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Drug Optimization & Pharmaceutical Profiling

# Protein Characterization in Drug Development

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# Personal Background

3 years in **Biopharma**: Swedish Orphan Biovitrum, SOBI (2017-2020)

-A **biopharmaceutical** company focused on rare diseases

-Department of Biopharmaceutical **Research** and **Development** (BRD)

-Section of **Protein Characterization** (**mass spectrometry**)

Currently at **facility** focused on ADME of therapeutics at Uppsala university

-**ADMEoT**, Drug Discovery and Development platform (**DDD-P**), **SciLifeLab**

-Uppsala University Drug Optimization and Pharmaceutical Profiling Platform (**UDOPP**)

-Focused on **mass spectrometry of therapeutic proteins**

# Candidate Drug to Clinical Trials

- Going from having a **candidate drug** in the lab to supplying material for a clinical trial requires the development of a process for **large scale manufacturing**

- The process is tightly regulated by the regulatory agencies in the different countries
    - USA: Food and Drug Administration (**FDA**)
    - EU: European Medicines Agency (**EMA**)

- The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (**ICH**)
    - **Harmonize** the requirements
    - Detailed **guidelines** for each step of the drug development

# Definitions

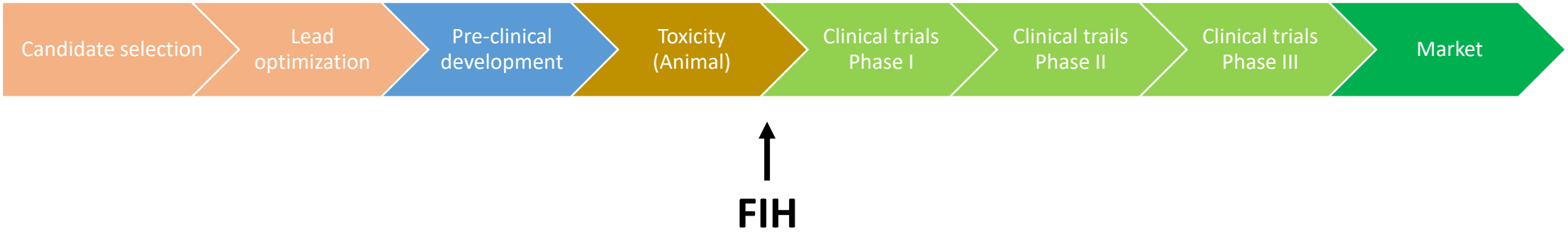
- Drug substance
  - Active pharmaceutical ingredient
  - End product from the purification process
  - Raw material for drug product
- Drug product
  - One or more drug substance
  - Excipients/additives
  - Raw material for the finished product
- Finished product
  - The sold product

# Process Development


Scale up process: lab -> factory
ml -> 1000(s) liters

Generate process that is: Robust
Yield same quality each time
**High protein yield**
Pure, with a minimum of contaminants
**Potent**
Simple process
**Cost efficient**

# Process Development




The **process** should largely be **finished** by "**first in human**" (FIH), since the final product need to have the **same characteristics** as was used in toxicity and clinical trials.

If production is increased (e.g. 1000 L batches -> 2000 L batches), the company needs to show that the product is not affected.

# Process Development

- Four functions:
  - Upstream
  - Downstream
  - Analysis
  - Formulation

# Upstream Process Development

- Develop bioreactor process
  - Scalable
  - Robust
  - Consistent product at variable scales
  - Suitable for GMP manufacturing
  - Not too complex
- High productivity, with low levels of impurities
- Maintain quality and function of the protein

- Expression systems:
  - Bacteria
  - Yeast
  - Mammalian cells

GMP = Good Manufacturing Practice

# Upstream Process Development

- Compare cell lines and clones
- Optimize growing conditions (e.g. media, temperature, pH)
- Optimize harvesting conditions

- Harvest
  - Clarification
  - Removal of cells and debris
  - Filtering

# Aim of the Purification Process (Downstream)

- Remove impurities
  - Host cell protein (ppm)
  - Host cell DNA (ppb)
  - Product aggregation

ppm = parts per million
ppb = parts per billion

- Remove contaminants
  - Bioburden (amount of viable microorganisms)
  - Endotoxins
  - Virus

- Maintain product function and yield

# Downstream Process Development

Purification

1) Reduce volumes and impurities
2) Ion exchange chromatography step(s)
   -To reduce impurities to target levels
3) Final concentration and buffer exchange

Virus reduction steps

-Introduced from cell line, raw material, or operator

-inactivation and filtering

# Protein Characterization is Needed at All Stages

- At each stage of the **purification**, and through out the **process development** the product is characterized using a variety of methods

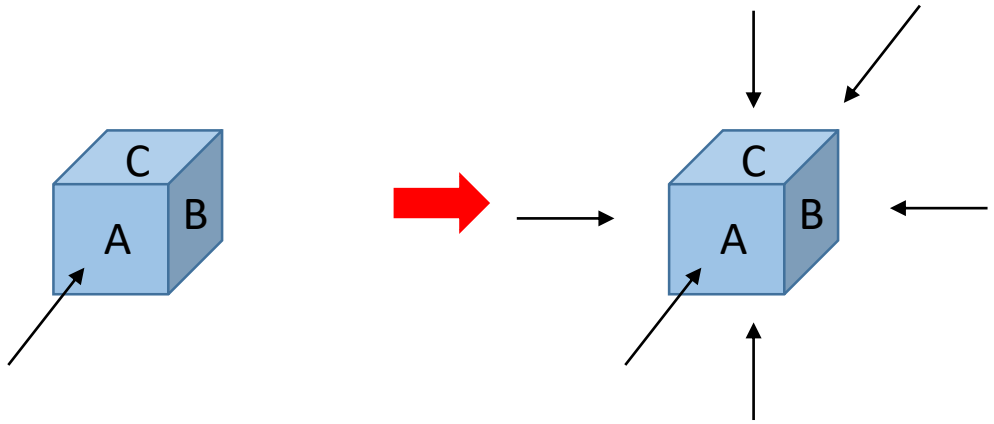
- Aim: Defining and Monitoring Quality Attributes
  - Characteristics that can be detected and quantified
  - Correlation to stability, potency, etc.

- The optimization of the process development is evaluated using these quality attributes

# Finding the "Right" Characteristic to Assay

- To know what is on each side of a cube, it is **not enough to check one side**, all sides need to be checked.

- Similarly, it is not enough to analyze a therapeutic protein with only one method. **Many methods** need to be used to characterize the protein before understanding which **characteristic(s)** is/are **most important**.




**Characteristics**
Retention time
Size
Glycosylation pattern
…

**Effect**
Yield
Potency

# Protein Characterization of Product

**Quality Attributes for mAb**

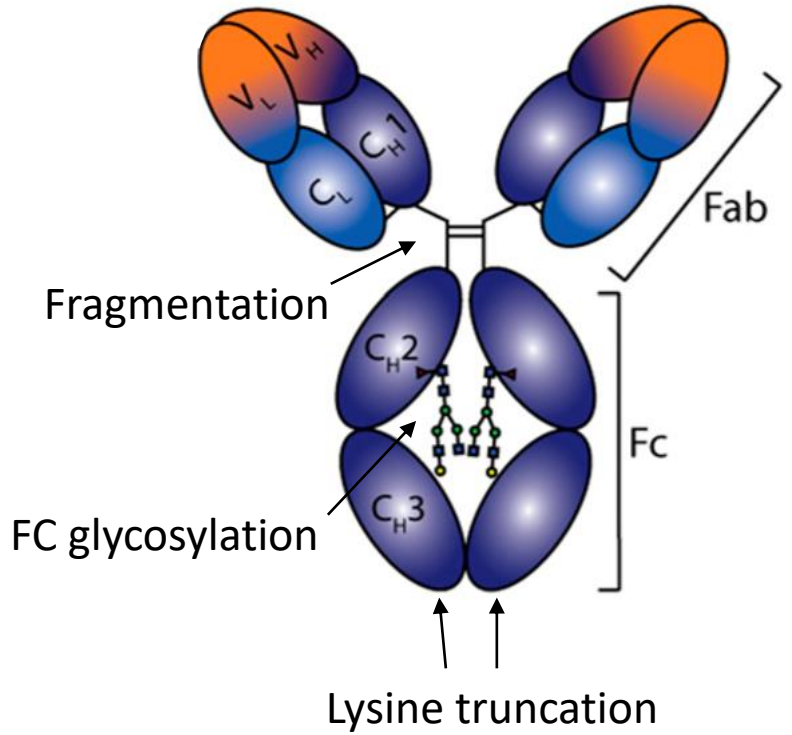
**Amino acid modifications**

- Deamidation
- Oxidation
- Lysine truncation
- Disulfide shuffling
- FC glycosylation

**Structural abnormalities**

- Aggregation
- Misfolding
- Fragmentation

**Monoclonal antibody**




Possible correlation to activity, stability and/or immunogenicity

j immunol, 2016, 196:1435-1441

# Methods Used for the Characterization

**Quantification**

| | |
|---|---|
| UV spectrophotometry (A280 nm) | -Amount |

**Purity**

| | |
|---|---|
| • Gel electrophoresis (1D SDS PAGE): | -Molecular weight, sample complexity |
| • Size exclusion chromatography: | -Complex size: monomer, dimer, trimer, etc. |
| • Reverse phase chromatography: | -Hydrophobic interactions: pre-peak(s), main peak, post-peak(s) |
| • Isoelectric focusing/Ion exchange chromatography: | -Charge based heterogeneity |

**Physical properties**

| | |
|---|---|
| • Dynamic light scattering: | -Aggregation |
| • Differential scanning fluorimetry (DSF) | -Melting temperature/structural stability |

# Impurities

- Cell related
  - Host cell DNA: qPCR
  - Host cell proteins: ELISA, LC-MS
- Process related (mAb)
  - Residual protein A: ELISA
  - Insulin: ELISA
- Contaminants
  - Bioburdan*: TAMC/TYMC
  - Endotoxin: LAL assay

Can cause response in patient!



*Amount of viable microorganisms; ELISA = enzyme-linked immunosorbent assay; TAMC, total aerobic microbial count; TYMC, total yeast/mold count

# Potency Assays

- Crucial to be able to assay mechanism of action
- Often cell based
- Exact type will depend on the mechanism of action of the protein

- Examples of assays mAb:
  - Antibody-dependent cell mediated cytotoxicity
  - Complement dependent cytotoxicity
  - Apoptosis
  - Binding to target on cells
  - Binding to target – binding or blocking soluble target

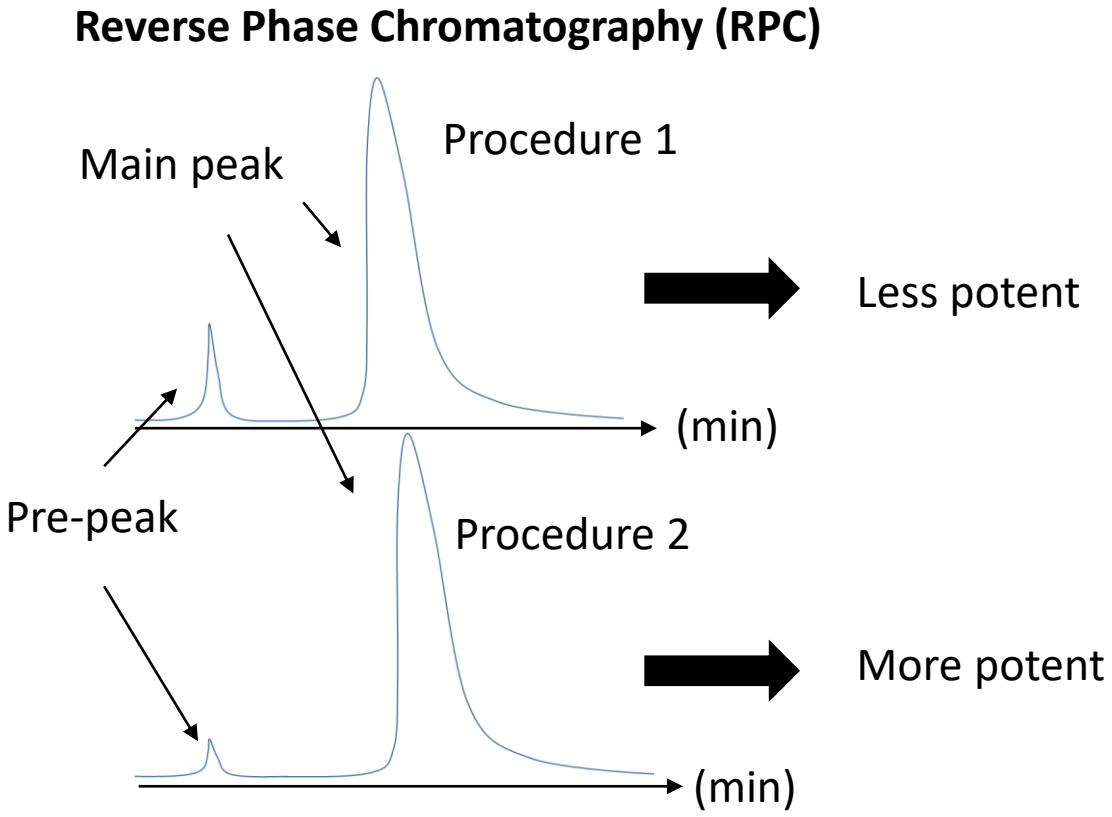
# Mass Spectrometry Based methods

- Protein mass

- Peptide mapping:
  - Sequence coverage
  - Post-translation modifications (PTMs)
  - Glycopeptides
  - Terminal sequence analysis
  - Non-reduced peptide mapping for disulfide bridge mapping

- N-linked oligonucleotide analysis
  -Global carbohydrate profile

# Hypothetical Example One: Loss of Activity Due to Pre-Peaks in RPC



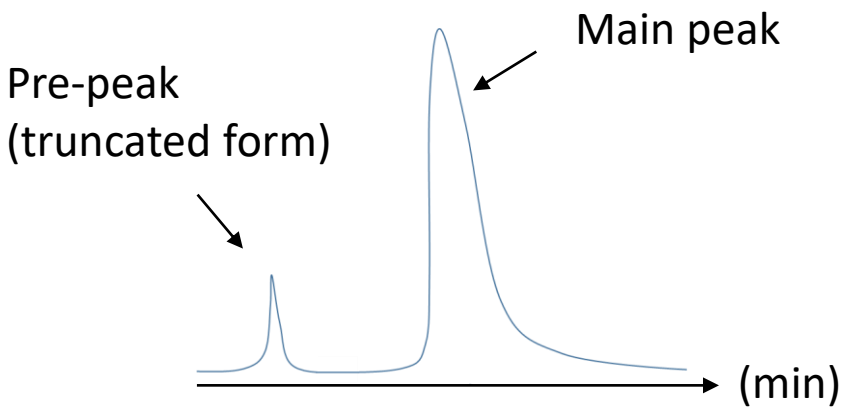

**Observation**
More pre-peak yield lower potency
(e.g. inactive form of protein)

**Solution**
Only collect fractions with main peak
(increase purity, but lower yield)

**Step to optimize**: Downstream/Purification

# Hypothetical Example Two: Loss of Activity Due to Truncation

**Reverse Phase Chromatography (RPC)**




**Observation**
A truncated form of the protein is generated during the purification process -> reduced yield + impurity that needs to be removed

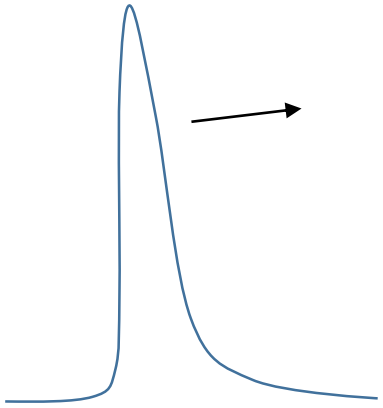
**Solution**
1) Change the amino acid sequence to remove the vulnerable site for cleavage or

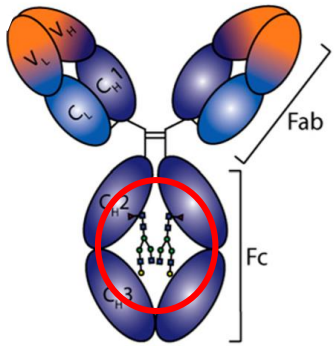
2) Change the cell line to one without the responsible protease

**Step to optimize**: Upstream/clone or cell line

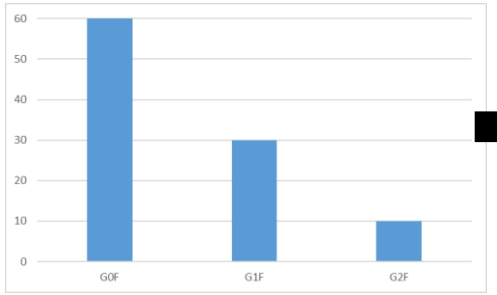
# Hypothetical Example Three: Increase Activity by Modifying the Carbohydrate Profile

Main peak (RPC)



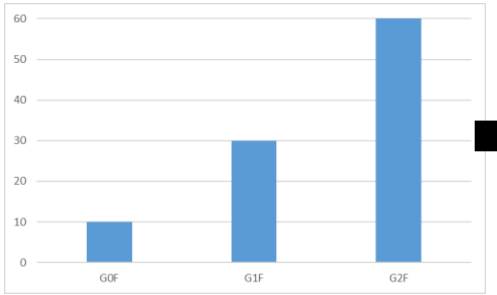



Carbohydrate Profile 1




Less Activity

Carbohydrate Profile 2




More Activity

G0F G1F G2F

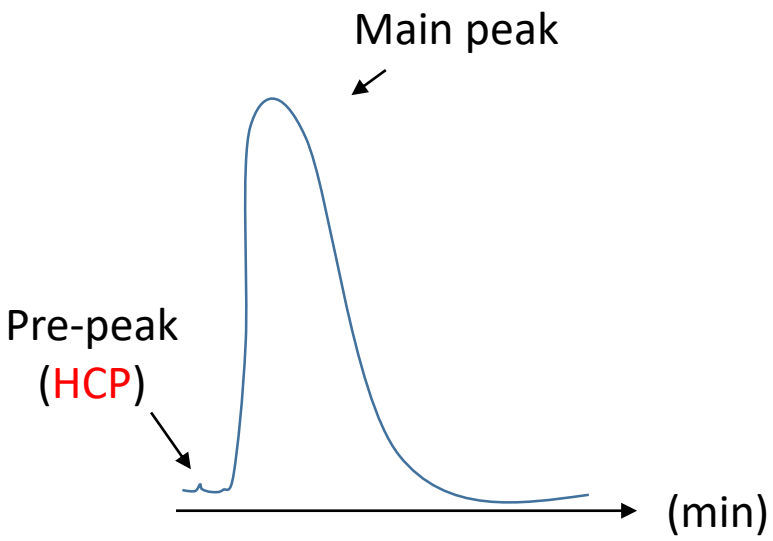
**Observation**
The half life can be extended by modifying the carbohydrate profile
(knowledge from experimentation or literature)

**Solution**
Change the media composition

**Step to optimize**: Upstream/Growing conditions

# Hypothetical Example Four: Presence of Host Cell Proteins (HCP)

**Reverse Phase Chromatography**




**Observation**

The product contain a minor component of HCP which is difficult to remove completely:

e.g. Annexin A2 during mAb purification
-> Risk for immunogenicity

**Solution**

Generate (or buy) deletion/knock-down version of the expression system

**Step to optimize**: Upstream/expression system

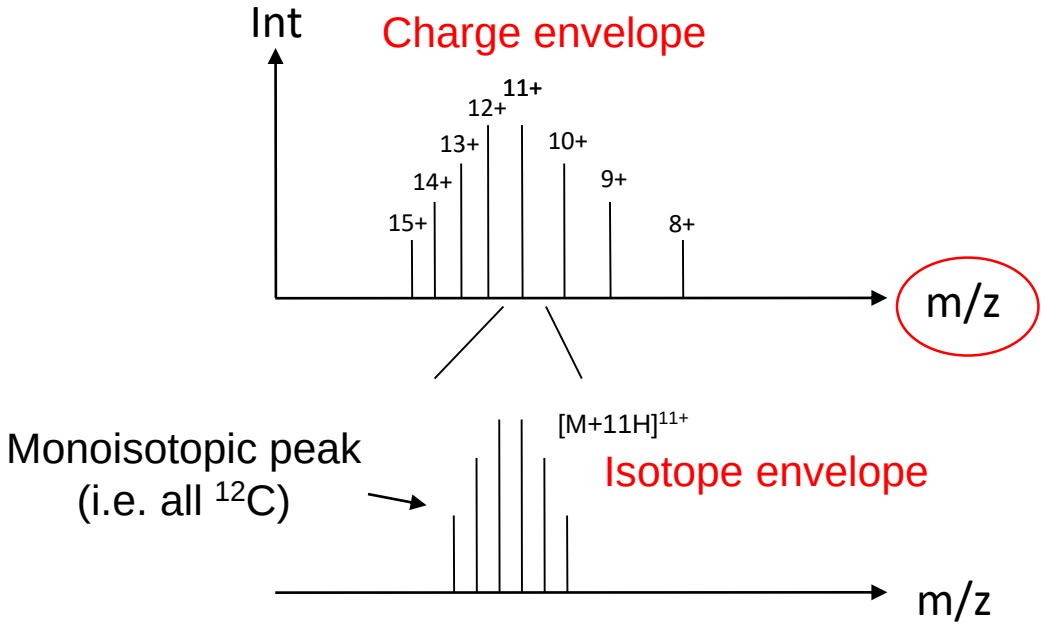


Biotechnology Progress. 2019;35:e2820.

# Mass Spectrometry Based Methods

# Mass Spectrometry – Intact Mass

- Mass spectrometry (MS) measures **charged particles** - mass divided by charge (m/z)
- Proteins are large and can accept many charges (protons) during ionization
  => **Charge envelope**



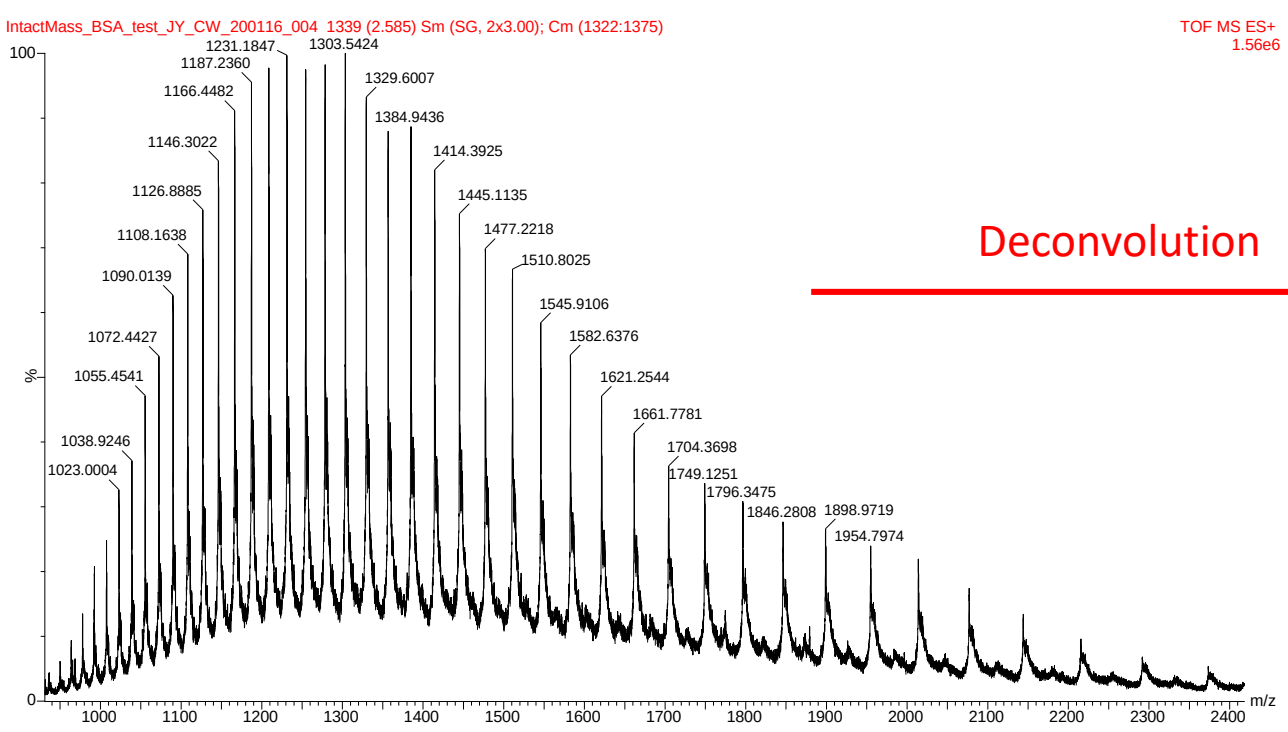

The distance between peaks vary and depend on the charge (shorter to larger)

The distance between isotopes is all the same 1 Da / charge state

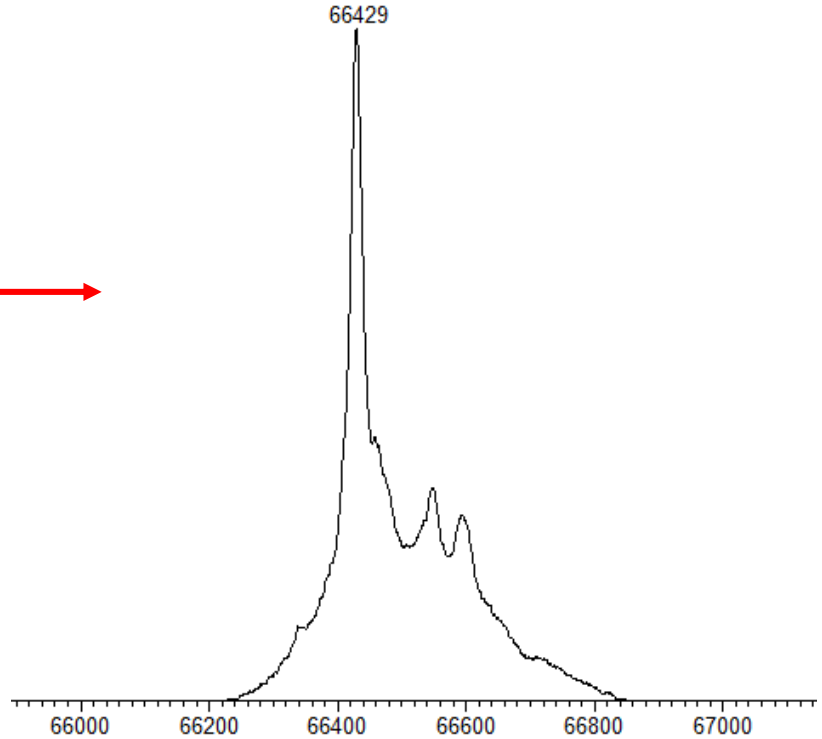
**The charge of each peak in the charge envelop can be determined by:**
1) The distance between isotopes
2) The distance between charge states

→ **Neutral mass**

# MS of Intact BSA




Deconvolution



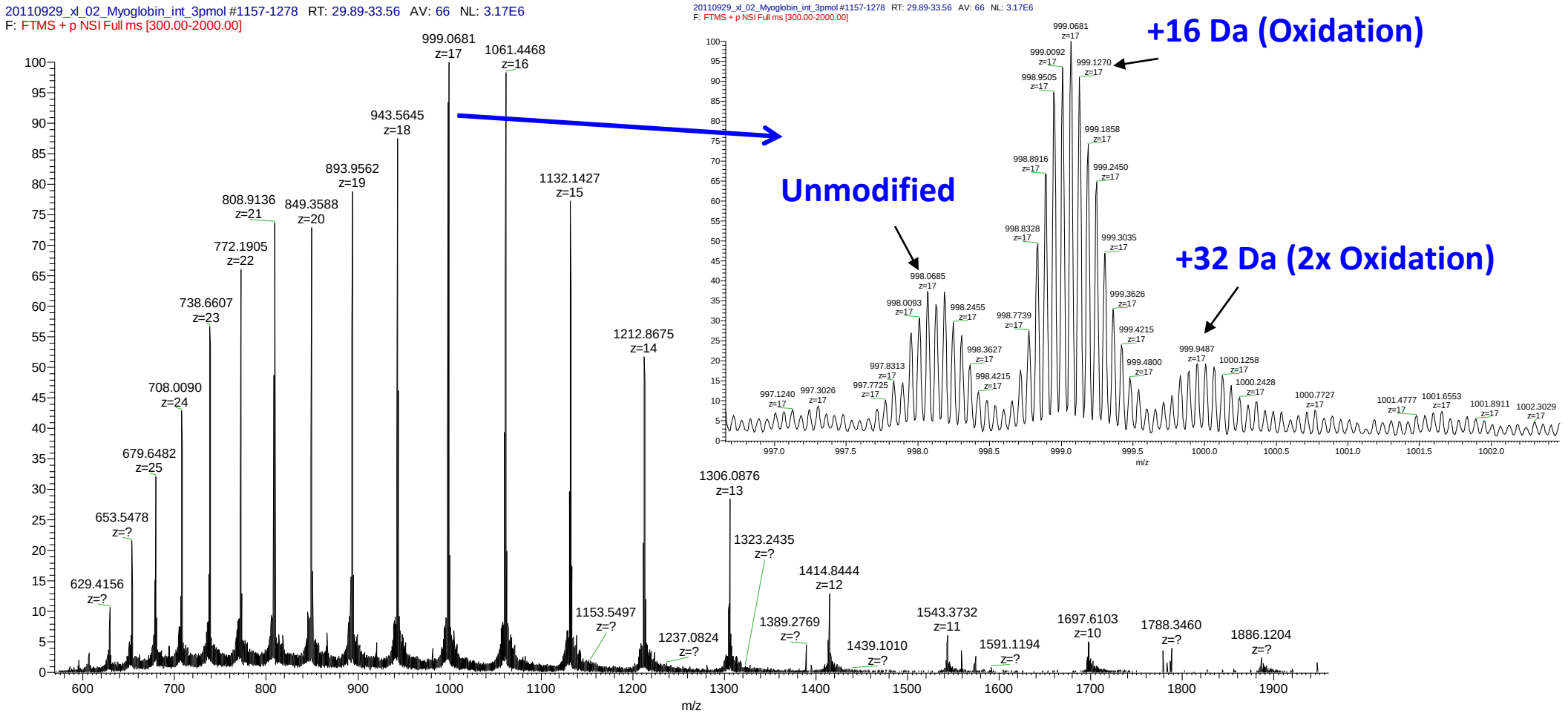

Measured mass: 66,429 Da
Expected mass [1]: 66,430 Da

Deviation: -1 Da (-15 ppm)

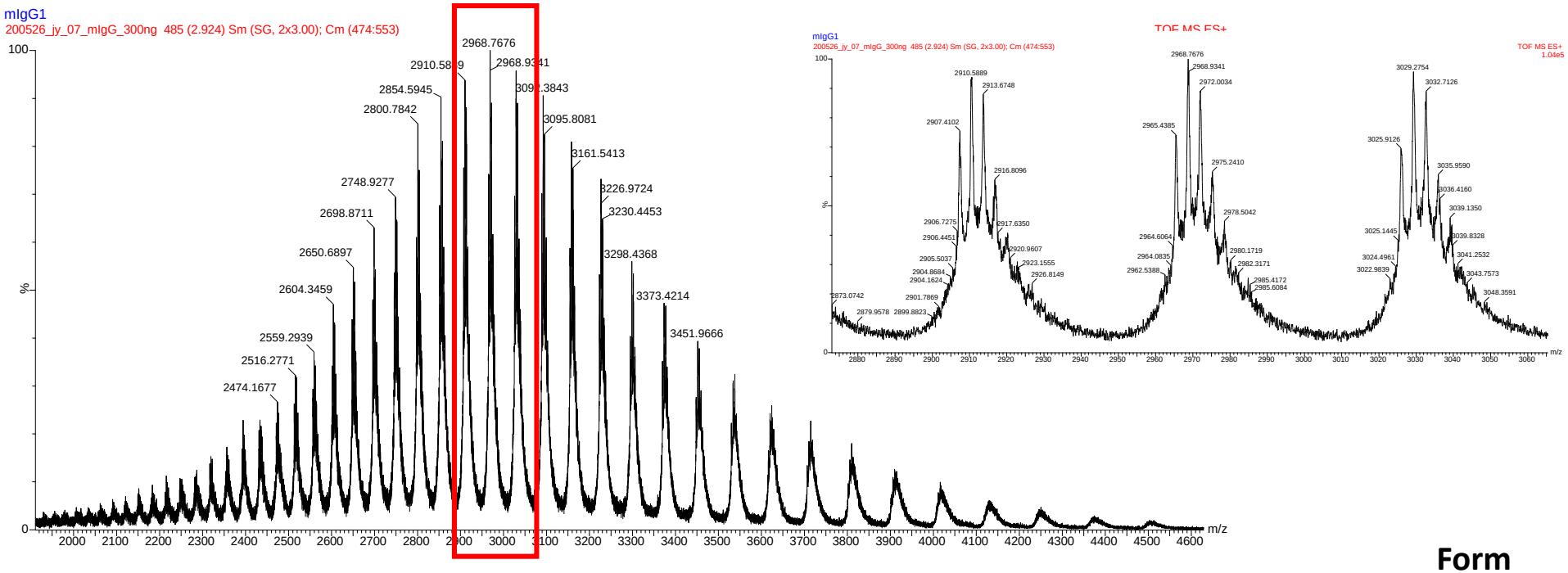


[1] Hirayama et al., Biochem. & Biophys. Res. Comm.,1990, 639-646

# MS of Myoglobin, 17kDa protein



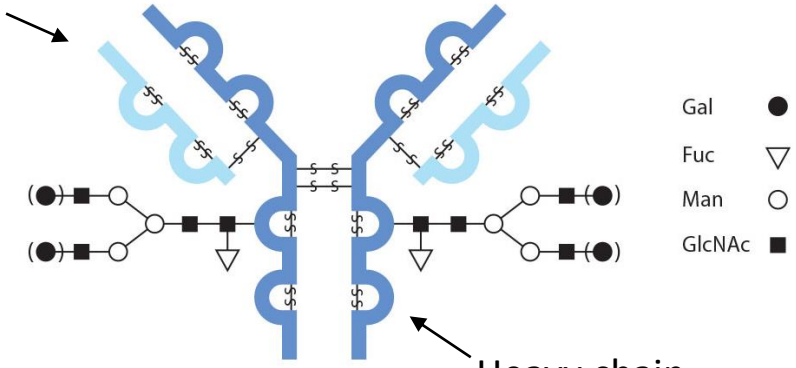
# MS of Intact mouse IgG1





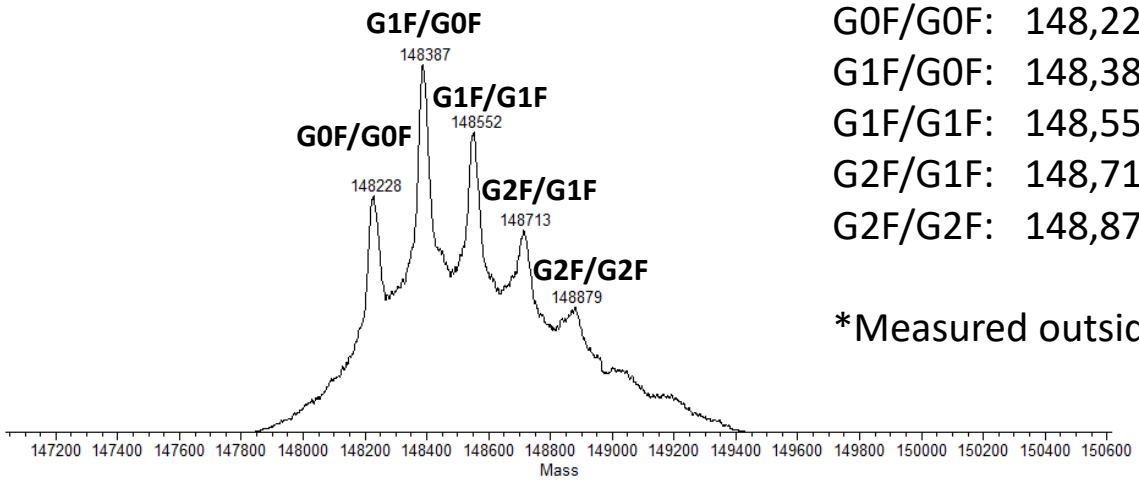


Light chain



Heavy chain

Gal ●
Fuc ▽
Man ○
GlcNAc ■



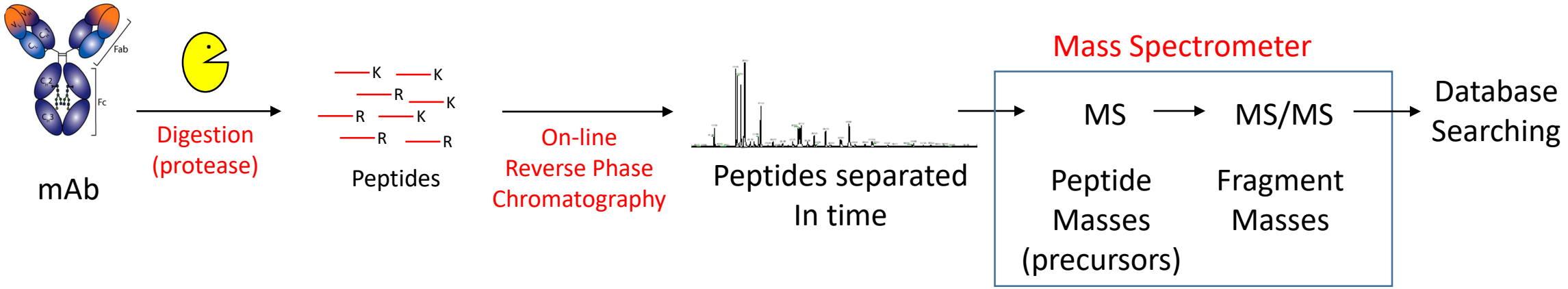

| Form | Mass | Deviation* |
|---|---|---|
| G0F/G0F: | 148,228 | 7.7 Da |
| G1F/G0F: | 148,387 | 5.4 |
| G1F/G1F: | 148,552 | 9.1 |
| G2F/G1F: | 148,713 | 7.1 |
| G2F/G2F: | 148,879 | 10.1 |

*Measured outside calibrated range

# Peptide Mapping Using LC-MS: 1) Peptide Identification

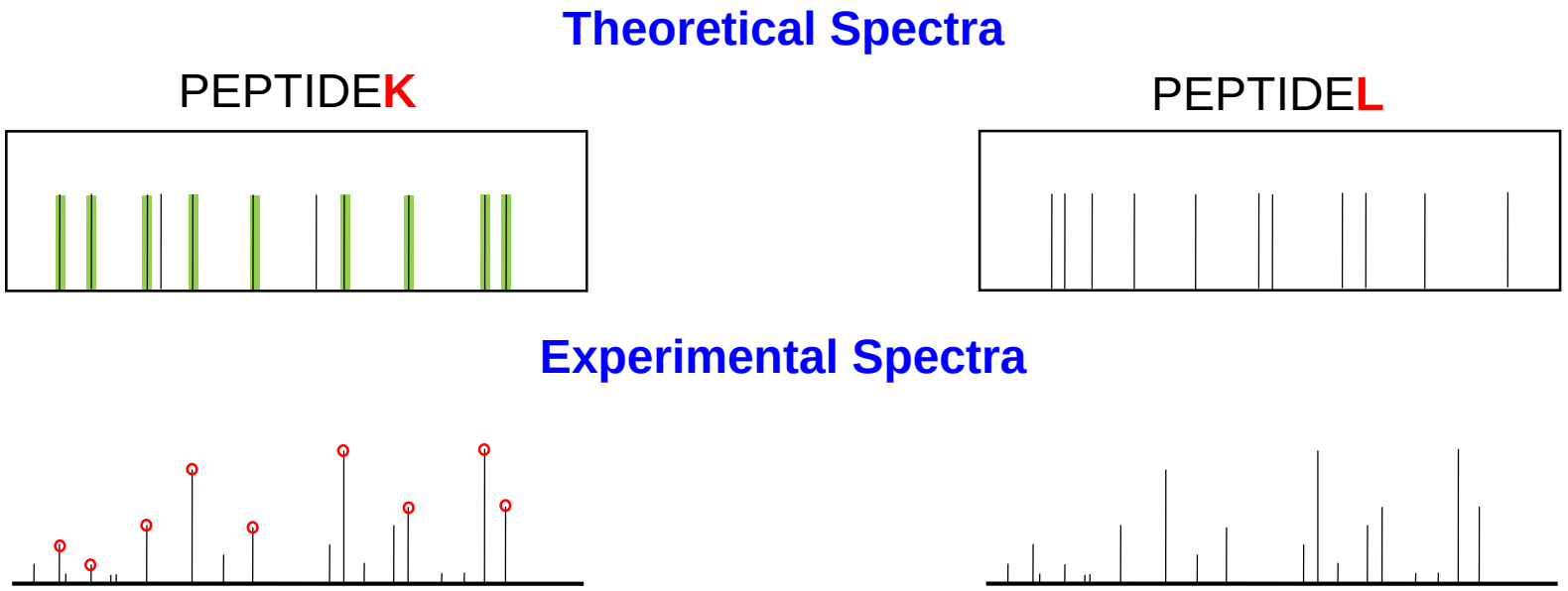
**Peptide identification**

- Proteins are digested by proteases (trypsin) to generate peptides
- The peptides are separated using on-line reverse phase chromatography
- The precursor mass is determined in MS
- The fragment masses are determined in MS/MS
- The peptide sequence is determined by database searching



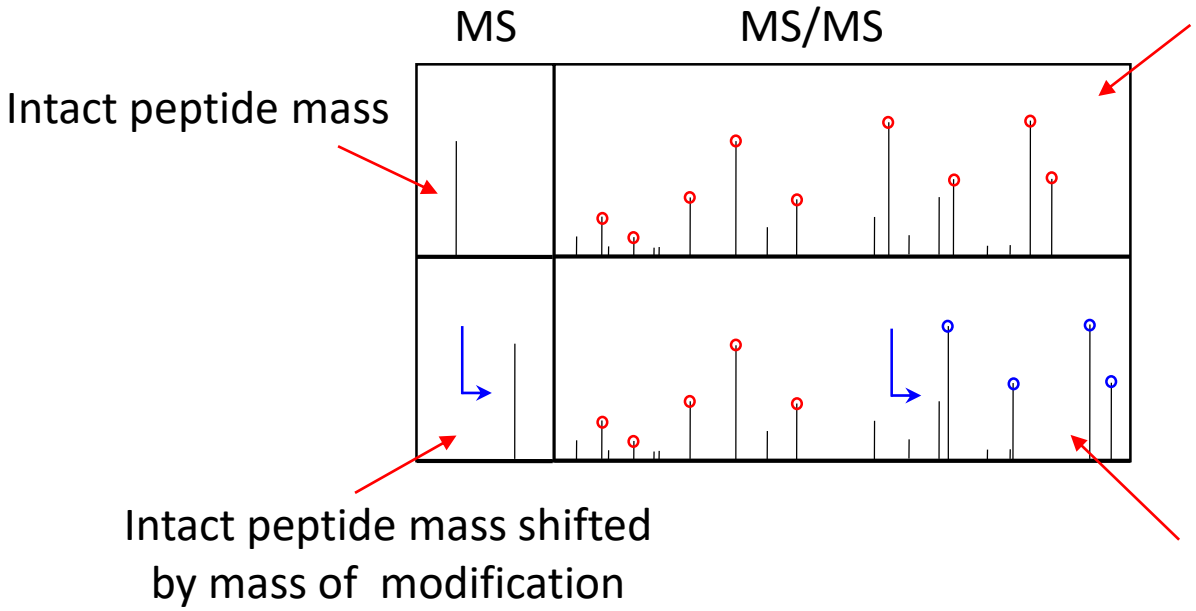

LC-MS = liquid chromatography coupled on-line to mass spectrometer

# Peptide Mapping Using LC-MS: 2) Peptide Identification




During the database search, each **MS/MS spectrum** together with the **precursor** (peptide) mass is **compared** to **theoretical spectra** and ranked according to number of matched fragments. Best rank = **peptide ID** (sequence)

# Peptide Mapping Using LC-MS: 3) Identification of Modified Peptides




Fragment masses matching to theoretical masses indicated by circles (◦/◦)

PEPTIDEK
(unmodified)

PEP[**T**]IDEK
(modified **T**)

All fragments containing modification are shifted by modification mass (**blue**)

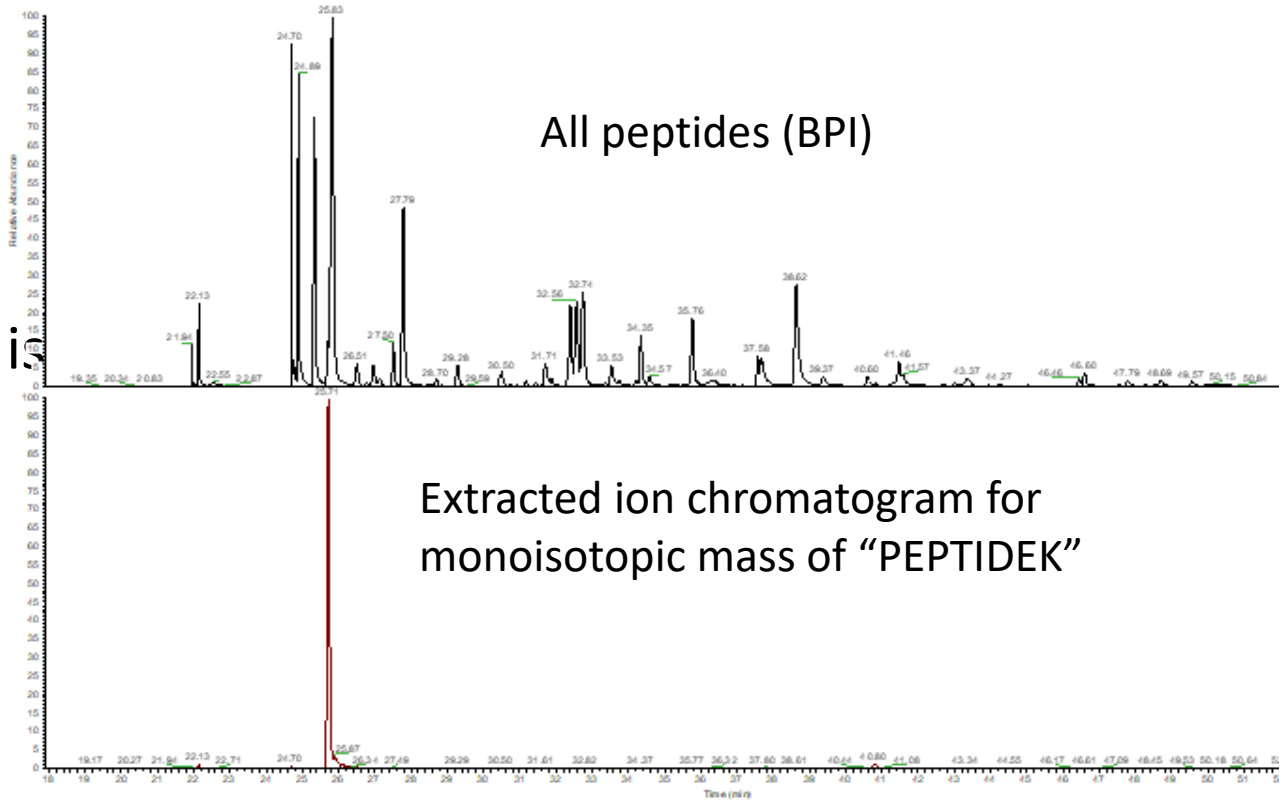
Modified peptides are identified in the same way as unmodified peptides, with the exception of allowing for the modification mass

# Peptide Mapping Using LC-MS: 4) Quantification of Identified Peptides

- The peptide mass (m/z) of each identified peptide is extracted
- The area is integrated
- The relative amount of each peptide is calculated (%)

**Relative amount:**

| | |
|---|---|
| PEPTIDEK | 70% |
| PEP[T]IDEK | 30% |





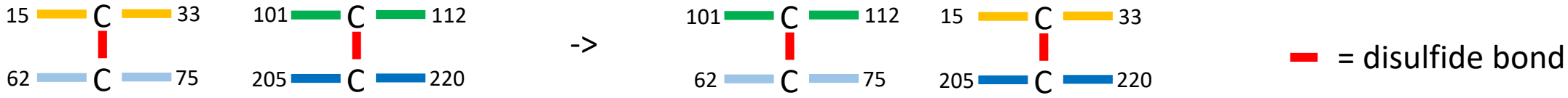

BPI = Base Peak Intensity

# Peptide Mapping

- **Sequence coverage** = identified sequence compared to whole sequence (%)
- **Modifications**
  - Relative amount (% of total peptide) per site
  - **Oxidation**: XXX**M**XXXK -> XXX**M$^{ox}$**XXXK (+16 Da)
  - **Deamidation**: XXX**N**XXXK -> XXX**D**XXXK (+1 Da)
    XXX**Q**XXXK -> XXX**E**XXXK (+1 Da)

X = amino acid

- **Disulfide shuffling**
  - Peptides cross-linked by disulfide bonds are identified in a similar way as unmodified peptides




Two pairs of cross-linked peptides

Switched pairs

# Formulation

- **Purpose:** To find the best possible buffer to **stabilize** the purified **product** during further processing, filling, distribution, storage and administration

**Buffers:**

- Detergents
- Excipients/Additives
- pH, ion concentration
- Buffer type (e.g. phosphate)

**To prevent:**

- Aggregation
- Fragmentation/truncation
- Modifications
- Compatibility problems

# Formulation - Stability testing

- Establish shelf life
  - Can only be based on real data
  - Year 1: every 3 months; Year 2: every 6 months; Year 3+: annually
- Establish adequate storage conditions
  - Liquid (5, 25, 40°C), frozen, dried
- Determine container system suitability
  - Does the protein interact with the container?
- Dose and safety
  - Chemical degradation of drug product

    -> reduce the concentration of active substance

    -> can lead to formation of toxic products

# Forced Degradation Studies (FDS)

- FDS is one of the key stages
  - Identification of degradation pathways and stability indicating methods
  - Try multiple stress conditions to determine appropriate stress for the specific product
  - Stressing to 10-15% instability
  - Correlate instability to potency
  - Define relevant method set, before evaluating large number of formulations

- Container and closure
  - Same as in the commercial product
  - Interaction with container and/or closure may occur

# Summary

- Pre-clinical drug developement involves of upstream, downstream, analysis, and formulation
- Protein characterization is needed at all steps to ensure quality
- Optimized formulation is needed for optimal stability
- Shelf life and storage conditions are determined through stability testing
- FDS is an important way to find out what atributes need to be measured, and what methods to use.