UMR-CNRS 8612 - Institut Galien Paris-Saclay

University Paris Saclay

# Quality control of biopharmaceuticals

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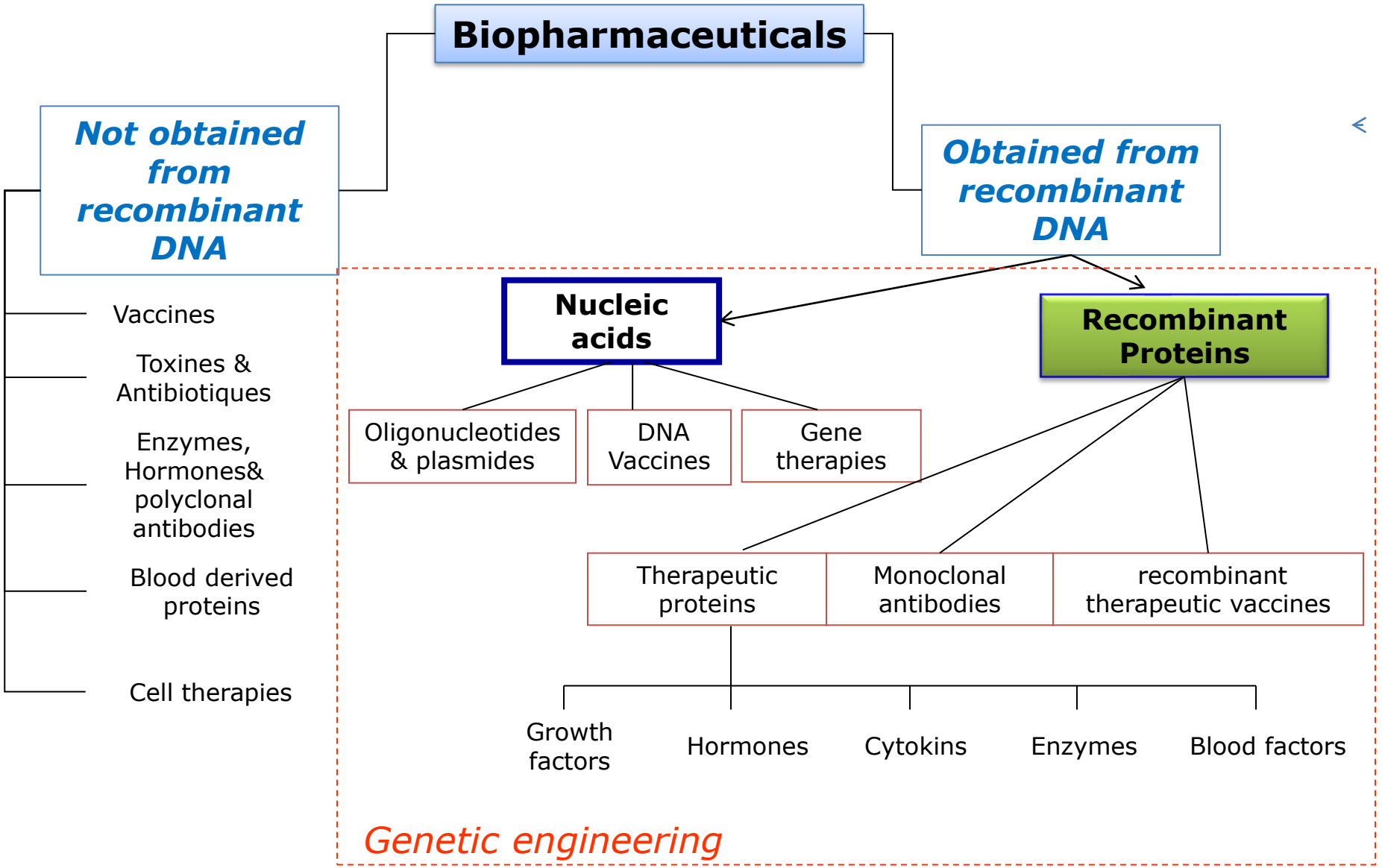
# • Classification of Biopharmaceuticals

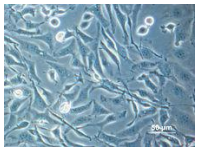


# • Different sources of biopharmaceuticals

➢ **Definition**: Biopharmaceuticals are defined as pharmaceuticals manufactured by biotechnology methods, with the products obviously having **biological sources**, usually **involving live organisms or their active components**

**1-Extraction** : from tissues, animal or human fluids (blood, urines, cells, tissues, milk…)

**2-Cell culture production** **:** Prokaryotic cells (bacteria) or eukaryotes (fungi, insects, mammals)



- **natural secretion** : toxins or bacterial enzymes *(*Botulinum toxin, streptokinase *…)*
- **after genetic modification :** expression of a foreign gene

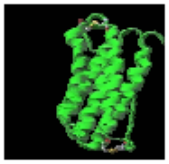
**3- From transgenic animals or plants**

**4- Live Organisms** (larvae; eggs…)

# •Specificities of therapeutic proteins

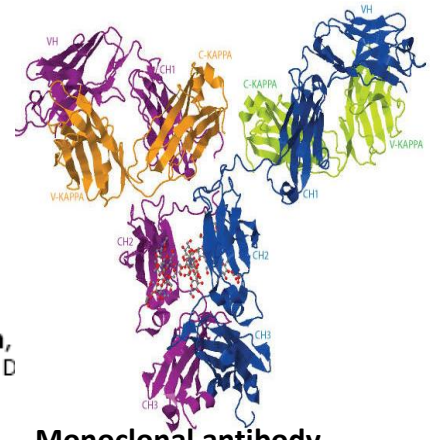
**Therapeutic proteins differ greatly from chemically synthetized molecules by:**

- **Their size**



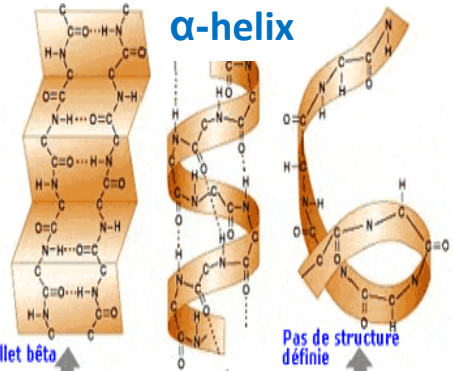
**Interferon alfa,**
165AA, MW: 19 625 Da

**Aspirin,**
MW: 180 D



**Monoclonal antibody**
150kDa

**Secondary structure**

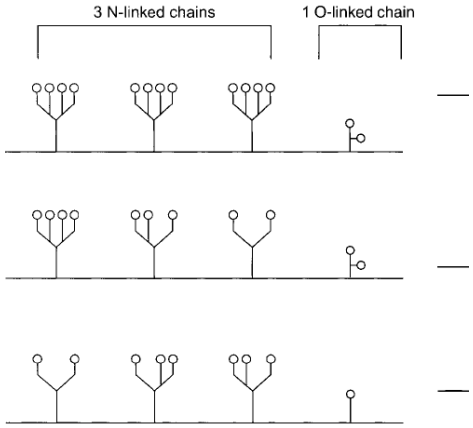



**Tertiary structure**

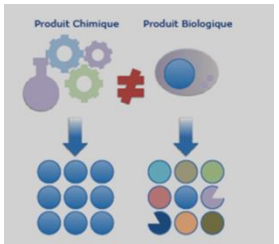
- **High structural complexity which is mandatory for their activity**

- **Heterogeneity**

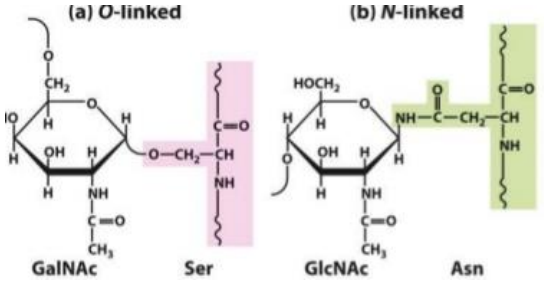
*One glycoprotein = mixture of glycoforms in a given and constant proportion*




- ***and by the way to produce them***



# **Structure/activity:** Glycosylation a major post-translational modification (PMT)



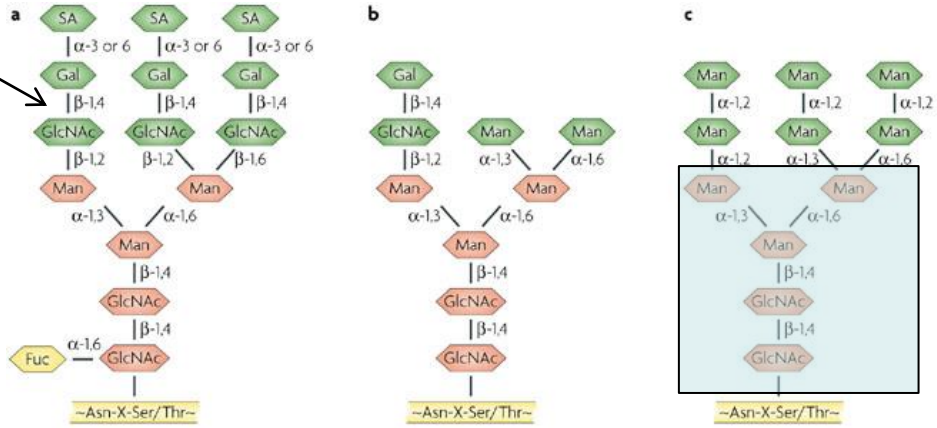

## N and O-glycosylation

N-glycosylation: Asn (consensus sequence: Asn-X-Ser/thr)

O-glycosylation: Ser; Thr

## N-glycans: 3 types

antenna




core

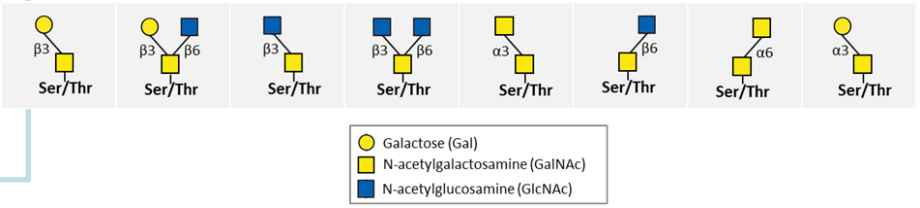
Complex hybrid oligomannose

balzarini ;Nature Reviews Microbiology 5, 583-597 (2007)

## Why glycosylation is important for biopharmaceuticals?

- Solubility and stability
- Folding
- Bioactivity:
  - Cell recognition (ligand/receptor)
  - Pharmacokinetics
  - in-vivo clearance
  - Antigenicity

## *O-glycans*




- Many cores
- Shorter
- GalNac

## • Quality control of recombinant proteins

- is mandatory to ensure safety and efficiency
- The active product is heterogeneous and structurally complex
- needs several complementary techniques to address :
  - Purity
  - Identity
  - Dosage
  - Stability
  - Activity

## • At which step?

- In-process

*In-process tests are performed at critical decision-making steps and at other steps where data serve to confirm consistency of the process during the production of either the drug substance or the drug product*.

- Purified protein
- Formulated product
- Compounding product (hospital)

# • Quality control - Objectives

**QC testing:**

- **To assess and ensure the safety and efficacy of the therapeutic protein**
- **To verify lot-to-lot consistency**

## Identity test

- To establish that the product batch contains the correct therapeutic protein
- Biological activity, immunogenicity

## Purity

- A biopharmaceutical product must be free of contaminating substances

- Impact the performance of the protein
- Cause unwanted and serious side effects (immunogenicity)

## Dosage

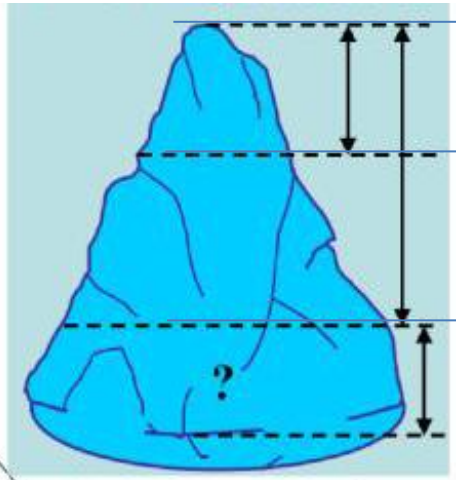
- To determine accurately the concentration of the purified protein / in the final product
- To ensure that the correct amount of active pharmaceutical is delivered to the patient

## Stability

- To evaluate the susceptibility of the protein toward different stresses
- To define optimal storage conditions

# • Why the evaluation of the desired product is a real challenge?

**A protein:**
**a complex and heterogenous mixture**




Lot release tests

Characterization

**Purity profile**

**Desired protein**

polypeptide variants

PTM variants

**Natural heterogeneity**

**Impurity profile**

**« unwanted » heterogeneity**

**Product related impurities**

**Process related impurities**

*Kozlowski S et al, Adv Drug Del Rev 2006*

PTM: post-translational modifications

# • What are the impurities?

## Process-related impurities:

Expected to be removed during the downstream process

- **Cell-derived**

Host cell proteins, nucleic acids, endotoxins…

- **Cell-culture medium derived**

Inducers, antibiotics, enzymes, serum, bacteria…

- **Downstream derived**

Chemicals and biochemicals, inorganic salts, solvents, residual components

## Product-related impurities:

- **Related forms and variants**

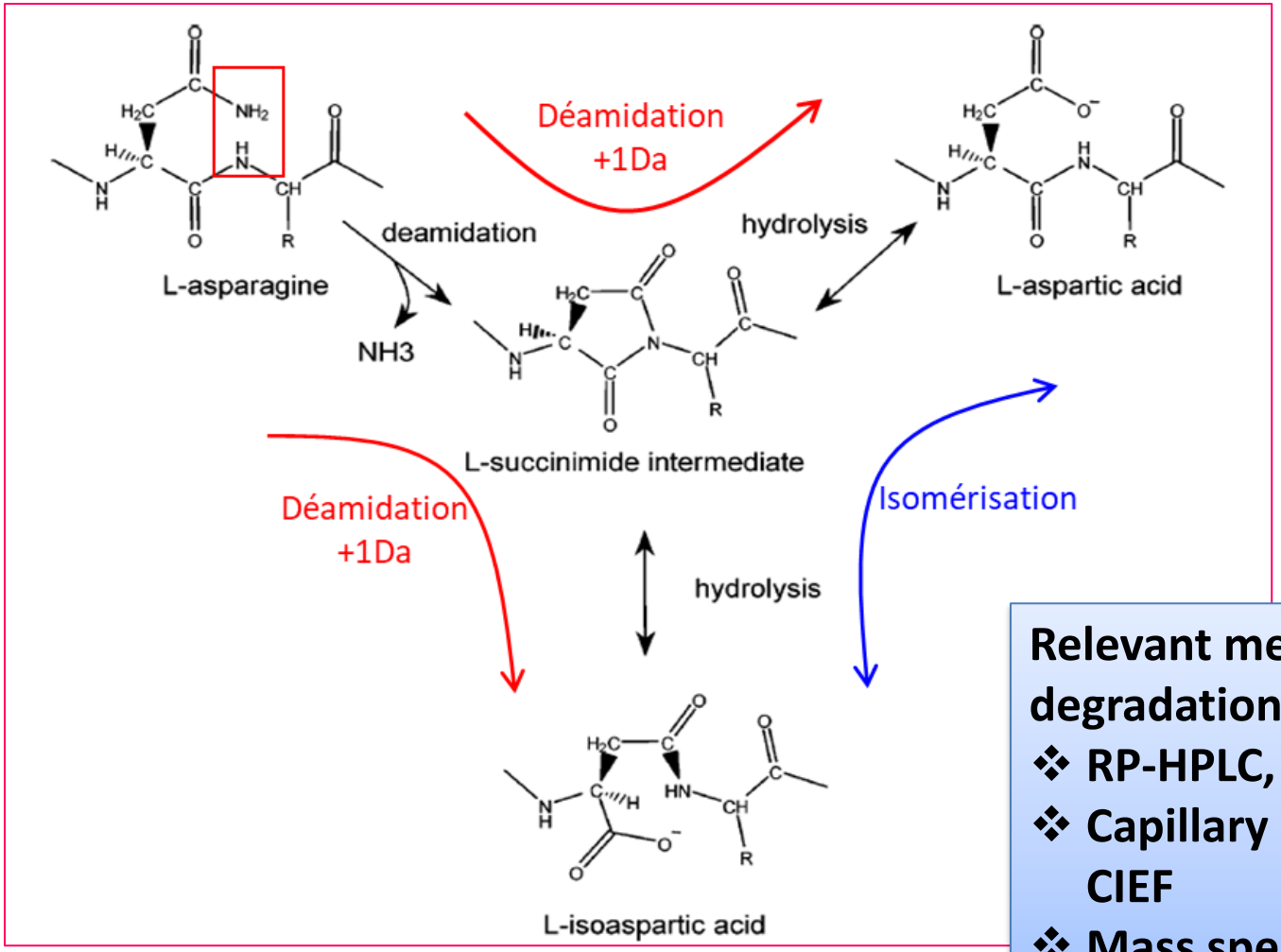
Deamidation, oxidation, isomerisation, racemization, glycated, altered PTMs….

- **Truncated forms, fragments;**
- **Dimers, oligomers, aggregates,**
- **Misfolded or unfolded forms**
- **Cys linked variants, scrambling.**

# • Chemical Degradation pathways – charge variants

## Deamidation (Gln, Asn), Isomerization (Asp)

- Very frequent during production, formulation, storage
- Catalyzed by high temperatures and ionic strengths, at neutral and basic pHs



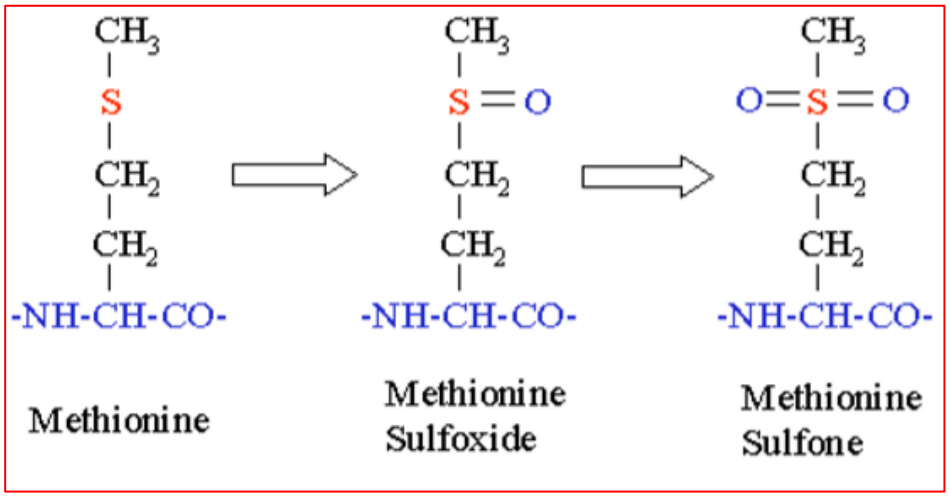

**Relevant methods to detect these degradations:**

- ❖ **RP-HPLC, IEX-HPLC**
- ❖ **Capillary electrophoresis: CZE, CIEF**
- ❖ **Mass spectrometry**

# • Chemical Degradation pathways – size variants

## Oxidation Met, Cys, (Thr, Phe, Lys)

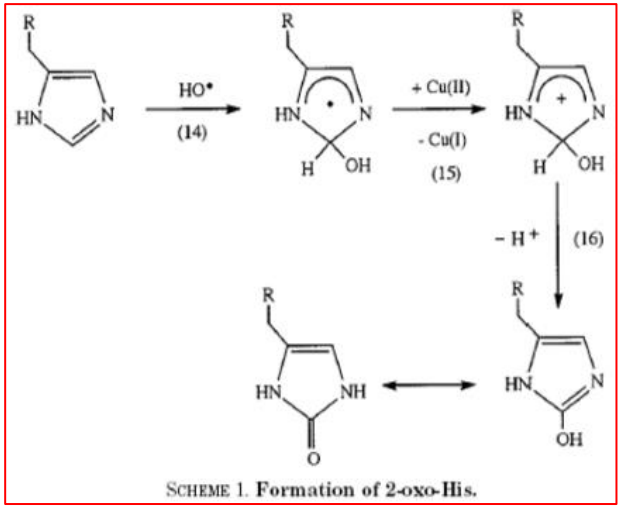
- Very frequent during purification, formulation, storage
- Site specific, metal catalyzed (Met, Cys)
- pH catalyzed (except Met)




**Relevant methods:**

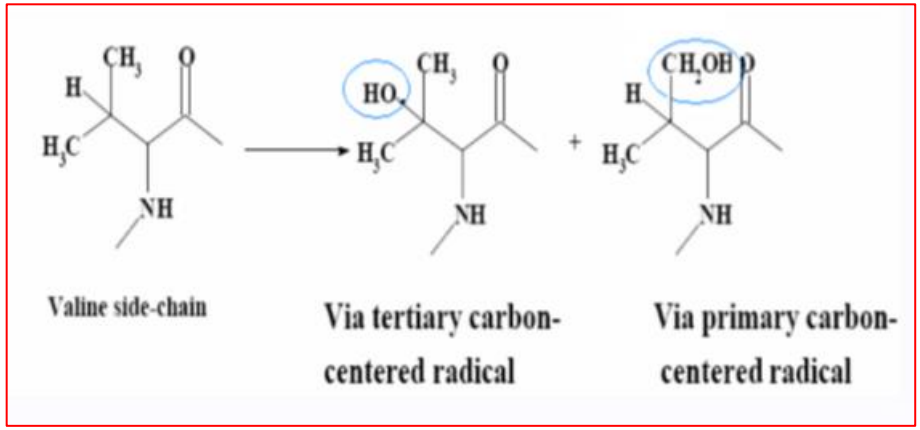
- ❖ **RP-HPLC, IEX**
- ❖ **Capillary electrophoresis**
- ❖ **Mass spectrometry**

## His oxidation catalyzed by metal

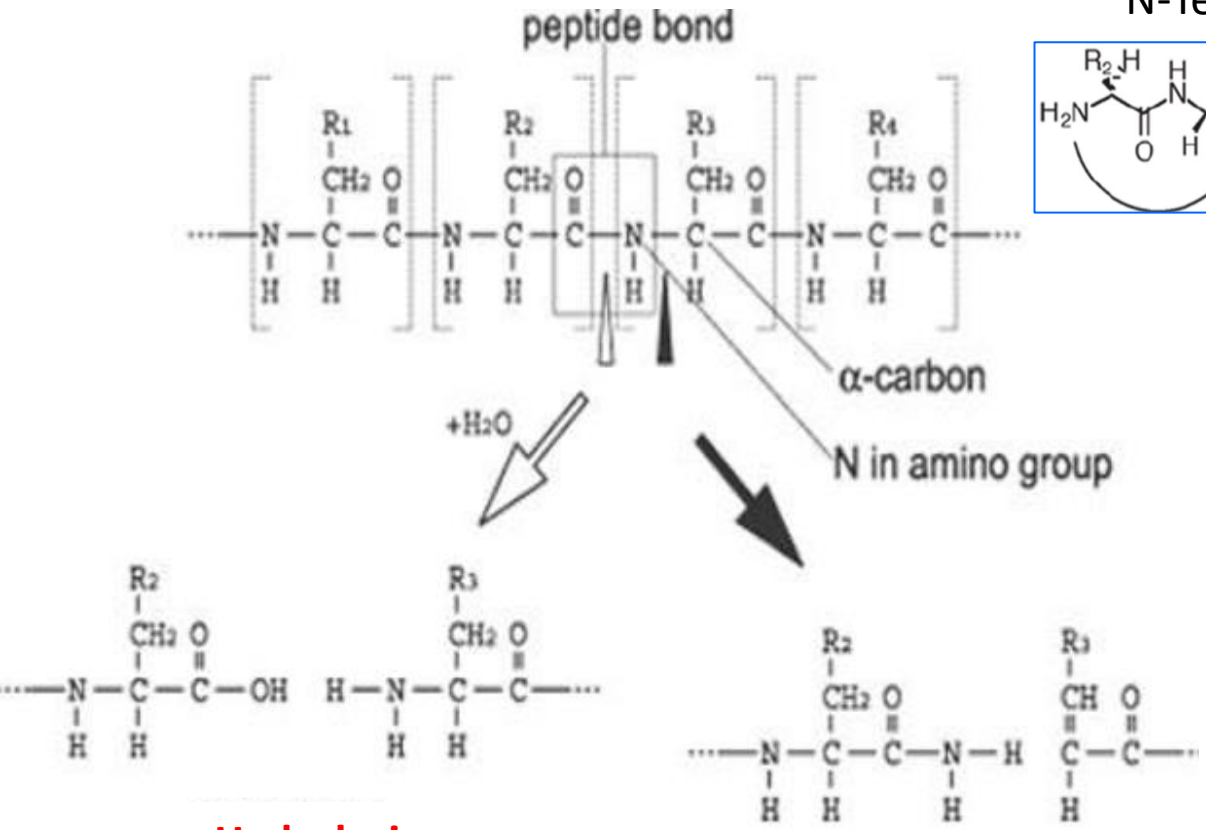



SCHEME 1. Formation of 2-oxo-His.

## Aliphatic amino acid oxidation



# • Chemical degradation pathways : fragments /oligomers




**Hydrolysis**

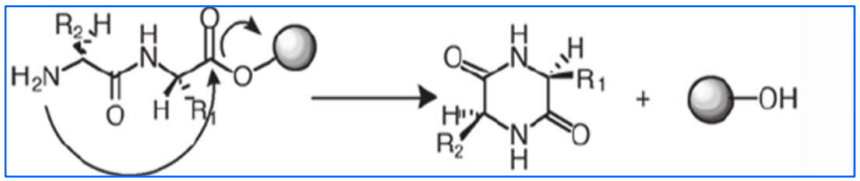
Fragmentation of the peptide binding

**β Elimination**

Cys, Ser, Thr, Phe, Lys

**Formation of a diketopiperazine (DKP)**

N-Term



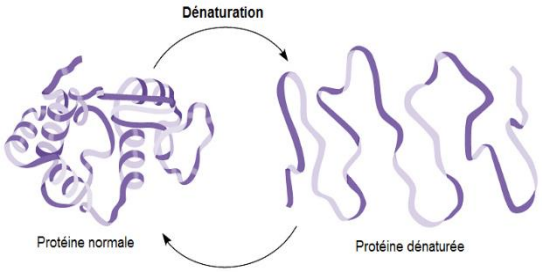
**Covalent Oligomerisation**
**Exchange of disulfide bond**

**Relevant methods:**

- **RP-HPLC, size exclusion chromatography (SEC)**
- **Capillary gel electrophoresis (CGE)**
- **Mass spectrometry**

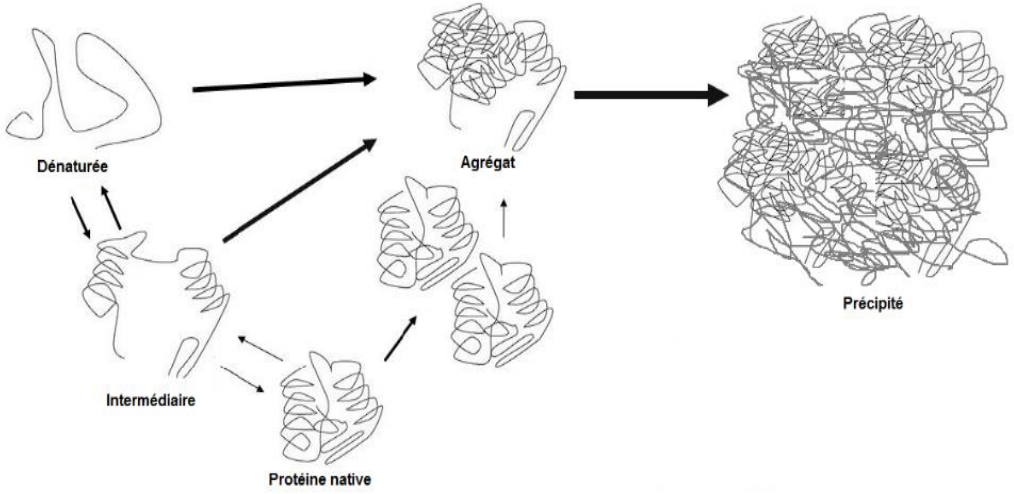
# • Physical degradation pathways : agregates, oligomers

➢ **Denaturation**




**Critical parameters :** Temperature, solvents, buffer pH and ionic strenght, chaotropic agents

➢ **Agrégation, precipitation**

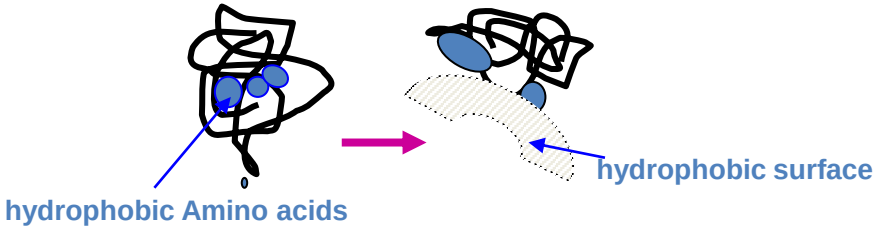



**Factors that favor the agrégation:**

High concentration of proteins

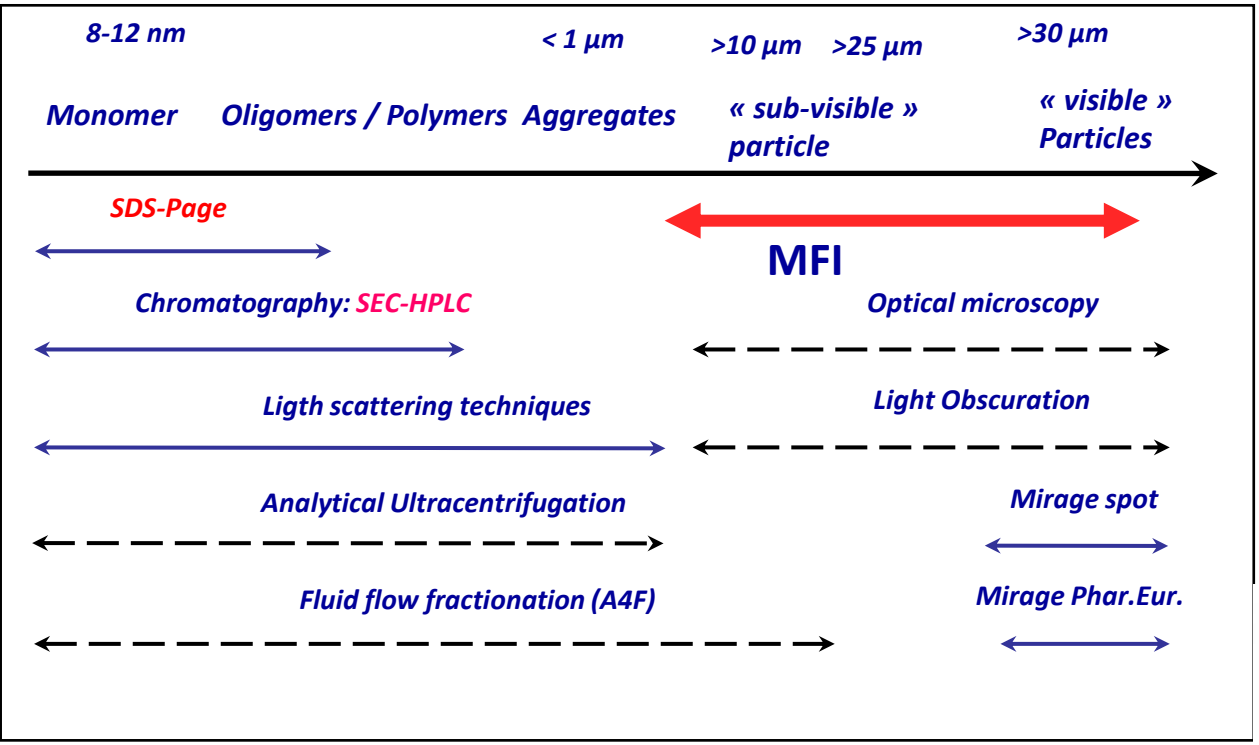
Stirring, freezing freeze-drying, thawning….

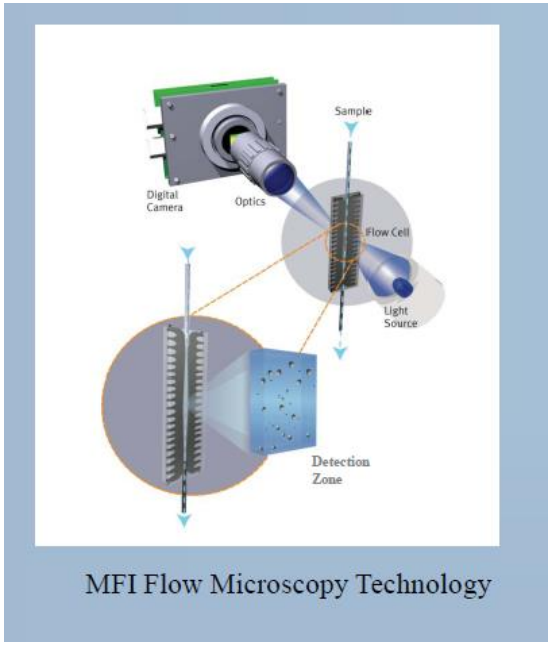
➢ **Adsorption**



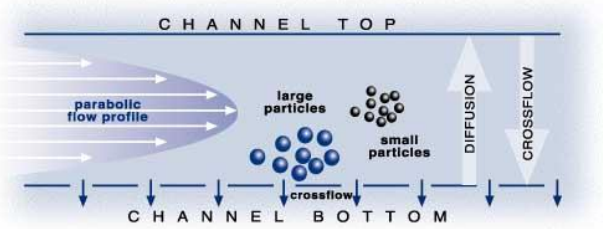
# Aggregates and particules detection

- Aggregates and particles are impurities among others , they can generate undesirable side effects (immunogenicity).
- Analytical techniques to be used depend on their size



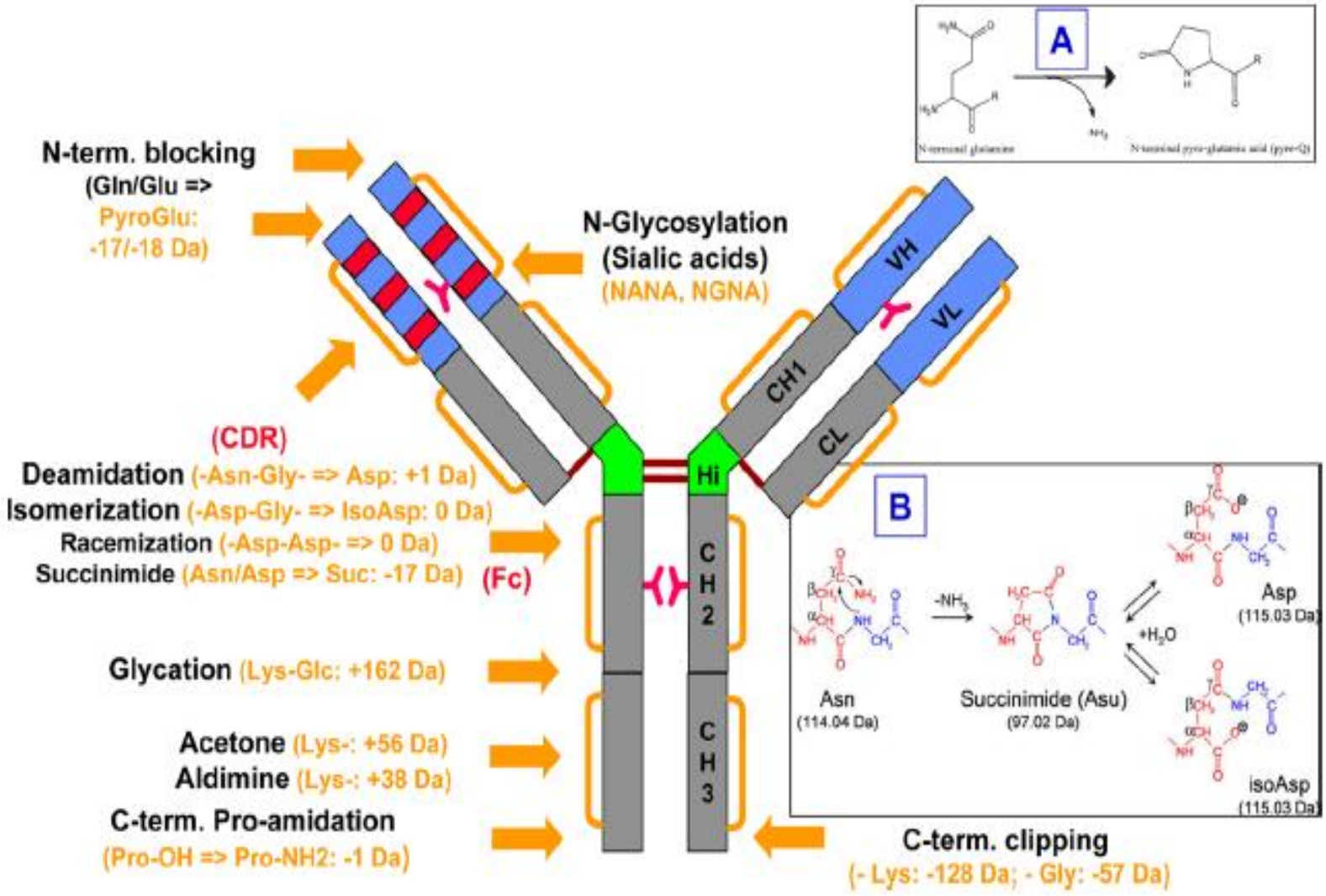

MFI Flow Microscopy Technology




Assymetrical field flow fractionation (A4F)

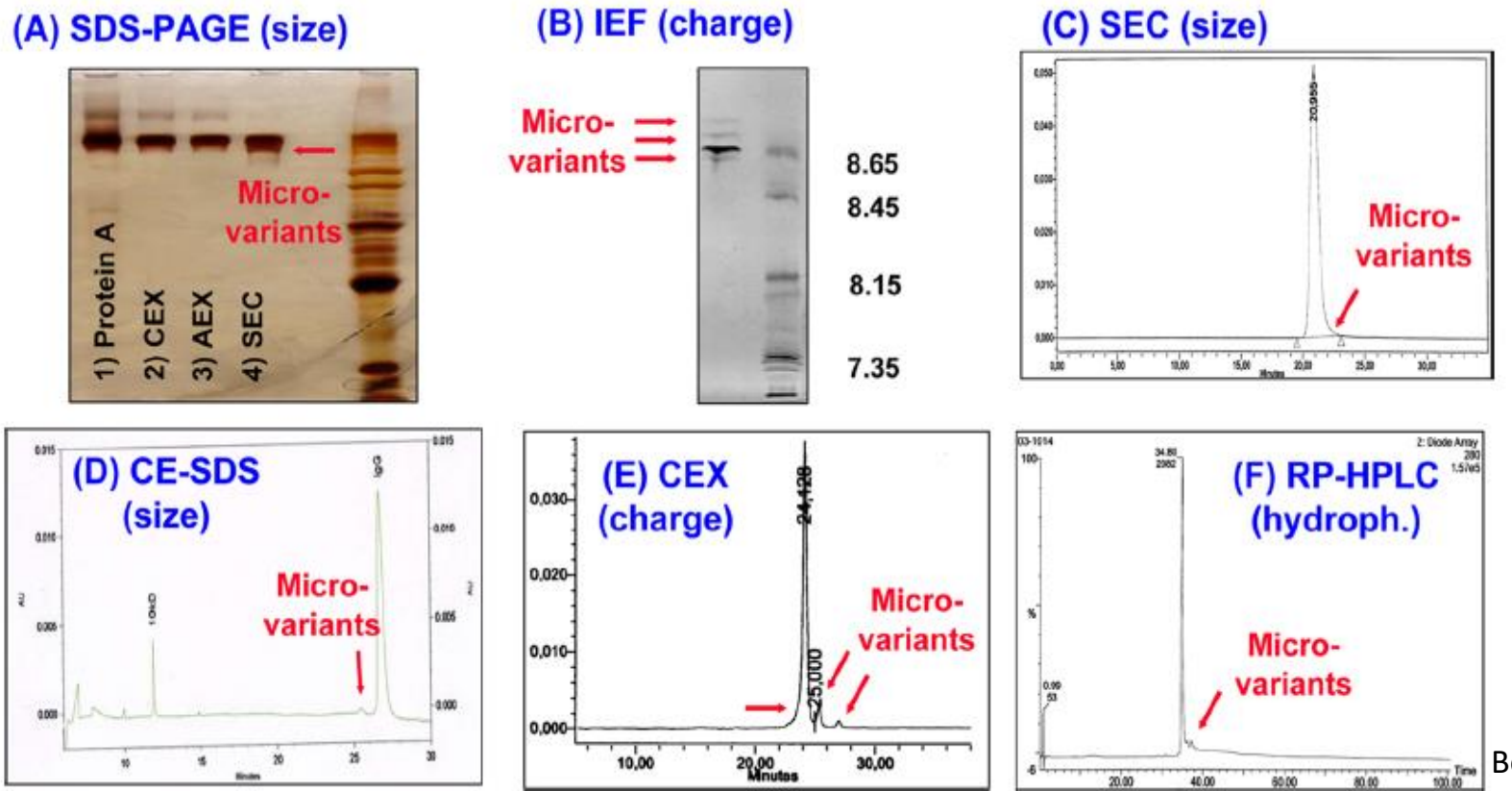
# How many microvariants are we looking for?

## *Exemple of a monoclonal antibody*



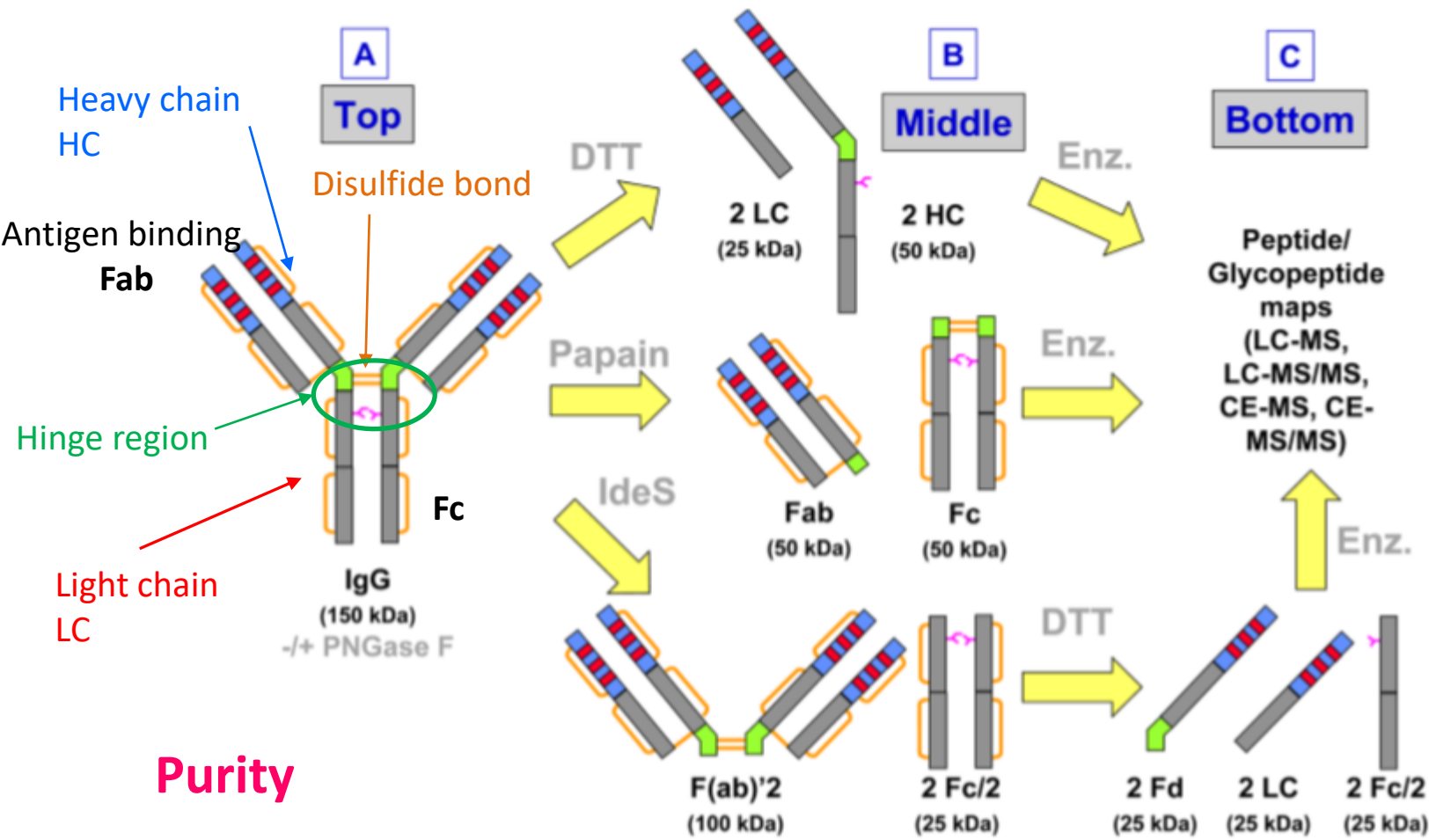
# Complementary techniques are required for a QC

- **Electrophoresis-based techniques** (gel electroph., CE, CE-MS…)
- **Liquid Chromatography techniques** (all modes/MS)
- **Spectrophotometrics techniques**: Circular dichroism, Fluorescence and UV absorbance, IRTF, DSC, Light diffusion…




Beck et al 2013

# • 3 Approaches for mAbs analysis



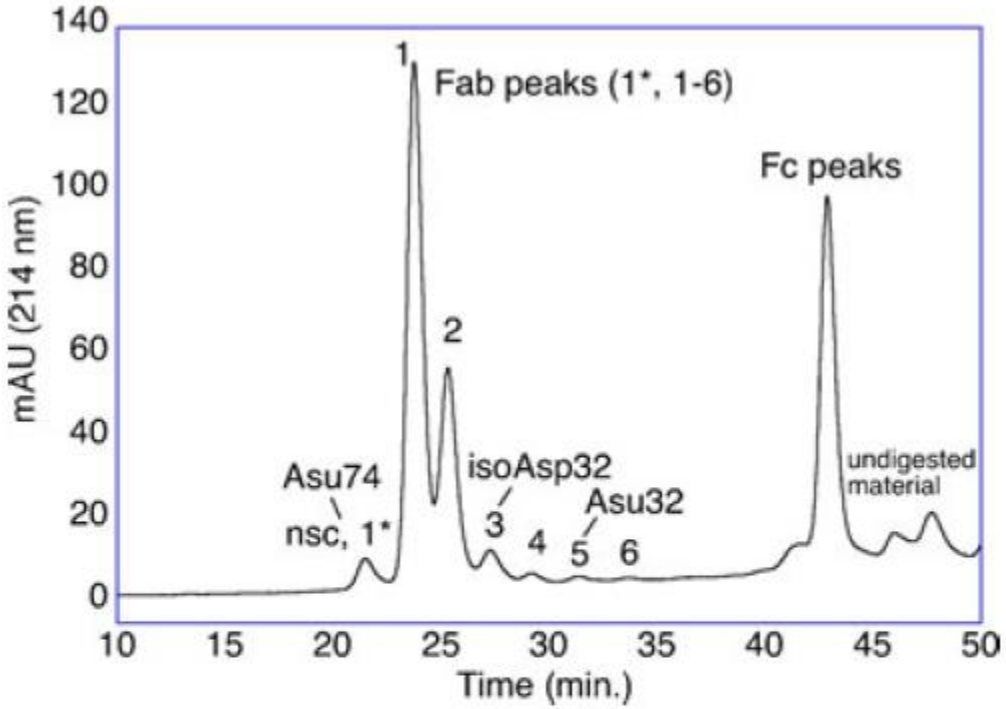

**Top down, Middle up, Bottom up**

DTT: dithiothreitol

*Beck A et al, Anal Chem2012*

# Middle up approach to detect Isomerisation and succinimide by HIC-chromatohraphy

HIC: Hydrophobic interaction chromatohraphy



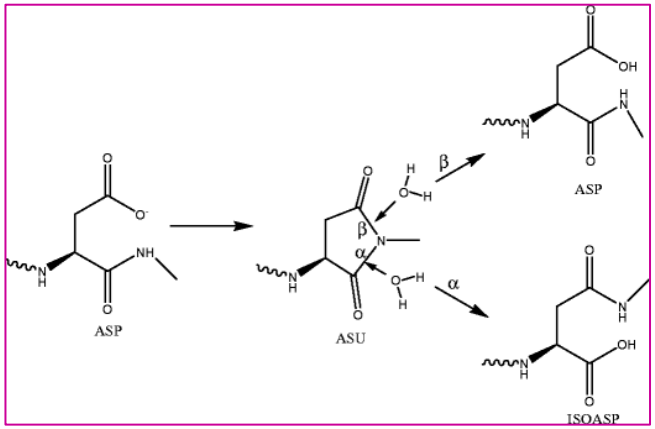




**Fig. 1** HIC elution profile of a papain digested MAb1 sample. Peak 1* corresponds to Asu74; nsc = non-specific clip due to papain cleavage, coelutes with peak 1*; Peak 2 is Asp32 with a free thiol group; Peak 3 corresponds to isoAsp32; and Peak 5 corresponds to Asu32.

Pharm Res (2012) 29:187–197

# • Quality control - Objectives

**QC testing:**

- **To assess and ensure the safety and efficacy of the therapeutic protein**
- **To verify lot-to-lot consistency**

## Identity test

- To establish that the product batch contains the correct therapeutic protein
- Biological activity, immunogenicity

## Purity

- A biopharmaceutical product must be free of contaminating substances

## Dosage

- To determine accurately the concentration of the purified protein / in the final product
- To ensure that the correct amount of active pharmaceutical is delivered to the patient

## Stability

- To evaluate the susceptibility of the protein toward different stresses
- To define optimal storage conditions

# Impurities and methods to control the purity

**1-Proteines from the Host cell : non spécific methods required**

-SDS-PAGE/Coomassie blue staining

- «host cell proteins» HCP tests

**2- Degraded proteins : resolutive techniques**

- RP-CLHP , IEX (deamidated, oxidized forms….), HIC (isomerisation)

-isoelectric focusing (IEF), Capillary zone electrophoresis (glycoforms, deamidated, oxidized forms….)

- Size exclusion chromatogaphy (fragments, agrégates)

-capillary gel electrophoresis (fragments, agrégates, non glycosylated sites…)

- hydrophobic interaction chromatography (HIC) : misfolding , unfolding:

- techniques specific for aggregates and particles detection

**3-DNA**

-Hybridization method using radiolabelled probes

**4- Pyrogenic substances, endotoxins :**

*- LAL test: in vitro test based on activation of the complement cascade by endotoxin in Limulus Polyphemus*

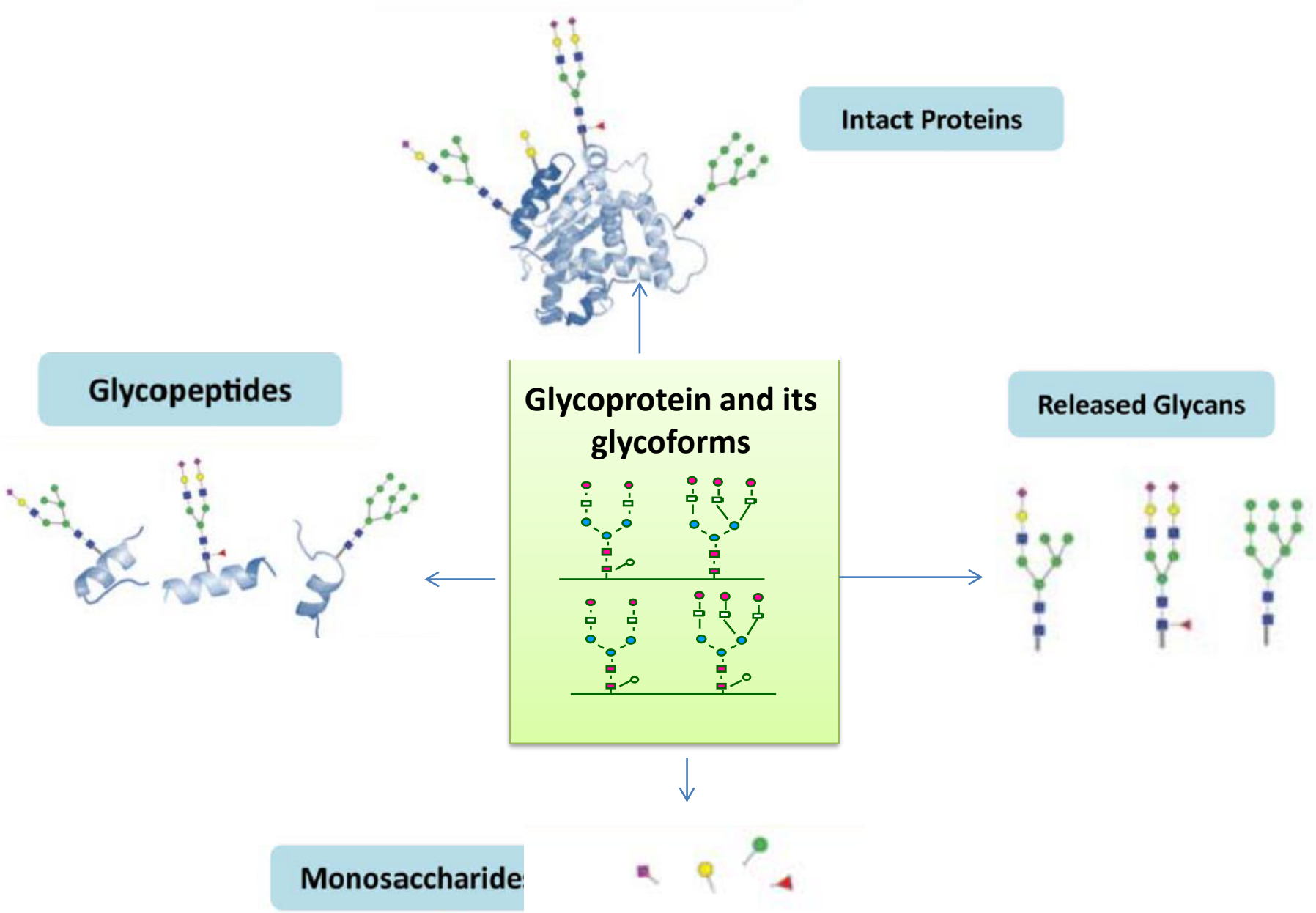
*- New coming "RP-HPLC-based technique"*

**5- Virus**

# Approaches for Identity control

- **Primary sequence**
  - amino acid composition
  - automated sequencing (EDMAN)
  - peptide mapping
- **Physicochemical parametrers estimation**
  - Molecular mass: SDS-PAGE, size exclusion chromatography, capillary Gel Electrophoresis mass spectrometry (MALDI HPLC-ESI-MS using ion trap or FTICR mass spectrometry)
  - Isoelectric point : gel isoélectrofocusing (IEF) or capillary isoelectrofocusing (CIEF)
  - Hydrophobicity : RP-HPLC
  - Combination of several parameters : PAGE , Capillary zone electrophoresis
- **Secondary and tertiary structure**
  - Circular dichroïsm
  - Infra-Red -based techniques
- **Post translationnal modifications : Glycosylation mostly**
  - Monosaccharide composition, sialic acid…
  - Glycan mapping
  - Glycopeptide mapping
  - Glycoform analysis: heterogeneity profile
- **Biological and immunological activity**

# Glycosylation analysis can be performed at different levels



# Dosage and activity

- **Colorimetric methods**

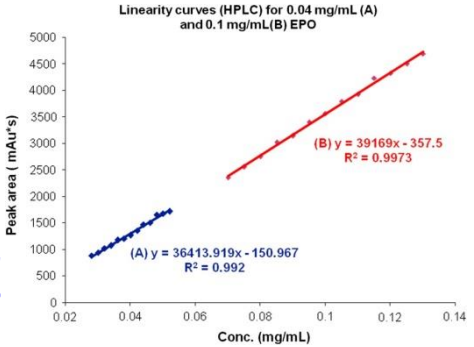
- *Méthodes du biuret et dérivées* :
  **Biuret** : $CuSO_4$ ($OH^-$) à 540 nm ⟶ 0,1 g/l
  **Lowry** : + Folin-Ciocalteu (Tyr) à 650-750 nm ⟶ 0,05 g/l
  **BCA** : + acide bicinchoninique (inverse) à 560 nm ⟶ 0,01 g/l
- *Méthodes par fixation de colorants* : **Bradford** au bleu de Coomassie G250 ($H^+$) à 600-650 nm ⟶ 0,01 g/l

- **Spectrophotometry (UV at 280nm)**

- **Immunochemical methods : ELISA..**

- **HPLC, CE techniques can be quantitative too!**

EPO dosage




*Rane et al., 2012*

- **Biological activity:**
  - Administer a known quantity of the product to a biological system (cells, animal...) and measure a quantitative response that will be transcribed into a unit of activity.
  - In Vivo, Ex vivo, in vitro (cell cultures), biochemical or enzymatic assays, binding tests (SPR.....)

# • Analytical techniques for QC of common variants of biopharmaceuticals

| | Modifications | Analytical techniques |
|---|---|---|
| Charge variants | Deamidation<br>Oxidation<br>Glycosylation | CEX, IEF, CIEF, CZE ;<br>HILIC-HPLC |
| | | |
| Size variants | Fragmentation, Aggregation | SEC-HPLC, SDS-PAGE, CGE |
| | | |
| Conformers | Misfolding / Denaturation | HIC (not in a QC context) |

# Homework for the 5th of November

- Prepare **15 min** oral presentation PPT

(groups of 3/4 students; each group assisted by one teacher T1 or T2)

**Group 1: Acidic and basic variants analysis of Monoclonal Antibodies by IEX-HPLC- (T1)**
**Group 2: Purity control of therapeutic proteins by Size Exclusion Chromatography (SEC-HPLC) (T1)**
**Group 3: SDS-PAGE for purity and identity controls of therapeutic proteins (T1)**
**Group 8: detection of chemical-Degradation variants of therapeutic proteins by RP-HPLC**

**Group 5: Peptide mapping of therapeutic proteins by RP-HPLC as an identity Control (T2)**
**Group 6: N- Glycan mapping of therapeutic glycopoteins by HILIC-HPLC (T2)**
**Group 7: Capillary gel electrophoresis for identity and purity control of Therapeutic proteins (T2)**
**Groupe 4: Control of Secondary structure of therapeutic proteins- (T2)**

# Oral presentation

- **3-5 slides/ 15 Min/ PPT presentation: use the template for each topic on ecampus**
  - Principle/Mechanism of the technique/approach used for this specific application
  - Type of information provided **for quality control purposes only** , Limits and advantages of the technique
  - A few exemples illustrating this technique/method **for quality Control of therapeutic proteins**
  - 30 min of group works (now)

    BE CAREFUL : **15 min for presentation is a maximum!**

  - **Documents available on ecampus plateform**; USE PREFERENTIALLY the selected ARTICLES for your presentation (figures or info…)
    - TUO8- biotechnology <supporting files< one folder per topic
  - PPT presentation , to be sent by email to your teacher (T1 or T2) before the 5th of November 2023 12:00am
  - Presentation the 6th of November 9-12am