Native and IM-MS characterization of ADCs

Alain VAN DORSSELAER

BioOrganic Mass Spectrometry Laboratory, IPHC
University of Strasbourg, CNRS
Strasbourg - France

ASMS 2015 Waters User’s meeting
mAbs as therapeutics

- mAbs: a very important class of human therapeutics
  - > 50 mAbs & derivatives currently approved (EMA/FDA)
  - indications: inflammation, autoimmunity, cancer, transplantation,…
  - > 400 mAbs & derivatives in pre-clinical development and clinical trial

- mAbs are complex therapeutic proteins
  (150 kDa glycoproteins)

- anti-cancer mAbs in clinical studies
  - 84 unmodified IgG (51%)
  - 10 bispecific (6%)
  - >35 ADC (15%)
  - 17 engineered (10%)
  - 16 fragments (10%)

- Next generation empowered mAbs:
  Bispecific mAbs (bsAbs) and Antibody-Drug-Conjugates (ADCs)

Double-barreled shotgun: two antigens targeted

2 different half mAbs

mAb + cytotoxic

A highly cytotoxic drug
mAb characterization is a nightmare

Peptide mapping (700-7000 Da)

Fragment analysis (25-100 kDa)

Intact mAb analysis (150 kDa)

Higher Order Structure Aggregates mAb/Ag complexes (>150 kDa)

The MS toolbox

Native MS

Native IM-MS

HDX-MS

Crosslinking MS

(nano)LC-MS/MS enzymatic digestion

Top-down MS (FT-ICR + Orbital trap)

LC-MS
Native MS: a brief history

1991 Native MS

1991
Holo-myoglobin
17.5 kDa

Katta et al. JACS 1991

2000 Automated nanoESI

2002
Haemocyanins
2.3 MDa

Sanglier et al. JASMS 2002

2006 Ion Mobility MS

2006
Ribosomes
2.3 MDa

McKay et al. JACS 2006

2012 High resolution native MS

2013 Bacteriophage HK97 capsid 18 MDa

Sniijder et al. Angew. Chem 2013

Increase in size, nb of partners and heterogeneity

Binding

Conformation

2006

Synapt G1

Synapt G2
Native MS analysis

Native MS gives information on assemblies maintained by noncovalent interactions

Solution

Sample preparation step = desalting step
- Buffer exchange
- Use of volatile aqueous buffer at neutral pH
- Ammonium buffer (0-1M) with controlled pH

Instrumental setting optimizations
- Control of the energy communicated to the ions in the 1st pumping stage region of the instrument (Vc, Pi adjustment)

Gas phase

Data interpretation
- Binding stoichiometries
- Binding specificity
- Solution affinities
- Dynamics of assembly/disassembly

Be careful!
Native Mass Spectrometry: a recursive question

We dream to get from Native MS and IM-MS experiments:
- Accurate mass measurements, stoichiometries,…
- Information on interactions forces
- Subunit spatial arrangement, structural information, shape, conformation changes,…

... but all this information concerns molecules in water solution
Native MS workflow for mAb analysis

For mAbs minimal and easy sample preparation are well described.

Purified mAb, bsAb or ADC

Deglycosylation Step
(PNGase F, IgZero, etc.)

Buffer Exchange
(spinn columns, microconcentrators, SEC etc.)

Native MS and IM-MS
(with ESI, needles or Chip-based introduction)

Data Interpretation

Synapt G2 HDMS (Waters) + nanoMate Triversa (Advion)
How do we jumped into the mAb field?

- **2008-2012: Reference Monoclonal Antibodies (mAb)**
  - mAb native MS
  - mAb/Ag binding stoichiometries
  - Native IM-MS

- **2013: towards bispecific mAbs (bsAbs)**
  - Native MS for monitoring of bsAb formation
  - IM-MS for bsAb
  - Real-time IM-MS to monitor bsAb formation

- **2014: immunoconjugate analysis (ADC)**
  - Native MS mandatory for ADCs’ analysis
  - Native MS for drug load profiles, average DAR
  - IM-MS for ADC heterogeneity assessment
  - IM-MS for drug load profiles, average DAR
1. Native MS and IM-MS for reference mAb analysis

2. Native MS and IM-MS for bsAb formation

3. Native MS and IM-MS for ADC formation
Intact mAb analysis: benefits of native MS for intact mAb analysis

**Denaturing Conditions**
mAb 2µM in H2O:ACN:FA (50:50:1)

*Full MS spectrum*

**Native Conditions**
mAb 5µM in AcNH4 150mM pH7.5

*Full MS spectrum*

Non covalent dimer not detected

In Native conditions sensitivity and mass accuracy are as good as in Denaturing conditions. But spectra are more simple, and you see interactions !!!

Native MS analysis of immune mAb/Antigen complexes

Direct determination of mAb/Ag binding stoichiometries

mAb (5 µM)

mAb (5 µM) + JAM-A (10 µM)

mAb (5 µM) + JAM-A (40 µM)

Unexpected 1:4 mAb:Ag stoichiometry

Native Ion Mobility MS (IM-MS) for conformational studies

By IM-MS: simultaneous measurement of drift times and m/z ratios

Instrumental setting optimizations
- Wave height and velocity
- Pressure in the IM cell

Data interpretation
- Conformation of biomolecules
- Conformational changes
Native Ion Mobility MS (IM-MS) for conformational studies

- Ions separation takes place according to ion mobility

\[ \Omega = \frac{3e}{16N} \times \frac{T}{273.15} \times \frac{760}{P} \times \frac{2\pi}{k_BT} \times \sqrt{\frac{1}{M_{gas}} + \frac{1}{M_{ion}}} \times \frac{z \times tD \times E}{L} \]

\[ \Downarrow \] Drift times can be related to collisional cross sections (CCS)

\[ \Downarrow \] Information on ion gas phase conformation (2D representation of 3D structure)
Ion Mobility Mass Spectrometry (IM-MS)

For mAb conformational characterization

Denaturing MS

IM-MS

Native MS

IM-MS

Denaturing IM-MS

Native IM-MS

2010: the first separation of 2 mAb isoforms by IM-MS reported.

Lc214  S-S  Hc127  
Hc232  S-S  Hc232  
Hc233  S-S  Hc233  

Figure 1. Illustration of the hinge region disulfide bonding pattern of human (a) IgG2-A and (b) IgG2-B antibodies.
1. Native MS and IM-MS for reference mAb analysis

2. Native MS and IM-MS for bsAb formation

3. Native MS and IM-MS for ADC formation
Native MS analysis for analysis for bsAb formed by Fab Arm Exchange

- **In vivo**, IgG4s can exchange half molecules by a dynamic process called **Fab-arm exchange (FAE)**. **In vitro**, the FAE process can be mimicked by a reaction with glutathione (GSH).

- 2 mAb features are important for the exchange reaction of IgG4:
  - CH3 domain
  - Ser at position 228

- Serine 228 in IgG4 introduces flexibility in the core hinge region. Besides the usual disulfide bonds connecting two heavy chains (covalent between HC), intrachain disulfide bonds may form instead (only non-covalent bonds between HC).

- The 2 arms are only maintained by non-covalent bonds between HCs.
Native MS and IM-MS for bispecific mAb characterization


IgG4 Fab Arm Exchange monitoring
Sample: Natalizumab and Hz6F4-2 IgG4

Native MS and IM-MS to monitor:
- Dynamics of Fab Arm Exchange
- Bispecific antibody formation

1. Native MS and IM-MS for reference mAb analysis

2. Native MS and IM-MS for bsAb formation

3. Native MS and IM-MS for ADC formation
ADCs are next generation empowered therapeutic mAbs

- **ADCs are tripartite molecules**, made by the chemical conjugation of cytotoxins to mAbs
  - cell-killing capabilities
  - targeting delivery of the conjugated drug to tumor cells
  - Highly cytotoxic drug

- **2 already approved ADCs’ on the market:**
  - brentuximab vedotin (Adcetris®, Seattle Genetics)
  - ado-trastuzumab emtansine (Kadcyla®, Genentech)
  - > 30 in clinical trials*

- **3 main types of conjugation:**
  - **at cysteine** -SH groups after reduction of the interchain disulfide bonds (brentuximab vedotin)
  - **at lysine** side chains amine (ado-trastuzumab emtansine)
  - at engineered cysteine residues at specific sites without reduction (thiomabs)

*Beck A. and Reichert JM., mAbs 2014
The analytical toolbox for ADC analysis

- Bottom up (nano)LC-MS/MS
- Middle-up LC-MS
- Middle- and Top-down MS (MS/MS on intact ADCs)

Site of conjugation

- Higher order structures
- Native MS ?
- Ion mobility MS?
- Average DAR

Drug load profile
Unconjugated D0

Determining the Drug Antibody Ratio (DAR) with MS?

\[ \text{DAR} = \frac{\sum_{n=0}^{S} nA_{\text{DAR},n}}{\sum_{n=0}^{S} A_{\text{DAR},n}} = 4.0 \]

- Hydrophobic Interaction (HIC)
- Ion Exchange (IEC)
- Size exclusion chromatography
- Reversed phase (rPHPLC)
- CE-SDS

Minutes

3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00

D0 D2 D4 D6 D8

- Cristallography
- HDX-MS
- SEC-MS
ADCs’ analytical characterization is more challenging than unconjugated mAbs

- Cysteine-conjugation generates heterogeneous population of drug-loads

  - Compared to “unconjugated” mAbs, ADCs have increased level of complexity:
    - a mixtures of species with different nb of drug molecules (0, 2, 4, 6, 8) at different positions
    - a mixture of covalent and non-covalently associated populations

**Need for translational complementary analytical techniques**
Our reference Cys-linked ADC: brentuximab vedotin

mAb
Brentuximab
anti-CD30
(chIgG1)
-Cys-SH (Hinge)

Linker
Peptide protease-clivable linker
-Cit-Val-

Drug
antimicrotubule drug
monomethylauristatin E

- indicated for treatment of hematological malignancies (Hodgkin lymphoma, systemic anaplastic large cell lymphoma)
3 main ADC quality attributes (QA) can be determined by Hydrophobic Interactions (HIC):
- average DAR
- drug load distribution
- Relative amount of unconjugated mAb (D0)

\[
\overline{\text{DAR}} = \frac{\sum_{0}^{8} nA_{\text{DAR}_{n}}}{\sum_{0}^{8} A_{\text{DAR}_{n}}} = 4.0
\]
Native MS: mandatory for cysteine-linked ADC analysis

Denatured conditions
IgGZero treated ADC @ 2 µM in H₂O:ACN:FA (50:50:1)

- Very minor ion signals correspond to intact ADC
- Non-covalent interactions are disrupted in denatured conditions
Native MS: mandatory for cysteine-linked ADC analysis

Native conditions
IgGZero treated ADC @ 5 µM in 150 mM AcONH₄ pH 7.5

Full scan mass spectrum

Intact ADC signals:
D0, D2, D4, D6, D8?

Clarification/simplification of native mass spectra due to fewer charges (increased sensitivity)

Contamination (PEG)
free LC + 1 payload
Cysteines

Native MS: mandatory for cysteine-linked ADC analysis

Native conditions
IgGZero treated ADC @ 5 µM in 150 mM AcONH₄ pH 7.5

- ADC is almost detected as an intact molecule
- Mass measurement affords drug binding stoichiometries to be assessed
- Native MS maintains non-covalent interactions in the gas phase

MaxEnt deconvoluted mass spectrum

<table>
<thead>
<tr>
<th>MWexp (Da)</th>
<th>Δmass (Da)</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0</td>
<td>145903</td>
<td>7.3</td>
</tr>
<tr>
<td>D2</td>
<td>148539</td>
<td>8.1</td>
</tr>
<tr>
<td>D4</td>
<td>151172</td>
<td>5.8</td>
</tr>
<tr>
<td>D6</td>
<td>153814</td>
<td>12.5</td>
</tr>
<tr>
<td>D8</td>
<td>156446</td>
<td>9.2</td>
</tr>
</tbody>
</table>
Native MS analysis to assess average DAR

Comparison between HIC and Native MS for brentuximab vedotin

- Native MS data are in good correlation with HIC data for all QA
- Native MS affords in one single run
  1) drug load profile determination and 2) average DAR calculation
Off-line HIC native MS coupling

Native MS allows to confirm mass homogeneity of each HIC fraction: no mixtures of different drug binding stoichiometries are observed within one HIC fraction.
Advantages of native MS compared to HIC

Cysteines

- **HIC profile**
  - Broad HIC peaks
  - Difficulty to unambiguously assess drug binding
  - No odd drug load expected

- **Native MS profile**
  - Native MS mass accuracies allows unambiguous drug binding stoichiometry assessment
  - Odd drug load are clearly detected
  - No peak broadening as on HIC

Native MS allowed to precise unclear HIC results
Native IM-MS of intact cysteine-linked ADC

**Deglycosylated Parent mAb**

**Deglycosylated ADC**

---

**SynaptG2, 3µM,**

AcNH₂ 150mM pH7.4

Vc=80v, Pi=6mbar, Bias25,

WH=40V, WV=923m/s,

He=130mL/min, N₂= 45mL/min
IM-MS enlighten and characterize the heterogeneity of ADCs

**Cysteines**

Native IM-MS of intact cysteine-linked ADC

**Deglycosylated Parent mAb**

**Deglycosylated ADC**

*SynaptG2, 3µM, AcNH₄ 150mM pH7.4, Vc=80V, Pi=6mbar, Bias25, WH=40V, WV=923m/s, He=130mL/min, N2= 45mL/min*
- ADC heterogeneity in drug binding is observed on native IM-MS plots
- Each individual even-drug load can be separated in IM-MS
- No positional isomers were detected (to low IM cell resolution on intact ADC)

*SynaptG2, 3µM, AcNH₄ 150mM pH7.4, Vc=80v, Pi=6mbar, Bias25, WH=40V, WV=923m/s, He=130mL/min, N2= 45mL/min*
### Native IM-MS of intact cysteine-linked ADC

#### Drug binding induces constant and reproducible \( \Delta t_D \) and \( \Delta CCS \) differences

#### Resolving power of IM cell

\[ t_D/\Delta t_D \text{ at FWHM : } 16.4 \pm 0.8 \]

#### Each individual even-drug load can be separated in IM-MS

### Table

<table>
<thead>
<tr>
<th>Parent mAb (24+ charge state)</th>
<th>ADC (24+ charge state) (brentuximab vedotin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent mAb (24+ charge state)</td>
</tr>
<tr>
<td></td>
<td>ADC (24+ charge state) (brentuximab vedotin)</td>
</tr>
<tr>
<td>( t_D ) (ms)</td>
<td>( \Delta(t_D) ) (ms)</td>
</tr>
<tr>
<td>( \Delta CCS ) (nm(^2))</td>
<td>( \Delta CCS ) (nm(^2))</td>
</tr>
<tr>
<td>CCS from IM-MS (nm(^2))</td>
<td>Predicted CCS (nm(^2))</td>
</tr>
<tr>
<td>Predicted ( \Delta CCS )  (nm(^2))</td>
<td></td>
</tr>
<tr>
<td>D0 14.2</td>
<td>D0 14.2 \pm 0.0</td>
</tr>
<tr>
<td>D2 14.9</td>
<td>D2 14.9 \pm 0.1</td>
</tr>
<tr>
<td>D4 15.6</td>
<td>D4 15.6 \pm 0.1</td>
</tr>
<tr>
<td>D6 16.3</td>
<td>D6 16.3 \pm 0.1</td>
</tr>
<tr>
<td>D8 17.0</td>
<td>D8 17.0 \pm 0.1</td>
</tr>
</tbody>
</table>

### Notes

- Each individual even-drug load can be separated in IM-MS.
- Drug binding induces constant and reproducible \( \Delta t_D \) and \( \Delta CCS \) differences.
For each charge state, intensities of the extracted drift peaks were plotted across a series for each drug binding stoichiometry (D0, D2, D4, D6 and D8) and a Gaussian curve fitted.

To give a relative intensity of each conformer, the area underneath each curve was calculated as a percentage of the total observed signal, here (D0+D2+D4+D6+D8).

\[
DAR = 3.7 \pm 0.1 \quad \text{(triplicates)}
\]
Average DAR determination and drug-load profiles obtained from native MS or native IM-MS are in good agreement with HIC data.

mAbs have more than 60 surface exposed Lys residues (compared to 32 Cys) available for reactions but only 32 cysteines, among which 8 only are involved in the interchain disulfide bridges of chimeric, humanized and human IgG1.

Lysine conjugation generates even more heterogeneous samples even if all species are covalent.

![Diagram of Lysine residues conjugation and distribution](image)
**Benefits of native MS for lysine conjugate analysis**

- **Denatured conditions**
  IgGZero treated ADC @ 2 µM in H₂O:ACN:FA (50:50:1)

- **Native Conditions**
  IgGZero treated ADC @ 5 µM in 150 mM AcONH₄ pH7.5

Very complex mass spectrum: overlapping charge state distribution hamper appropriate Dn quantification

Deconvolution fails in detecting D8 species

---

Decomplexification of the mass spectrum
Deconvolution fails in detecting D8 species

---

* + 220 Da linker adducts
Lysines

Benefits of charge state reduction for lysine ADC analysis

Deconvoluted mass spectrum

MaxEnt1 deconvolution

n=8

Deconvoluted mass spectrum = 3.0

DAR = 3.0

Detection of D8 species

T-DM1 (2µM) + 10 mM imidazole

m/z

5000 6000 7000 8000 9000 10000 11000

%

0 100

m/z

5000 6000 7000 8000 9000 10000 11000

%

0 100

m/z

5000 6000 7000 8000 9000 10000 11000

%

0 100

m/z

5000 6000 7000 8000 9000 10000 11000

%

0 100
Native MS and IM-MS for Lysine-linked ADCs

Sample: trastuzumab emtansine (Kadcyla)

$\text{DAR} = 3.5 \pm 0.1$

Average DAR and drug load profile from native MS

Average DAR and drug load profile from IM-MS
What’s coming next?

- **Trastuzumab**

- **Trastuzumab-B**

**Special feature:**
Head comparison of trastuzumab and cetuximab with corresponding biosimilar and biobetter candidates

Native MS is a rapid, robust and reliable method for first-line semi-quantitative mAb characterization.

Native IM-MS for semi-quantitative global conformational characterization of mAbs.

HDX for structural mAb characterization (epitope mapping, comparability studies, etc.)
Many Thanks for your attention!

The native MS group of LSMBO
Julien MARCOUX
Guillaume TERRAL
Johann STOJKO
François DEBAENE (past)
Cédric ATMANENE (past)
Alain VAN DORSELAER
Sarah CIANFERANI

The Antibody Physico-Chemistry group
Elsa WAGNER-ROUSSET
Olivier COLAS
Nathalie CORVAÏA
Amandine BŒUF (past)
Daniel AYOUB (past)
Alain BECK