For instance, native LC-MS methods recently developed are now used as first line for routine characterization of many mAb formats, in addition or as alternative to more classical reversed phase LC-MS methods. In particular, size-exclusion chromatography coupled to mass spectrometry in native conditions (SEC-nMS) is developed to analyze mAbs and ADCs [31–33], and is now used in research and development (R&D) laboratories to monitor their critical quality attributes (CQAs) including the amount of unconjugated mAb (DO), the average drug-to-antibody ratio (avDAR), the drug load distribution (DLD) and the different mAb variants (charge-, size- or hydrophobic-variants mainly) [21]. Those analytical workflows serve not only for main product characterization but also for detection, identification and quantification of minor impurities that might lead to immunogenicity. While many mAb formats can be readily assessed using a combination of analytical methods, monitoring rebridging reaction is still difficult [12]. This can be explained by the plethora of by-products that might occur upon incomplete partial rebridging reactions (Fig. 1c). Commonly, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is used for routine analysis of rebridged mAbs as it can reveal the approximate size of the reaction products, either after or before disulfide reduction [34]. However, SDS-PAGE suffers from different limitations, such as low sensitivity, low resolution and low mass accuracy. In this context, LC-MS approaches seem highly promising alternatives for the characterization of newly developed rebridged mAbs and ADCs.

In this study, we investigated the advantages and limitations of classical reversed phase LC-MS (rpLC-MS) and SEC-nMS in order to propose a new hybrid LC-MS method to gain in-depth information on rebridged mAbs and their by-products. We propose here a method combining SEC performed in denaturing conditions (dSEC) with MS detection (dSEC-MS). Based on our understanding, dSEC has only been

utilized for online desalting of proteins [35] and mAbs [36,37] in classical denaturing conditions followed by MS detection to achieve precise mass measurement. However, our method is specifically adapted for the characterization of rebridged mAbs and Fab fragments to afford in one unique analysis assessment of the purity, the homogeneity and the proper stoichiometry of the main products, coupled with the identification of the side-products.

2. Experimental

2.1. Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (France): acetonitrile (ACN), ammonium acetate (AcONH₄), boric acid (H₃BO₃), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), formic acid (FA), tris(2-carboxyethyl)phosphine (TCEP) and trifluoroacetic acid (TFA). Immobilized pepsin (Agarose Resin) and Immobilized papain (Agarose Resin) were obtained from Thermo Fischer Scientific (France). EndoS enzyme was obtained from New England Biolabs (Ipswich, MA, USA). All aqueous solutions were prepared with ultra-pure water system (Sartorius, Göttingen, Germany). LockMass and RDa calibrant solutions were obtained from Waters (Manchester, UK). Trastuzumab (Trazimera) was purchased from Pfizer (Germany). Ethynylbenziodazolones (EBZ) reagent was synthesized at Prof. Waser's group (Laboratory of Catalysis and Organic Synthesis, Ecole Polytechnique Fédérale de Lausanne, EPFL, Lausanne, Switzerland).

2.2. Preparation of intact rebridged mAb and rebridged Fab fragments

Fab#A rebridging– 80 µg of Fab-trastuzumab in BBS was treated with 5 equivalents TCEP (in 15 mM H₂O solution) and was reacted with 5 equivalents EBZ (in 10 mM DMSO solution).

Fab#B rebridging– 80 µg Fab-trastuzumab in BBS was treated with 5 equivalents TCEP (in 15 mM H₂O solution) and was reacted with 5 equivalents EBZ (in 10 mM ACN solution).

Trastuzumab rebridging– 120 µg of trastuzumab solubilized in BBS was reacted with 10 equivalents TCEP (in 15 mM H₂O solution) and was reacted with 10 equivalents EBZ (in 10 mM DMSO).

The Fab fragments and intact mAb rebridging was performed in a one-pot reaction process. The samples were incubated at 37 °C under agitation (650 rpm) during 5 h for Fab#A and during 6 h for rebridged

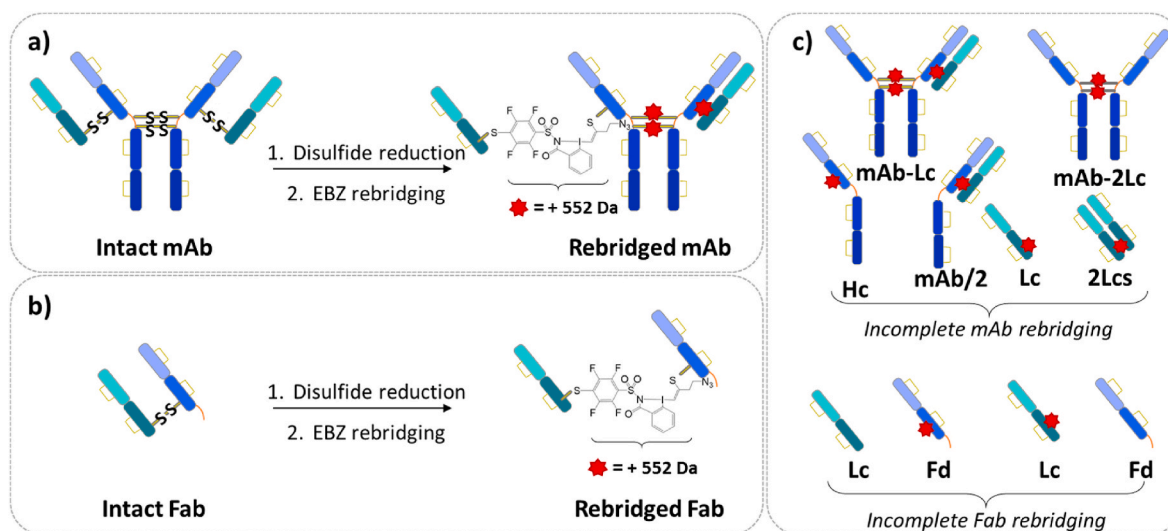


Fig. 1. (a) Schematic representation of rebridged mAb formation following disulfide bonds reduction. (b) Schematic representation of Fab rebridging. (c) Schematic representation of incomplete rebridging of reduced mAb and Fab fragment leading to the development of heterogeneous products. Disulfide bonds are depicted in yellow lines. The payload is depicted as a red star.

trastuzumab and for Fab#B. The excess of reagent was removed by gel filtration chromatography on Zeba™ Spin Desalting Columns (Thermo Fisher Scientific, Pierce Biotechnology, USA), 7K MWCO. The final solutions had a yield of >95 % (1.5 mg/mL, 50 µL for Fab#A and Fab#B and 2.4 mg/mL, 50 µL for rebridged trastuzumab). BBS buffer contained 25 mM H₃BO₃ and 2 mM EDTA at pH 8.0.

2.3. Mass spectrometry analyses

All analyses were performed on a BioAccord LC-MS system (Waters, Manchester, UK). It comprises an Acquity UPLC M-Class system; including a binary solvent manager, a sample manager at 4 °C, a column oven at room temperature for SEC separation and at 80 °C for rpLC and an UV detector operating at 214 nm and 280 nm, coupled to an RDa detector. The mass spectrometer was calibrated in the 400–7000 *m/z* range in the positive mode using a solution containing; 50 ng/µL of sodium iodide in isopropanol/water (80/20 *v/v*) and 0.5 ng/µL of rubidium iodide in isopropanol/water (80/20 *v/v*). A LockMass solution containing 3.75 ng/µL of leucine enkephalin, 12.5 ng/µL of caffeine and 2.5 ng/µL of 1-pentanesulfonic acid in ACN/water (80/20 *v/v*) was injected automatically prior to each analysis.

SEC-nMS – Between 5 to 10 µg were injected on a MaxPeak Premier Protein SEC 250 Å, 1.7 µm, 4.6 × 150 mm (Waters, Manchester, UK) used with an isocratic gradient of 150 mM AcONH₄ (pH 6.9) at a flowrate of 250 µL/min over 6 min. The mass spectrometer was operated with a capillary voltage of 3.5 kV and a backing pressure of 2 mbar. The cone voltage was set to 80 V. Acquisitions were performed on the *m/z* range 400–7000 with a 1 s scan time.

rpLC-MS – Less than 1 µg was injected on a Bioresolve RP mAb polyphenyl (450 Å, 2.7 µm 2.1 × 50 mm column, Waters) at a flowrate of 300 µL/min at 80 °C. Mobile phases consisted of 0.1 % FA in H₂O (mobile phase A) and 0.1% FA in ACN (mobile phase B). The separation was carried out using a gradient from 5 to 95% of mobile phase B in 25 min. The column was washed with 95% mobile phase B for 1 min and then was equilibrated with 5% mobile phase B for 3 min. The BioAccord was operated in the positive mode with a capillary voltage of 1.5 kV. Desolvation temperature was set to 330 °C, the cone voltage to 60 V and the source pressure was fixed at 2 mbar. Acquisitions were performed on the 400–7000 *m/z* range with a 1 s scan time.

dSEC-MS – SEC analysis was performed using the AdvanceBioSEC3 (4.6 × 150 mm, 2.5 µm, 300 Å, Agilent) or the Maxpeak Protein SEC (4.6 mm × 150 mm, 1.7 µm, 250 Å, Waters) column kept at room temperature. The separation was carried out using an isocratic gradient of mobile phase (20% ACN + 0.1% FA + 0.1% TFA) at a flowrate of 0.1 mL/min for 15 min. For dSEC-MS, 1–3 µg of protein samples were injected. The BioAccord was operated in the same conditions than for rpLC-MS. Full scan acquisition was performed on the high mass range (400–7000 *m/z*) with a 1 s scan time.

Data processing – All MS data interpretations were performed using UNIFI v1.913.9 (Waters, Manchester, UK) and MassLynx V4.1 (Waters, Manchester, UK). The avDAR values were calculated based on the relative peak intensities measured from the raw mass spectra (four charge states) using the equation below:

$$avDAR = \frac{\sum_{k=0}^n k \times I_k}{\sum_{k=0}^n I_k}$$

Where *k* is the number of drugs and *I_k* is the relative peak intensity of DAR_{*k*}.

To relatively quantify the amount of by-products we used integration of the peak areas from UV chromatograms, using the following equation:

$$Amount\ of\ by\ -\ products\ (\%) = \frac{\sum Peak\ area_{free\ species}}{\sum Peak\ area_{all\ species}} \times 100$$

Where the free species correspond to the heavy chain (Hc), the light chain (Lc), half mAb species in case of mAb rebridging, or Lc and Fd in

case of Fab rebridging.

3. Results and discussion

3.1. SEC-nMS analysis of rebridged mAb-based formats

The Fab/mAb underwent a one-pot reaction, involving first a reduction under mild controlled conditions, followed by subsequent rebridging allowing the incorporation of one or more hypervalent iodine (EBZ) molecules as payloads. These molecules were covalently inserted between the heavy chain (Hc) and the light chain (Lc) regions of the Fab/mAb [38], leading to a mass increase of +552 Da per rebridged drug incorporation (Fig. 1a and b). In the case of the Fab domains, two different reaction conditions selected from an exhaustive screen [38] were used to obtain different avDAR values and reaction yields to challenge the different LC-MS methods.

As multiple examples have demonstrated the advantages of using nMS individually or hyphenated to SEC for the analysis of multiple therapeutic mAb-based protein formats [21,28,31,33,39,40], the rebridged Fab/mAb compounds were first analyzed with SEC-nMS (Fig. 2). Similar chromatographic profiles are observed for unreacted and rebridged Fab samples. Peak 1 was identified as a dimeric aggregate (~90 kDa) and the most intense peak (peak 2) was attributed to the monomeric forms of Fab with D1 and D0 species being detected for both Fab#A and Fab#B. Although the D1 species is the most intense population (48 192 ± 1 Da and 48 188 ± 1 Da for Fab#A and Fab#B, respectively) in both cases, D1/D0 relative intensities are significantly different, leading to different avDAR values for Fab#A (0.8 ± 0.0) and Fab#B, (0.5 ± 0.0), thus corroborating that the different reaction conditions do not lead to the same degree of conjugation. Of note, an additional minor species is observed, corresponding to a truncated form of the Fab domain (-KTH residues in the C-terminal side of the Fd domain) due to trastuzumab papain over-digestion (peaks labelled * in Fig. 2b, 47 271 ± 1 Da, 47 274 ± 1 Da and 47 275 ± 1 Da for unreacted Fab, Fab#A and Fab#B, respectively). Considering the relative intensities of D1/D0, a rebridging yield of 79 ± 1 % and 55 ± 5 % was calculated for Fab#A and Fab#B, respectively.

Similarly to rebridged Fab, SEC-UV chromatogram of rebridged mAb (Fig. 2c) revealed two peaks where peak 1 was attributed to the dimer (~290 kDa), while the most intense one (peak 2) was identified as the monomer. The chromatographic profile of the rebridged trastuzumab (theoretical targeted avDAR 4.0) is rather similar to that of the unreacted trastuzumab with a slightly higher retention time and peak width (FWHM = 0.1 min for unreacted mAb and FWHM = 0.2 min for rebridged mAb). These effects have already been described in the literature when comparing the SEC profile of an ADC and its unconjugated mAb counterpart [22], suggesting that the hydrophobicity of the cargo molecules enhances the non-specific interactions between the ADC and the stationary phase of the column, thus leading to greater retention times and broader chromatographic peaks.

While one unique species with a mass of 145 864 ± 2 Da was detected for unmodified trastuzumab, at least four different populations could be identified in the SEC-UV main peak of the rebridged mAb, corresponding to D1, D2, D3 and D4 (Table S1) species and an avDAR value of 2.5 ± 0.1 (instead of 4.0 expected). The nMS data thus pinpoint an incomplete/partial rebridging reaction, with the most intense reaction product being D2.

Although SEC-nMS coupling offers a fast, robust, and straightforward analysis of rebridged mAb-derived formats providing useful information like the accurate mass measurement of all species, the DLD, and the avDAR, some inconsistencies were also revealed. Indeed, for both Fab and mAb samples, nMS highlighted partial/incomplete rebridging, but no experimental evidence of unreacted species (free Lc and Fd parts for Fab or free Lc and Hc for mAb) were detected neither at the chromatographic nor at the MS levels. One possible explanation might be that residual non-rebridged chains of the Fab and the intact mAb remain

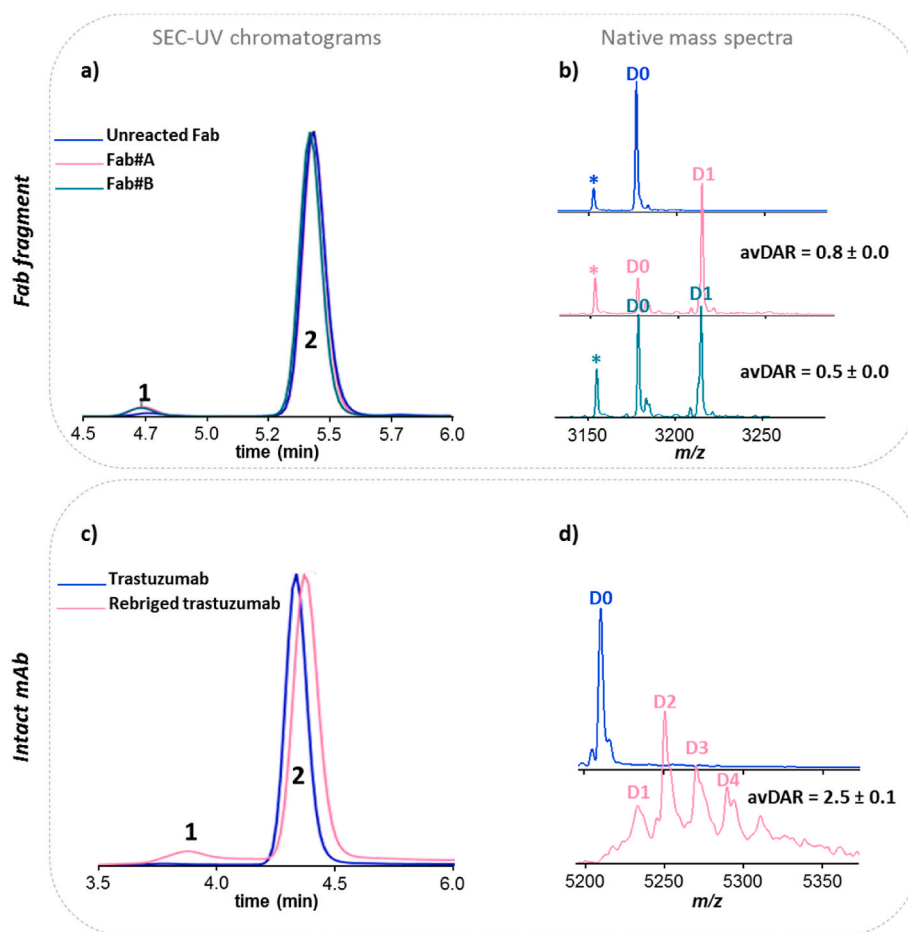


Fig. 2. Online SEC-nMS analysis of rebridged Fab/mAb samples. (a) Overlay of SEC-UV signals of Fab samples: 1 = dimers, 2 = main products, and (b) corresponding native mass spectra (zoom on 15+) of reference untreated Fab (blue), Fab#A (pink) and Fab#B (green). (c) Overlay of SEC-UV signals of intact mAb samples: 1 = dimers, 2 = main products and (d) corresponding native mass spectra (zoom on 28+) of reference trastuzumab (blue) and rebridged (pink) trastuzumab. The masses of the different species are summarized in Table S1.

maintained, under native conditions, due to the preservation of non-covalent interactions.

3.2. rpLC-MS analysis of rebridged mAb-based formats

To further test our hypothesis of coexistence of covalent (expected) and non-covalent (unexpected) rebridged assemblies, we next performed rpLC-MS analysis in denaturing conditions, the most routine LC-MS method that relies on separation of protein populations as a function of their apparent hydrophobicities. Since the combination of acidic pH and chaotropic agents used in rpLC induces the denaturation of the proteins, the non-covalent interactions between the different chains will be disrupted. The chromatographic profiles of the different unreacted Fab and mAb samples along with their rebridged counterparts are depicted in Fig. 3, while masses of the observed species are summarized in Table S2.

For unreacted Fab, Fig. 3a exhibits two peaks at 12.71 min and 13.20 min. The first most intense peak (72%, $47\,638.9 \pm 0.4$ Da) corresponds to the expected Fab fragment consisting of Lc and Fd chains held together by one disulfide bond (D0). The second peak (28%, $47\,272.5 \pm 0.6$ Da) corresponds to over-digested Fab already observed in SEC-nMS. For rebridged Fab#A and Fab#B (Fig. 3b and c), three different regions could be identified on the LC chromatograms: the Lc region (before the main peak); the Fab region (main peak) and the Fd region, the latest one partially co-eluting with Fab species. The main chromatographic peak (peak 3, ~ 12.87 min) corresponds to the D0 and D1 Fab covalent populations (Fig. 3). Of note, 2 Da mass difference is systematically

observed between theoretical and experimental masses of Fab domains (even for the unreacted Fab). Thereby, the mass difference along with the fact that the Lc, and the Fd subunits on the Fab are covalently linked, leads to the conclusion that Fab production induces the reduction of one intra-chain SS bond. The relative MS intensities led to avDAR values of 0.8 ± 0.0 , and 0.6 ± 0.0 for Fab#A and Fab#B, respectively, in agreement with SEC-nMS results. In the Lc region, two populations are detected corresponding to unmodified Lc (10.77 min) and Lc+457 Da (11.57 min). In the Fd region (13.20 min), two populations are co-eluting corresponding to Fd+93 Da and Fd+457 Da, species that are considered as “partially rebridged” moieties as they are attached to only parts of the drug-payload (Fig. S1a). The formation of these +93 Da (alkynyl adduct) and +457 Da (sulfonamide) arises most likely from the fragmentation of the EBZ reagent upon thiolate addition through an elimination reaction (Fig. S1b) [38]. Of note, unmodified Lc might originate from unreacted EBZ.

Despite optimizations of the chromatographic method, no better separation of Fab and Fd peaks ($R_s = 0.95$) could be obtained due to similar hydrophobicities of Fab and Fd fragments. The observation of free Lc and Fd subunits only in the rebridged samples (and not in the control unconjugated Fab) undoubtedly confirms that the rebridging reaction is not complete, corroborating our hypothesis that species detected in SEC-nMS consist of a mixture of covalently rebridged Fab and non-covalently maintained Fab. Thereby, the intensities of the unreacted species might serve as an indirect method to relatively quantify the rebridging efficiency. Given that the by-products account for $22 \pm 1\%$ (Fab#A) and $41 \pm 1\%$ (Fab#B) of the overall signal, it

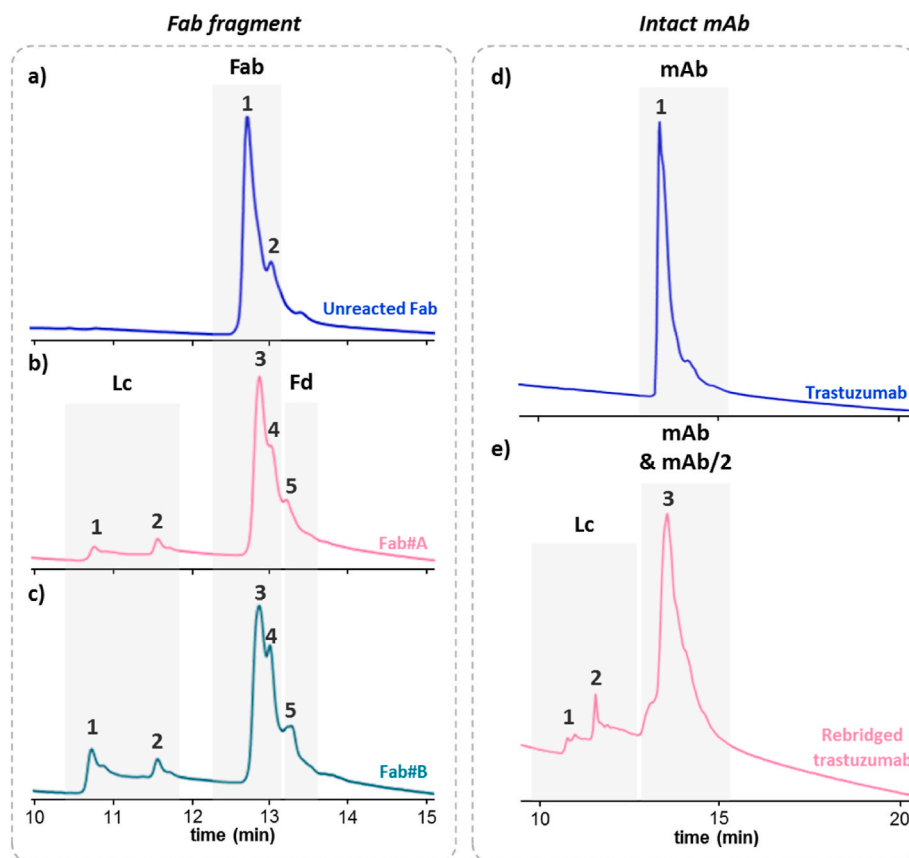


Fig. 3. UV-chromatograms obtained by rpLC-MS analysis of (a) unreacted Fab, (b) Fab#A and (c) Fab#B. UV signal of (d) trastuzumab and (e) rebridged trastuzumab. Mass measurements of the different species are summarized in Table S2.

could be inferred that the rebridging was more effective for Fab#A.

Similar rpLC analysis was performed on the rebridged intact mAb. While one unique chromatographic peak (13.30 min, $145\,864.8 \pm 0.6$ Da) is observed for intact deglycosylated trastuzumab (Fig. 3d), the chromatogram of the rebridged mAb exhibits additional signals in the 10–12 min range along with a broader main peak (FWHM = 0.6 min) at 13.30 min (Fig. 3e). Peak 1 (at 10.75 min) could be assigned to the unconjugated Lc (2%, $23\,439.9 \pm 0.1$ Da); peak 2 to Lc+457 Da corresponding to “partially rebridged Lc” (13%, $23\,896.9 \pm 0.1$ Da) overlapped with dimeric Lc species mainly 2 Lc D0 and 2 Lcs D1 ($48\,877.9 \pm 0.1$ Da and $47\,430.2 \pm 0.4$ Da, respectively). Peak 3 exhibits several co-existing populations, comprising different forms of species of mainly mAb/2 species (D1 and D2) along with intact mAb products (D0, D1, D2, D3, and D4). The different conjugated species detected at the intact trastuzumab level led to an avDAR value of 2.4 ± 0.1 , which is in line with the results obtained using SEC-nMS. In addition, MS signals corresponding to the release of one (123 kDa, mAb-Lc) and 2 Lcs (99 kDa, mAb-2Lc) were also observed within the broad chromatographic peak (Table S2).

Altogether, our results highlight that rpLC-MS is far more informative than SEC-nMS for the characterization of both main and side products resulting from a partial rebridging reaction. Intact Fab/mAb signals allow determination of the DLD and the avDAR. In addition, free Lc (unmodified and bearing one linker-payload molecule) along with mAb/2 species detection support incomplete rebridging reaction hypothesis, with several side-products identified. However, the high number and the co-elution of some rpLC-MS detected entities hampers an easy estimation of the rebridging reaction yield/efficiency. As ionization efficiencies of co-eluting half and intact mAbs are significantly different, the accurate calculation of the ratio between covalently and non-covalently rebridged species is also difficult.

3.3. Hybrid dSEC-MS for rebridged mAb-based formats characterization

In order to improve available LC-MS methods and with the idea to develop a method that provides a maximum of information in a straightforward manner, we next aimed at performing SEC in denaturing conditions followed by classical denaturing MS analysis (dSEC-MS). We took advantage of the benefits of SEC-nMS and rpLC-MS for rebridged mAb analysis, namely SEC capabilities of size variants separation along with denaturing conditions necessary to distinguish between covalent and non-covalent rebridged assemblies. Chumsae *et al.* (2009) have reported the use of SEC-MS in denaturing conditions for the analysis of reduced mAbs, yielding to subunits (Hc and Lc) separation within ~80 min [41]. We have thus adapted the previously described protocol (run duration, gradient and column choice) with the aim of reducing the analysis time while keeping the LC separation by using cutting-edge sub-3 μ m SEC columns. Separation capabilities of two different SEC columns (AdvanceBioSEC3 300 Å $2.7\ \mu\text{m}$, 4.6×150 mm column, Agilent and Maxpeak Protein SEC 250 Å $1.7\ \mu\text{m}$, 4.6×150 mm column, Waters) were tested on Fab#A sample along with the optimization of the chromatographic conditions (Fig. S2). In both cases, two main peaks are detected. The use of the AdvanceBioSEC3 column provided a clear separation ($R_s = 0.73$) of the covalently rebridged Fab populations (~50 kDa, D0 and D1), while Lc and Fd subunits (~25 kDa) co-elute in the second peak. Conversely, the Maxpeak Protein SEC column exhibits enhanced baseline separation ($R_s = 5.80$) with lower retention times, more symmetrical chromatographic peak shapes ($A_s = 1.74$ for the major peak) and less peak tailing, which consequently, leads to partial separation of Lc and Fd fragments. Altogether, these results clearly demonstrate that secondary interactions between the analytes and the stationary phase of the Maxpeak Protein SEC column are drastically reduced. This is due to the particle technology of the Maxpeak Protein

SEC column that is built following the Ethylene Bridged Hybrid (BEH) technology with an additional coverage of hydroxyl-terminated polyethylene oxide (PEO) that acts as a barrier surface to eliminate nonspecific interactions. Thus, the inertness of the Maxpeak Protein SEC column highlights the suitability of the latter column to separate populations that can be generated during the rebridging process.

For the reference Fab sample, one sharp peak (FWHM = 0.19 min) is observed at 10.88 min on the dSEC-UV chromatogram corresponding to the mass of the Fab fragment ($47\,638.9 \pm 0.1$ Da) co-eluting with Fab-KTH species ($47\,273.3 \pm 0.3$ Da). Interestingly, for Fab#A and Fab#B (Fig. 4a), three different peaks were detected according to dSEC-UV signal (versus only one peak observed in SEC-nMS, Fig. 2a). The major peak for both samples (10.88 min) corresponds to co-elution of covalently rebridged Fab D1 species ($48\,190.8 \pm 0.3$ Da for both, Fab#A and Fab#B) and D0 species ($47\,638.6 \pm 0.3$ Da and $47\,638.8 \pm 0.3$ Da for Fab#A and Fab#B, respectively). Fab-KTH species were also observed in this region ($47\,272.0 \pm 0.4$ and $47\,272.0 \pm 0.6$ Da for Fab#A and Fab#B, respectively). The dSEC separation shows two partially resolved ($R_s = 5.8$ and $R_s = 5.5$ for Fab#A and Fab#B, respectively) additional peaks for both samples: peak 2 (~13.08 min) and peak 3 (~13.37 min) corresponding to Fd and Lc species, respectively. The deconvoluted mass spectra for peak 2 pinpoints the co-elution of both unmodified Lc ($23\,439.5 \pm 0.2$ Da and $23\,439.6 \pm 0.2$ Da for Fab#A and Fab#B, respectively) and Fd+93 Da fragment ($24\,292.3 \pm 0.5$ Da and $24\,292.6 \pm 0.5$ Da for Fab#A and Fab#B, respectively) species. The peak 3 shows the presence of partially rebridged species namely: Lc+457 Da ($23\,896.6 \pm 0.4$ Da and $23\,896.7 \pm 0.4$ Da for Fab#A and Fab#B, respectively) and Fd+457 Da ($24\,657.6 \pm 0.3$ Da and $24\,657.7 \pm 0.5$ Da for Fab#A and

Fab#B, respectively). The experimental masses and the relative intensities corresponding to the Fab conjugates are overall in line with the masses reported with SEC-nMS, leading to a very similar avDAR value (0.8 ± 0.0 and 0.6 ± 0.1 for Fab#A and Fab#B, respectively). Similarly to SEC-nMS, relative intensities of chromatographic peaks of intact Fab versus non-rebridged fragments (such as Fd and Lc) could serve to easily approximate the ratio of covalent versus non-covalent rebridged Fab. Therefore, upon integration of the chromatographic peak area of the fragments signals, the relative amount of by-product species represents $11 \pm 0\%$ and $25 \pm 2\%$ of the total signal for Fab#A and Fab#B samples, respectively. However, as it can reasonably be assumed that Fab D0 and D1 species have similar ionization efficiencies, D1 species represent $75 \pm 3\%$ and $62 \pm 2\%$ of the Fab main peak area, respectively.

Performances and benefits of the dSEC-MS method were even more obvious for intact rebridged trastuzumab analysis (Fig. 4e). While one unique peak is observed for unreacted trastuzumab, dSEC-UV shows 6 peaks corresponding to rebridging products of different sizes: intact mAb species (~150 kDa) which are baseline resolved from mAb/2 species (~75 kDa), Hc (~50 kDa) and Lc (~25 kDa) species, which significantly improves the ease of data interpretation. The most intense peak was assigned mainly to different intact rebridged trastuzumab species (from D1 to D4) with an avDAR of 2.5 ± 0.1 , similar to the value calculated with the previous LC-MS methods. Of note, the chromatographic peak of rebridged trastuzumab is slightly larger compared to the reference trastuzumab (FWHM = 0.35 min versus 0.23 min for reference mAb). In this case, the peak broadening is caused by the co-elution of structures where either one (~123 kDa, mAb D1 lacking the Lc+457 Da fragment) or two Lcs (~99 kDa, mAb D1 lacking 2 Lcs) have been

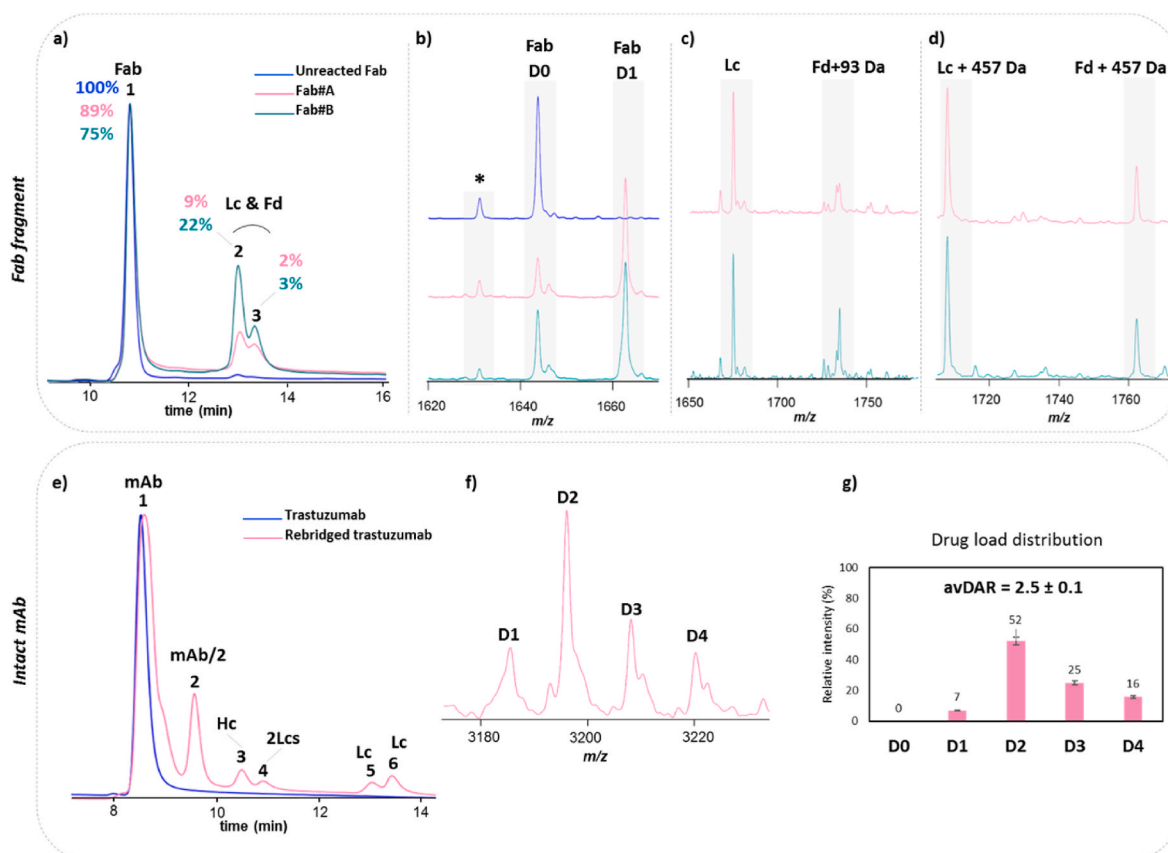


Fig. 4. dSEC-MS analysis of reference Fab (blue), Fab#A (pink) and Fab#B (green). (a) Overlay of the SEC-UV signals of the three samples. The right side of the figure represents the corresponding mass spectra of each sample with (b) the zoom on the charge state 30+ for the Fab peak (~10.8 min), (c) zoom on the 14+ for the second peak (~12.9 min) and (d) the zoom on 14+ for the last peak (~13.3 min). (e) SEC-UV profiles in denaturing conditions of naked trastuzumab (blue) and rebridged trastuzumab (pink). Peaks from 1 to 6 represent the different observed species with their corresponding masses detected further in the MS and summarized in Table S3. (f) Mass spectrum of the intact rebridged mAb species zoomed on the 48+ and (g) represents the drug load distribution of the intact rebridged mAb.

released as a result of an incomplete rebridging reaction. This is consistent with the detection of free Lc species (11–14 min). However, in our dSEC-MS conditions, the peak representing the intact trastuzumab species is noticeably less complex to its counterpart in rpLC-MS analysis. This is due to non-overlapping mAb/2 nor free Hc with intact mAb species (Figs. S3 and S4).

For relative quantification of covalently rebridged species, extracted ion chromatograms (XIC) of expected rebridged mass species in peak 1 (Fig. 4e, m/z 3128.10) allowed us to determine the amount of the completely rebridged species (16% of D4 species) versus partially rebridged ones (Fig. S5). Moreover, our study revealed the presence of other conjugates on free subunits. Particularly, the mAb/2 (bearing one or two payloads $73\,486.5 \pm 0.7$ Da and $74\,038.7 \pm 0.5$ Da) along with the two rebridged Lc subunits ($47\,429.1 \pm 1.0$ Da) as already observed in rpLC-MS (Fig. 4c). More species were observed bearing increment masses corresponding to incomplete conjugation, mainly for the Hc ($50\,140.9 \pm 1.1$ Da) and Lc ($23\,896.4 \pm 0.3$ Da) (see Table S3).

Although this sample is very challenging due to its high heterogeneity, our method could clearly assign the avDAR along with the DLD of the rebridged intact trastuzumab. More importantly, we could relatively quantify the covalent rebridging efficiency even if it is tricky to separate the mAb, the mAb-2Lc and the mAb-Lc, leading to monitor the reaction of intact trastuzumab rebridging.

4. Conclusions

We have evaluated the advantages and limitations of two LC-MS methods broadly used for mAb and ADC characterization, namely SEC-nMS and rpLC-MS, for the characterization of conjugated Fab fragments (Fab-drug conjugates, FDCs) and ADCs resulting from a rebridging reaction. While native MS has been reported as a very important method for several ADC-formats characterization (Lys, Cys) [42,43], we highlight that it is not optimal for a comprehensive in-depth characterization of FDC/ADC rebridging products. Despite the straightforward determination of avDAR and DLD, native MS failed in distinguishing expected covalently rebridged species from non-covalently rebridged assemblies resulting from an incomplete rebridging reaction. To do so, harsh denaturing conditions used in rpLC-MS are preferred and afforded differentiation between fully rebridged mAb/Fab fragment and rebridging by-products (mAb/2, Lc, Hc, Fd). However, one main limitation of rpLC-MS relies in co-elution of species with similar hydrophobicities, such as intact mAb or mAb/2 that exhibit very different ionization efficiencies in MS analysis. Size-variant separation capabilities offered by SEC thus seemed to be of utmost importance to separate intact mAb species (~150 kDa) from mAb/2 species (~75 kDa), Lc and Fd subunits (~25 kDa) and all by-products resulting from incomplete Fab or mAb rebridging. By synergizing the potentialities of SEC for size variant separation and denaturing conditions in the chromatographic dimension to disrupt non-covalently maintained rebridged species, we have developed a straightforward hybrid dSEC-MS method for size-variant separation followed by precise mass measurement in denaturing conditions. This method provides invaluable rapid assessment of the main CQAs requested for ADCs (avDAR, unreacted D0, and DLD) along with thorough identification of all reaction by-products through accurate mass measurements. Thus, allowing a more comprehensive understanding about the chemical mechanism of the bioconjugation process. Ultimately, the capability of using cutting-edge inert sub- $3\mu\text{m}$ columns with an adaptable benchtop LC-MS system enables our method to be adopted by biopharmaceutical R&D laboratories for routine analytical characterization of newly developed ADCs resulting from rebridging reactions.

CRedit authorship contribution statement

Rania Benazza: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Ilias Koutsopetras:**

Writing – review & editing, Methodology, Formal analysis. **Valentine Vaur:** Writing – review & editing, Methodology, Formal analysis. **Guilhem Chaubet:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Oscar Hernandez-Alba:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Sarah Cianféroni:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the CNRS, the University of Strasbourg, the “Agence Nationale de la Recherche” (BioconjUgi; ANR-19-CE07-0045 and Conformabs; ANR-21-CE29-0009-01), the French Proteomic Infrastructure (ProFI; ANR-10-INBS-08-03) and the Interdisciplinary Thematic Institute IMS (Institut du Médicament Strasbourg), as part of the ITI 2021–2028 supported by IdEx Unistra (ANR-10-IDEX-0002), SFRI-STRAT’US project (ANR-20-SFRI-0012). R. B. and I. K. acknowledge the European Union’s Horizon 2020 Research and Innovation Program Marie Skłodowska-Curie ITN for funding their PhDs under Grant Agreement No. 859458. V. V. acknowledges the “Agence Nationale de la Recherche” for funding her PhD (BioconjUgi; ANR-19-CE07-0045). The authors would also like to thank Prof. Jérôme Waser for providing them with the rebridging reagents to study the reaction.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2024.125727>.

References

- [1] K.J. Norsworthy, C.W. Ko, J.E. Lee, J. Liu, C.S. John, D. Przepiora, A.T. Farrell, R. Pazdur, FDA approval summary: Mylotarg for treatment of patients with relapsed or refractory CD33-positive acute myeloid leukemia, *Oncol.* 23 (2018) 1103–1108.
- [2] C. Dumontet, J.M. Reichert, P.D. Senter, J.M. Lambert, A. Beck, Antibody-drug conjugates come of age in oncology, *Nat. Rev. Drug Discov.* 22 (2023) 641–661.
- [3] Z. Fu, S. Li, S. Han, C. Shi, Y. Zhang, Antibody drug conjugate: the “biological missile” for targeted cancer therapy, *Signal Transduct. Targeted Ther.* 7 (2022) 93.
- [4] H. Kaplon, A. Chenoweth, S. Crescioli, J.M. Reichert, *Antibodies to watch in 2022*, *mAbs* 14 (2022) 2014296.
- [5] P.J. Carter, G.A. Lazar, Next generation antibody drugs: pursuit of the ‘high-hanging fruit’, *Nat. Rev. Drug Discov.* 17 (2018) 197–223.
- [6] P. Tarantino, B. Ricciuti, S.M. Pradhan, S.M. Tolane, Optimizing the safety of antibody-drug conjugates for patients with solid tumours, *Nat. Rev. Clin. Oncol.* 20 (2023) 558–576.
- [7] H. Kaplon, S. Crescioli, A. Chenoweth, J. Visweswaraiah, J.M. Reichert, *Antibodies to watch in 2023*, *mAbs* 15 (2023) 2153410.
- [8] N. Jain, S.W. Smith, S. Ghone, B. Tomczuk, Current ADC linker chemistry, *Pharm. Res. (N. Y.)* 32 (2015) 3526–3540.
- [9] V. Kostova, P. Desos, J.B. Starck, A. Kotschy, The chemistry behind ADCs, *Pharmaceuticals* 14 (2021) 442–449.
- [10] T. Rady, L. Turelli, M. Nothisen, E. Tobaldi, S. Erb, F. Thoreau, O. Hernandez-Alba, S. Cianferani, F. Daubeuf, A. Wagner, G. Chaubet, A novel family of acid-cleavable linker based on cyclic acetal motifs for the production of antibody-drug conjugates with high potency and selectivity, *Bioconjugate Chem.* 33 (2022) 1860–1866.
- [11] A.N. Marquard, J.C.T. Carlson, R. Weissleder, Expanding the scope of antibody rebridging with new pyridazinedione-TCO constructs, *Bioconjugate Chem.* 31 (2020) 1616–1623.

- [12] B.T. Ruddle, R. Fleming, H. Wu, C. Gao, N. Dimasi, Characterization of disulfide bond rebridged fab-drug conjugates prepared using a dual maleimide pyrrolbenzodiazepine cytotoxic payload, *ChemMedChem* 14 (2019) 1185–1195.
- [13] J.P. Nunes, M. Morais, V. Vassileva, E. Robinson, V.S. Rajkumar, M.E. Smith, R. B. Pedley, S. Caddick, J.R. Baker, V. Chudasama, Functional native disulfide bridging enables delivery of a potent, stable and targeted antibody-drug conjugate (ADC), *Chem. Commun.* 51 (2015) 10624–10627.
- [14] F.F. Schumacher, J.P. Nunes, A. Maruani, V. Chudasama, M.E. Smith, K.A. Chester, J.R. Baker, S. Caddick, Next generation maleimides enable the controlled assembly of antibody-drug conjugates via native disulfide bond bridging, *Org. Biomol. Chem.* 12 (2014) 7261–7269.
- [15] E.M.D. Allouche, E. Grinhagena, J. Waser, Hypervalent iodine-mediated late-stage peptide and protein functionalization, *Angew. Chem., Int. Ed. Engl.* 61 (2022) e202112287.
- [16] C. Bahou, V. Chudasama, The use of bromopyridazinedione derivatives in chemical biology, *Org. Biomol. Chem.* 20 (2022) 5879–5890.
- [17] S.J. Walsh, S. Omarjee, W. Galloway, T.T. Kwan, H.F. Sore, J.S. Parker, M. Hyvonen, J.S. Carroll, D.R. Spring, A general approach for the site-selective modification of native proteins, enabling the generation of stable and functional antibody-drug conjugates, *Chem. Sci.* 10 (2019) 694–700.
- [18] S. Sun, P. Akkapeddi, M.C. Marques, N. Martinez-Saez, V.M. Torres, C. Cordeiro, O. Boutoureira, G.J.L. Bernardes, One-pot stapling of interchain disulfides of antibodies using an isobutylene motif, *Org. Biomol. Chem.* 17 (2019) 2005–2012.
- [19] C.H. Chau, P.S. Steeg, W.D. Figg, Antibody-drug conjugates for cancer, *Lancet* 394 (2019) 793–804.
- [20] A. Beck, E. Wagner-Rousset, D. Ayoub, A. Van Dorsselaer, S. Sanglier-Cianferani, Characterization of therapeutic antibodies and related products, *Anal. Chem.* 85 (2013) 715–736.
- [21] E. Desligniere, H. Diemer, S. Erb, P. Coliat, X. Pivot, A. Detappe, O. Hernandez-Alba, S. Cianferani, A combination of native LC-MS approaches for the comprehensive characterization of the antibody-drug conjugate trastuzumab deruxtecan, *Front. Biosci.* 27 (2022) 290.
- [22] E. Desligniere, A. Ehkirch, B.L. Duivelshof, H. Toftevall, J. Sjogren, D. Guillaume, V. D'Atri, A. Beck, O. Hernandez-Alba, S. Cianferani, State-of-the-Art native mass spectrometry and ion mobility methods to monitor homogeneous site-specific antibody-drug conjugates Synthesis, *Pharmaceuticals* 14 (2021).
- [23] F. Fussl, L. Strasser, S. Carillo, J. Bones, Native LC-MS for capturing quality attributes of biopharmaceuticals on the intact protein level, *Curr. Opin. Biotechnol.* 71 (2021) 32–40.
- [24] F. Ma, F. Raoufi, M.A. Bailly, L. Fayadat-Dilman, D. Tomazela, Hyphenation of strong cation exchange chromatography to native mass spectrometry for high throughput online characterization of charge heterogeneity of therapeutic monoclonal antibodies, *mAbs* 12 (2020) 1763762.
- [25] R.L. Shi, G. Xiao, T.M. Dillon, M.S. Ricci, P.V. Bondarenko, Characterization of therapeutic proteins by cation exchange chromatography-mass spectrometry and top-down analysis, *mAbs* 12 (2020) 1739825.
- [26] E. Desligniere, A. Ehkirch, T. Botzanowski, A. Beck, O. Hernandez-Alba, S. Cianferani, Toward automation of collision-induced unfolding experiments through online size exclusion chromatography coupled to native mass spectrometry, *Anal. Chem.* 92 (2020) 12900–12908.
- [27] V. Le-Minh, F. Halgand, G. Van der Rest, M. Taverna, C. Smadja, Conformation assessment of therapeutic monoclonal antibodies by SEC-MS: unravelling analytical biases for application to quality control, *J. Pharm. Biomed. Anal.* 185 (2020) 113252.
- [28] A. Ehkirch, O. Hernandez-Alba, O. Colas, A. Beck, D. Guillaume, S. Cianferani, 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.
- [29] A. Ehkirch, A. Goyon, O. Hernandez-Alba, F. Rouviere, V. D'Atri, C. Dreyfus, J. F. Haeuw, H. Diemer, A. Beck, S. Heinisch, D. Guillaume, S. Cianferani, A novel online four-dimensional SECxSEC-IMxMS methodology for characterization of monoclonal antibody size variants, *Anal. Chem.* 90 (2018) 13929–13937.
- [30] F. Fussl, A. Trappe, K. Cook, K. Scheffler, O. Fitzgerald, J. Bones, Comprehensive characterisation of the heterogeneity of adalimumab via charge variant analysis hyphenated on-line to native high resolution Orbitrap mass spectrometry, *mAbs* 11 (2019) 116–128.
- [31] E. Desligniere, M. Ley, M. Bourguet, A. Ehkirch, T. Botzanowski, S. Erb, O. Hernandez-Alba, S. Cianferani, Pushing the limits of native MS: online SEC-native MS for structural biology applications, *Int. J. Mass Spectrom.* 461 (2021) 116502.
- [32] F. Debaene, A. Boeuf, E. Wagner-Rousset, O. Colas, D. Ayoub, N. Corvaia, A. Van Dorsselaer, A. Beck, S. Cianferani, Innovative native MS methodologies for antibody drug conjugate characterization: high resolution native MS and IM-MS for average DAR and DAR distribution assessment, *Anal. Chem.* 86 (2014) 10674–10683.
- [33] J. Jones, L. Pack, J.H. Hunter, J.F. Valliere-Douglass, Native size-exclusion chromatography-mass spectrometry: suitability for antibody-drug conjugate drug-to-antibody ratio quantitation across a range of chemotypes and drug-loading levels, *mAbs* 12 (2020) 1682895.
- [34] T.L. Kirley, A.B. Norman, Unfolding of IgG domains detected by non-reducing SDS-PAGE, *Biochem. Biophys. Res. Commun.* 503 (2018) 944–949.
- [35] A.O. Bailey, R. Huguet, C. Mullen, J.E.P. Syka, W.K. Russell, Ion-ion charge reduction addresses multiple challenges common to denaturing intact mass analysis, *Anal. Chem.* 94 (2022) 3930–3938.
- [36] L.J. Brady, J. Valliere-Douglass, T. Martinez, A. Balland, Molecular mass analysis of antibodies by on-line SEC-MS, *J. Am. Soc. Mass Spectrom.* 19 (2008) 502–509.
- [37] A.C. Lazar, L. Wang, W.A. Blattler, G. Amphlett, J.M. Lambert, W. Zhang, Analysis of the composition of immunoconjugates using size-exclusion chromatography coupled to mass spectrometry, *Rapid Commun. Mass Spectrom.* 19 (2005) 1806–1814.
- [38] I. Koutsopetras, A.K. Mishra, R. Benazza, O. Hernandez-Alba, S. Cianferani, G. Chaubet, S. Nicolai, J. Waser, Cysteine-cysteine cross-conjugation of both peptides and proteins with a bifunctional hypervalent iodine-electrophilic reagent, *Chemistry* (2023) e202302689.
- [39] B.L. Duivelshof, A. Beck, D. Guillaume, V. D'Atri, Bispecific antibody characterization by a combination of intact and site-specific/chain-specific LC/MS techniques, *Talanta* 236 (2022) 122836.
- [40] M. Habberger, 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.
- [41] H. Liu, G. Gaza-Bulseco, C. Chumsae, Analysis of reduced monoclonal antibodies using size exclusion chromatography coupled with mass spectrometry, *J. Am. Soc. Mass Spectrom.* 20 (2009) 2258–2264.
- [42] J. Marcoux, T. Champion, O. Colas, E. Wagner-Rousset, N. Corvaia, A. Van Dorsselaer, A. Beck, S. Cianferani, Native mass spectrometry and ion mobility characterization of trastuzumab emtansine, a lysine-linked antibody drug conjugate, *Protein Sci.* 24 (2015) 1210–1223.
- [43] J.F. Valliere-Douglass, W.A. McFee, O. Salas-Solano, Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues, *Anal. Chem.* 84 (2012) 2843–2849.