Methoden zur Bestimmung von Antikörper Aggregation und Identifizierung unterschiedlicher Ladungs- und Oxidationszustände

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Agenda

- Vorstellung von Agilent Lösungen in der Proteinanalytik
- Analytik Monoklonaler Antikörper, ihre Herausforderungen
- Teil 1: Analytik von Aggregaten mittels Größenausschlusschromatographie
- Teil 2: Untersuchung von Ladungsvarianten von mABs mittels Ionenaustauschchromatographie und Kapillarelektrophorese
- Zusammenfassung
Agilent’s Biologic Characterization and QA/QC Solutions

Glycan Analysis
- mAb-Glyco-Chip + Chip LC/MS
- Capillary Electrophoresis HPLC/FLD

Aggregation
- Size Exclusion Columns + HPLC/UV
- Reversed Phase Columns + LC/UV or LC/MS

Biologic Molecular Weight Determination
- CE/MS

Charge Variants
- Ion Exchange Columns + LC/UV
- Agilent CE + CE Kits
- Agilent CE/MS + CE Kits

Peptide Characterization/Mapping
- Reversed Phase Columns + LC/UV or LC/MS

Oxidation
- CE/MS

Amino Acids
- Reversed Phase columns, derivitization, eluents and standards + LC/UV or LC/MS

Oligonucleotides
- Reversed Phase or Ion Exchange columns + LC/UV or LC/MS
Important to know upfront

- we speak about **recombinant** biotherapeutics here!!

- there is **always heterogeneity** in a biodrug

- **assays** are done to
  a.) **characterize** the drug molecules profile
  b.) confirm that the **profile is the same** as before in order to ensure drug safety and efficacy

- in different phases of drug development different techniques are used in order to investigate for the same attribute
  a.) focus on **MS-based** techniques in **discovery** and development
  b.) if possible **non MS-based** techniques in **QA/QC**
Monoclonal antibodies as example

The structure of antibodies
-oh how complex they are-

From: Vlasak et al. *Current Pharmaceutical Biotechnology, 2008, Vol. 9, No. 6*
1. Aggregation Analysis

Why is there aggregation of biologic therapeutics?

• proteins are marginally stable in solution
• due to various stresses (heat, thawing, shearing) during purification, processing and storage precipitation and aggregation can occur

Result: Aggregates (dimers, trimers and higher order multimers) are formed

Consequence: Aggregates can cause anaphylactoid reactions or loss of drug efficacy

Current Analytical Methods to Detect Aggregates:

- SEC/UV, SEC/FLD, SEC-MALS, SEC= size exclusion chromatography
- AUC = analytical ultra centrifugation
- FFF = free field fractionation
- DLS, MALS DLS= dynamic light scatter, MALS= multi angle light scatter
Basics of Bio-Chromatography

Size Exclusion Chromatography

- Protein/peptide sample contains molecules of different size
- Column contains porous silica particles
- Particles act as molecular sieve
- Larger proteins elute first, passing through the column more rapidly, followed by smaller proteins

Isocratic Separation
What can Agilent offer for Aggregate Analysis?

Instruments suited for SEC:
1260 Infinity Bio-inert quaternary LC
1260 Infinity isocratic, binary or quaternary LC
1220 Isocratic

Columns for aggregate analysis:
- Unique, 3 μm particle
- Proprietary hydrophilic coating to prevent secondary interaction
- 100Å, 150Å, 300Å pore sizes
- Highest resolution
- Highest efficiency
- Faster SEC separations
- Can be run with low salt buffers

Bio-inert fraction collector available

The choice for bioanalytical and biopurification up to 10 ml/min
Agilent Bio SEC-5 and Bio SEC-3
Monoclonal Antibody Aggregation Monitoring

- faster separation
- higher resolution
- longer lifetime
- superior repeatability
- less unspecific interaction

Choice of different pore sizes
Unique hydrophilic coating
3 µm and 5 µm particle columns
Fast SEC – Without NaCl in the Buffer

**Column:** Agilent Bio SEC-3, 7.8 x 150mm

**Sample:** mAb (2mg/ml)

**Injection:** 5ul

**Flow rate:** 1.0, 1.5 and 2ml/min (56 bar, 75 bar, 105 bar)

**Eluent:** 150mM sodium phosphate + 100mM Na-sulfate

**Detection:** 220nm

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>Resolution Monomer/Dimer</th>
<th>Monomer Efficiency</th>
<th>Percentage Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0ml/min</td>
<td>1.53</td>
<td>3,510</td>
<td>0.64</td>
</tr>
<tr>
<td>1.5ml/min</td>
<td>1.43</td>
<td>2,502</td>
<td>0.47</td>
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<tr>
<td>2.0ml/min</td>
<td>1.13</td>
<td>1,917</td>
<td>0.64</td>
</tr>
</tbody>
</table>

**Monomer Efficiency**

**Dimer Efficiency**

**Percentage Dimer**

**Resolution Monomer/Dimer**

**Detection:** 220nm

**Flow Rate:**

- 2.0ml/min
- 1.5ml/min
- 1.0 ml/min

**4 Minutes**
Detection Modes in SEC other than UV: MALS and DLS

- determines the absolute molar mass and the average size of particles in solution, by detecting how they scatter light in solution

- MALS measurements work by calculating the amount of scattered light at each angle detected

Agilent 390 Multi detector suite
- Refractive Index
- Viscometer
- Dual Angle Light Scattering detector
- Coming soon:
  - Dual angle LS with Dynamic Light Scattering

Principle of MALS
- Dynamic light scattering is used to determine $R_H$, the hydrodynamic radius
- Static light scattering can determine $R_g$, the radius of gyration

Wyatt, Mini Dawn, Dynamic light scatter
Are there any IEX or SEC columns requiring 1000 bar equipment?

No

• You do not run large proteins too fast due to slow mass transfer !!!
• Excessive pressure might destroy protein conformation
• No real bio-inert system can tolerate 1000 bar
• Benefit of small particles: yes, same resolution with shorter columns and shorter runtimes

Example calculations for the bio-columns generating highest pressure:

**SEC:** BEH200 1.7, 4.6 mm ID, 300 mm
→ Recommended flow rate 0.4 ml/min, resulting pressure: 341 bar
→ Even at 0.6 ml/min: 512 bar

**IEX:** Agilent Bio mAB 1.7 um, 4.6 mm ID, 250 mm ID (most challenging column now)
→ Optimum flow at 0.5 ml/min, resulting pressure: 355 bar
→ Even at 0.75 ml/min : 533 bar

The 1260 bio-inert LC (600 bar system) is perfectly suited to handle the pressures of all current Bio columns
2. Charge Variant Analysis

Why is there charge heterogeneity in biologic therapeutics?

Amino acids can undergo alterations during production and storage

Charge heterogeneity in therapeutic proteins in mABs can be caused by a variety of factors:

- Incomplete C-terminal processing of Lys and Asn
- Deamidation of Asn and Gln and isomerization
- Oxidation of Met, Trp, Cys, His
- Sialylation of terminal glycans
- Disulfide mediated conformational change
- Glu cyclization = pyroglutamate
- Adduct formation/control

Example for charge heterogeneity: deamidation of asparagin
Two Different Major Techniques to Analyze Charge Heterogeneity

Charge heterogeneity information has to be quantitative

**HPLC:** *Cation Exchange Chromatography*
- **pro:** easy to use
  - excellent quantitation and reproducibility
  - can be used to isolate impurities
- **con:** resolution
  - speed

**CE:** *Capillary Isoelectric Focusing (cIEF)*
- **pro:** speed
  - resolution
- **con:** reproducibility
  - sensitivity
  - not suitable to isolate impurities
Workflow for Characterization of Charge Heterogeneity

Charge profile obtained by LC or CE

- different: Isolation of impurities
- similar: No further action

LC/MS based characterization
2a. LC-based Approaches to Charge Heterogeneity

Problems that can be associated with LC-based charged variant analysis:

- high salt or pH changes
- unspecific interaction of molecule with surfaces
- metal ions eluting from instrument can cause disturbance of chromatography or column poisoning
- **Long runs!**

![Graph showing resolved basic and acidic variants](image1)

![Graph showing massive fronting and tailing](image2)

**How a charge profile should look like and how not!**

Resolved basic and acidic variants

Massive fronting and tailing makes quantitation impossible
• Sample is injected in a mobile phase buffer with low salt
• Analytes are typically eluted at constant pH with increasing salt (ionic strength)
• Higher charge proteins bind more strongly, more salt is needed for elution
• A typical mobile phase will contain NaCl (corrosive)
• Alternatively elution can be done with a pH gradient
Charge Variants

Charge Isoform Analysis of Monoclonal Antibodies
- a typical example how it should look like -

Agilent 1260 Bio –inert LC, Agilent Bio MAb, NP5, 4.6mm x 250mm
Buffer A: 10 mM Sodium Phosphate, pH 7.50
Buffer B: A + 100 mM NaCl, pH 7.50
Gradient: 15-95% B in 60 min
Flow rate: 0.8 mL/min.
Sample: 5 ul 5 mg/mL, mAb
Why a Bio-inert HPLC Solution for Biopharma?

Reasons for having a dedicated Bio-inert HPLC:

- Corrosion and pH issues with standard LC
- Unspecific surface binding
- Low throughput, low resolution
- Decreased column lifetime and chromatographic performance due to column fouling
- Avoid peak tailing for critical proteins

• More confidence out of your analysis
• No passivation required
What can Agilent offer?

The Agilent 1260 Infinity bio-inert quaternary LC

*The New Standard in Bioanalysis*

**100% bio-inert**
- Precious sample does not touch metal surfaces
- Extended pH range 1-13 (shortterm 14)
- High salt tolerance: 2 M salt, 8 M urea
- No stainless steel in mobile phase flow path
- New capillary technology

**UHPLC capability**
- 600 bar

**Superior Ease of Use and Robustness**
- Active seal wash
- Quaternary buffer mixing
- Superior Bio-HPLC columns for biotherapeutic characterization
- Column compartment for up to 30 cm columns

*The choice for bioanalytical and biopurification up to 10 ml/min*
Materials used in the sample flow path

Agilent 1260 Infinity bio-inert LC (guaranteed metal-free):

• new PEEK-capillaries (PEEK inside to provide bio-inertness, metal outside to allow for 600 bar)

• new fitting design

• ceramic autosampler needle

Other materials used in the industry:

• **MP35N**: Cobalt, Nickel, Chromium, Molybdenum

• **Inconel 300**: Nickel, Chromium, Iron

→ this is not bio-inert since those metals are complex formers
Agilent’s Column Portfolio: NEW Ion Exchange Columns

**Agilent Bio MAb**
High Resolution Separations of Monoclonal Antibodies

- Non-porous PS/DVB particles
- Uniform polymeric coating and WCX layer, specifically designed for antibody separations
- Available in 10 µm, 5 µm, 3 µm, 1.7 µm particle sizes
Agilent Bio MAb HPLC columns: superior performance from the inside out.

- Particles, coating and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Hydrophilic coating eliminates most non-specific interactions
- A highly uniform, densely packed, weak cation exchange (WCX) layer chemically bonded to the hydrophilic, polymeric coating
Workflow for Characterization of Charge Heterogeneity with LC

- Charge profile obtained by LC
  - different: Isolation of impurities by fraction collection
  - similar: No further action
- LC/MS based characterization
2. CE Based Separations: What can we offer?

**3100 OGE – Offgel Electrophoresis**
- Fractionation system for protein/peptide separation
- IEF based separation & sample prep
  (for 2D: combine with LC-MS, 2100 or CE)

**2100 Bioanalyzer – Microfluidic system**
- Fast analytical system dedicated to bio- molecules and cells
- Predefined methods and reagent kits

**7100 CE – Capillary Electrophoresis**
- Flexible analytical system for all CE modes
- Broadest range of detectors, DAD, MS, LIF, CCD,...

New! **Lab901 TapeStation**
- DNA, RNA, Protein
- 96 Wellplate format
Capillary Electrophoresis: Advantages

Ideal for charged or polar samples

- Highest resolution, often > 100,000 plates!
- Minimal sample prep, e.g., filter and dilute

- High resolution due to non-laminar flow
- Fast separations
- Smallest sample volumes required
- Can handle crude matrices
- Low consumption of solvents (buffers)
- Orthogonal to HPLC
Agilent CE-ESI-MS Setup

On a single PC run:
- MassHunter
- CE-ChemStation / Open Lab CDS

PC-system controlling
- 7100 CE
- MS systemes
- Sheath liquid pump

The Agilent CE-MS Advantage

Single vendor solution: direct and competent support
Sheath Liquid Interface: robust and reliable, offering efficient control on chemistry
Capillary outlet on ground: no compromises on voltages for CE or ESI-MS
CE methods for charge heterogeneity analysis

cIEF = capillary iso electric focusing

**Steps:**
1. **Focusing:** formation of a pH gradient with ampholytes, proteins migrate to respective pH zone according to their pl
2. **Mobilization of gradient** (pressure, chemically) to move zones through detector

*Figure 4.11 The Principle of Isoelectric Focusing*

What to do if you have an aberrant charge profile?

G3100 OFFGEL: Charge Variant ID of NBE’s

OFFGEL Key Features

- Preparative IEF of native or denatured mAb
- Flexibility in pl Resolution
  OFFGEL is using industry-standard IPG strips.
- Throughput
  Process up to 16 samples in parallel to

Meert, et. al., Anal. Chem. 2010
Summary

• Agilent has the most comprehensive portfolio for all applications shown

• There are application notes available for all applications presented

• A primer (August/September) will summarize all available techniques that we suggest

• A primer on "validation in the Biopharmaceutical Lab" is available

• check at www.Agilent.com (bio-inert quaternary LC or CE)
Thank you for your attention !!!

Any Questions?