Multilevel Characterization of Biotherapeutics using CESI-MS: from Intact Protein to Peptide Mapping Approach

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Separation in capillary electrophoresis

- Analytes are separated depending on their charge and size.
- CE provides fast separation, great efficiency, low sample consumption.
CE-ESI-MS Coupling

Advantages of CE-MS

- Great efficiency
- Ultra-low flow rate
- Selectivity
- Sensitivity
- Structural information
CE-ESI-MS Coupling

Advantages of CE-MS

- Great efficiency
- Ultra-low flow rate

- Increase sensitivity\(^1\)
- Minimize ion suppression\(^2\)
- Maximize ionization efficiency

\(^1\)Wilm, Mann International Journal of Mass Spectrometry 1994, 136, 167–180
1. Monoclonal antibodies primary structure characterisation by CESI-MS

2. Biosimilarity assessment by CESI-MS

3. Antibody Drug Conjugate characterization by CESI-MS

4. Glycoform Separation and Characterisation of Cetuximab Variants by Middle-up Off-line CE-UV/ESI-MS
CESI-MS coupling

- CESI-MS allows to be operated using nano flowrates
  Favorable to ESI ionization

- CESI-MS showed improved sensitivity compared to sheath liquid interface
  
  - Fasearl et al., Anal. Chem. 2011, 83, 7297-7305
  - Busnel et al., Anal. Chem. 2010, 82, 9476-9483

Diagram and picture of the CESI interface
mAbs characterisation by CESI-MS/MS

- Monoclonal antibody (mAb) therapeutics attract the most interest due to their strong therapeutic potency.
  - In 2015, over than 43 are marketed
  - more than 30 mAbs in clinical trial phase III

- mAbs specificity for its antigen opens new avenues for therapeutic treatments
  - oncology
  - autoimmune diseases
  - Transplant rejection prevention

- mAbs are complex and heterogeneous glycoproteins representing a challenge to analytical sciences
  - Characterization on different level of the mAbs
  - Necessity of precise and high throughput characterization

Monoclonal Antibody

Trastuzumab

Average mass: 148,057 Da (1,328 a.a.)

A. Beck et al., Anal. Chem. 2012, 84, 4637-4646
mAbs characterisation workflow

- Primary structure characterisation workflow based on bottom-up proteomics strategy

In-solution tryptic digestion → Analysis by t-ITP CESI-MS/MS →
- Amino acid sequence characterisation
- Glycosylations (structure)
- PTMs hot spots characterisation

CESI8000 coupled to 5600 TripleTOF MS
Amino acid sequence characterisation

- **MS/MS amino acid sequence characterisation (trastuzumab)**

  100% sequence coverage achieved in a single injection through only purely tryptic unmodified peptides

  EVQLVESGGGLVQPGGLVLSCAASGFNIKDT
  YIHVRQAPGKGEWVARIPNYTRYADSV
  KGRFTISADTSKNTAYLQNSLRAEDTAVYYC
  SRWGGDFGYAMDYWGQGTTLVSSASTKGP
  SFVFLAPSSKSTGTAALGCLVKDYFPEPVTV
  SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV
  PSSSLGTQNYCNAHKPSNTKVDKVEPKSC
  DKTHTCPPCPAPELLGGPSVFLFQPDKDTLLMI
  SRTPETCVVVDVSHEDPEVKFNWYVDGVEVH
  NAKTKPREEQNYSTRYVSVLTVHQLDWNK
  EYKCKVSNKALPAIEKTISAKAGQPREPQVYL
  PPSREEMTKNQVSLTCLVKGFYPSDIAIWESEN
  GQPENNYKTTPVLSDGSFFLYSKLTVDKSRW
  QQGNVFSCSVMHEALHNHYTQKSLSLSPG
  DIQMTQSPSLSASVGRVTITCRASQDVNTA
  VAWYQQPGAPKCLLYSFLYSVPSRFSG
  SRSGTDFTLTISLQPEDFATYYCQHYTTTP
  FQQGTKVEIKRTVAAPSVFIFPSDEQLSGT
  SVVCLLNFPREALKVQWKVDNLQSGNSOE
  SVTEQEYDKSTYSSSTLTSKADYKHKVY
  ACEVTHQLSSPVTKSFNGEC

  variable domain
  complementarity determining region
  constant domain
  identified peptides

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
Amino acid sequence characterisation

APK
(m/z 315.2039 ; 2+)

DYFPEPVTVSVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
(63 amino acids ; m/z 1119.898 ; 6+)

Implementation of CE allows separation and successful detection of a larger variety of peptides
Trastuzumab (Herceptin)

Average mass: 148,057 Da (1,328 a.a.)
Glycosylations characterization

- mAbs glycosylations are characterized simultaneously using the same CESI-MS/MS data

Gahoual R. et al., mAbs, 2013 (5), 479-490
Glycosylations characterization

- Glycopeptides MS signal intensity used to estimate glycoforms relative abundances

15 different glycoforms identified in trastuzumab case

Possibility to detect weakly abundant glycosylation

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
PTMs hot spots characterization

N-terminal glutamic acid cyclization characterization

- CE mechanism separates of peptide with N-terminal glutamic acid cyclization from the unmodified peptide

Results suggest partial modification of sample. Favorable conditions to estimate sample modification level.

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
PTMs hot spots characterization

Methionine oxidation

- Methionine oxidation causes peptide mass shift (+15.99 Da) leading to the separation of the modified peptide in CZE

confirmed by MS/MS spectra

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
PTMs hot spots characterization

Asparagine deamidation

- Deamidation (+ 0.98 Da) involves mobility change in CZE enabling the separation of the unmodified peptide

CE separation of deamidated peptides eases the identification of the modification by MS

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
PTMs hot spots characterization

Aspartic acid isomerization

OEIs and MS/MS spectra of peptides HT23 (intact and modified)

CE separation prior to MS analysis allows in this particular case to include aspartic acid isomerization in the overall characterization workflow

Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081
Aspartic acid separation in CZE

- Aspartic acid isomers separation by CZE confirmed using a synthetic peptide

Synthetic peptides sequences:

\[ \text{NH}_2\text{-GLEWIGYSY} \text{ D} \text{ GTNNYKPSLKO} \text{H} \]
\[ \text{NH}_2\text{-GLEWIGYSY isOD} \text{ GTNNYKPSLKO} \text{H} \]
1. Monoclonal antibodies primary structure characterisation by CESI-MS

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mAbs biosimilarity assessment

- As several mAbs patent are ending in the next few months/years, other companies should have the possibility to commercialize « unprotected » mAbs

- mAbs complexity and production process (cell line selection) makes it nearly impossible to produce strictly the same product as the innovator company

- FDA and EMA are introducing guidelines to help biopharma companies to determine the key features needed for a biosimilarity between two products in term of structure, PK and PD => reducing clinical trials

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EMA, CHMP/437/04
EMA, CHMP/437/04 Rev 1
$2^{nd}$ case

Cetuximab vs. candidate biosimilar
Amino acid sequence similarity

- A single analysis of each sample sufficient to conclude on the complete similarity regarding AA sequence

- Complete sequence coverage is obtained through peptides without miscleavages or PTMs

- CESI-MS/MS enabled to confirm an error, recently reported in the literature

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Gahoual R. et al., mAbs, 2014 (6), 1464-1473

D. Ayoub et al., mAbs 2013, 5, 699-710
Glycoforms characterisation

- Fc/2 glycosylation site characterisation

Heterogenous glycoforms could be identified

Difference in glycoforms distribution could be observed

Cetuximab possess two different N-glycosylation sites

Significant number of glycans could be characterized

Gahoual R. et al., mAbs, 2014 (6), 1464-1473
Glycoforms characterisation

- Fd glycosylation site characterisation

Glycoforms exhibited by the candidate biosimilar are significantly different from cetuximab

- 30 % of glycans contains N-acetylneuraminic acid

Rejected as biosimilar

Gahoual R. et al., mAbs, 2014 (6), 1464-1473
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ADC characterisation by CESI-MS/MS

- Antibody drug conjugates (ADCs): New class of biopharmaceutical drugs for cancer treatment

ADC characterisation by CESI-MS/MS

- Antibody drug conjugates (ADCs): New class of biopharmaceutical drugs for cancer treatment

[Cysteine-linked ADCs]

Drug

Monoisotopic mass of drug: 1316.7869 Da
MMAE: 716,4962 (+2H)
ADC characterisation by CESI-MS/MS

Objectives:

A. Characterization of intact ADCs: Determination of average DAR et DAR Distribution

B. Peptide mapping:

Identify

- Amino acid sequence
- PTMs: N-glycosylation, deamidation, oxidation, cyclization...
- Drug conjugate peptides

<table>
<thead>
<tr>
<th>Chain</th>
<th>Disulfide interchain</th>
<th>Séquences</th>
<th>Nb of vcMMAE</th>
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<tr>
<td>HC</td>
<td>LC-HC</td>
<td>219SCDK222</td>
<td>1</td>
</tr>
<tr>
<td>LC</td>
<td>LC-HC</td>
<td>212GEC214</td>
<td>1</td>
</tr>
<tr>
<td>HC</td>
<td>HC-HC</td>
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<tr>
<td>HC</td>
<td>HC-HC</td>
<td>223THTCPPCPAPELGGPSVFLFPPKPK248</td>
<td>2</td>
</tr>
</tbody>
</table>
Characterisation of Intact ADCs

- **DAR Distribution**

![Graph showing Deconvoluted Mass spectra](image)

- **Average DAR**

\[
DAR = \frac{\sum_{n=0}^{8} nA_{Drug}}{\sum_{n=0}^{8} A_{drug}} = 3.7
\]
Amino acid sequence characterisation

- MS/MS amino acid sequence characterisation

94% sequence coverage achieved in a single injection using tryptic digestion


**Amino acid sequence characterisation**

- **MS/MS amino acid sequence characterisation**

QIQLQSGPEVVK PGASVK ISCK ASGYTFDYYITWVK QK PGQGLEWIGWIYPGSNKT YNEK FK GK
ATLTVDTSSSTAFMLQLSSLTSEDATYFCANYGNYWFAYWG
QGTQVTSAASTKGPSVFPLAPSSK STSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHK PSNTK VDK KVEPK SCDK
THTCPCCPAPELLGGPSVFLFPPK PK DTLMSK
TPEVTCVVDVSHEDPEVK FNWYVDGVEVHNAK TK PR
EEQYNSTYR VVSVLTVLHQDWLNGK EYK CK VSNK
ALPAPIEK TISK AK GQPR EPQVYTLPPSR DELTK
NQVSLTCLVK GFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSK LTVDK SR WQQG
NVFSCSVMHEALHNHYTQK SLSLPG

- **DIVLTQSPASLAVSLGQR** ATISCK
  ASQSVDFDGSYMNWYQQK PGQPPK
  VLIYAASNLESGIPAR
  FSGSGTGTDFTLNIHPVEEEDAATYYCQSNED
  PWTFGGGTK LEIKR TVAAPSVFIFPPSDEQLK
  SGTASVVCLNNFYPR EAK VQWK
  VDNALQSGNQESVTEQDKS
  DSTYSLSSTLTSK ADYEK HK
  VYACEVTHQGLSSPVTK SFNRGEC

98.8% sequence coverage achieved in a single injection using chymotryptic digestion
Detection and characterization of the 4 modified peptides using MS/MS data

Drug characterization

N. Said et al. Anal. Chem. 2015, Submitted
Drug characterization

**Conjugate peptide:**  LC-HC interchain $^{208}$SFNRGEC$^{14}$

**Extracted ion electropherogram**

**MS spectra**

**MS/MS spectra:** precursor 710.05 Da

**Drug fragmentation**
**Conjugate peptide:** LC-HC interchain $^{219}$SCDK$^{222}$

**Drug characterization**

CE separation of drug peptides eases the identification of the modification by MS

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Introduction

- mAbs are complex and heterogeneous glycoproteins representing a challenge to analytical sciences
  - Characterization on different level of the mAbs

Intact protein analysis
Introduction

- mAbs are complex and heterogeneous glycoproteins representing a challenge to analytical sciences
  - Characterization on different level of the mAbs

**Intact protein analysis**

- Classical condition described in the literature\(^1,2\)
  - 400 mM ε-amino-caproic acid pH 5.7
  - Triethylenetetramine as additive

**Not compatible with ESI-MS detection**

\(^1\) He et al, Anal. Chem. 2010, 82, 3222-3230  
\(^2\) Gassner et al, Electrophoresis 2013, 34, 2718-2724
Off-line coupling strategy

PACE MDQ  
Beckman Coulter

On-line

Proteineer FC  
Bruker

Off-line

CESI8000 - MS

M. Biacchi et al. *Electrophoresis* 2014, 35 (20), 2986-2995
mAbs characterisation workflow

```
IdeS digestion → Analysis by CE-UV/Fraction collection → Analysis by NanoESI infusion → Fc/2 variant characterization → F(ab’)2 characterization
```

Cetuximab (ChlgG 1) → IdeS Enzymatic cleavage → F(ab’)2
Characterisation of $\text{F}_{\text{C/2}}$ variants

- Characterization of 7 Fc/2 glycoforms.
- Separation due to C-terminal lysine truncation
  - Peak 1 $\text{F}_{\text{c/2}}$
  - Peak 3 $\text{F}_{\text{c/2}}$-K

Peak 2: Mixture $\text{F}_{\text{c/2}}$ and $\text{F}_{\text{c/2}}$-K

Characterisation of $F_{C/2}$ variants

- Characterization of 7 $F_{c/2}$ glycoforms.
- Separation due to C-terminal lysine truncation
  - Peak 1 $F_{c/2}$
  - Peak 3 $F_{c/2}$-K

Separation of $F_{c/2}$ aggregates

Characterisation of F\(_{(ab')}\)_2 variants

- Characterization of 8 F(ab’)2 glycoforms.
- Separation of F(ab’)2 glycoforms

Separation based on the presence of sialic acid

Conclusion

• Monoclonal antibodies primary structure characterisation by CESI-MS
  - 100% amino acid sequence characterisation
  - 15 glycoforms characterisation
  - All PTMs hot spots characterisation

• CESI-MS/MS allowed to conclude in each case on the biosimilarity assessment

• ADC characterisation by CESI-MS
  - Average DAR measurement
  - 98.8% amino acid sequence characterisation
  - MS/MS characterisation of the 4 modified peptides

• Charaterisation and Glycoform Separation of Cetuximab Variants by Middle-up Off-line CE-UV/ESI-MS
  - Separation and characterisation of $F_{C/2}$ variants ($F_{C/2}$ and $F_{C/2}$–K)
  - Separation and characterisation of glycoform $F_{(ab')}_2$ variants (presence of sialic acid)
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Thank you for your attention
# mAbs characterisation by CESI-MS/MS

<table>
<thead>
<tr>
<th></th>
<th>Trastuzumab</th>
<th>Cetuximab</th>
<th>mab in-dev #1</th>
<th>mab in-dev #2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sequence coverage</strong></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>% MS2 y/b ions</strong></td>
<td>&gt; 90%</td>
<td>&gt; 70%</td>
<td>&gt; 90%</td>
<td>&gt; 70%</td>
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<tr>
<td><strong>identified glycosylations</strong></td>
<td>15</td>
<td>15</td>
<td>10</td>
<td>16</td>
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<tr>
<td><strong>other PTMs hotspots</strong></td>
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<tr>
<td>glutamic acid cyclization</td>
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<td>1 / 1</td>
<td>1 / 1</td>
<td>1 / 1</td>
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<tr>
<td>methionine oxidation</td>
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<td>0 / 0</td>
<td>2 / 2</td>
<td>0 / 0</td>
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<td>4 / 4</td>
<td>2 / 2</td>
<td>4 / 4</td>
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<tr>
<td>aspartic acid isomerization</td>
<td>6 / 6</td>
<td>2 / 2</td>
<td>3 / 3</td>
<td>2 / 2</td>
</tr>
</tbody>
</table>

*Results summary obtained with the t-ITP CESI-MS/MS method*

The t-ITP CESI-MS/MS method developed demonstrated its robustness on different samples including technical replicates in each case.

*Gahoual R. et al., Anal. Chem., 2014 (86), 9074-9081*
• Glycosylations relative quantification was performed using maximum intensities or peak area.

Similar results with both methodologies

Maximum intensity was selected for the quantification.
Glycoforms relative abundances

- Glycosylations relative abundances estimated from the CESI-MS/MS data were confronted to other techniques if data were available

D. Ayoub et al., mAbs 2013, 5, 699-710
## PTMs hot spots characterization

<table>
<thead>
<tr>
<th>position</th>
<th>sequence</th>
<th>trastuzumab</th>
<th>biosimilar</th>
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<tr>
<td></td>
<td></td>
<td>unmodif</td>
<td>modif</td>
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<tr>
<td><strong>N-term glutamic acid cyclization</strong></td>
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<tr>
<td>1 - 19</td>
<td><strong>EVQLVESGGGLVQPGGSLR</strong></td>
<td>98.2</td>
<td>1.8</td>
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<tr>
<td><strong>Asparagine deamidation</strong></td>
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<tr>
<td>51 - 59</td>
<td><strong>IYPTNGYTR</strong></td>
<td>89.4</td>
<td>10.6</td>
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<tr>
<td>374 - 395</td>
<td><strong>GFYPDIAVEWESNGQPENNYK</strong></td>
<td>85.8</td>
<td>14.3</td>
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<tr>
<td>25 - 42</td>
<td><strong>ASQDVNTAVAWYQQPKG</strong></td>
<td>96.1</td>
<td>3.9</td>
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<td><strong>Methionine oxidation</strong></td>
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<tr>
<td>252 - 258</td>
<td><strong>DTLMISR</strong></td>
<td>95.3</td>
<td>4.7</td>
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<tr>
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<td><strong>Aspartic acid isomerization</strong></td>
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<tr>
<td>99 - 124</td>
<td><strong>WGGDFGYAMYWGQTLVTSSASTK</strong></td>
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<td><strong>TTPPVLDSDGSFFLYSK</strong></td>
<td>75.3</td>
<td>24.7</td>
</tr>
</tbody>
</table>

_Gahoual R. et al., mAbs 2014, in press_
$1^{\text{st}}$ case

trastuzumab vs. candidate biosimilar
Amino acid sequence similarity

- Complete sequence coverage obtained for trastuzumab
- Biosimilar candidate sequence could be successfully identified except HC K²¹⁷

Suggesting an amino acid substitution between the two samples

Gahoual R. et al., *mAbs* 2014, 6, 1464-1473
Amino acid sequence similarity

Interpretation of unidentified MS/MS spectra

Unambiguous characterisation of the amino acid substitution of biosimilar candidate

VDK R^{217} V E P K

rejected candidate

CESI-MS/MS spectra of trastuzumab biosimilar candidate

Gahoual R. et al., mAbs 2014, 6, 1464-1473
Glycoforms characterisation

- Glycosylation distribution evaluated for each sample using CESI-MS/MS data

Identification of a significant number of glycoforms

Minor differences of glycoforms could be distinguished

Gahoual R. et al., mAbs 2014, 6, 1464-1473
Poor crystallization = No MALDI-MS signal

Outlet BGE: EACA 200 mM; AcNH4 25 mM pH 5.7

Outlet BGE: AcNH4 25 mM pH 5.7

Biacchi et al., Anal. Chem., 2015 submitted