CESI-MS Comparison of Tryptic Digests from Different Monoclonal Antibodies in an Assessment of Biosimilarity

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Introduction

Several of the 10 top selling drugs are antibody-based therapeutics that have lost or will lose patent protection soon.[1] As highly complex glycoproteins, monoclonal antibodies (mAbs) have a wide range of micro-variants. Therefore, mAbs require extensive characterization through multiple methods for structure assessment, manufacturing control, and biosimilarity studies. Biosimilars contain a version of an active substance that is already an authorized original biological drug. Biosimilars require additional analytical and statistical characterizations for FDA approval.[2] As drugs lose patent protection these biosimilars have started to enter the market.

Capillary electrospray Ionization (CESI) is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device (Figure 1).[3] CESI-MS operates at low nL/min flow rates and offers several advantages, including increased ionization efficiency and a reduction in ion suppression. CESI-MS separates analytes by their charge and size, and it is therefore a separation mechanism that is complementary to more traditional techniques, such as reversed-phase LC. CESI-MS has been previously used to characterize mAbs.[4]

This document summarizes the work recently published by the research group at LSMIS at the Université de Strasbourg.[5] In this application note we will show how CESI-MS can be used to characterize and compare a marketed reference mAb with other biosimilar candidates. In a single injection the CESI-MS will be used to simultaneously detect post translational modifications (PTMs) as well as provide sequence coverage. This data will then be used to compare the reference mAb with a biosimilar.

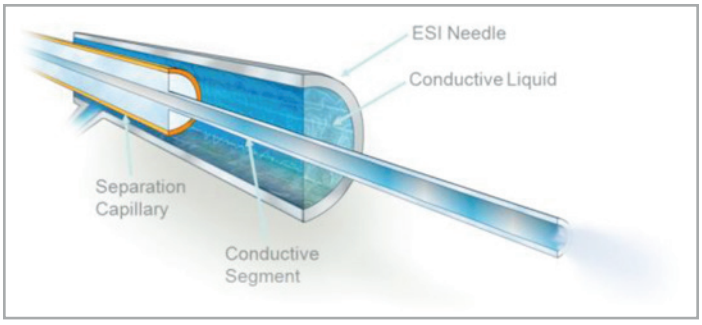

Figure 1. The OptiMS cartridge provides an ultra-low-flow ESI interface.

Materials and Methods

Chemicals: All chemicals were reagent grade and purchased from Sigma Aldrich or alternative suppliers. Trastuzumab and cetuximab samples are European Medicines Agency (EMA) approved products purchased from Roche (Penzberg, Germany) and Merck KGaA (Darmstadt, Germany), respectively. The biosimilar versions were produced at Pierre Fabre (Saint-Julien-en-Genevois, France) for analytical method development. RapiGest SF surfactant was purchased from Waters (Milford, MA).

Sample Preparation: Samples were first diluted with Milli-Q water, 0.1% RapiGest, then reduced and alkylated using dithiothreitol followed by iodoacetamide. The proteins were then digested using trypsin (overnight at 37 °C). After digestion, 1% (v/v) formic acid (FA) was added to cleave the surfactant and samples were diluted to a final concentration of 2.2 µM of protein using 50 mM ammonium acetate (pH 4.0).[5]

CESI-MS method: For the analysis, a bare fused silica OptiMS cartridge (30 µm ID x 91 cm) was used, and was thermostatted using recirculating liquid coolant (20 °C). Samples were injected hydrodynamically (10 psi, 60 s) and the peptides separated using a background electrolyte of 10% acetic acid (Table 1).

For MS analysis, a SCIEX TripleTOF® 5600+ LC-MS/MS System was fitted with the NanoSpray® III Ion Source. Gas 1 and 2 were not used, the curtain gas was set to 5 psi and the temperature of the interface was set at 150 °C. Ionization at these very low-flow rates occurred by simply applying the ionspray voltage (1,750 V). MS data was acquired using a TOF survey scan (accumulation time 250 ms, m/z 50–2,000 amu), which triggered MS/MS acquisition (accumulation time 100 ms per scan, 100–2,000 amu) and led to a total duty cycle time of 1.75 sec.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Action	Time (min)	Pressure (psi)	Direction	Voltage (kV)	Solution
Rinse	3.5	100	Forward	0	0.1 M NaOH
Rinse	1.5	100	Forward	0	Water
Rinse	3	100	Forward	0	0.1 M HCl
Rinse	5	100	Forward	0	10% acetic acid
Rinse	2	75	Reverse	0	10% acetic acid
Injection	60 s	10	Forward	0	Sample vial
Injection	10 s	5	Forward	0	10% acetic acid
Separation	35	0	Forward	20	10% acetic acid
Voltage	2	10	Forward	1	10% acetic acid

Table 1. CESI separation conditions used for the analysis of tryptic digests.

Results

Amino acid sequence is a key criterion for demonstrating biosimilarity, so in this study, 2 reference mAbs (trastuzumab and cetuximab in their commercial formulation) were compared to 2 potential biosimilars (trastuzumab-B and cetuximab-B). For trastuzumab and trastuzumab-B, a single analysis of a tryptic digest of each protein provided 100% sequence coverage on both the heavy chain (HC) and light chain (LC), confirming the results of a previous study that demonstrated CESI-MS/MS is capable of 100% coverage in a single analysis (Figure 2).[4]

When these sequences were compared, only 1 difference between the primary sequence of the biosimilar and the reference mAb could be identified on the HC at position 217. Analysis of MS/MS spectra was able to confirm that trastuzumab-B, in position 217, underwent a lysine residue substituted by an arginine (Figure 3).

The same experiment was performed for cetuximab and cetuximab-B, and once again 100% sequence coverage was obtained for the reference mAb and the biosimilar (cetuximab-B), but in this case the sequences of these 2 mAbs were identical.

To confirm these results, experiments were repeated in triplicate and the same results were obtained each time. In addition, more than 70% of the y/b fragment ions in MS/MS spectra of the peptides were observed, and in the case of trastuzumab, it was more than 90%. This high MS/MS fragment ion coverage was

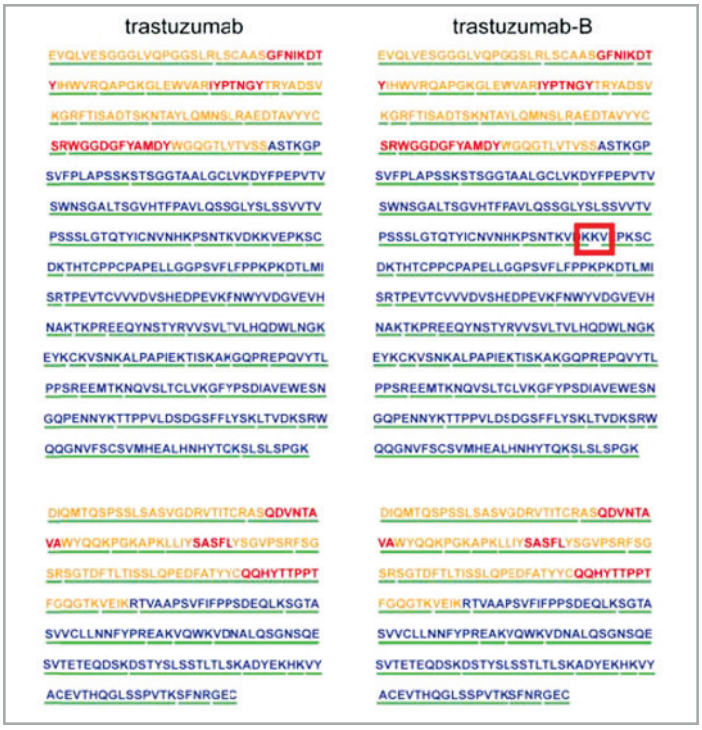

Figure 2. Sequence coverage obtained by CESI-MS/MS for trastuzumab (left-hand side) and trastuzumab-B (right hand side).

only possible due to the high ionization efficiency of CESI-MS/MS (running at a flow of < 40 nL/min) in combination with the short duty cycle time of the MS system. This combination is well suited for the detection of post translation modifications (PTMs), which often help to distinguish reference mAbs from biosimilars.

Glycosylation is important to the structural conformation and stability of mAbs. In this study, glycan release was not performed during sample preparation, so glycans could be characterized directly by detecting the glycopeptides, enabling identification of the site of glycan modification. In the case of the trastuzumab/trastuzumab-B analysis, the injection of the reference mAb identified 16 different glycoforms. When the reference mAbs and biosimilars were semi-quantitatively compared (Figure 4), both biosimilars exhibited different glycoforms distribution compared to the reference mAb.

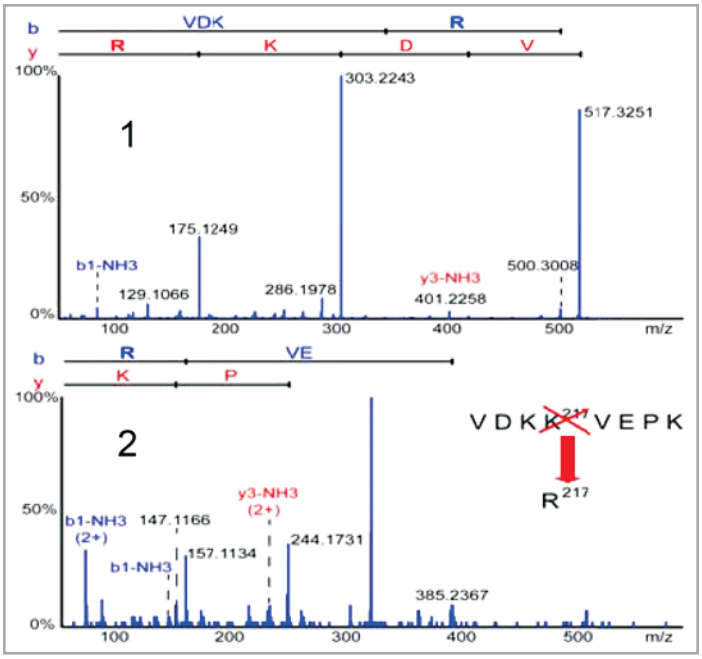

Figure 3. MS/MS spectra for trastuzumab (1) and trastuzumab-B (2), showing the absence of the fragmentation ions m/z 517.3095 (1+) for trastuzumab-B and the presence of m/z 314.6937 (2+) characterizing the amino acid substitution of lysine to arginine.

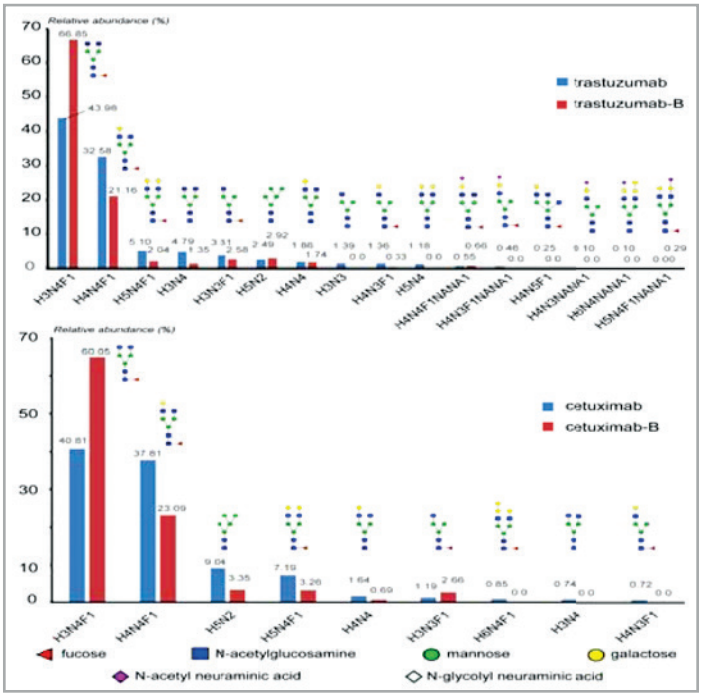

Figure 4. Glycoform profiling results obtained from the CESI-MS/MS data for the Fc peptide of trastuzumab/trastuzumab-B and cetuximab/cetuximab-B.

In addition to glycosylation, other critical quality attributes (CQAs) are considered important in biosimilarity assessment. These CQAs, referred to as PTM hot spots, may influence the immunogenicity and the PK/PD of the protein[2] and include N-terminal glutamine/glutamic acid cyclization, leading to the formation of N-terminal pyroglutamic acid (pE), asparagine deamidation (deaN), methionine oxidation (oxiM) and aspartic acid isomerization (isoD) on various positions, depending on the mAb. The CESI-MS/MS data was also used to characterize these PTM hot spots on the different mAbs. Table 2 highlights that hot spots could be monitored successfully in the cetuximab/cetuximab-B comparison.

Modification	Cetuximab distribution		Biosimilar distribution	
	% mod.	% unmod.	% mod.	% unmod.
Q1 / pQ1	100	0	100	0
N161 / deaN161	25.6	74.4	59.5	40.5
D282 / isoD282	95.2	4.8	96.3	3.7
N386 / deaN386	45.6	54.4	95.7	4.3
D403 / isoD403	100	0	100	0
N41 / deaN41	87.8	12.2	94.7	5.3
N158 / deaN158	96.6	3.4	100	0

Table 2. Summary of the CESI-MS/MS study of PTM hot spots occurrence levels for cetuximab/cetuximab-B. Percent distribution and modifications were determined through signal intensity for each targeted PTM.

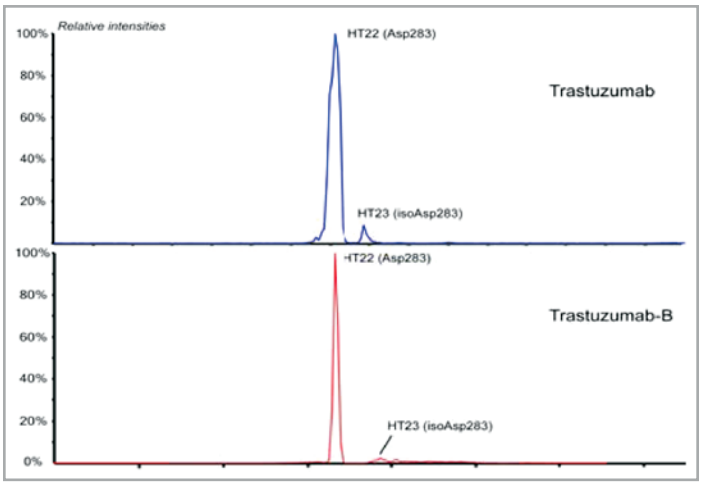

Figure 5. Extracted ion electropherogram (EIE) corresponding to m/z (839.408, 2+) for digested peptide HT22 (position 278-291), which experiences aspartic acid isomerization.

In CQA analysis, the change in structure by a PTM often significantly changes the electrophoretic mobility of the peptide (e.g., from 0.5 min in the case of deaN to several minutes for pE), aiding separation and identification by CESI-MS/MS. The use of CE as a separating technique is clearly of great interest. This is emphasized in Figure 5, where it is possible to separate the same peptide differing solely by the conformation of its aspartic acid, which is not possible by MS alone, as they exhibit the same mass. Again, all identifications were aided by the high-quality MS/MS spectra obtained in this study.

Throughout this study, it was important that sequence coverage, PTM hot spot detection and glycosylation site mapping information was obtained by just a single injection of each digested mAb.

Conclusions

A CESI-MS/MS protocol for biosimilarity studies of mAbs has been developed. Using this protocol, and with a single injection of a tryptic digest of an mAb, several important structural properties could be obtained, including:

- 100% amino acid sequence coverage.
- Glycosylation site mapping and identifications.
- PTM hot spot mapping and identification.

For further information on this topic, we would like to refer readers to the full scientific publication on which this application note is based.[5]

References

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