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Combination of intact, middle-up and bottom-up levels to characterize 7 therapeutic monoclonal antibodies by capillary electrophoresis – Mass spectrometry

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ABSTRACT

Significant growth of biopharmaceuticals requires powerful analytical methods to better understand their structure by establishing a complete characterization. To this end, a combination of bottom-up, middle-up and intact molecule levels with a capillary electrophoresis-mass spectrometry coupling has been performed to have a comprehensive picture of monoclonal antibodies. In this study, 7 worldwide health authorities approved mAbs have been analyzed to get information about their charge heterogeneity, the identification of post translational modifications (PTMs), their location and relative quantitation. Intact mAbs isoforms have been partially separated in less than 12 min and enabled to have a global illustration of mAbs heterogeneity and high masses PTMs characterization notably major N-glycosylation forms. Particularly, 2X-glycosylated and 1X-glycosylated forms have been partially separated. To deepen characterize PTMs carried by the backbone structure, advanced investigations at a middle-up level have been performed. Limited IdeS proteolysis allowed to study independently Fc/2 and F(ab)'2 fragments. Following the same separation conditions, isoforms of these fragments have been separated and data interpretation allowed to disclose additional PTMs as K-clip, oxidations or deamidations. A second intermediate level has been examined by adding a reduction step to establish a more precise assessment of PTMs and isoforms from the F(ab)'2 fragment. This reduction step released the light chains from the Fd fragment to get only 25 kDa fragments to analyze. CE-ESI-MS coupling allowed to get more information particularly about low masses PTMs. The precise location and relative quantitation of each PTM has been investigated at the peptidic level induced by a tryptic digestion of the studied mAbs. The concordance of the results shows the efficiency of the CE-ESI-MS coupling to characterize mAbs and highlight the need of the multi-level combination to get a comprehensive characterization of biotherapeutics.

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1. Introduction

Over the last decades, the expanding development of monoclonal antibodies (mAbs) has led to numerous solutions employed in therapeutic treatment. These glycoproteins become one of the main class of biopharmaceuticals involved in various treatment such as cancers, auto-immune disease but also to prevent transplant rejection [1–3]. Nowadays, more than 70 mAbs have been approved by worldwide health authorities and commercially available and 80 candidates are in the last phase of clinical trials [4]. The increasing number of candidates is supported by the expanding range of biological targets identified and high specificity of mAbs to the corresponding antigens. MAbs production processes are based on cell lines inducing their own modifications that irremediably elicits a diversity of isoforms. The mixture of proteoforms involved by the variety of macro and micro-heterogeneities leads to a charge heterogeneity. It can be explained by post-translational modifications (PTMs) such as N-glycosylations, partial amino-acid sequence cleavage, deamidations or oxidations which can occur during fabrication, purification, conditioning or storage steps. Combining certain of these PTMs may result in a difference of biological activity of the treatment or affect the stability and safety of the drugs.

Currently, a large panel of analytical techniques are required to perform an advanced characterization of mAbs among biotherapeutics [5,6]. Mass spectrometry (MS) is now recognized as a major tool in protein structural characterization [7] and requested for example in ICH Q6B guideline (https://www.ich.org/

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page/search-index-ich-guidelines). Recent advances in high resolution MS instrumentation and have even led to the use of MS-based methods for quality control testing in a current Good Manufacturing Practice environment [8,9]. The continuous evolution of separative techniques and MS hyphenation enhances protein mixtures understanding and is widely applicable in mAbs assessments [10–12]. On-line capillary electrophoresis-mass spectrometry coupling *via* an electrospray ionization interface (CE-ESI-MS) has already proved its ability to separate and characterize microvariants of mAbs [13–15]. The effectiveness of miniaturized systems oriented our strategy to CE-ESI-MS to separate and characterize mAbs proteoforms and rank Critical Quality Attributes [16] during early developability [17], forced degradation [18] and comparability studies [19]. It offers the possibility to combine protein separation and analysis at different levels such as bottom-up, middle-up and intact levels that justifies its use in biotherapeutic characterization [20–22]. Indeed, the intrinsic heterogeneity of mAbs involves the need of a comprehensive characterization in order to obtain a model the most realistic of the real drug.

Traditional mAb characterization has been operated following a "bottom-up" strategy with a LC—MS/MS coupling or more recently with a sheathless CE-ESI-MS/MS coupling [11,23]. Although the important ability to identify different proteoforms with minor micro-heterogeneities, this approach can occur some artifactual modifications due to sample preparation and chemical treatment. This limitation can be surpassed by a comprehensive structural characterization at higher levels as middle-up and intact molecule approaches. These respectively intermediate and ultimate levels seem to be particularly interesting for biopharmaceutical companies because of their ability to illustrate the heterogeneity of produced mAbs. Middle-up level can be performed by digesting mAbs with IdeS protease which cleaves Immunoglobulins (IgGs) under the hinge region between 2 Glycine residues at a specific amino-acid sequence CPPCPAPELLG/GPSVF to obtain Fc/2 fragments (25 kDa) and F(ab)'2 fragment (100 kDa). Optional reducing step can be done to reduce intra-chain and inter-chain disulfide bonds to cleave the F(ab)'2 fragment by releasing the light chain (LC) (25 kDa) from the Fd fragment (25 kDa), facilitating the mAb assessment. To complete mAbs characterization, an intact protein level corresponding to the highest analytical level of mAbs has been investigated. Based on the CE-ESI-MS development previously described by our group, a partial separation of intact mAbs has been performed and spectra interpretation highlighted a charge heterogeneity which may be explained by a combination of PTMs [24]. As expected, PTMs characterized at this upper level are essentially high weight major modifications as N-glycosylations but surprisingly it also disclosed some common low masses PTMs as oxidations or deamidations.

In this work, we report the development of a multi-level combined approach implementing a complete structural characterization of mAbs based on a CE-ESI-MS coupling. Adalimumab (huIgG1, CHO), natalizumab (hzIgG4, NS0), nivolumab (huIgG4, CHO), palivizumab (hzIgG1, SP2/0), infliximab remicade® (chIgG1, SP2/0), rituximab (chIgG1, CHO) and trastuzumab (hzIgG1, CHO) were the seven commercial mAbs considered in this multi-level assessment.

2. Experimental

2.1. Chemicals

Chemicals used were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO, USA). Water used to prepare buffers and sample solutions was obtained using an ELGA purelab UHQ PS water purification system (Bucks, UK). Adalimumab, Natalizumab, Nivolumab, Palivizumab, Infliximab, Rituximab and Trastuzumab were obtained as European Union pharmaceutical-grade drug product from their respective manufacturers.

2.2. Sample preparation

2.2.1. Intact level

For intact mAbs analysis, samples were desalted to remove all residual components of the storage solution. Samples were buffer exchanged with water three times at 4 °C on Amicon centrifugal filters with a 10 kDa cut off (Merck, Darmstadt, Germany). Each centrifugation was made at 14′000 g speed during 20 min to claw back mAbs at 33.3 μM. Then, mAbs were led to a final concentration of 6.7 μM using formic acid 1 % - methanol 30 % (v/v).

2.2.2. Middle-up level

MAbs were clived under the hinge region with IdeS enzyme (FabRICATOR®, Genovis), to obtain two Fc/2 fragments and one f(ab)'2 fragment. Samples were diluted in 200 mM ammonium bicarbonate pH 6.5 to a final concentration of 1 g/L. A volume of 2.24 μL of IdeS (67 U/μL) was added to each sample and left at 37 °C for 45 min. After digestion, three desalting steps were performed with water on Amicon centrifugal filters with a 10 kDa cut off at 4 °C and 14'000 g for 20 min. Then, mAbs were led to a final concentration of 6.7 μM using formic acid 1 % - methanol 30 % (v/v).

2.2.3. Middle-up reduced level

After IdeS digestion, 20 mM TCEP was added to samples then left 30 min at room temperature to reduce disulfide bonds on F(ab)'2 fragments. The reduction of F(ab)'2 fragments results in the dissociation of the light chain (LC) and the Fd fragment. After reduction, three desalting steps were performed with water on Amicon centrifugal filters with a 10 kDa cut off at 4 °C and 14'000 g for 20 min. Then, mAbs were led to a final concentration of 6.7 μM using formic acid 1 % - methanol 30 % (v/v).

2.2.4. Bottom-up level

A volume corresponding to 100 μg (0.67 nmol) of protein was used. Samples were first diluted from stock solution to a concentration of 45.6 μM by using water. A second dilution to a concentration of 22.2 μM was performed by using 0.1 % RapiGest surfactant an incubation at 40 °C for 10 min. Reduction of the samples was then achieved by the addition of dithiothreitol (DTT, final concentration of 25 mM) and incubation at 95 °C for 5 min. To perform tryptic digestion, a volume of 1 μL of trypsin (0.5 μg/μL) was added to the samples that were left at room temperature for 3 h. Then another volume of 1 μL was added afterward and digestion was performed overnight at 37 °C. In order to cleave the surfactant, formic acid (FA) was added to the samples at a final concentration of 1 % (v/v) and samples were left at room temperature (RT) for 2 h. Samples were finally diluted to a final protein concentration of 2.2 μM using ammonium acetate 200 mM (pH 4.0).

2.3. Capillary electrophoresis

All of the CE experiments have been done on a CESI8000 capillary electrophoresis system from Sciex Separation (Brea, CA, USA) hyphenated to a timsTOF Pro (Bruker Daltonics, Bremen, Germany). For Intact and middle-up levels analysis, bare fused silica capillary (total length 100 cm; 30 μm i.d.) with porous tip on its end have been positively coated with polyethyleneimine (PEI) following Sciex protocols. A second capillary (total length 80 cm; 50 μm i.d.) allowed electric contact after a filling step of both with 3 % acetic acid background electrolyte (BGE). Before each run, capillaries were flushed 3 min at 50 psi with BGE. Samples were injected at

2 psi 10 s and separations were performed at −30 kV during 15 min. No capillary wash was done between each run in order to avoid any degradation of PEI coating. For tryptic digests, analyses have been performed on a bare fused silica capillary (total length 100 cm; 30 μm i.d.). BGE was acetic acid 10 % and 100 nL of 2.2 μM of mAbs have been injected in the capillary. Runs length was set at +20 kV during 60 min each.

2.4. Mass spectrometry

For each analysis level of mAbs, CE was hyphenated to a timsTOF Pro (Bruker daltonics, Bremen, Germany). This equipment is a hybrid mass spectrometer composed of an ion mobility cell, a quadrupole followed by a time-of-flight (TOF) analyzers. Contrary to bottom-up level, ion mobility cell was switch off for intact and intermediate analysis levels. Nano-ESI source parameters were set as follow: nanoESI voltage +1.5 kV while dry gas supplies was 1.2 L/min, source heating temperature 150 °C. Mass/charge (*m/z*) range was 1000–5000 in MS for intact mAbs analysis, 1000–4000 in MS for intermediate levels and 400–3000 for peptide level. Data processing was performed with DataAnalysis 5.1. Deconvolution of mass spectra was done using ESI Compass Maximum Entropy deconvolution option. All spectra were calibrated by external calibration using Peptide mix from Bruker.

2.5. Data analysis

MS data were analyzed with the dissect mode of DataAnalysis 5.1 software with an internal S/N threshold of 3 and a maximum of 10 overlapping compounds. Cut-off intensity of mass spectrum calculation was set at 0.1 %. After an automatic interpretation of the results, a manual validation has been performed on the results. MS spectra have been extracted from each peak represented on the base peak electropherogram. For intact mAbs analyses, each profile has been selected and deconvoluted between 140 and 160 kDa with the maximum entropy algorithm of DataAnalysis 5.1. to calculate the mass of corresponding compounds. Spectra obtained at the middle-up have been deconvoluted between 20 and 30 kDa for Fc/2, Fd and LC fragments and 90–100 kDa to identify f(ab)'2 fragment. Finally, fragments earned with reduction step added to the partial digestion with IdeS were sought only between 20 and 30 kDa. Manual and automatic results have been confronted to theoretical masses to detect any mass modification and get additional information about the characterized compounds.

3. Results and discussion

3.1. Intact level

Regarding capillary zone electrophoresis (CZE)-based methods for the characterization of mAbs charges variants, until 2016, almost all literature described quality control application in terms of product heterogeneity using CZE with UV detection [23,25]. However, strategies with optical detection didn't allow to obtain structural information for the basic and/or acidic variants representing the limitation of these methodologies. These last years, some reports revealed the usefulness of CZE-MS for the assessment of macro-heterogeneity [15,24,26]. MS resolving power coupled CZE efficiency exhibited as well the baseline separation of 2X-glycosylated, 1X-glycosylated and aglycosylated population as the characterization of various other PTMs such as deamidation, oxidation or lysine clipped forms. In each cases, CZE experimental conditions are based on the use of a neutral or positive coated capillaries to avoid protein adsorption on the inner surface of the capillary wall. Indeed, due to the negative charge surface of the silanol groups, electrostatic interactions imply peak tailing phenomena degrading separation efficiency. Different strategies as positive polyethylenimine (PEI) or uncharged polyacrylamide coatings have been investigated [24,26]. In a previous study, our group detailed a CZE-MS methodology to perform the analysis of mAbs at the intact level. Based on the use of a PEI coated capillary, acidic background electrolyte (BGE) and acidic methanol/water sample buffer, micro-variant separations of intact mAbs have been observed in less than 20 min (RSD < 3 % on migration times (n = 10)). In the present study and in order to assess the methodology, this CZE-MS method has been applied to seven world-wide health authorities approved mAbs. Separation obtained (Fig. 1), showed differences of profile depending on the nature of the mAbs. This expected result can be explained by the difference of heterogeneity of each mAbs added to the difference of pI values (from 7.1–9.1 [27]). However, a general profile can be observe with the main peak surrounded by acidic variants before and basic variants after. This order of separation is explained by the use of positive coated capillary which confers a reverse electroosmotic mode. MS data processing consisted to integrate and deconvolute each peak of the based peak electropherogram. Results of identified isoforms for the seven mAbs are given in the Table S1 of the supporting information. As a first PTMs characterization step about cyclisation of glutamine (Glu) or glutamic acid (E), seven mAbs have to be compared in two groups. Indeed, three analyzed mAbs (adalimumab, infliximab, trastuzumab) own N-terminal Glu residue while four others (nivolumab, palivizumab, natalizumab, rituximab) own N-terminal Gln. Concerning the Glu residue's group, no cyclisation has been observed while 100 % of 2X-pyroGln have been identified for the Gln residue's group. These results mean that either the mAbs are not modified or MS sensitivity is not sufficient to detect small amounts of modified mAbs. Only the analysis at a lower level of complexity can answer this question. Concerning the other PTMs, adalimumab will be select as a representative example to illustrate these obtained MS results (Fig. 2). For each peak, deconvoluted mass spectra exhibited a glycoform pattern corresponding to 2X-glycosylated and 1X-glycosylated forms. Focused on the 2X-glycosylation populations, measured average masses (Table 1) corresponding to G0F/G0F, G0F/G1F and G0F/G0F-GlcNac while G0F and G1F are identified for the 1X-glycosyled forms. As expected, no separation of mAbs isoforms has been observed whereas separation obtained between 2X-glycosyled and 1X-glycosylated forms [24,26]. Concerning possible acidic or basic variants, mass difference between 3 and 15 Da were observed. As already described in the literature, the most important antibody basic charge variants are iso-Asp modification, C-terminal Lys truncation, aglycosylation, incomplete cyclization of the N-terminal Gln to pyroGln or methionine oxidation; whereas sialylation, asparagine deamidation, glycation, cysteinylation are the most commonly observed acidic variants [28]. Primary structure of adalimumab carries several residues (Asp, Asn, Met...) which can be potentially isomerized, deamidated or oxidized. However, due to the mass accuracy limitation of the mass spectrometer, it is impossible to conclude without any ambiguity on the exact modification. Only hypothesis can be formulated on the real nature of acidic and basic variant. To alleviate this limitation, only a multi-level analysis involving partial enzymatic digestion could be an alternative solution to ensure the identification and deeper characterize mAbs isoforms.

3.2. Middle-up level

An increasingly number of reports from worldwide drug agencies such as the FDA and the EMA highlight the importance of multi-level characterization of mAbs [29–31]. Middle-up analysis of mAbs involving IdeS partial digestion has been performed

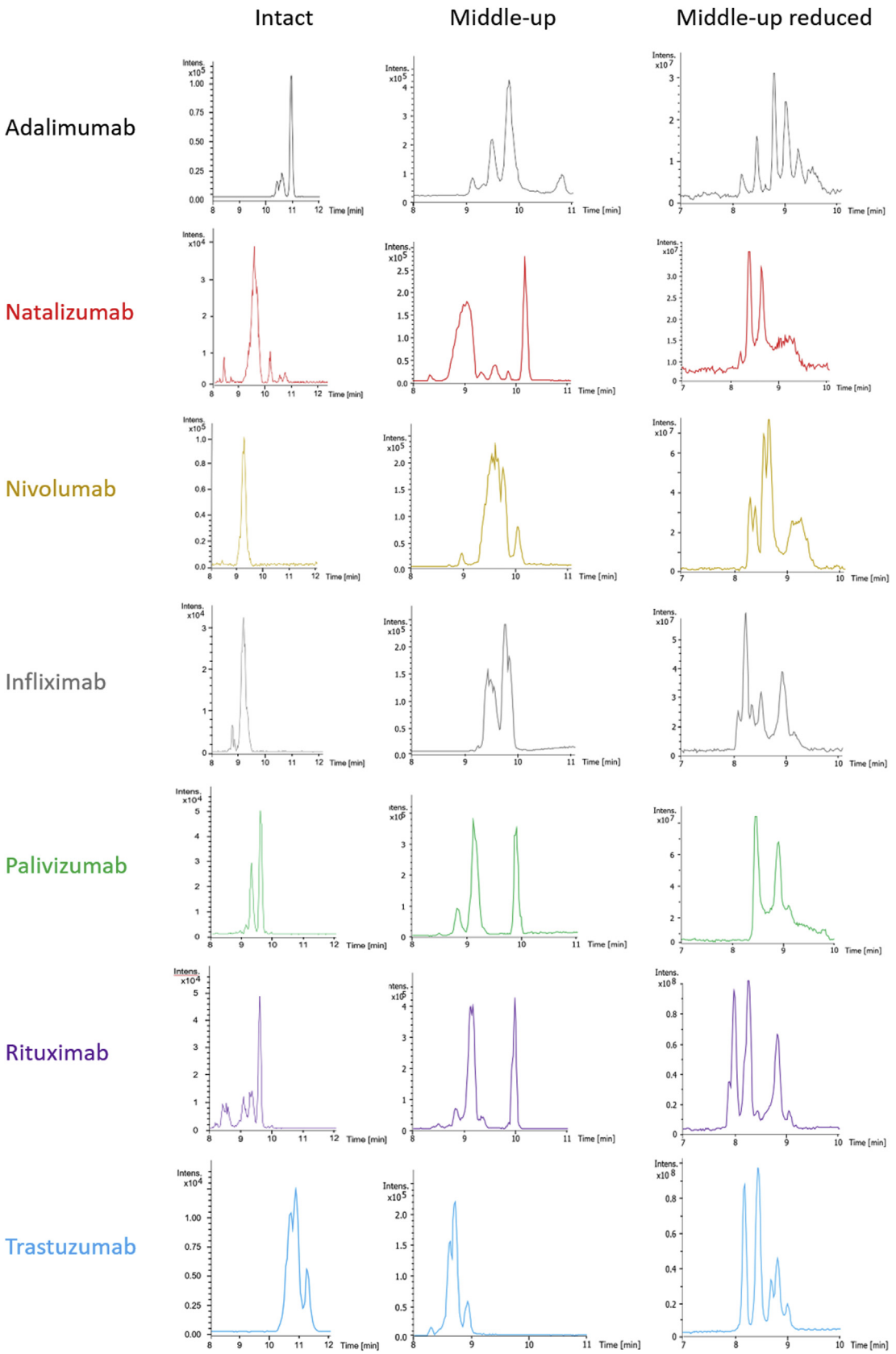


Fig. 1. CZE-ESI-MS separation of intact, middle-up and reduced middle-up of the seven mAbs.

to get additional information and confirm results earned with the intact protein strategy. This intermediate level consists in cleaving the immunoglobulin (IgG) specifically under the hinge region to obtain F(ab)'2 and Fc/2 fragments. The two fragments are respectively around 100 kDa and 25 kDa. Method development has been carried out to separate and analyze fragments with such a mass

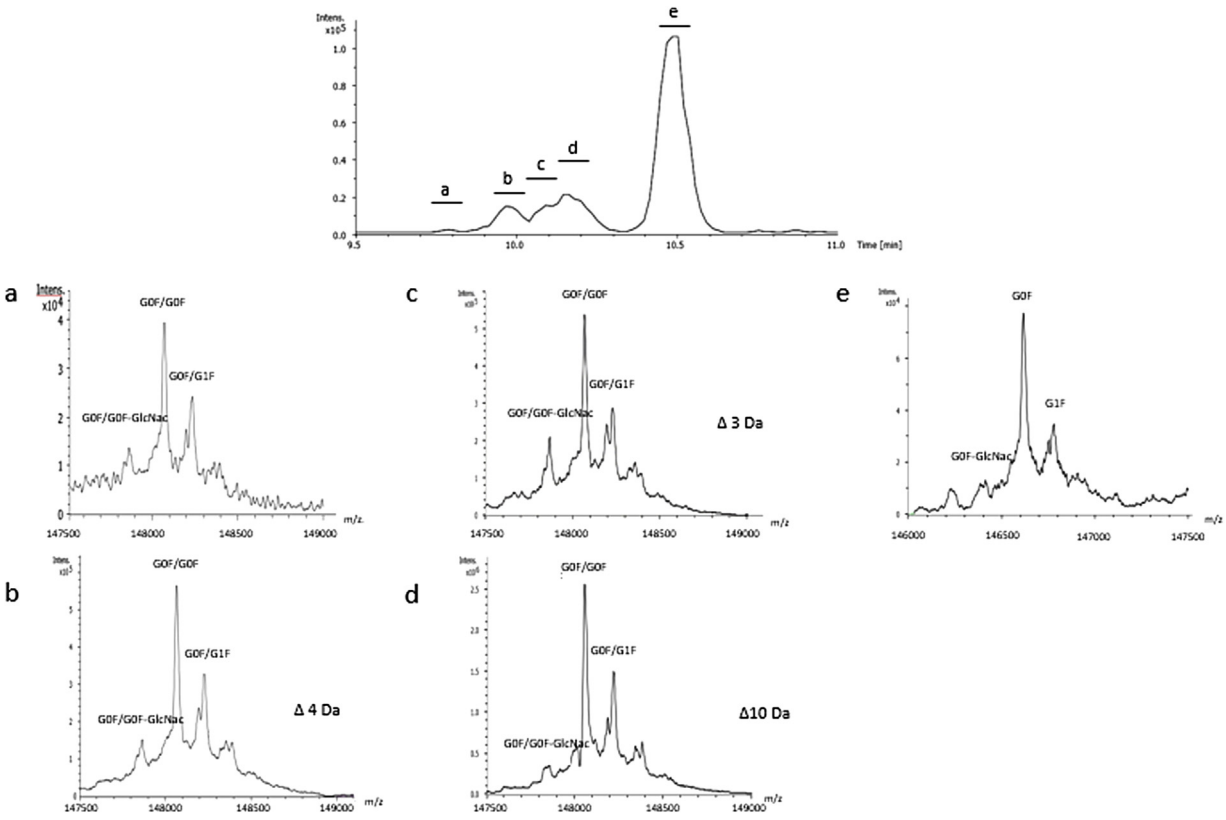


Fig. 2. CZE-ESI-MS separation of intact adalimumab and corresponding deconvoluted mass spectra.

Table 1
Assignment of species observed for intact Adalimumab after CZE-ESI-MS analysis.

Fragment	glycan species	observed mass (Da)	theoretical mass (Da)	assigned PTM	migration time (min)	Δ mass (Da)
	G0F	146619	146635		10.55	16
	G1F	146781	146797		10.55	16
	G0F/G0F-GlcNac	147861	147877		10.45	16
	G0F/G0F-GlcNac	147867	147876		10.15	9
	G0F/G0F-GlcNac	147869	147877		10.05	8
	G0F/G0F-GlcNac	147871	147877		9.95	6
	G0F/G0F	148064	148080		10.45	16
	G0F/G0F	148070	148080		10.15	10
	G0F/G0F	148071	148080		10.05	9
	G0F/G0F	148075	148080		9.95	5
	G0F/G1F	148227	148242		10.45	15
	G0F/G1F	148232	148242		10.15	10
	G0F/G1F	148232	148242		10.05	10
	G0F/G1F	148236	148242		9.95	6

Annotation: see supporting information Fig. S1.

variance. CZE conditions have not been subject to modification between each level of analysis. After mAb infusion, the mass range has been fixed between 1000 and 4000 Thomson to incorporate the charge envelope of the two kinds of fragments. Separations have been performed in less than 11 min for each mAbs (Fig. 1) (RSD < 3 % on migration times (n = 3)). In each case, 4–6 peaks have been separated, with 2–5 Fc/2 isoforms and 1–4 F(ab)'2 isoforms (Table S1). After a comparison between theoretical masses and experimental ones, major glycoforms as Man5, GlcNac, G0F, G1F and G2F already found at the intact molecule level are also recovered. Supplementary glycoforms are linked with Fc/2 fragments of the different mAbs. For example, G0 glycoform has been found on each mAb except natalizumab, G1 and G2 forms have been identified on respectively 5 and 3 mAbs including natalizumab, nivolumab and trastuzumab (Table S1). In addition, each mAb spectra deconvolution highlighted the presence of lower mass PTMs well defined such as K-clip which is a loss of the terminal lysine on the HC, methionine oxidation but also potential asparagine deamidation. Concerning the Glu/PyroGlu and Gln/PyroGln groups of mAbs described in the intact level section, similar results have been observed at the middle-up level, namely, 100 % N-terminal Glu and 100 % PyroGln respectively.

As at the intact level analysis, adalimumab was chosen to illustrate these results. Electropherogram in Fig. 3 shows a separation of the IdeS digest of adalimumab with several peaks, reflecting a separation of F(ab)'2 and Fc/2 fragments. Deconvoluted spectra revealed a baseline separation of F(ab)'2 and Fc/2 fragments and a partial separation of Fc/2 isoforms (Table 2). Results showed Fc/2 fragment with G0F-GlcNac, Man5, Man6, G0F, G1F and G2F glycoforms. These observation are in agreement with the main glycloforms observed at the intact level. Moreover, additional glycosylation has been obviously characterized due to the difference of weight between

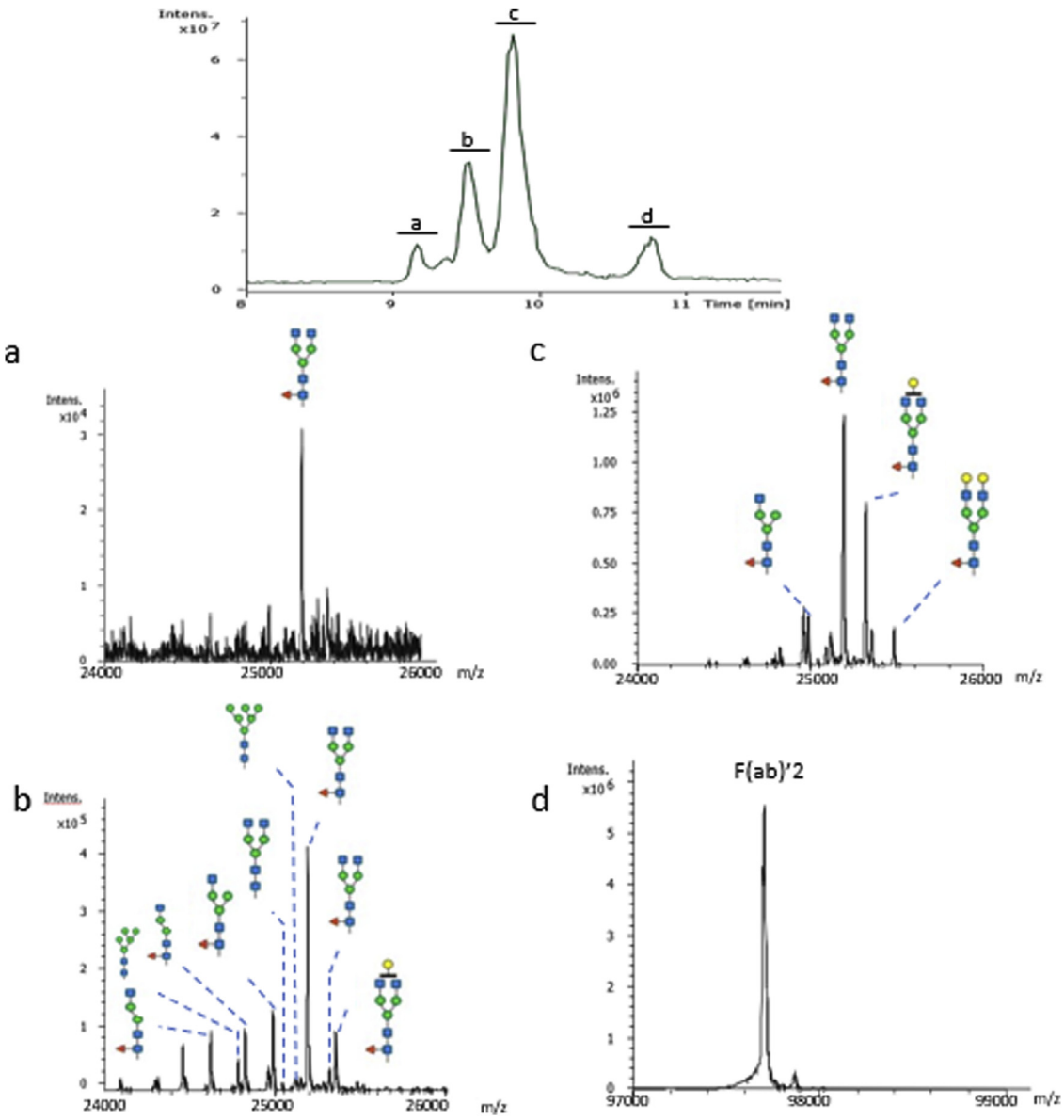


Fig. 3. CZE-ESI-MS separation of IdeS digest of adalimumab and corresponding deconvoluted mass spectra.

Table 2
Assignment of species observed for IdeS digest of Adalimumab after CZE-ESI-MS analysis.

fragment	glycan species	observed mass (Da)	theoretical mass (Da)	assigned PTM	migration time (min)	Δ mass (Da)
Fc/2	non-glycosylated	23754.0	23754.6	Kclip	10.00	0.6
Fc/2	[H1N2F1]	24467.8	24468.3	Kclip + 1 deam *	9.55	0.5
Fc/2	[H2N2F1]	24631.0	24631.4	Kclip	9.55	0.4
Fc/2	[H2N3F1]	24833.6	24833.6	Kclip + 1 deam*	9.80	0.0
Fc/2	G0F [H3N4F1]	25200.1	25199.9	Kclip	9.65	−0.2
Fc/2	G0F [H3N4F1]	25328.1	25328.1	–	9.90	0.0
Fc/2	G0F [H3N4F1]	25327.1	25327.1	deam*	10.30	0.0
Fc/2	G1F [H4N4F1]	25361.0	25361.1	Kclip + 1 deam*	9.80	0.1
Fc/2	G1F [H4N4F1]	25361.6	25362.1	Kclip	10.30	0.5
Fc/2	G1F [H4N4F1]	25490.1	25490.2	–	10.00	0.1
Fc/2	G2F [H5N4F1]	25524.3	25524.2	Kclip	9.55	−0.1
Fc/2	G0F-GlcNac [H3N3F1]	24995.7	24995.8	Kclip + 1 deam*	9.65	0.1
Fc/2	G0F-GlcNac [H3N3F1]	24996.3	24996.7	Kclip	10.30	0.5
Fc/2	G0F-GlcNac [H3N3F1]	25125.3	25124.9	–	10.00	−0.7
Fc/2	G0 [H3N4]	25052.6	25052.8	Kclip + 1 deam*	9.55	0.2
Fc/2	Man5 [H5N2]	24971.7	24971.7	Kclip	9.55	0.0
Fc/2	Man6 [H6N2]	25132.0	25132.8	Kclip + 1 deam *	9.55	0.8
f(ab)'2	–	97713	97712	4 deam *	10.00	1
f(ab)'2	–	97711	97711	5 deam *	10.30	0
f(ab)'2	–	97708	97708	8 deam *	10.70	0

Annotation: see supporting information Fig. S1.

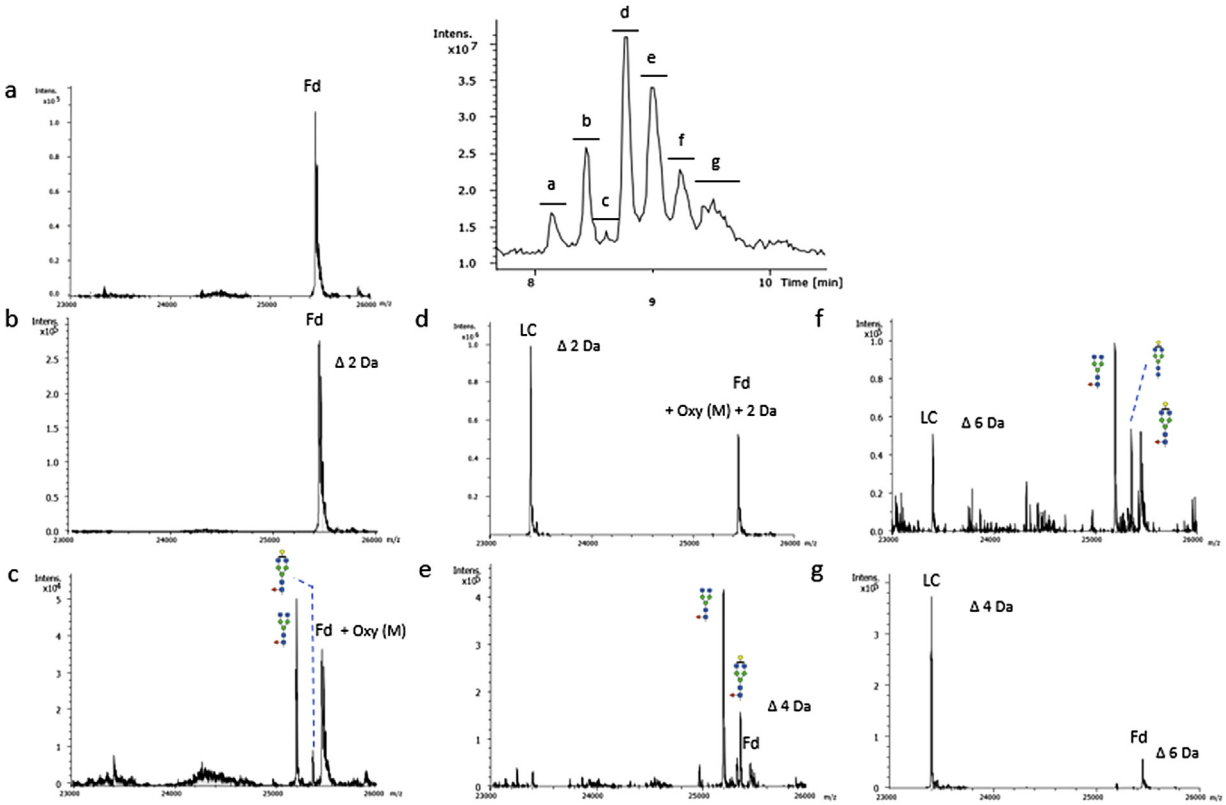


Fig. 4. CZE-ESI-MS separation of reduced IdeS digest of adalimumab and corresponding deconvoluted mass spectra.

Fc/2 fragment (25 kDa) and intact mAbs (150 kDa). Another PTMs such as K-clip on C-term of Fc/2, correlated to a difference of mass of 128 Da, have been observed in a large number of glycoform. As at the upper level, low mass differences were detected on Fc/2 and F(ab)'2 fragments corresponding to acidic or basic variants. Regarding the Fc/2 fragments, difference of 1 Da corresponding to one deamidation of Asn have been observed in the majority of the identified glycoforms. Concerning F(ab)'2, difference between 4–8 Da have been observed which can correspond to several deamidation. However, despite the compatibility of results between theoretical and experimental data, same limitation of the mass accuracy of mass spectrometer can be underline for 100 kDa fragments. Even if there is sufficient Asn residues in F(ab)'2 fragment to obtain several deamidation, only hypothesis can be formulated on the real nature of the modification. A possibility to overcome this limitation is to reduce F(ab)'2 fragment. Indeed, reduction of disulfide bonds anchored in F(ab)'2 fragment could release the LC from the Fd part of the mAb and thus allow a deeper structural investigation.

3.3. Reduced middle-up level

Reduction workflow consisted to break the disulfide bonds with the reducing agent tris(2-carboxyethyl)phosphine (TCEP) to release the light chain LC from the Fd part. Hence, the masses of the three fragments earned with this sample treatment were all about 25 kDa and enabled a more comprehensive analysis of the F(ab)'2 fragment. MS analytical parameters have been optimized to redefine the *m/z* range and key factors such as the accumulation time of ions in the mass spectrometer but also the pre-pulse storage time in order to enhance both detection and mass accuracy. As expected at this lower level, for each mAbs, 3–7 peaks have been observed on the electropherogram in less than 10 min, thus proving the efficiency and fastness of the separation method (Fig. 1). For each mAb, the quantity of peaks drawn by the total ion electropherogram is increasing and respective peaks' efficacy are better, more intense and better resolved than results at the upper levels.

Besides the presence of the three theoretical fragments, information has been obtained by deconvoluted spectra regarding the nature of PTMs found on the mAbs, such as low mass differences and oxidations. As shown at higher analytical levels, spectra interpretation of Fd fragments revealed pyroGln in Fd N-terminal position for mAbs bearing a Gln on N-term HC, and Glu in the same position for mAbs bearing a Glu on N-term HC. In addition, oxidation and deamidation have been clearly located on LC or Fd fragments, completing the characterization of PTMs at the different level. On a global basis of the seven mAbs, in respect of Fc/2 fragments, besides previously identified glycoforms, additional ones have been partially separated but unambiguously characterized such as Man5, Man6, G0F-GlcNac as indexed in Table S1 with or without K-clip. Moreover, uncomplete glycosylation forms have been detected and separated, extending the characterization of PTMs on mAbs.

Using the example of adalimumab results illustrated in Fig. 4, numerous peaks are drawn on the total ion electropherogram (TIE) arising from a separation of LC, Fd and Fc/2 fragments. Some glycosylation forms like G1F, G0 or G1 already found at intact and middle-up levels are also recovered at this level. Low masses PTMs as asparagine deamidation and methionine oxidation are also found at this level, enhancing the interpretation of MS spectra and more widely mAbs characterization (Table 3). This secondary intermediate level led to new limitations concerning the identification of PTMs on the 3 formed fragments. Indeed, all three Fd, Fc/2 and LC fragments earned by an Ides digestion added with a reduction step have a molecular weight about 25 kDa. Hence, major peaks on MS spectra may interfere with some minor peaks representing fragments carrying PTMs with a lower intensity. More precisely, conglomeration of many species differing slightly on their mass can be co-eluted. Ions detection is clearly dependent from ionization, transfer and detection so hidden peaks can sometimes reveal some

Table 3
Assignment of species observed for reduced IdeS digest of Adalimumab.

fragment	glycan species	observed mass (Da)	theoretical mass (Da)	assigned PTM	migration time (min)	Δ mass (Da)
Fc/2	G0F [H3N4F1]	25201.9	25202.0	Kclip + 2 deam *	8.90	0.1
Fc/2	G0F [H3N4F1]	25199.7	25200.0	Kclip + 4 deam *	9.25	0.3
Fc/2	G1F [H4N4F1]	25362.8	25363.2	Kclip + 3 deam *	9.05	0.4
Fc/2	G1F-GlcNac [H4N3F1]	25157.9	25157.9	Kclip + 1 deam *	9.25	0.0
Fc/2	G1 [H4N4]	25217.7	25218.0	Kclip + 2 deam *	9.25	0.3
LC	–	23409.7	23409.9	2 deam *	8.80	0.2
LC	–	23406.2	23406.0	6 deam *	9.35	−0.2
Fd	–	25456.2	25456.4	2 deam *	8.45	0.2
Fd	–	25454.3	25454.4	4 deam *	9.50	0.1
Fd	–	25472.3	25472.4	oxy + 2 deam *	8.45	0.1

Annotation: see supporting information Fig. S1.

strong present PTMs. Moreover, even if fragments have a reduced mass allowing a better characterization, they can possess numbers of different possible site of modification as Asn, Asp or Met for example. At this level of characterization and without fragmentation process, it is impossible to locate precisely the modification. At the moment, the bottom-up level remains the better alternative totally complementary with higher levels. This third mAb analytical level enables to get precise structural information on peptides. The protein backbone slicing with an enzymatic tool allows to isolate the PTMs and opens a way to characterize and relatively quantify their presence.

3.4. Bottom-up level

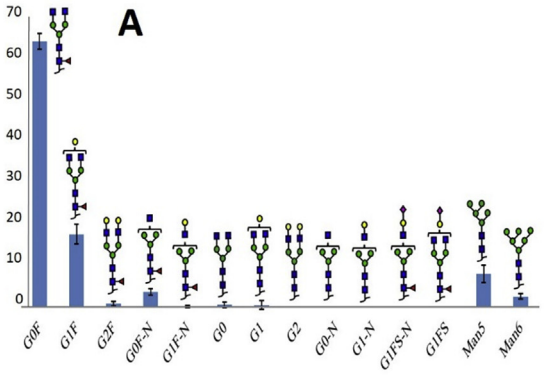
One of the reference method to determine protein amino acid sequence and to locate or quantify PTMs is the MS based bottom-up analysis. For primary structure characterization, this methodology is particularly crucial in early development of therapeutic antibodies as well as during long-term life cycle management of the biopharmaceutical products. This level 3 of mAb analysis allows to perform their characterization with respect to several aspects that define their primary structure, including amino acid sequence, glycosylation and other types of PTMs such as oxidation of Met, deamidation of Asn, cyclisation of Gln/Glu an isomerization of Asp [21].

Based on previous work [32], mAbs samples were treated by an in solution tryptic digestion. Peptides earned had a length between two and about sixty amino acids. For each mAbs, tryptic peptide mixtures were separated using the sheathless CZE-ESI-MS/MS system based on their charge state in solution as well as their hydrodynamic radius. Peptides were identified from MS/MS spectra in an approach similar that used in bottom-up proteomic experiments.

For each mAbs, sequence coverage of 100 % of both the heavy chain and light chain was obtained from a single injection of 200 fmol (Fig. S2). Regarding MS/MS spectra, remarkable number of y and b ions allows peptide identification in peptide fragment fingerprinting strategy. Identification of more than 80 % of the y and b ions composing the seven different mAbs are systematically obtained.

Concerning N-glycosylation characterization, using the same set of CZE-ESI-MS/MS data, 14 of the main Fc N-Glycan species typically found in therapeutic IgG mAbs produced in CHO, NS0 and SP2/0 cell lines were systematically identified. Peak assignment of glycostructures was performed based on accurate mass measurement in MS1, provided by high resolution MS (mass accuracy below 2 ppm) and MS/MS spectra, respectively. As the intact and middle-up analysis, results of adalimumab have been selected to illustrate the CZE-ESI-MS method performance (Fig. 5), however, results on the other six mAbs are done in the supporting information Fig. S2). The glycan profiling of Adalimumab (Fig. 5a) has been designed using the evaluation of the relative occurrence levels of each glycopeptide. Reproducibility of the method has been already described in the literature by our group [33]. Briefly, triplicates of mAbs digestion were performed using different experimenters and triplicates injections of each digested samples were carried out. Calculated values of standard deviation never exceeding 4 % were observed, and a systematic comparison of the glycosylation patterns obtained for each mAbs was compared with the HILIC-FD reference method. This validated CZE-ESI-MS/MS method as a powerful approach to perform relative quantitation of N-glycan species for mAb characterization. Moreover, N-glycan species identified at this bottom-up level are in accordance with those forms characterized at intact and intermediate levels. G0F, G1F, G2F, G0, G1, G2, Man5, Man6, G0F-GlcNac are Figured out at all levels but the bottom-up level allows to get the precise localization and relatively quantify them. Besides glycosylation, other PTMs must also be considered following potential influence in the affinity between a mAb and its corresponding antigen. Same protocol has been processed for the identification and the relative quantification of selected PTMs such as methionine oxidation, N-terminal glutamic acid cyclization, asparagine deamidation and isomerization of aspartic acid. Data showed in Fig. 5b for adalimumab (supporting information Fig. S2 for the six other mAbs) that every single PTM monitored could be successfully characterized and relatively quantified. Advantages of bottom-up as compared to intact of middle-up approaches, is the faculty to locate precisely the modification on the backbone of the mAbs using MS/MS spectra. Moreover, the ability of CE to separate in each case the intact peptide to the modified peptide allowed to calculate the abundance level of the modification. It is interesting to note that for the 3 mAbs with N-terminal Glu in the HC, low abundance level of cyclisation is observed (<4 %) while for the 4 mAbs with N-terminal Gln in the HC, peptide is almost totally modified (<90 %). The presence or not of amino acid cyclization is in accordance with the other analytical levels however only bottom-up level has sufficient sensitivity to detect small amount of modified peptide. Concerning oxidation of methionine, focusing on DTLMISR peptide, which is common to every analyzed mAbs, less than 5 % modified peptide was observed in each case. However, depending of the mAbs, other oxidized methionine could be characterized elsewhere on the protein backbone with relative abundance until 10 %. This kind of occasional modification has also been found at the middle-up level, reinforcing the quality and robustness of the methods employed at the 3 levels. Finally, all deamidation of asparagine and isomerization of isoaspartic acid have been identified and characterized. Except for one peptide in rituximab bearing a D/isoD modification (abundance level of the modified peptide >20 %), calculated abundance level is included between 0–12%. Just as the previous results, the bottom-up strategy corroborate the information gathered at the intact and middle-up levels, where identical masses proteins were separated, nonetheless.

The consistency of the cross checked-results of combined intact, intermediate and bottom-up levels demonstrate the power and robustness of the CZE-ESI-MS to fully characterize therapeutics. Highest levels could be used first to have a global illustration of



B

Sequence	PTM	mAb distribution Unmodif (%)	mAb distribution Modif (%)	SD (%)
EVQLVESGGGLVQPGGSLR	E/pE	**98.46**	**1.54**	0.10
DTLMISR	M/oxiM	**94.60**	**5.40**	0.62
WQQGNVFSCSVMHEALH NHYTQK	M/oxiM	**98.69**	**1.17**	0.20
GFYPSDIAVEWESNGQPEN NYK	N/deaN	**97.68**	**2.32**	1.07
VDNALQSGNSQESVTEQD SK	D/isoD	**100.00**	**0.00**	0.00
DSTYSLSSTLTLSK	D/isoD	**99.64**	**0.36**	0.11
TPEVTCVVVDVSHEDPEVK	D/isoD	**99.09**	**0.91**	0.91
FNWYVDGVEVHNAK	D/isoD	**95.20**	**4.80**	1.43
TTPPVLDSDGSFFLYSK	D/isoD	**100.00**	**0.00**	0.00

Fig. 5. Glycoform semiquantitative analysis (A) and other PTMs occurrence levels (B) for adalimumab obtained by CE-ESI-MS.

the charge heterogeneity but also to get a qualitative information about the different N-glycosylations and PTMs attendance. Lower levels and particularly the third level (bottom-up) should be preferred to get quantitative information and precise localization of the PTMs and to ensure the amino acid structure.

4. Conclusion

In this work, seven commercial mAbs have been thoroughly characterized by a sheathless CZE-ESI-MS coupling. MAbs are glycoproteins which can be analyzed at three complementary analytical levels known as intact level, middle-up level and bottom-up level. Based on a previous work from our group to separate intact mAbs isoforms, supplemental levels have been investigated to get a deeper characterization and detect additional information such as PTMs combination and location. Contrary to the bottom-up analysis performed on a bare fused silica capillary, a PEI positive coating has been set up to avoid protein adsorption on the inner surface of the capillary at intact and middle-up levels. These upper levels analyses have been surveyed to get structural information as realistically as possible by avoiding artifactual modifications due to heavy sample preparation. A partial separation of intact isoforms has been obtained in less than 12 min. MS spectra interpretation of intact mAbs exposed the presence of major high molecular weight PTMs as N-glycosylations. Intermediate level has been investigated using an IdeS digestion to facilitate the characterization of low mass PTMs on mAbs. The separation of F(ab)'2 and Fc/2 fragments induced by the cleavage (respectively of 100 kDa and 25 kDa) has been performed following the same separation conditions used at the intact level. In each case, isoforms separation has been obtained in less than 11 min and disclosed a separation between F(ab)'2 and Fc/2 fragments. More precisely, isoforms of Fc/2 fragments have been elucidated carrying different N-glycosylations and other PTMs as methionine oxidations or K-clips. Mass accuracy enabled to get supplemental N-glycans structure. However, the F(ab)'2 fragments were still too imposing to undoubtedly assign very low mass PTMs such as asparagine deamidation. The solution employed in this study was to add a reducing step to release LC from Fd part by breaking up interchain disulfide bonds. All fragments induced by this workflow are about 25 kDa each. The separation has been performed in less than 10 min and gave numerous peaks in each case, corresponding to various isoforms of fragments. Here, high accuracy provided by the CE-ESI-MS coupling enabled the assessment of macro and micro-heterogeneities from N-glycosylations to asparagine deamidation. Nevertheless, the precise location of PTMs could only provide from a peptidic level assessment. The bottom-up level has been examined to identify, locate and relatively quantify all kind of PTMs. The synergy of PTMs identified at this lower level and the upper levels underline the paramount importance of the combination of multi-level analyses to establish a comprehensive characterization of mAbs and get an illustration as close as possible to the real protein mix administered to patients. However, the multi-level characterization of mAbs presented in this work could be qualified as a first step to integrate the quality control process of biotherapeutics. An exhaustive quality control of biomolecules could not be reached without the complete validation of CE-ESI-MS methods and the comparison of obtained results with orthogonal techniques.

CRediT authorship contribution statement

Jérémie Giorgetti: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Alain Beck:** Conceptualization, Resources. **Emmanuelle Leize-Wagner:** Conceptualization, Funding acquisition, Resources, Supervision, Validation. **Yannis-Nicolas François:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, 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

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113107.

References

[1] A. Beck, T. Wurch, C. Bailly, N. Corvaia, Strategies and challenges for the next generation of therapeutic antibodies, Nat. Rev. Immunol. 10 (5) (2010) 345–352.

[2] J.M. Reichert, Marketed therapeutic antibodies compendium, mAbs 4 (3) (2012) 413–415.
[3] M. Berger, V. Shankar, A. Vafai, Therapeutic applications of monoclonal antibodies, Am. J. Med. Sci. 324 (1) (2002) 14–30.
[4] H. Kaplon, J.M. Reichert, Antibodies to watch in 2018, mAbs 10 (2) (2018) 183–203.
[5] A. Beck, E. Wagner-Rousset, D. Ayoub, A. Van Dorsselaer, S. Sanglier-Cianférani, Characterization of therapeutic antibodies and related products, Anal. Chem. 85 (2) (2013) 715–736.
[6] D. Reusch, M. Haberger, B. Maier, M. Maier, R. Kloseck, B. Zimmermann, M. Hook, Z. Szabo, S. Tep, J. Wegstein, N. Alt, P. Bulau, M. Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 1: separation-based methods, Mabs 7 (1) (2015) 167–179.
[7] P. Lossl, M. van de Waterbeemd, A.J.R. Heck, The diverse and expanding role of mass spectrometry in structural and molecular biology, EMBO J. 35 (24) (2016) 2634–2657.
[8] S. Rogstad, H. Yan, X. Wang, D. Powers, K. Brorson, B. Damdinsuren, S. Lee, Multi-attribute method for quality control of therapeutic proteins, Anal. Chem. 91 (22) (2019) 14170–14177.
[9] I. Sokolowska, J. Mo, F. Rahimi Pirkolachahi, C. McVean, L.A.T. Meijer, L. Switzar, C. Balog, M.J. Lewis, P. Hu, Implementation of a high-resolution liquid chromatography–Mass spectrometry method in quality control laboratories for release and stability testing of a commercial antibody product, Anal. Chem. (2019).
[10] S. Rosati, Y. Yang, A. Barendregt, A.J.R. Heck, Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry, Nat. Protocols 9 (4) (2014) 967–976.
[11] S. Fekete, D. Guillarme, P. Sandra, K. Sandra, Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals, Anal. Chem. 88 (1) (2016) 480–507.
[12] D. Reusch, M. Haberger, D. Falck, B. Peter, B. Maier, J. Gassner, M. Hook, K. Wagner, L. Bonnington, P. Bulau, M. Wuhrer, Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: mass spectrometric methods, Mabs 7 (4) (2015) 732–742.
[13] E.A. Redman, N.G. Batz, J.S. Mellors, J.M. Ramsey, Integrated microfluidic capillary electrophoresis-electrospray ionization devices with online MS detection for the separation and characterization of intact monoclonal antibody variants, Anal. Chem. 87 (4) (2015) 2264–2272.
[14] A.M. Belov, R. Viner, M.R. Santos, D.M. Horn, M. Bern, B.L. Karger, A.R. Ivanov, Analysis of proteins, protein complexes, and organellar proteomes using sheathless capillary zone electrophoresis - native mass spectrometry, J. Am. Soc. Mass Spectrom. 28 (12) (2017) 2614–2634.
[15] R. Haselberg, T. De Vijlder, R. Heukers, M.J. Smit, E.P. Romijn, G.W. Somsen, E. Domínguez-Vega, Heterogeneity assessment of antibody-derived therapeutics at the intact and middle-up level by low-flow sheathless capillary electrophoresis-mass spectrometry, Anal. Chim. Acta 1044 (2018) 181–190.
[16] A. Beck, H. Liu, Macro- and micro-heterogeneity of natural and recombinant IgG antibodies, Antibodies 8 (1) (2019) 1–18.
[17] Y. Xu, D. Wang, B. Mason, T. Rossomando, N. Li, D. Liu, J.K. Cheung, W. Xu, S. Raghava, A. Katiyar, C. Nowak, T. Xiang, D.D. Dong, J. Sun, A. Beck, H. Liu, Structure, heterogeneity and developability assessment of therapeutic antibodies, mAbs 11 (2) (2019) 239–264.
[18] C. Nowak, J.K. Cheung, S.M. Dellatore, A. Katiyar, R. Bhat, J. Sun, G. Ponniah, A. Neill, B. Mason, A. Beck, H. Liu, Forced degradation of recombinant monoclonal antibodies: a practical guide, mAbs 9 (8) (2017) 1217–1230.
[19] A. Ambrogelly, S. Gozo, A. Katiyar, S. Dellatore, Y. Kune, R. Bhat, J. Sun, N. Li, D. Wang, C. Nowak, A. Neill, G. Ponniah, C. King, B. Mason, A. Beck, H. Liu, Analytical comparability study of recombinant monoclonal antibody therapeutics, mAbs 10 (4) (2018) 513–538.
[20] Y. Zhao, L. Sun, M.D. Knierman, N.J. Dovichi, Fast separation and analysis of reduced monoclonal antibodies with capillary zone electrophoresis coupled to mass spectrometry, Talanta 148 (2016) 529–533.
[21] R. Gahoual, A. Burr, J.M. Busnel, L. Kuhn, P. Hammann, A. Beck, Y.N. Franccois, E. Leize-Wagner, Rapid and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry, Mabs 5 (3) (2013) 479–490.
[22] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by capillary electrophoresis—mass spectrometry, J. Chromatogr. B 1011 (2016) 24–32.
[23] A. Lechner, J. Giorgetti, R. Gahoual, A. Beck, E. Leize-Wagner, Y.-N. François, Insights from capillary electrophoresis approaches for characterization of monoclonal antibodies and antibody drug conjugates in the period 2016–2018, J. Chromatogr. B 1122–1123 (2019) 1–17.
[24] J. Giorgetti, A. Lechner, E. Del Nero, A. Beck, Y.-N. François, E. Leize-Wagner, Intact monoclonal antibodies separation and analysis by sheathless capillary electrophoresis-mass spectrometry, Eur. J. Mass Spectrom. 25 (3) (2018) 324–332.
[25] R. Gahoual, A. Beck, E. Leize-Wagner, Y.-N. François, Cutting-edge capillary electrophoresis characterization of monoclonal antibodies and related products, J. Chromatogr. B 1032 (2016) 61–78.
[26] A.M. Belov, L. Zang, R. Sebastiano, M.R. Santos, D.R. Bush, B.L. Karger, A.R. Ivanov, Complementary middle-down and intact monoclonal antibody proteoform characterization by capillary zone electrophoresis – mass spectrometry, Electrophoresis 39 (16) (2018) 2069–2082.
[27] 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 1065 (Supplement C) (2017) 119–128.
[28] 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 (5) (2012) 578–585.
[29] A. Resemann, W. Jabs, A. Wiechmann, E. Wagner, O. Colas, W. Evers, E. Belau, L. Vorwerg, C. Evans, A. Beck, D. Suckau, Full validation of therapeutic antibody sequences by middle-up mass measurements and middle-down protein sequencing, mAbs 8 (2) (2016) 318–330.
[30] L. Fornelli, D. Ayoub, K. Aizikov, A. Beck, Y.O. Tsybin, Middle-down analysis of monoclonal antibodies with Electron transfer dissociation orbitrap fourier transform mass spectrometry, Anal. Chem. 86 (6) (2014) 3005–3012.
[31] J. Dai, Y. Zhang, A middle-up approach with online capillary isoelectric Focusing/Mass spectrometry for in-depth characterization of cetuximab charge heterogeneity, Anal. Chem. 90 (24) (2018) 14527–14534.
[32] R. Gahoual, J.-M. Busnel, A. Beck, Y.-N. François, E. Leize-Wagner, Full antibody primary structure and microvariant characterization in a single injection using transient isotachophoresis and sheathless capillary electrophoresis–Tandem mass spectrometry, Anal. Chem. 86 (18) (2014) 9074–9081.
[33] J. Giorgetti, V. D'Atri, J. Canonge, A. Lechner, D. Guillarme, O. Colas, E. Wagner-Rousset, A. Beck, E. Leize-Wagner, Y.-N. François, Monoclonal antibody N-glycosylation profiling using capillary electrophoresis – mass spectrometry: assessment and method validation, Talanta 178 (Supplement C) (2018) 530–537.