Journal of Pharmaceutical and Biomedical Analysis 185 (2020) 113218

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

In-depth analysis of monoclonal antibodies using microfluidic capillary electrophoresis and native mass spectrometry

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ARTICLE INFO

Article history:
Received 13 December 2019
Received in revised form 27 February 2020
Accepted 28 February 2020
Available online 29 February 2020

Keywords:
Native MS
Charge variant
Capillary electrophoresis
Rituximab
Trastuzumab
Bevacizumab

ABSTRACT

Charge variant profiling of therapeutic proteins is required by the International Council for Harmonisation guidelines and is traditionally performed by capillary electrophoresis or ion exchange chromatography. Recently, improvements in the hyphenation of capillary electrophoresis with mass spectrometry and the introduction of mass spectrometry compatible background electrolytes has allowed the implementation of native mass spectrometric determination of the charge variant profile obtained from the electrophoretic separation. The low flow operation of the microfluidic electrophoretic platform significantly boosts mass spectrometric sensitivity and increases the dynamic range, even when using sample amounts as low as 1 ng in capillary. In the current study, rituximab, trastuzumab and bevacizumab drug products were analysed using the ZipChip microfluidic CE-ESI-MS platform that facilitated confident identification of proteoforms with an average mass accuracy of <15 ppm. Up to 52 proteoforms were identified for trastuzumab drug product, while rituximab sample revealed the presence of fragments and sialylated N-glycans. Overall, the CE-ESI-MS platform proved to be a fast and robust tool for therapeutic protein charge variant profiling and facilitated efficient coupling with native mass spectrometry for the generation of highly informative characterisation data.

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1. Introduction

The biopharmaceutical industry continues to develop mAb (monoclonal antibody)-based biotherapeutics for several applications and disease treatments, progressing from oncology and autoimmune treatments to new medicines for inflammatory and cardiovascular disorders [1]. Nevertheless, the complexity of these molecules creates a significant challenge for analytical technologies to monitor product quality attributes (PQAs) that need to be controlled to guarantee safety and efficacy of the therapeutic product. According to International Council for Harmonisation guidelines, one of the features that needs to be monitored during biopharmaceutical development and batch release is the charge variant (CV) profile. Charge variation is generally caused by post translational modifications (PTMs), which can influence the overall surface charge and isoelectric point (pI) of the protein [2]. Standardized methods include the use of isoelectric focusing (iEF) or the use of ion exchange liquid chromatography using UV detection (IEX-UV) [2–6] as well as capillary electrophoresis, where electrophoretic mobility of the molecules depends on several factors such as their charge, size and conformation as a function of their hydrodynamic radius.

The development of native mass spectrometry (MS) hyphenated to IEX and CE separation methods, using electrospray ionization (ESI) and high resolution Orbitrap MS instruments, has facilitated the direct identification of CV proteoforms of these complex therapeutics [7,8]. Native MS offers several advantages that include fast analysis and minimal sample preparation; this results in reduced artificially induced modification on the analyte and avoids low reproducibility with respect to more laborious sample preparation heavy workflows (such as peptide mapping or released N-glycan analysis). Another key advantage of native MS is the lower amount of charges present on the protein in its native conformation; as a consequence the generated charge envelope is locate at higher *m/z* values, providing higher spectral spatial resolution for variants having small mass differences [9]. As such, for large proteins like mAbs, mass spectra in native mode need to be acquired on instruments able to assure high mass resolution also in this extended mass range, usually ranging between 4,000 and 8,000 *m/z*.

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https://doi.org/10.1016/j.jpba.2020.113218
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CV separation coupled with native mass spectrometry further enhances proteoform analysis, leading to the confident identification and quantitation of variants exhibiting very small mass shifts, such as deamidation, as it modifies the overall net surface charge of the mAb thereby enabling the modified form to be chromatographically or electrophoretically separated from the main proteoform [10,11].

In the present study, we investigated three widely used IgG1 monoclonal antibodies [12] using microfluidic CE-ESI-MS for the acquisition of native MS spectra. The three biotherapeutics analysed in this study are IgG1 mAbs expressed in CHO cell lines. In particular we analysed rituximab, bevacizumab and trastuzumab drug products, which ranked among the top-selling biotherapeutics in the last decade.

The native MS data acquired using the CE-ESI-MS platform was found to be robust and reliable for CV profiling having several advantages, which include fast and easy sample preparation and a universal MS method for several analytes. Moreover, it was possible to determine MS identity of each species with high data quality, allowing accurate determination of certain low abundant PTMs (<1%) with good reproducibility across replicate analysis. The amount of protein used was as low as 1 ng, proving sensitivity and robustness of the platform and an increased dynamic range with respect to other native methodologies. The features analysed using CE-ESI-MS included, but were not limited to, main PQAs such as deamidation, lysine clipping, pyro-glutamate formation as well as N-glycan profiling and identification of mAb degradation products.

The data presented herein, demonstrate the applicability of CE-ESI-MS for both routine sample analysis and comparability with standards for batch-to-batch control analysis making the CE-ESI-MS platform a solid tool for high throughput therapeutic protein analysis to obtain extensive structural information and for orthogonal analysis to other techniques such as peptide mapping.

2. Materials and methods

2.1. Materials

Rituximab, bevacizumab and trastuzumab drug products were kindly provided by the Hospital Pharmacy Unit of the University Hospital of San Cecilio in Granada, Spain and frozen at −80 °C in aliquots for further study.

2.2. CE-MS analysis

100 μg of each monoclonal antibody were buffer-exchanged with ZipChip® Native Diluent (908 Devices Inc., Boston, MA, USA) in 0.5 mL spin-filters with 10 KDa molecular weight cut-off and brought to a concentration of 0.5 mg·mL^{-1}. Microfluidic CE separation was carried on a ZipChip™ platform (908 Devices Inc., Boston, MA, USA) using an HRN (high resolution native) microchip and background electrolyte (908 Devices Inc., Boston, MA, USA) provided in the Native antibody Kit, compatible with native MS analysis. BGE provided with Native antibody kit is a proprietary formulation based on ammonium acetate at pH 5.5 and it was modified with 4% DMSO [13]. Chips feature a 22 cm long channel and are made of glass; channel surfaces are treated with a covalent coating to suppress the electroosmotic flow (EOF) and minimize adsorption of biomolecules [14]. No conditioning step is required and no regeneration step is performed between runs. Chips were only primed before the first use on each day and dried after data acquisition for overnight storage. CE method was optimized using the default method in ZipChip software as a starting point: in particular CE analysis time was adjusted to 15 min and an injection of 2 nL was used instead of 1 nL. Field strength of 500 V was used to perform CE analysis. The system was coupled to a Q Exactive™ Plus hybrid quadrupole Orbitrap mass spectrometer with extended mass Biopharma Option (Thermo Scientific, Bremen, Germany). Tune parameters for MS analysis were: sheath gas 2 au (arbitrary units), in-source CID 150 V to reduce the generation of adducts caused by salts present in BGE formulation, S-lens 200 V, acquisition gain control 1×10^6, inlet capillary temperature 200 °C, HMR mode on. ESI spray voltage at the emitter is not user defined and is fixed at 3.5 kV. Scan parameters were as follows: resolution 35,000, 5 microscans, max injection time 20 ms and mass range between 2,500 and 8,000 *m/z*. Each sample analysis was performed in triplicate after sample well wash.

2.3. Native MS spectra data analysis

Raw data were analysed using BioPharma Finder 3.0 software. Spectra were deconvoluted using Xtract™ algorithm for non-isotopically resolved spectra with the following settings: output mass range between 146,000 and 150,000 Da, charge state range between 20 and 50, minimum adjacent charge states set to 5, deconvolution mass tolerance is 15 ppm, noise threshold 5%. Retention time range of the peaks separated in the electropherograms were individually deconvoluted and same time range was used for triplicate analysis. Only components present in triplicates were evaluated. For each monoclonal antibody an N-glycan database obtained from previous analysis was employed to support identification, along with peptide mapping data generated in our laboratory.

3. Results and discussion

CV profiling of the three monoclonal antibodies analysed was performed within 15 min (Fig. 1A–C with excellent resolution and reproducibility across replicates. Charge envelopes from the native proteins were obtained in the range between 4,000 and 7,000 *m/z* (Fig. 1D–F) with baseline resolution of the variants deriving from N-glycosylation heterogeneity for each charge state (zoom in Fig. 1D–F). Mass measurement of intact proteins is challenging due to the isotopic distribution used for calculated theoretical average masses and the presence of adducts, especially in native mass spectrometry [15]. Mass accuracy expected for these analysis is strongly dependent on MS analyser technology [16], with Orbitrap based mass analysers capable of routinely achieving mass deviations <10 ppm [8,10]. In order to perform a confident identification, database of PQAs generated through released N-glycan and peptide mapping analysis was used to discriminate when more than one composition was possible. Moreover, knowing the elution order of CVs, it is possible to further discriminate between near-isobaric species and to allow a greater range for mass deviation that would not be acceptable for other techniques, such as reverse phase LC–MS analysis of intact mAbs.

3.1. CE-ESI-MS analysis of rituximab drug product

Rituximab is a biotherapeutic targeting the CD20 antigen present on malignant B lymphocytes. Since its patent expired, a number of biosimilars have been already approved for therapeutic treatment, also increasing the demand for the development of analytical tools for biosimilarity assessment at both the structural and functional levels. Previous studies have explored rituximab variants and heterogeneity also using charge-based separation methods, minimal data has so far been presented that allows efficient and confident identification and quantification of each proteoforms present in the profile [17–19]. Rituximab drug product is known to possess charge heterogeneity arising due to N-terminal pyro-glutamate formation on both light and heavy chain and

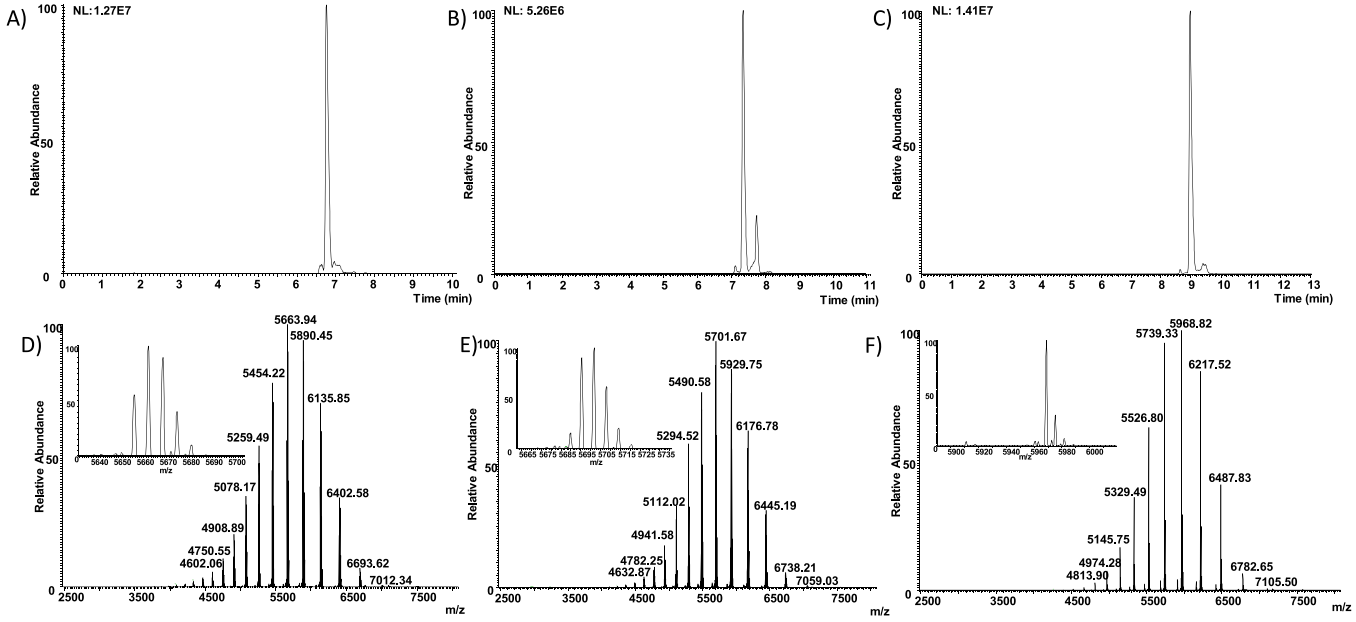


Fig. 1. Electropherograms of the CV profiles obtained for rituximab (A), trastuzumab (B) and bevacizumab (C). Panels D, E, F show the charge envelopes relative to each mAb and a zoom of the most abundant charge state.

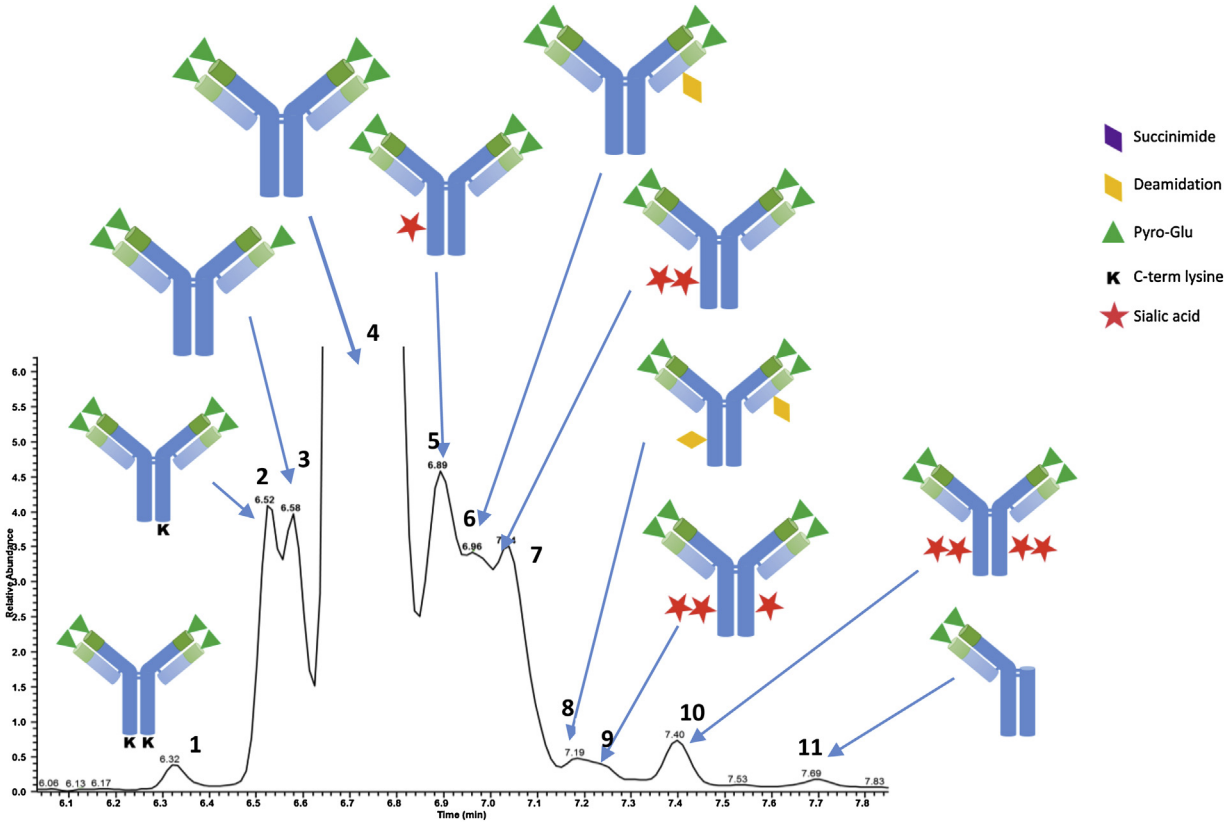


Fig. 2. Zoom of the CE-ESI analysis on rituximab drug product. The electropherogram shows the separation of the CVs; the 11 peaks identified are marked showing the modification responsible of the charge shift towards more basic or acids species with respect to the main peak.

incomplete cleavage of C-terminal lysine during bioproduction. Both modifications have not been reported to affect either product stability or efficacy and therefore are not considered critical quality attributes [20]. Nevertheless, these modifications can affect the CV profile of the drug product and as a result it is important to monitor the peaks generated by these modifications to ensure lot-to-lot consistency. More importance is given to rituximab Fc glycosylation where this may influence its mechanism of action and have serious impact on biological activity [21].

The data obtained using the CE-ESI-MS platform (Fig. 2, Table S1) allowed confident identification of 45 different proteoforms for rituximab, which include 10 different CVs with their intrinsic

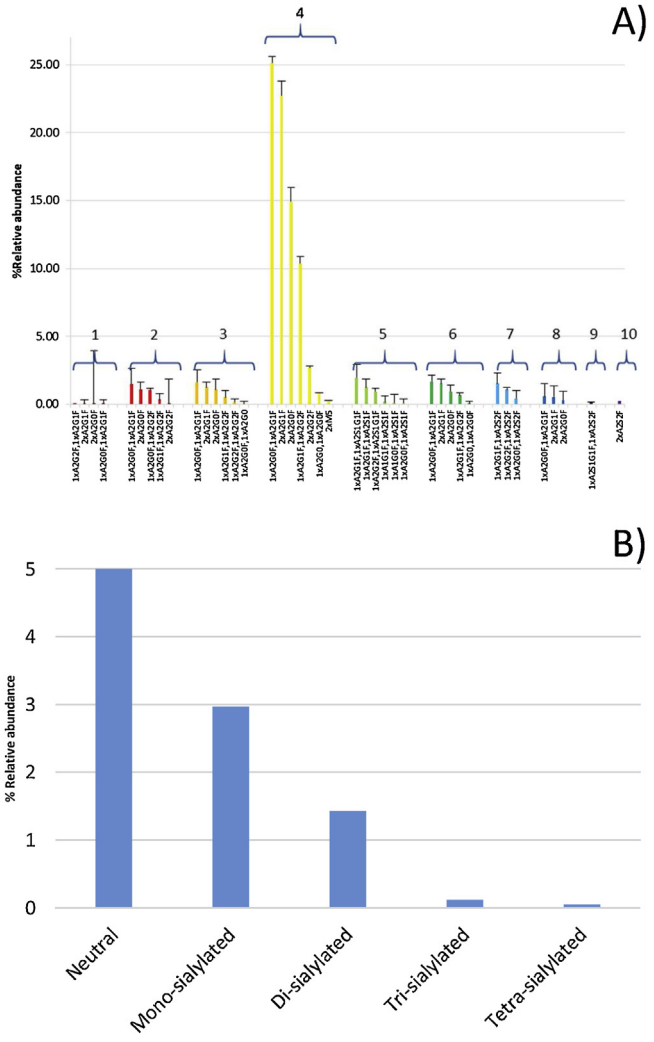


Fig. 3. Bar-graph for rituximab drug product charge variants relative abundance (A). The ten groups, indicated with different colors, correspond to the CV species separated in the CE-ESI analysis. Moreover, for each group, N-glycan heterogeneity is shown and proves to be reproducible across all the charge variants. Zoom of the bar-graph for N-glycan sialylated variants relative abundance (B) present on both Fc regions.

N-glycan heterogeneity and one mAb fragment. CVs in the basic region were identified as possessing one or both C-terminal lysines (Peak 1 and 2, Fig. 2) and charge contribution also derived from the absence of one N-terminal pyro-glutamic acid (Peak 3, Fig. 2). Acidic variants present in rituximab drug product were identified as species possessing one or two deamidation events (Peaks 6 and 9, Fig. 2) and presenting sialylated structures in the Fc N-glycan profile (Peaks 5, 7, 8 and 10, Fig. 2). For each peak it was possible to assign complex heterogeneity deriving from N-glycosylation of the Fc region, accounting for up to seven variants (Fig. 3A). Although sialylated variants are usually difficult to identify at intact level due to their lower ionization potential in positive mode and their low abundance on mAbs, it was possible to confidently identify and relatively quantify these species (Fig. 3 B, Table S1). The monoclonal antibody fragment (peak 11) showed to have a mass of ~ 100 KDa, possessing a complete Fc region and only one arm of the Fab region, where the hydrolysis site is located between T and H residues in upper hinge region; this site is known to be prone to hydrolysis and β-elimination but could also be the site of action for Cathepsin L1 enzyme, often present in biotherapeutics formulation as an host cell protein carried over during downstream process [10,22].

3.2. CE-ESI-MS analysis of trastuzumab drug product

Trastuzumab (Herceptin®) is a recombinant humanized IgG1 kappa targeting the extracellular domain of the human epidermal growth factor receptor protein [23]. Herceptin received approval from the FDA in 1998. A biosimilar of trastuzumab is already available on the market; the FDA approved Ogivri™ (trastuzumab-dkst) for the treatment of patients with breast or metastatic stomach cancer whose tumors overexpress the HER2 gene (HER2+) [23]. Trastuzumab presents two well-known sites for deamidation in the variable region [24]. Asn30 in the light chain is the most abundant site subject to deamidation in trastuzumab and is caused by the slightly acidic conditions of the drug formulation, though not leading to any significant impact on IgG1 activity [24]. Asn55 is a deamidation site commonly found in IgG1 and it is located in the CDR (complementarity determining region), thus important for antigen recognition [25]. CV profiling could help identify and quantify these variants; deamidation results in a decrease of pI value and a small mass increase of 0.98 Da, which is difficult to resolve at intact level without having upfront separation prior to native MS. CE-MS analysis performed in this study on trastuzumab drug product revealed the presence of 8 peaks, each presenting N-glycan heterogeneity for a total of 52 different proteoforms identified with high confidence, with an average deviation from the theoretical mass of 15 ppm (Fig. 4, Table S2). Peaks 1–3 represent more basic variant and include species presenting only one or both C-terminal lysine (peaks 1 and 3) and one proteoform presenting a succinimide residue which is an intermediate step in the deamidation/isomerization pathway. In the main peak (peak 4) it is possible to identify six N-glycan variants with the most abundant being the proteoforms presenting 2xA2G0F and A2G0F/A2G1F N-glycans. At higher migration times 4 peaks were identified; peak 5 corresponded to species presenting one sialic acid, while peak 6 represented the variant with one deamidation at Asn30 in the light chain and possibly one more deamidated variants forming a shoulder of peak 6 (Fig. 4). Peak 7 corresponded to the deamidated version of peak 5, presenting a sialic acid in the N-glycan moiety. Peak 8 was identified as a double deamidated proteoform, presenting the same split shape as peak 6.

Trastuzumab CV profile was recently characterized by mean of CVA-MS analysis [11]; Bailey et al. identified most of the species also identified in the present study while characterizing also isoaspartic acid variants where the conversion to isoaspartate, while not changing the overall pI, can influence the local tertiary structure modifying the surface protein charge. Through our CE-MS methodology it was not possible to identify these species. To verify their presence an extracted ion chromatogram (XIC) was generated setting a mass tolerance of 20 ppm to include any isobaric or near-isobaric species. In this way the XIC for the most abundant N-glycan variant of the most abundant charge state in the main peak (5,701.67 *m/z*) was obtained. In this simplified electropherogram it was possible to identify the deamidated and double deamidated species, together with the main peak and one additional shoulder on the main peak (data not shown). In this instance the CE-MS platform did not provide an efficient separation. Nevertheless, the dynamic range of our analysis is improved respect to previously reported data, as on each variant more proteoforms derived from N-glycosylation were identified for low abundant species.

3.3. CE-ESI-MS analysis of bevacizumab drug product

Bevacizumab (Avastin®) is recombinant humanized monoclonal antibody therapeutic targeting and inhibiting vascular endothelial growth factor A (VEGF-A) which is involved in angiogenesis stimulation across a number of diseases [26,27]. Bevacizumab was the first available angiogenesis inhibitor in the United States and it has

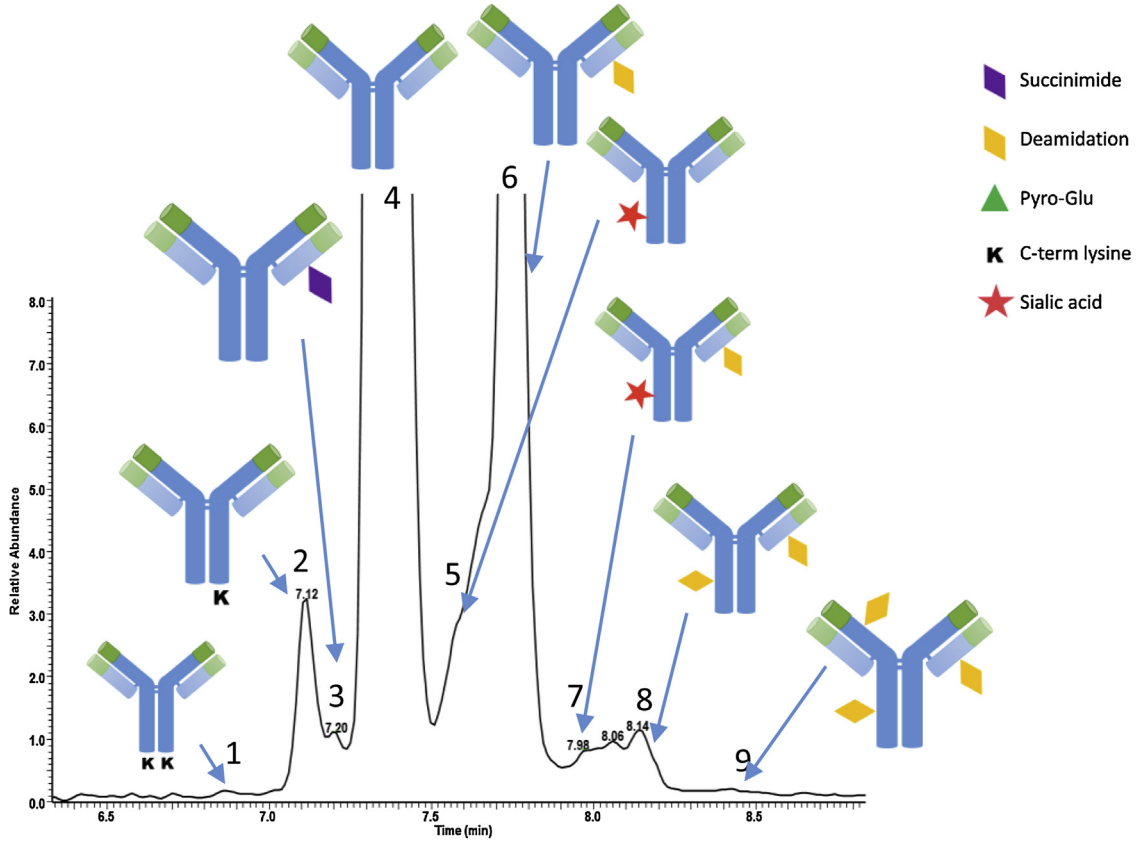


Fig. 4. Zoom of the CE-MS analysis of trastuzumab drug product. Peaks identified in Table S2 are indicated on the electropherogram and described with a picture having the corresponding CV modification.

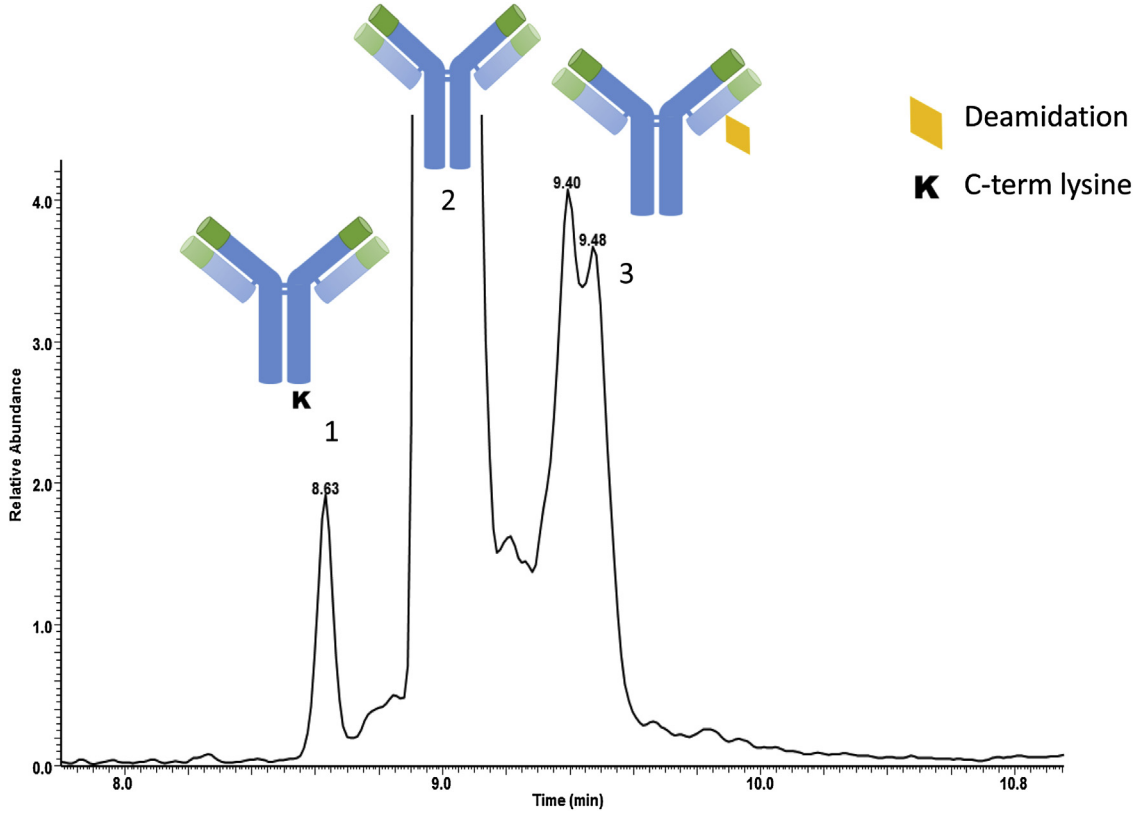


Fig. 5. Zoom of the CE-MS analysis of bevacizumab drug product. Peaks identified in Table S3 are indicated on the electropherogram and described with a picture having the corresponding CV modification.

been approved in 2004. CE-MS analysis of bevacizumab drug product revealed a simpler profile when compared to the other drugs analysed in this study. Only 3 peaks were detected (Fig. 5, Table S3), all with a relative abundance lower than 4% compared to the main peak. Peak 1 consisted of a lysine variant, with only one lysine on the C-terminal of the two heavy chains. Peak 2 was the main peak and was characterized by absence of C-terminal lysines and an extended N-glycan heterogeneity due to several degrees of galactosylation. Acidic peak 3 corresponded to deamidated species that were thought to be located on two different sites causing a split of the deamidated peak. Minor peaks were visible between the main and deamidated peaks though it was not possible to obtain their composition. This lead to the conclusion that they could be due to isoAsp variants, which was reported to have an abundance around 1% from previous data obtained through peptide mapping (data not shown). Previous studies [20] have also revealed the presence of species lacking one N-glycan for bevacizumab drug product, which was also observed herein with this heterogeneity noted in all three peaks. The same study also reveals additional heterogeneity due to the presence of glycation, however this modification was not investigated in the present study as the pI change due to glycation is not able to cause a shift in pI value of the protein.

3.4. Evaluation of CE-ESI-MS platform performance

The analysis of rituximab, trastuzumab and bevacizumab drug products by microfluidic CE-MS analysis showed high complexity and variety of proteoforms, ranging from deamidation to N-glycan variants bearing sialic acid and N- and C-terminal modifications. Overall the technique proved to be reliable, fast and especially sensitive; due to the low flow rate used in the analysis it was possible to improve CV analysis dynamic range and this allowed the identification with high confidence of species as low 0.01 % using only 1 ng of sample.

4. Conclusions

Following the widely accepted and implemented use of CE, CiEF and CZE for CV profiling of biopharmaceuticals, various approaches have been reported to hyphenate MS mass measurement to this separation technique [15,28–30]. Although many successful applications delivering robust and accurate data have been described, most of the platforms are complex in terms of assembly and operation, returning poorly resolved profiles with only proteoforms present in the main peak identified with acceptable mass accuracies [15]. The improved results of the platform presented herein are facilitated by the performance of the Orbitrap MS technology that enables operation at higher resolution settings and improved sensitivity and signal-to-noise ratio when compared to QTOF technology. The possibility to keep mAbs in their native conformation is a key feature of the CE-ESI-MS platform presented herein, with all the advantages of native mass spectrometry already listed in terms of MS signal spatial resolution. In this study, the heterogeneity of rituximab, trastuzumab and bevacizumab drug products was investigated. Simple and fast analysis was performed with minimal method optimization and sample preparation, achieving the confident identification of up to 52 proteoforms in trastuzumab drug product, as well as mAb fragments in rituximab drug product using the ZipChip microfluidic CE-ESI-MS platform for native CV profiling. CV profiles obtained in this study showed improved separation resolution, critical to support MS identification of near-isobaric variants such as deamidated forms, and good comparability with previously reported profiles obtained using IEX chromatography, even though slightly different selectivity could be observed, such as for iso-Asp variant in trastuzumab. A key advantage of the CE-ESI-MS platform was the low sample consumption and increased dynamic range, when compared to similar LC–MS applications; accurate MS determination with an average mass error of 15 ppm was achieved, identifying proteoforms as low as 0.01 %. The performance achieved during this study enables deep characterization of monoclonal antibodies, enhanced by the upfront separation of CVs prior native MS and full exploitation of MS capabilities in terms of sensitivity, enhanced by the use of low sample introduction flow rates when compared to LC–MS methods.

Moreover, the platform used in this study can enable a deeper characterization of therapeutic protein heterogeneity even at early stages of the development pipeline when samples amount is limited such as during cell line development, clonal selection or early stage developability assessment.

Author contributions

Sara Carillo and Jonathan Bones conceptualized the research and generated the experimental plan.

Sara Carillo and Craig Jakes contributed to the generation and processing of the experimental data.

Sara Carillo, Craig Jakes and Jonathan Bones drafted the manuscript.

All authors then edited the manuscript and contributed to the subsequent drafts and approved the final version for submission.

Declaration of Competing Interest

Authors declare that there are no conflicts of interest.

Acknowledgments

Ashley Bell, Erin Redman and Trent Basarsky from 908 Devices are gratefully acknowledged for instrument access and technical assistance with the ZipChip instrument used to generate the experimental data. S.C., C.J. and J.B. acknowledge collaborative funding support from Thermo Fisher Scientific and access to the Q Exactive MS instrumentation used during the current study.

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.2020.113218.

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