

# Full Antibody Primary Structure and Microvariant Characterization in a Single Injection Using Transient Isotachopheresis and Sheathless Capillary Electrophoresis–Tandem Mass Spectrometry

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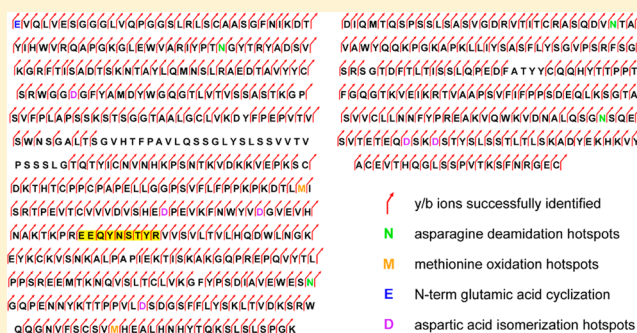
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## Supporting Information

**ABSTRACT:** Here we report the complete characterization of the primary structure of a multimeric glycoprotein in a single analysis by capillary electrophoresis (CE) coupled to mass spectrometry (MS). CE was coupled to electrospray ionization tandem MS by means of a sheathless interface. Transient isotachopheresis (t-ITP) was introduced in this work as an electrokinetically based preconcentration technique, allowing injection of up to 25% of the total capillary volume. Characterization was based on an adapted bottom-up proteomic strategy. Using trypsin as the sole proteolytic enzyme and data from a single injection per considered protein, 100% of the amino acid sequences of four different monoclonal antibodies could be achieved. Furthermore, illustrating the effectiveness and overall capabilities of the technique, the results were possible through identification of peptides without tryptic miscleavages or posttranslational modifications, demonstrating the potency of the technique. In addition to full sequence coverages, posttranslational modifications (PTMs) were simultaneously identified, further demonstrating the capacity of this strategy to structurally characterize glycosylations as well as faint modifications such as asparagine deamidation or aspartic acid isomerization. Together with the exquisite detection sensitivity observed, the contributions of both the CE separation mechanism and selectivity were essential to the result of the characterization with regard to that achieved with conventional MS strategies. The quality of the results indicates that recent improvements in interfacing CE-MS coupling, leading to a considerably improved sensitivity, allows characterization of the primary structure of proteins in a robust and faster manner. Taken together, these results open new research avenues for characterization of proteins through MS.



Capillary electrophoresis (CE) was commercially introduced as a separation technique during the early 1980s,<sup>1–3</sup> though electrokinetically driven separation strategies have been applied in laboratories since the beginning of the 20th century.<sup>4,5</sup> In CE, analytes are separated under an electrical field; this technique has some major advantages including the possibility to obtain separations within minutes while maintaining exceptional separation efficiency. This is partially explained by the absence of a stationary phase, tremendously reducing the longitudinal dispersion responsible for peak broadening. Despite the considerable effort made regarding instrumental development, platforms combining CE and electrospray ionization (ESI) mass spectrometry (MS) are still marginally used as compared to chromatography-based methods. This is mainly related to the difficulty to maintain the CE electrical field while positioning the capillary outlet inside the ESI source. Another aspect is related to the fact that CE-

ESI-MS platforms rarely provide optimal sensitivity, as common interfaces rely on strategies that by nature induce losses of sensitivity.<sup>6,7</sup> On the other hand, coupling of high-performance liquid chromatography (HPLC) with MS is more straightforward and tends to be preferentially used for separation ahead of MS due to its ease of coupling and excellent robustness. However, as ESI-MS has demonstrated its suitability for the study of biological samples such as protein and peptides,<sup>8,9</sup> electrophoresis should be theoretically the preferred separation technique for biological samples especially because it is a miniaturized technique which should favor the formation of a nanoESI, thus enhancing the ionization process. Biologists are routinely using electrophoresis to reduce the

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complexity of samples or to isolate proteins<sup>10</sup> and likewise DNA<sup>11,12</sup> or RNA.<sup>13,14</sup> Recently a novel sheathless interface was introduced for CE-ESI-MS hyphenation.<sup>15</sup> It is originally based on a sheathless design by Moini et al.<sup>16</sup> and is here referred to as CESI-MS. It allows the hyphenation of CE to ESI-MS without sacrificing the sensitivity because it does not require any sheath liquid to maintain the electrical contact, which would otherwise be responsible for analyte dilution. Separation performances and characteristics of CE are in terms of efficiency and selectivity, well applicable to the range of analytes that are typically well separated by reverse phase liquid chromatography (RP-LC). Additionally, CE could also alleviate some of the drawbacks usually encountered when using RP-LC such as separation and elution of very small and hydrophilic peptides that may elute with the dead volume in RP-LC or large ones that could be adsorbed irreversibly on the stationary phase. Other groups have recently shown that the implementation of an electrophoretic separation, prior to the MS analysis, could benefit bottom-up proteomics analysis compared to conventional methodologies.<sup>17,18</sup> Here we are reporting the capacity of t-IPTP CESI-MS/MS methodology to enable the complete amino acid (AA) sequence characterization for a protein in a single injection. Transient isotachopheresis (t-IPTP) is an electrokinetic-based preconcentration process, commonly used in CZE, which allows for larger sample injections without any detrimental effect on separation efficiency. Contrarily, the integration of t-IPTP often enables an improvement of separation efficiency as compared to conventional CZE.<sup>19</sup> In t-IPTP, the sample buffer used has an electrophoretic mobility superior to that of the background electrolyte (BGE); under the electrical field applied during the separation, the sample content is stacked in a reduced capillary volume compared to the actual injected volume. The use of t-IPTP allows for the injection of significantly larger volumes without losing separation efficiency: maximum of 25% of the total capillary volume while only 1–2% in conventional CZE.<sup>15</sup>

mAbs (monoclonal antibodies) are tetrameric glycoproteins having a molecular mass of approximately 150 kDa. They are composed of two heavy chains (HCs) and two light chains (LCs) linked to each other by several disulfide bonds. The HC bears at least one N-glycosylation site.<sup>20</sup> The first monoclonal antibody (mAb) studied here was trastuzumab, which is approved for the treatment of HER2-positive breast cancer,<sup>21</sup> and the second antibody studied was cetuximab, directed against epidermal growth factor receptor (EGFR) and used to treat colorectal, head, and neck cancer.<sup>22,23</sup> From an analytical standpoint, these proteins have an interesting trait due to their structural complexity. They present a large number of microheterogeneities commonly found in proteins such as posttranslational modifications (PTMs) including glycosylations and small chemical modifications.<sup>24</sup> Four mAbs were studied. Along with the characterization of the AA sequence, other aspects of the primary structure of the studied proteins could be characterized with an unprecedented reliability. Separation mechanisms provided by CE demonstrated their utility for protein characterization by MS, as it has been possible to separate peptides having only minor differences as small as one AA conformational change.

## EXPERIMENTAL SECTION

**Materials.** Chemicals used were of analytical grade or high purity grade and purchased from Sigma-Aldrich (Saint Louis, MO). Water used to prepare buffers and sample solutions was

obtained using an ELGA Purelab UHQ PS water purification system (Bucks, UK). Trastuzumab and cetuximab samples are EMA/FDA-approved formulations purchased, respectively, from Genentech (San Francisco, CA) and Merck (Whitehouse Station, NJ). RapiGest SF surfactant was purchased from Waters (Milford, MA).

**Sample Preparation.** For each mAb sample, a volume corresponding to 100  $\mu\text{g}$  of protein was sampled using the final formulation for the approved mAbs (trastuzumab and cetuximab) and samples coming directly from the bioreactor for the mAbs in development samples. Samples were diluted using Milli-Q water to a final concentration of 6.7  $\mu\text{g}/\mu\text{L}$ . Samples were then diluted using 0.1% RapiGest surfactant to a final concentration of 3.35  $\mu\text{g}/\mu\text{L}$  and heated to 40 °C during 10 min. Dithiothreitol (DTT) was added to the sample to obtain a final concentration of 25 mM. Samples were then heated to 95 °C during 5 min. After the sample was cooled to room temperature (RT), iodoacetamide (IDA) was added to a final concentration of 10 mM. Afterward, samples were placed in the dark for 20 min to allow alkylation of cysteine (Cys). A volume of 1  $\mu\text{L}$  of trypsin (0.5  $\mu\text{g}/\mu\text{L}$ ) was added to the sample, which was left at room temperature for 3 h, and another volume of 1  $\mu\text{L}$  was added afterward. Digestion was performed overnight at 37 °C. After digestion was complete, formic acid (FA) was added to the samples at a final concentration of 1% (v/v) to cleave the surfactant, and samples were left at RT for 2 h. Finally, samples were diluted to a final protein concentration of 2.2  $\mu\text{M}$  using 50 mM ammonium acetate (pH 4.0).

**Capillary Electrophoresis.** The CE experiments were performed with a PA 800 Plus capillary electrophoresis system from Beckman Coulter equipped with a temperature-controlled autosampler and a power supply able to deliver up to 30 kV. Hyphenation was realized using a CESI prototype made available by Sciex Separations (Brea, CA). The prototype of bare fused-silica capillaries (total length 100 cm; 30  $\mu\text{m}$  i.d.) had a characteristic porous tip of 3 cm on the end, and a second capillary (total length 80 cm; 50  $\mu\text{m}$  i.d.) filled during experiments with BGE allows electric contact. The new capillaries were flushed for 10 min at 75 psi (5.17 bar) with methanol and then 10 min with 0.1 M sodium hydroxide, followed by 10 min with 0.1 M hydrochloric acid and 20 min with water also at 75 psi. Finally, the capillary was flushed 10 min at 75 psi with BGE which was 10% acetic acid. Hydrodynamic injection (410 mbar for 1 min) corresponding to a total volume of 90 nL of sample injected was used. Separations were performed using a voltage of +20 kV.

**Mass Spectrometry.** For antibody characterization, the CESI system was hyphenated to a 5600 TripleTOF mass spectrometer (AB Sciex, Darmstadt, Germany). The 5600 MS was equipped with a hybrid analyzer composed of quadrupoles followed by a time-of-flight (TOF) analyzer. ESI source parameters were set as follows: ESI voltage –1.75 kV while gas supplies (GS1 and GS2) were deactivated, source heating temperature 150 °C, and curtain gas value 5. Experiments were performed in Top15 information-dependent acquisition (IDA), and accumulation time was 250 ms for MS scans and 100 ms for MS/MS scans, leading to a total duty cycle of 1.75 s. Mass/charge ( $m/z$ ) range was 100–2000 in MS and 50–2000 in MS/MS. Using those parameters, the mean resolution provided by the instrument was 40 000 in MS ( $m/z$  485.251) and 25 000 in MS/MS ( $m/z$  345.235).

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDT  
YIHVVRRQAPGKGLEWVARIYPTNGYTRYADSV  
KGRFTISADTSKNTAYLQMNSLRRAEDTAVYYC  
SRWGGDGFYAMDYWGQGLTIVTSSASTKGP  
SVFLAPSSKSTSGGTAALGCLVKDYFPEPVTV  
SWNSGALTSVHTFPAVLQSSGLYSLSSVTV  
PSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC  
DKTHTCPPCPAPELGGPSVFLFPPKPKDTLMI  
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH  
NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK  
EYCKVSNKALPAPIEKTISKAKGQPREPQVYTL  
PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN  
GQPENNYKTPPVLDSDGSFFLYSKLTVDKSRW  
QQGNVFSCSVMHEALHNHYTQKLSLSPGK

DIQMTQSPSSLSASVGRVTITCRASQDVNTA  
VAWYQQKPKGAPKLLIYSASFLYSGVPSRFSG  
SRSGTDFLTITSSLPEDFATYYCQQHYTTPPT  
FGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA  
SVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTETEQDSKSTYLSLSTLTLKADYEKHKVY  
ACEVTHQGLSSPVTKSFNRGEC

**Figure 1.** Sequence coverage obtained for trastuzumab by CESI-MS/MS methodology. Experimental conditions: 90 nL injected (200 fmol). CESI-MS/MS spectra recorded on 5600 TripleTOF (AB Sciex, San Francisco, CA). Constant domain (blue), variable domain (orange), and complementarity determining region (red) are represented in the heavy chain and light chain.

MS/MS data analysis: Data obtained from the CESI-MS/MS experiments were analyzed using Peakview software (AB Sciex, San Francisco, CA). Purely tryptic peptides (without miscleavages or PTMs except for cys carbamidomethylation) were determined theoretically from considered mAb amino acid sequences available through the literature. Additional peptides were identified using Mascot search engine provided by Matrix Science; tryptic cleavage rules were applied. Carbamidomethylation of cysteine (+57.02 Da) and N-deamidation of aspartic/isoaspartic acid (+0.985 Da) or succinimide intermediate (−17.03 Da) were selected as variable modifications. Methionine oxidation (+15.99 Da) and N-terminal glutamic acid cyclization (−17.02 Da) were also selected as variable modifications. The mass tolerance allowed for search algorithm identification was set to  $\pm 5$  ppm for precursor ions and  $\pm 0.05$  Da for fragmentation ions.

## RESULTS AND DISCUSSION

mAbs were characterized in a bottom-up proteomic adapted strategy, and samples were digested by trypsin using an in-solution digestion protocol. We reported previously the development of a method using sheathless CE-ESI-MS/MS for monoclonal antibody characterization.<sup>25</sup> In the current work the methodology was significantly modified to improve the level of characterization, especially regarding the amino acid sequence and glycoforms. The digestion protocol was modified to enhance proteolytic digestion efficiency. Additionally, the changes increased the compatibility of the sample's content to capillary zone electrophoresis (CZE) and transient isotachopheresis (t-ITP) while also controlling the matrix effect. The sample preparation was conducted without any desalting treatment to prevent any potential loss of peptides due to either poor or irreversible retention during reverse-phase solid-phase extraction (SPE). After digestion, the sample was diluted to a final concentration of 2.2  $\mu$ M in ammonium acetate (50 mM, pH 4.0). Ammonium acetate was chosen as a sample matrix for its compatibility with both ESI-MS and t-ITP. The separation was performed under an electrical field of 210 V/cm

in a background electrolyte (BGE) composed of 10% acetic acid. This BGE has two advantages; it presents a rather low conductivity, as it is not a strong acid, and it is fully compatible with the ESI ionization process. mAb digests were analyzed through CESI-MS/MS, and the injection volume corresponded to a quantity of 200 fmol of digested peptides. Peptide identification was performed through a peptide fragment fingerprinting (PFF) strategy where peptides are identified based on their complete molecular mass and fragmentation pattern with a mass accuracy systematically better than 5 ppm.

As emphasized in Figure 1, the CESI-MS/MS analysis of the mAb tryptic digest allowed us, in a single injection, to obtain 100% sequence characterization for both the HC and LC. Furthermore, the full sequence characterization could be performed exclusively through identification of peptides without PTMs or miscleavages. To our knowledge, this is the first time that a protein tryptic digest could be entirely characterized in a single injection in such a manner. As expected, additional peptides exhibiting miscleavages or various PTMs could also be identified during the experiment; they can be used to confirm parts of the AA sequence if necessary. This result was achieved by the use of t-ITP CESI-MS/MS for the method along with the sample preparation which was adapted to allow highly efficient digestion. Additionally, sample preparation enables a complete compatibility with the CE separation conditions. Indeed, sample preparation exploits the electrokinetic separation and ESI ionization to the fullest extent. The same experiment was performed by nanoLC-MS/MS on trastuzumab digest using the same instrumental settings. Identifications from a single nanoLC-MS/MS analysis did not result in complete sequence coverage (Supporting Information Figure S-1). A simple solution to complete the sequence coverage would be to use a different proteolytic enzyme such as chymotrypsin and concatenate all peptides identified in each digest.

The capacity to characterize, without restrictions, every peptide comprising the digest opens new possibilities for protein primary structure characterization. In particular, the

possibility to go beyond the DNA sequence usually used for peptide identification and also identify mutations and/or transcription mismatches. This capability is enabled by the low pH (2.2) of the BGE, which induces every peptide to be positively charged in solution. Under this condition, all peptides migrate toward the MS inlet, regardless of their chemical nature, under electrophoresis, as it can be demonstrated by the separation and identification, under the same experimental conditions, of peptides having from 2 to 63 AAs and a large range of isoelectric points (pI). Results point out additional advantages provided by the CESI-MS technique for protein characterization; N-terminal as well as C-terminal parts of the protein could be completely and robustly characterized. Moreover in this case, the N-terminal parts known as the variable domain of the antibody are involved in antibody-antigen recognition and require a high level of characterization. Tandem MS (MS/MS), through gas-phase fragmentation of tryptic peptides, allows precise identification of the AA order of a peptide depending on the spectra quality.<sup>26</sup> Results demonstrated the capacity of the CESI-MS technique to obtain almost all y/b ions of peptides from mAb variable domains and even for trastuzumab in its totality. Over the whole protein, systematically more than 70% of the y/b fragment ions could be obtained during the experiment and more than 90% in the case of trastuzumab (Table 1), depending largely on the size of

**Table 1. Summarized Results Obtained for a Single Analysis of Each Antibody Studied Using CESI-MS/MS Analysis, Showing the Robustness of the Methodology Developed and the Extension of the Primary Structure Characterization**

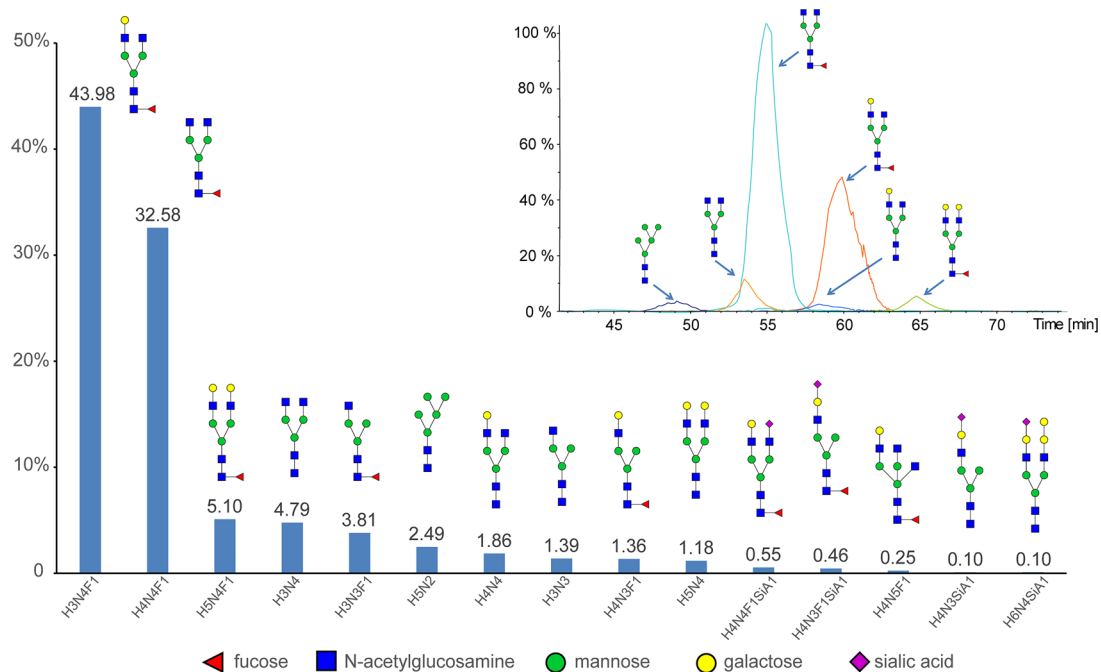
	trastuzumab	cetuximab	mAb in-dev #1	mAb in-dev #2
sequence coverage	100%	100%	100%	100%
%MS2 y/b ions	>90%	>70%	>90%	>70%
identified glycosylations	15	15	10	16
Other PTM Hotspots				
glutamic acid cyclization	1/1	1/1	1/1	1/1
methionine oxidation	2/2	0/0	2/2	0/0
asparagine deamidation	4/4	4/4	2/2	4/4
aspartic acid isomerization	6/6	2/2	3/3	2/2

the tryptic peptides generated. The y and b ions are generated by peptide fragmentation in collision-induced dissociation (CID) in MS/MS.<sup>27</sup> Peptide identifications are partially based on those fragment ions. The possibility to detect nearly all of the fragment ions allows, on one hand, increased confidence in the identification. On the other hand, fragment ions give precious information about the precise succession order of AAs along the sequence and allow determination of the exact AAs experiencing chemical modifications. This capability could be confirmed for both the HC and LC of the four different mAbs studied (trastuzumab, cetuximab, mab 1, and mab 2). Additionally, three different digestions were characterized for each sample and considered as technical replicates, allowing us to obtain similar results and proving the robustness of the designed methodology. MS/MS results describe the superior spectra quality obtained while coupling CE to MS by means of the CESI interface. Spectra quality is a direct consequence of the ionization efficiency which directly impacts the achievable

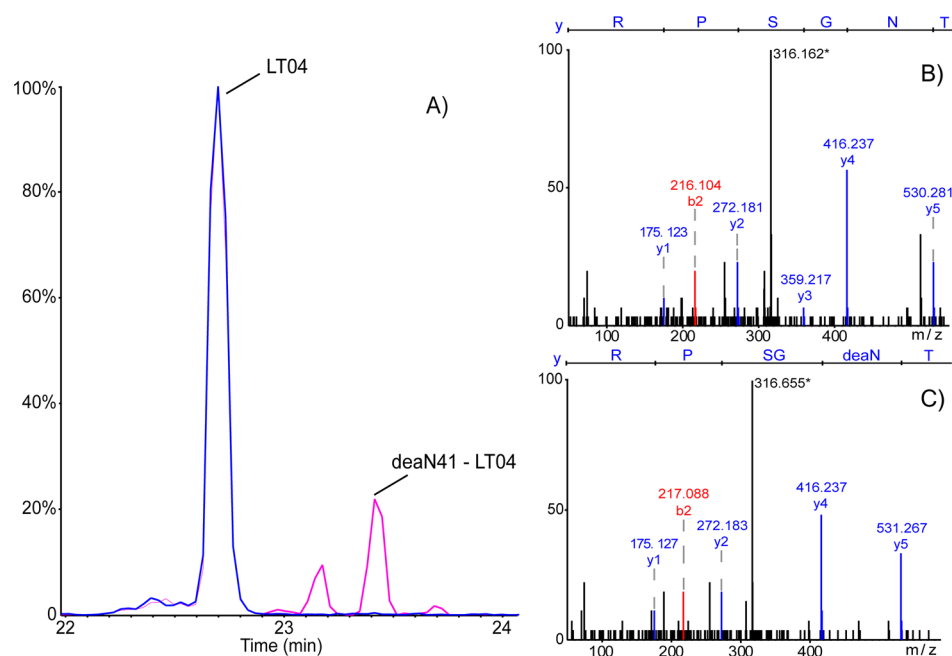
sensitivity and signal/noise ratio. In the case of the CESI interface, the ability to generate a very stable spray at quite low flow rates enables robust operation in the nanoESI regime. Intrinsic characteristics of the CESI interface have a key role in the ionization yield of the interface.<sup>28</sup> Briefly, in nanoESI, smaller droplets are initially formed, favoring Rayleigh division but also a desolvation process and finally resulting in readily improved ionization and signal/noise ratio compared to standard ESI.<sup>29</sup>

mAbs are glycosylated proteins, and those glycans are naturally incorporated in the protein during secretion into the extracellular environment.<sup>30</sup> Glycosylation has been implicated in mAb safety and pharmacokinetics/pharmacodynamics (PK/PD) and is one of the main sources of heterogeneity among this type of protein. Therefore, extensive characterization in terms of structure and relative abundance are mandatory. Concomitantly to primary sequence characterization, using the same CESI-MS/MS data, in-depth characterization of glycosylation was possible. For example, in the case of trastuzumab, 15 different glycoforms were identified, demonstrating the outstanding sensitivity of the CESI-MS method (Figure 2). Glycopeptides were identified based on accurate mass measurement in MS1 provided by high resolution MS (sub 2 ppm) and, additionally, fragmentation spectra. Indeed, MS/MS spectra exhibited the fragmentation of glycans present on the glycopeptide, giving structural information on the glycans along with reinforcing the confidence of the identification. Furthermore, the electropherogram obtained showed partial separation of the different glycopeptides, demonstrating the benefit of using CE as the separating technique for this type of characterization. As displayed in Figure 2, particular glycopeptides having a difference of one galactose (meaning a mass difference of 162 Da) could be baseline separated. The capacity to separate peptides having such small differences is clearly interesting because they tend to compete against each other during the ionization process, potentially interfering with relative quantification. Therefore, their separation participates to ease their ionization, imparting a rare sensitivity with regard to glycosylation characterization with the CESI-MS/MS methodology developed. Such sensitivity could be achieved while the entire peptide digest mixture was characterized without glycan release followed by extraction which is commonly performed in glycan analysis by MS.<sup>31</sup> Cetuximab contains a second N-glycosylation site on the HC: one is located in the Fc/2 domain (Asn<sup>299</sup>) similarly to trastuzumab while the second one is located in the Fd domain on Asn<sup>88</sup>.<sup>32</sup> The CESI-MS/MS experiments on cetuximab indicated the two different sites, and each glycosylation site could be precisely located based on the CESI-MS/MS data. Additionally, structural glycan characterization as well as relative quantification could both be established independently for each site in the same experiment.

Additional PTMs were also analyzed in the same run. For example, the trastuzumab HC N-terminal extremity contains a glutamic acid which can undergo partial cyclization leading to pyroglutamic acid.<sup>20</sup> The *m/z* ratios corresponding to the native N-terminal peptide and the pyroglutamic acid variant migrate as two different peaks separated by several minutes. This result can be explained by the fact that glutamic acid cyclization entails for the AA a mass loss of 17.02 Da. As CE separates compounds on the basis of their size and charge state in solution, this PTM involves a significant modification of the electrophoretic mobility.



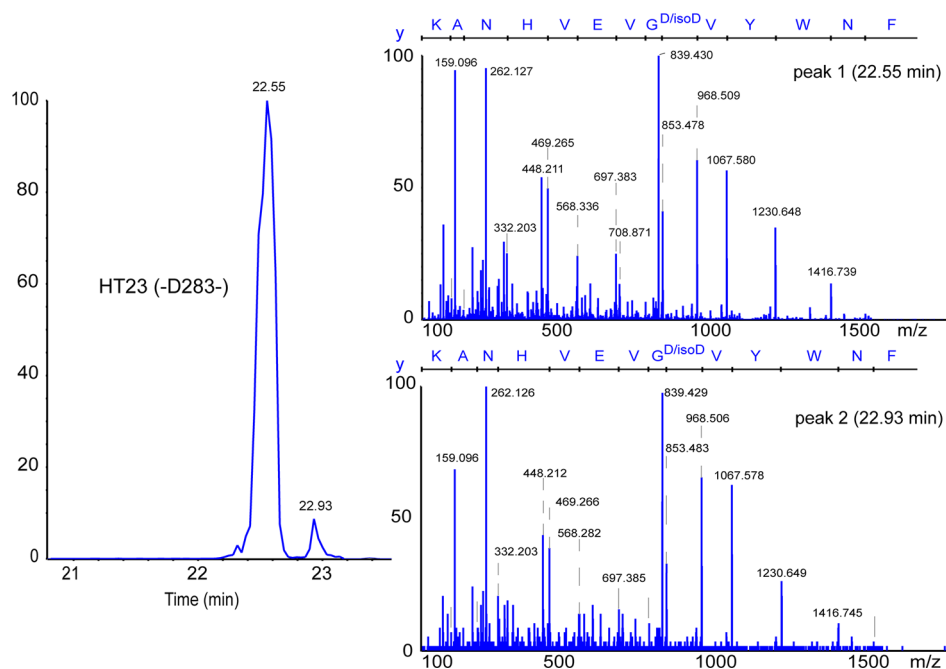
**Figure 2.** Glycoform determination obtained for trastuzumab using the CESI-MS/MS method in a single analysis (left-hand side). Extracted ion electropherogram (EIE) corresponding to the  $m/z$  of the most abundant glycoforms, illustrating the separation selectivity obtained with CE regarding mAb glycopeptides (right-hand side).



**Figure 3.** (A) EIE corresponding to the  $m/z$  of peptide LT04 (light chain, position 40–45) and LT06 with deamidated Asp41. Deconvoluted MS/MS spectra corresponding to (B) peptide LT04 and (C) LT04 deamidated Asp41 (deamidation represented by deaN).

Another common microvariant is methionine oxidation. This modification implies for the peptide a mass increase of 15.99 Da while the charge density remains the same. In a similar way as previously, the same CESI-MS/MS data highlight the capacity of CE to separate the modified peptide undergoing methionine oxidation from the native peptide (Supporting Information Figure S-2). Those results open perspectives for improved relative quantification regarding the level of occurrence of those modifications similarly to glycosylation characterization.

Deamidation is associated with the removal of the amide group present on the side chain of asparagine (Asn) and, to a lesser extent, of glutamine (Gln) residues.<sup>33</sup> These modifications are observed by separation methods such as isoelectric focusing (IEF) and cationic exchange chromatography (CEX) in combination with offline MS methods. In contrast, the CESI-MS/MS method afforded complete separation between the parent and the degraded peptide (Figure 3). That characteristic in separation could be confirmed for every deamidation hot-spot identified on both trastuzumab and cetuximab (four



**Figure 4.** EIE corresponding to  $m/z$  for digested peptide HT23 (heavy chain; position 278–291) experiencing aspartic acid isomerization. Raw MS/MS spectra for both peaks (right-hand side) demonstrated the same fragmentation pattern in addition to precursor  $m/z$  and charge state values.

different sites each). The ability to separate those modified peptides is particularly important. Deamidation involves a loss of only 0.98 Da; such a small difference would lead, during the ionization process, to competition, lowering the sensitivity of the MS signal in the case that both peptides could not be separated. That appears to be quite relevant, as the deamidation sites on the studied mAbs exhibited a low level of modification usually below 5%, urging the necessity to prevent ionization competition. The excellent separation provided by CE enables, in the case of this characterization, the best sensitivity for both peptides. One direct consequence is that the fragmentation spectra quality was significantly improved, allowing precise location of the modified AA even if other Asn or Gln were present in the peptide.

The last considered PTM is aspartic acid isomerization; this modification is particularly difficult to characterize. Indeed, the change of conformation of aspartic acid (Asp) could not induce a significant variation of affinity toward the reverse stationary phase and requires particular analytical methodologies giving access only to a specified aspect of the protein.<sup>33–35</sup> Furthermore, the conformation change does not induce a change in the mass of the peptide; thus, ESI-MS using hybrid analyzers such as a quadrupole-time-of-flight (Q-TOF) does not allow a determination of potential Asp isomerization. From the CESI-MS/MS data, extraction of the  $m/z$  ratio corresponding to a peptide potentially presenting Asp isomerization systematically exhibited two consecutive peaks as shown in Figure 4. The important acquisition rate capacity provided by MS therefore enabled us, from the CESI-MS/MS data, to obtain the fragmentation spectra for both peaks. From the fragmentation pattern, MS/MS spectra presented in Figure 4 unambiguously proved that the two peaks correspond to the same peptides. Fragmentation is obtained inside the MSCID; in this fragmentation mode the energy conveyed to neutral particles (usually  $N_2$  or Ar) is limited to a few tenths of an electron volt (eV). Such energy levels allow the activation of the fragmentation of the peptide backbone, enabling the

detection of specific b and y fragments ions.<sup>36</sup> In the context of this study, two consecutive peaks leading to the same fragmentation spectrum suggest that those CE conditions enable the separation of the same peptide having different Asp isomers. From a theoretical aspect, electrophoretic mobility is significantly influenced by the hydrodynamic radius of the molecule. Two similar peptides with the same AA sequence containing different aspartic acid isomers should be differently oriented. This would most likely induce a difference in their respective hydrodynamic radius, therefore implying a difference of electrophoretic mobilities between them. Also the different potential Asp isomerization sites studied, on both samples, exhibited the same behavior while peptides having no Asp did not present this characteristic, reinforcing the assertion on separation based on Asp isomerization.

To validate with certainty the capacity of the developed CE method to separate peptides with regard to Asp isomerization, two peptides were specially synthesized. Those peptides, composed of 20 AAs, have exactly the same AA sequence and contain one Asp, each synthetic peptide bearing a different Asp isomer. As emphasized in Supporting Information Figure S-3, several samples composed of a mixture of both synthetic peptides in different ratios were analyzed using the same t-ITP-CESI-MS/MS conditions as in the mAb characterization. Results obtained for the different mixture ratios exhibit two consecutive peaks for the  $m/z$  ratios corresponding to the synthetic peptide. On the contrary, when only a single peptide is injected, the extracted ion electropherogram (EIE) showed only one peak. To reinforce the result, peak heights illustrate relatively the evolution in proportion of one peptide to the other. These results demonstrate without ambiguity the selectivity of the separation in the case of a peptide experiencing Asp isomerization. These results further emphasize the relevance of using CE separation for protein primary structure by MS. It indeed allows the discrimination of peptides having Asp isomerization in a robust manner, thereby further

enriching the information that can be obtained by MS on a given protein molecule.

## CONCLUSION

To summarize, we report here the use of t-ITP-CESI-MS/MS for the characterization of four different therapeutic mAbs. The instrumental components used include in particular a CE-ESI-MS interface which has been recently developed. Using a single injection, we were able to characterize the primary structure of those antibodies in a robust manner at an unprecedented level. We managed to obtain the complete AA sequence characterization while only relying on tryptic peptide without miscleavages or exogenous modifications. It is the first time that this level of characterization has been achieved in a single injection/run, suggesting new approaches for bottom-up proteomics in particular. Simultaneously to the AA sequencing, PTMs including glycosylation were also characterized. Results highlighted the benefit of using electrophoretic separation in complement to chromatographic separation which is conventionally applied in this type of study. CE separation selectivity showed the ability to separate peptides having only minor differences while the sensitivity provided by CESI-MS led to the improvement of the MS/MS characterization. Indeed, the opportunity to separate peptides having only an isomerization of one AA or a difference of 0.98 Da enables the ability to cancel ionization competition between the different peptides and explains the capacity of the CESI-MS/MS methodology to characterize, in the same experiment, the intact and the modified peptide. Similarly, CE proved through MS to ease primary structure characterization, as it was possible to detect aspartic acid isomerization on several peptides from the same analysis along with its other attributes. Glycosylations were also characterized from the same experiment; thus, 15 different glycans could be characterized for trastuzumab, showing that using CESI-MS improved the sensitivity. Note that no glycan release was necessary, reducing the sample treatment and the necessity to use different experimental conditions to characterize glycosylation along with the other characteristics of the primary structure of the protein. Finally this experiment could be achieved by injecting a quantity of sample corresponding to 200 fmol of digested peptide, illustrating the suitability of the CESI-MS/MS method for small amounts of sample. The CESI-MS/MS data reported here indicate that electrophoretic separation, combined with the highly efficient CESI interface, becomes a viable alternative to LC-ESI-MS/MS for innovative approaches in MS proteomics such as identifying AA mutations or transcription mismatches.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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