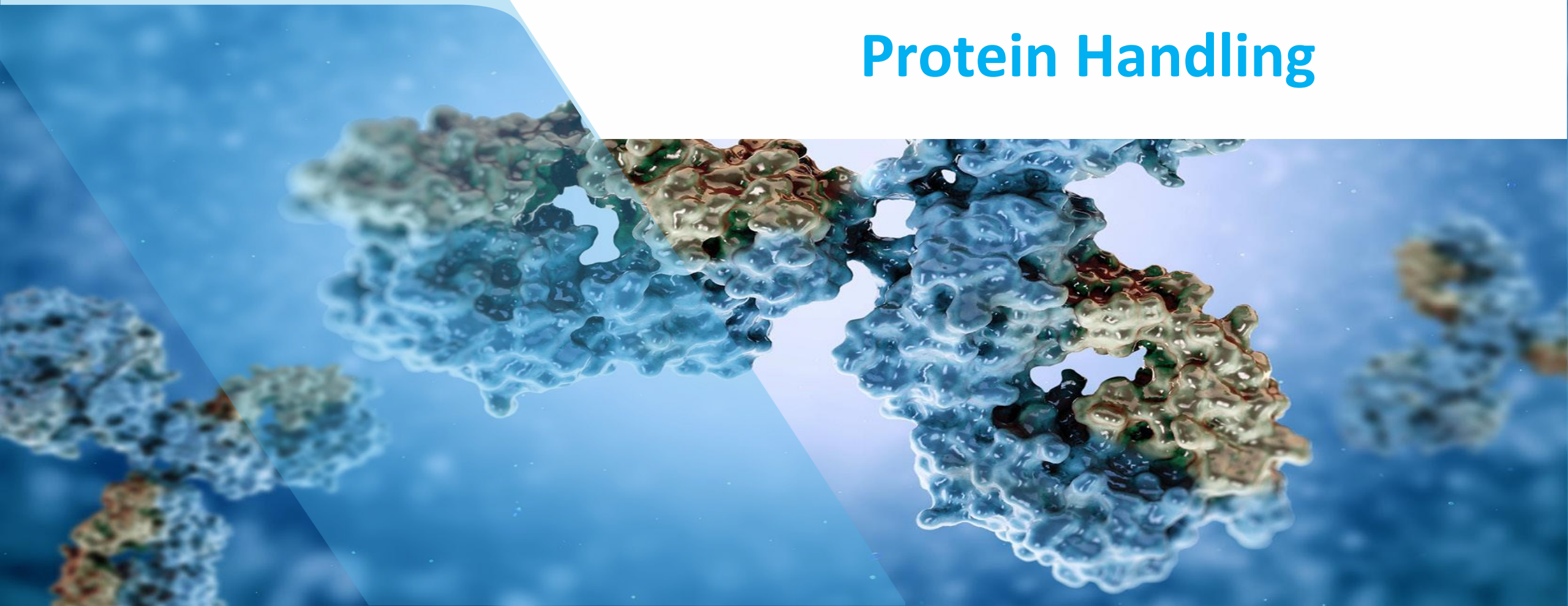
nibrt

National Institute for Bioprocessing Research and Training

Protein Handling



Learning Objectives

1. Describe the chemical structure of proteins

2. Describe the relationship between protein structure, stability and function

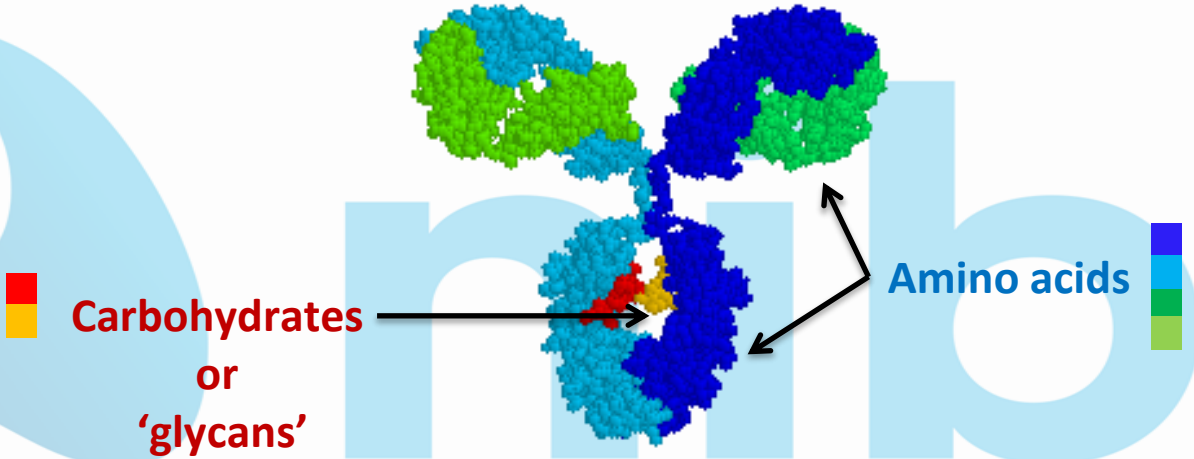
3. Define how manufacturing/processing conditions significantly impact biopharmaceutical stability

4. Explain why protein stability is critical to the production of biopharmaceuticals

Topics

- Introduction to Protein Chemistry
- Protein Structure
- Post Translational Modifications
- Protein Stability

What are Proteins?



- Proteins are large, 3D, molecules that have many functions inside the body
- Proteins are primarily composed of molecular building blocks called '**amino acids'**
- Many biopharmaceuticals also contain carbohydrates (sugars) called '**glycans'**

How are Proteins Made in the Body?

- The ‘recipe’ for a protein is encoded as DNA (master copy of the recipe)
- The DNA is **transcribed** into RNA by the cell (working template)
- RNA can be understood by cellular machinery and **translated** into the language of amino acids
- The cell then assembles a protein molecule from the code

https://www.youtube.com/watch?v=gG7uCskUOrA

Topics

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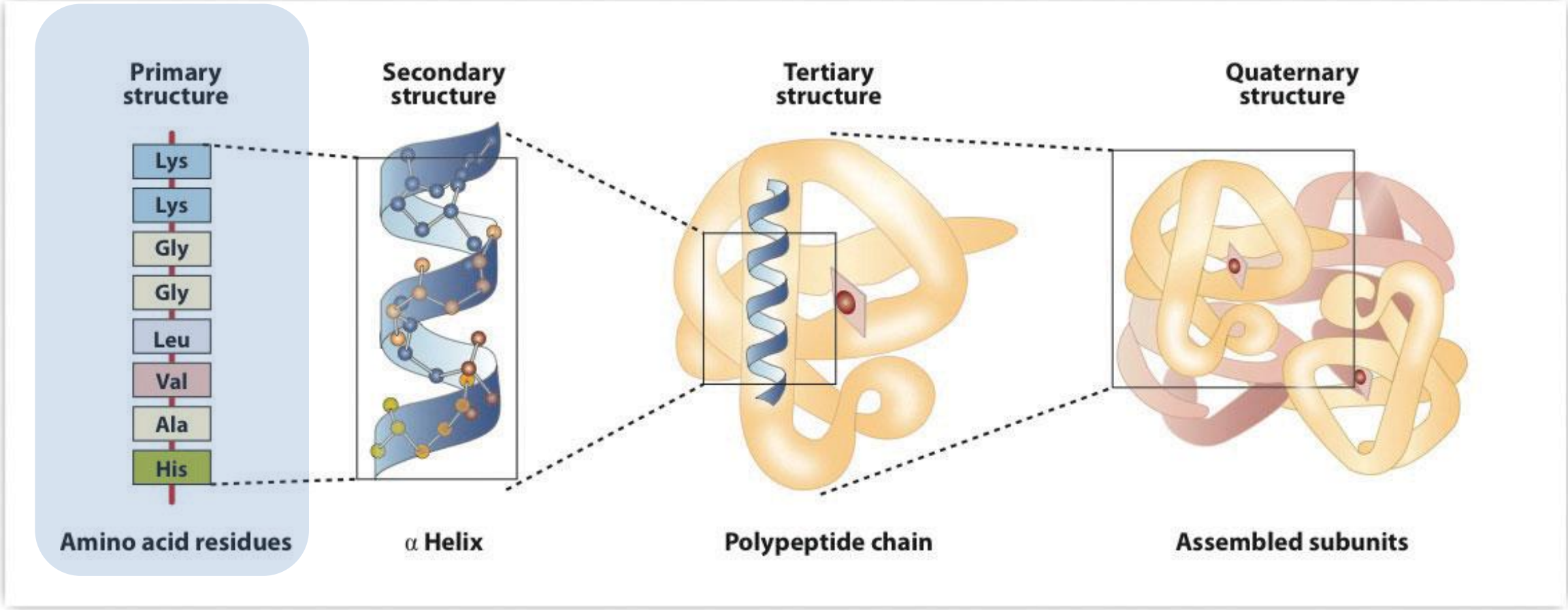
- Protein Structure
- Post Translational Modifications
- Protein Stability

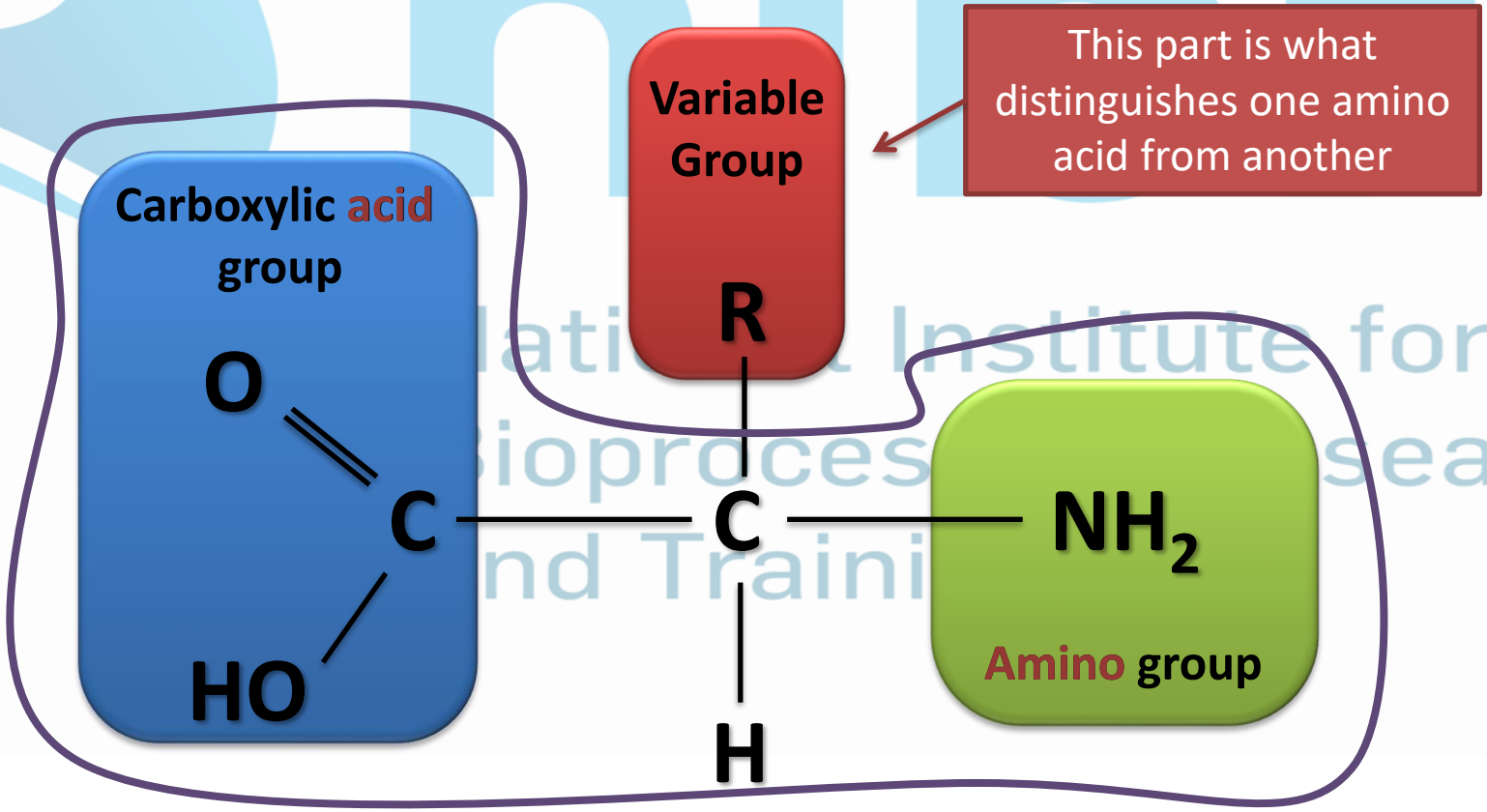
Protein Structure: Four Levels of Organisation

Proteins have complex 3D structures. This structure is very important – it allows the protein to do it's job.

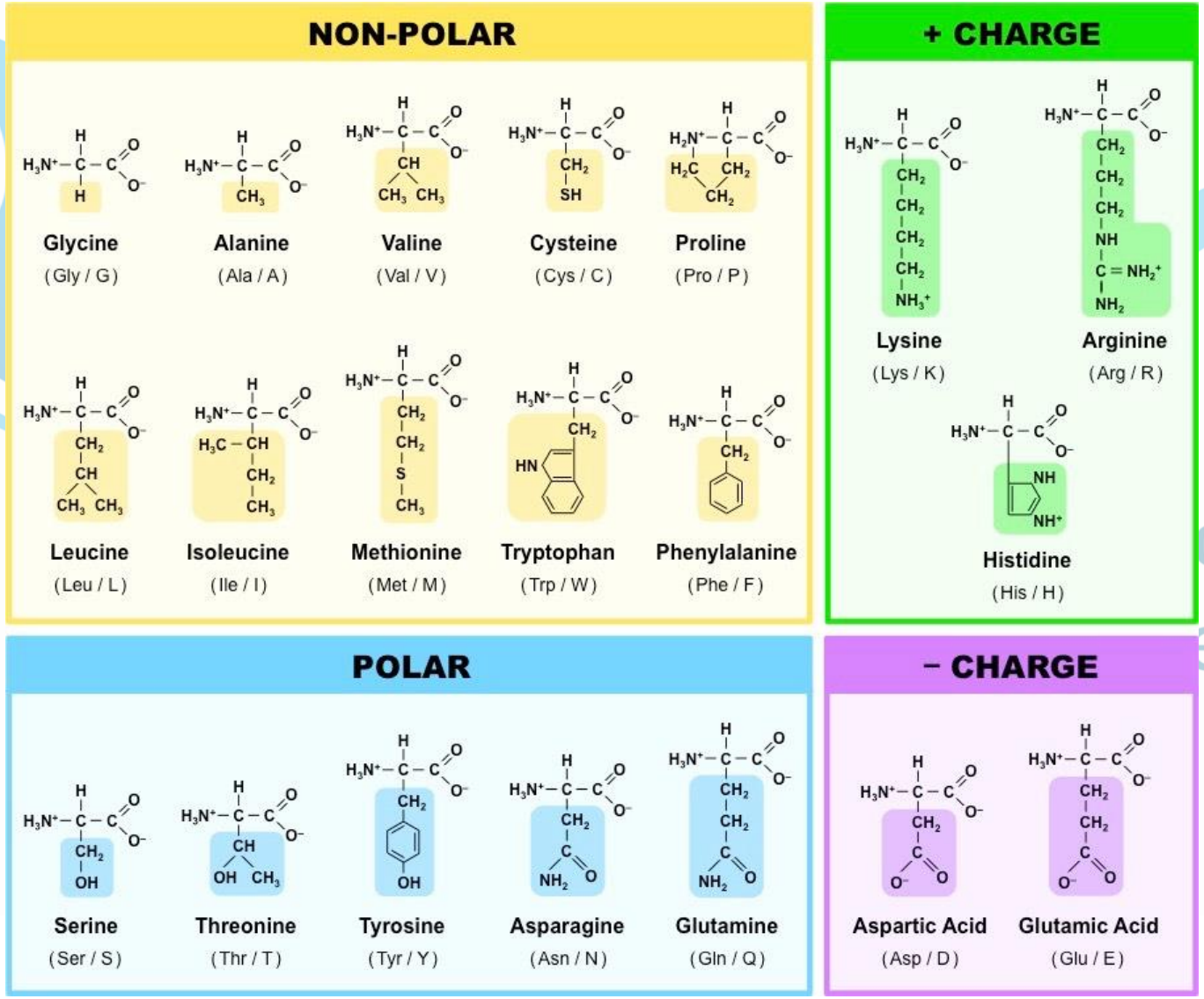


Primary Structure

- Proteins consist of chains of **amino acids**
- Amino acids are molecules consisting of a carbon with a primary amine, a carboxylic acid, a hydrogen & a **variable side chain group** (R)



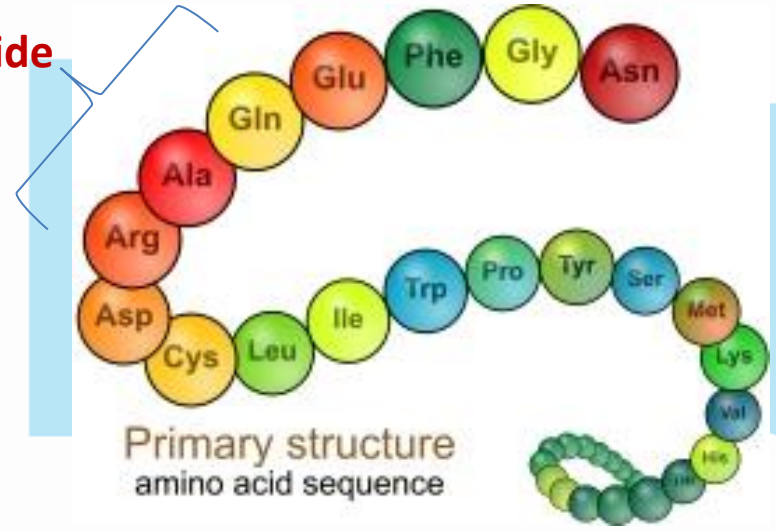
Primary Structure

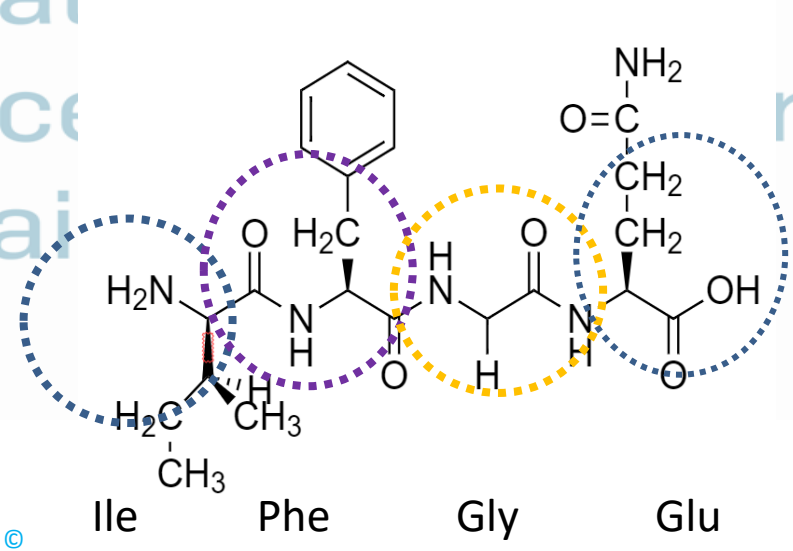


www.bioninja.com

Primary Structure

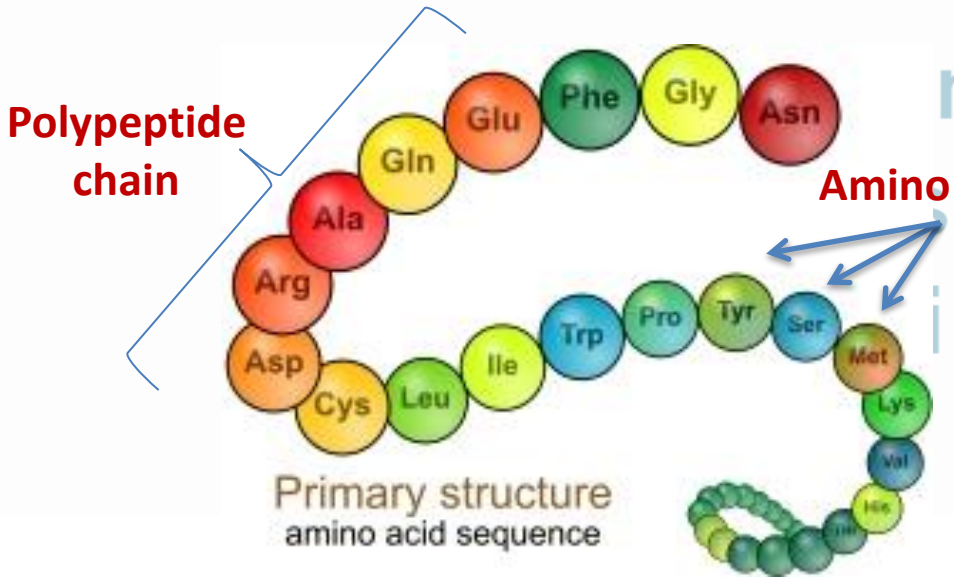
- To make a protein, amino acids are linked together in a chain to form a **polypeptide.** This process is carried out by an enzyme complex in the cell
- Each type of protein will have amino acids linked together in a unique order
- The linkage between each amino acid is called a covalent bond called a **'peptide bond'**
- This is an **extremely strong** inter-atomic linkage – not easily damaged during processing





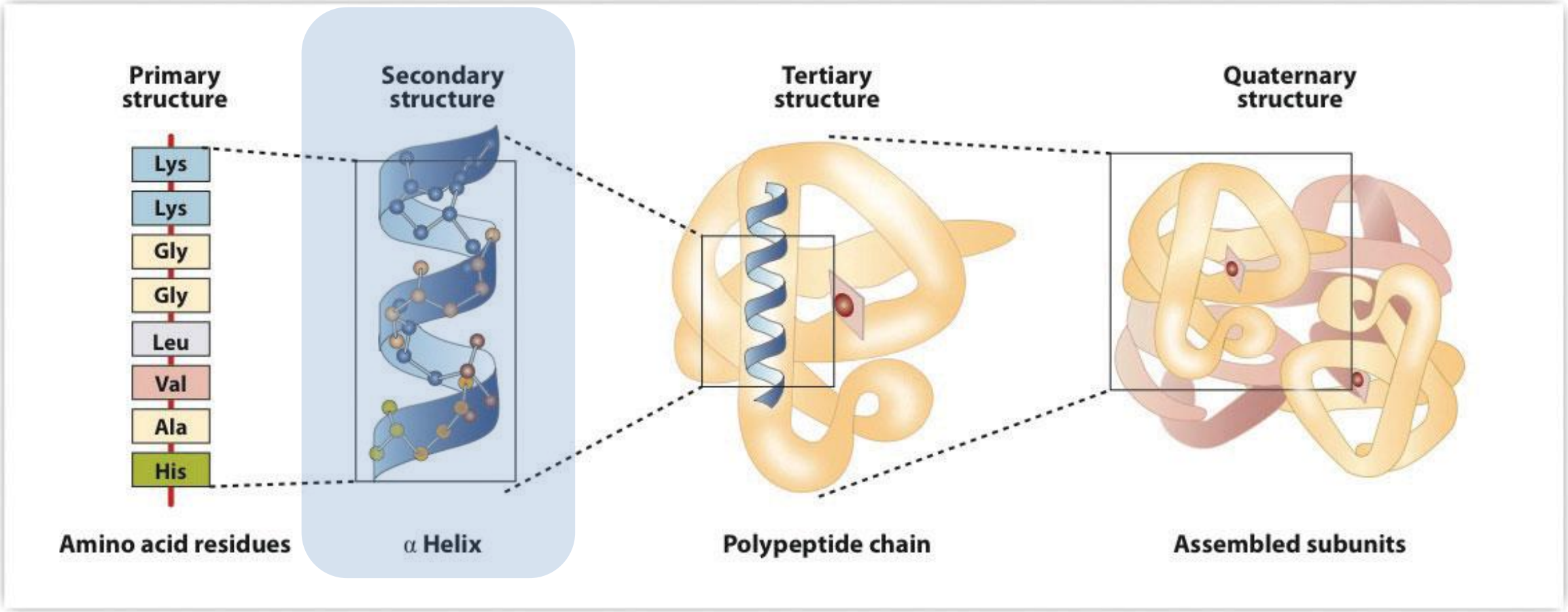
Primary Structure

- The **sequence** of amino acids in the polypeptide is determined by the **genetic code**
- **Mutations** in the gene will lead to **incorrect amino acid** sequence and ultimately **incorrect protein structure**

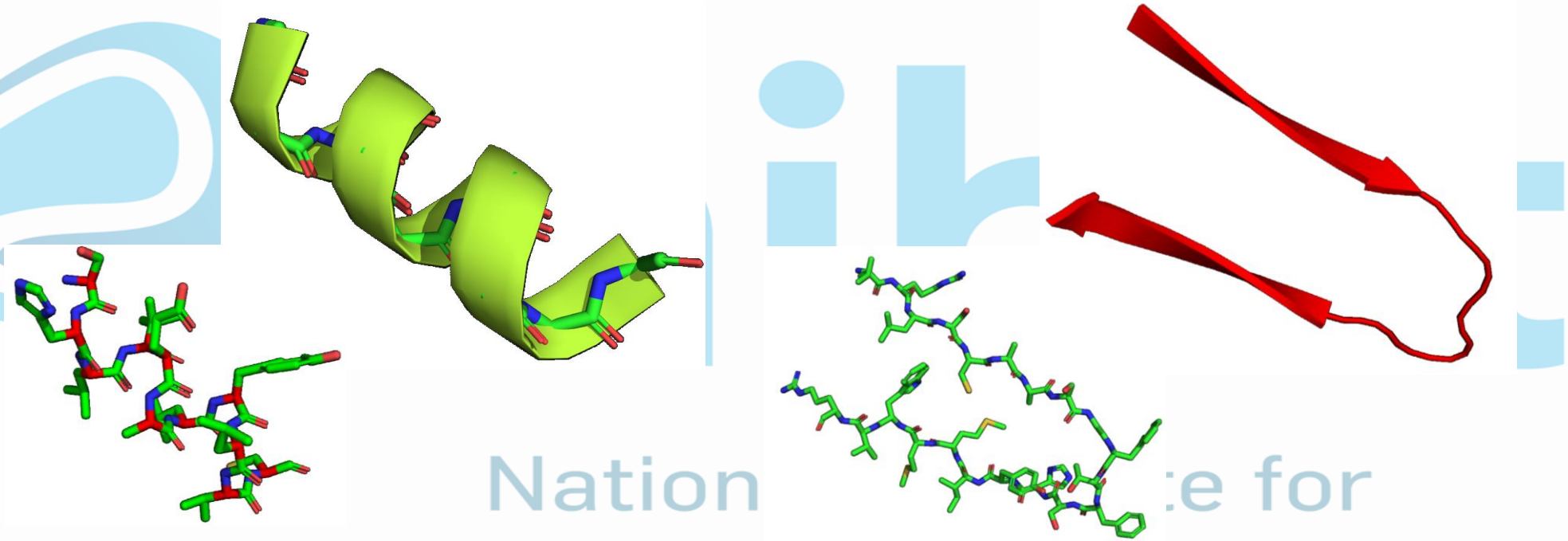


Amino acids can also be chemically changed during processing, formulation, filling storage and sample handling!

Protein Structure: Four Levels of Organisation



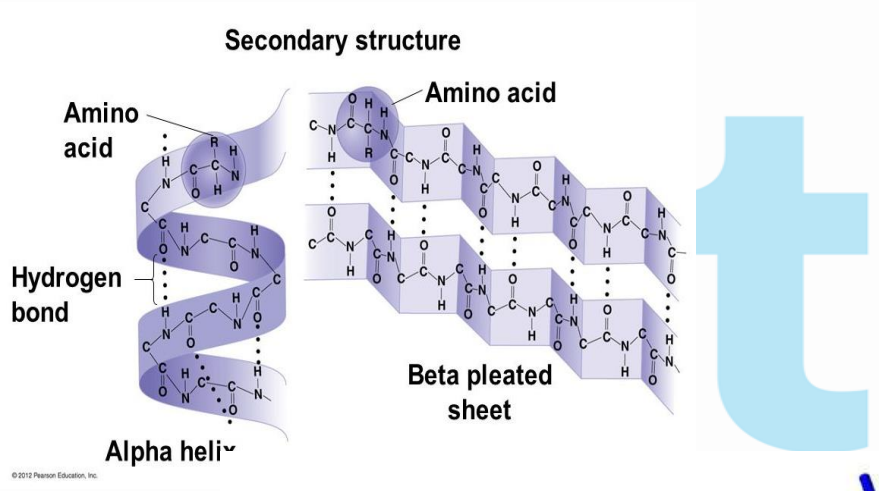
Secondary Structure



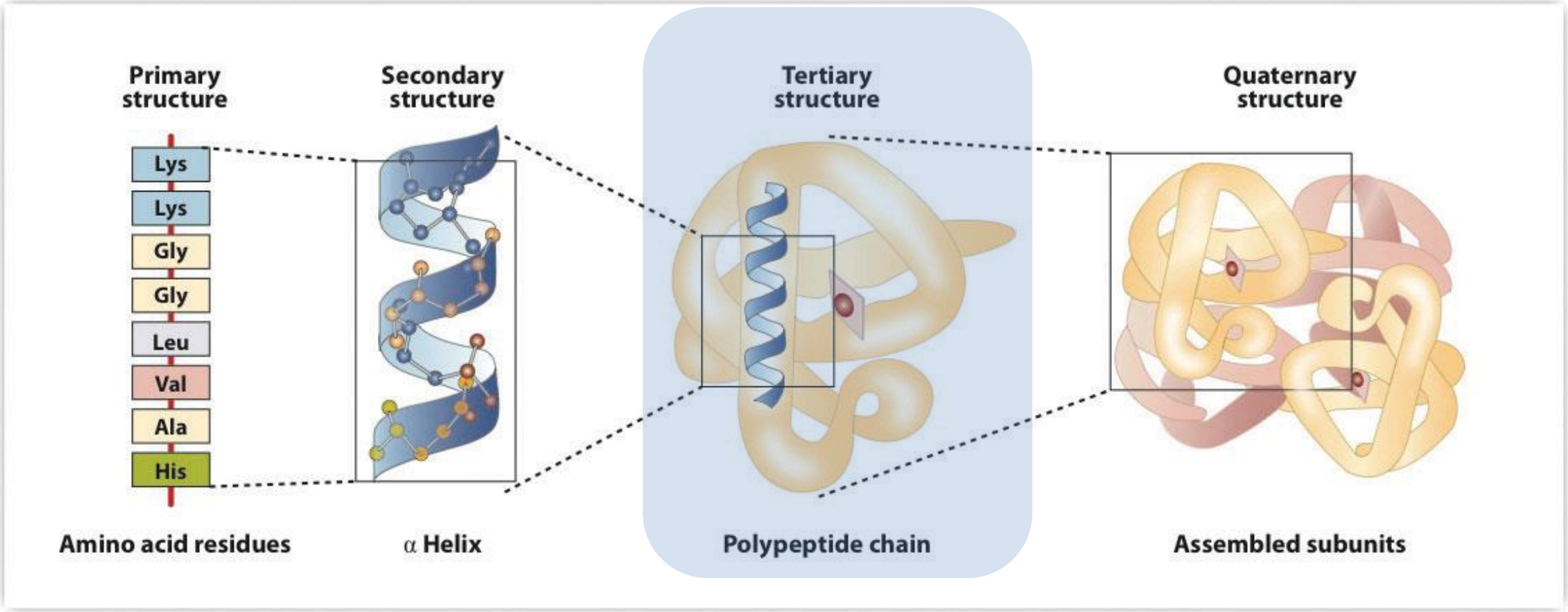
- Amino acids which are **close together** in the sequence (primary structure) start to interact and form secondary structures.
- Secondary structure consists of **repeating patterns**– the peptide winds up into **α-helicies** or folds into **β-sheets**

Secondary Structure

- Secondary structures are held together by hydrogen bonding: **negative** end of one molecule becomes **weakly attached to the hydrogen** of another
- these interactions are called **hydrogen bonds**
- Individual hydrogen bonds are **very weak**, it takes lots of them working together to hold the secondary structure in place
- Hydrogen bonds **can easily be broken** by **incorrect protein handling** during processing

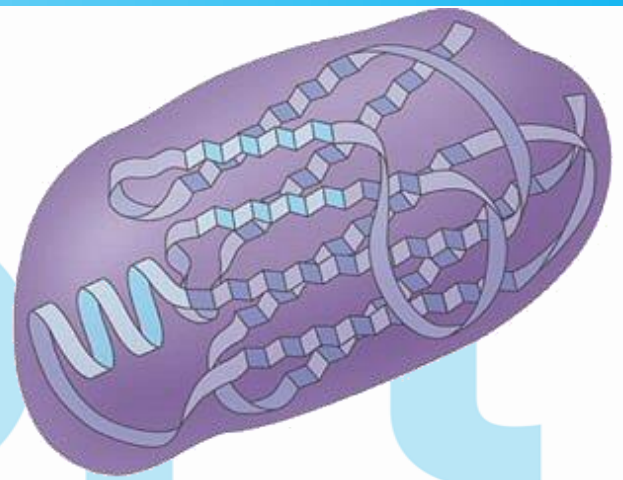


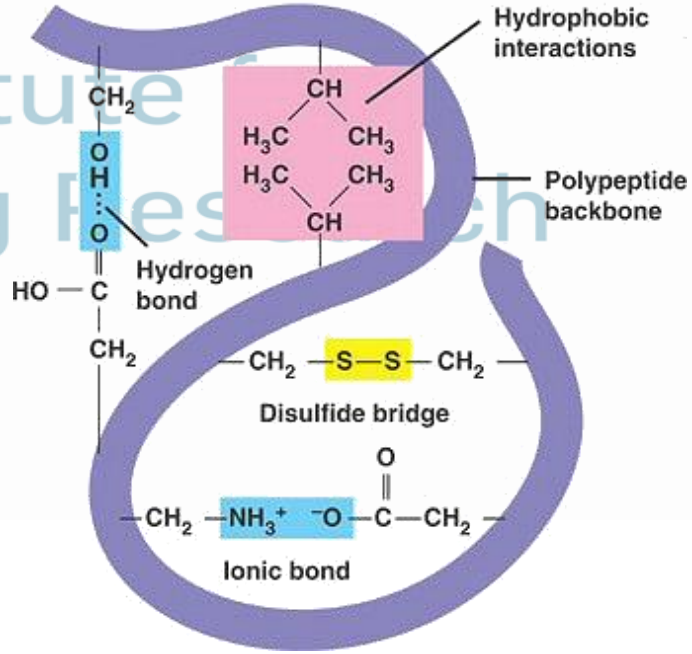
Protein Structure: Four Levels of Organisation



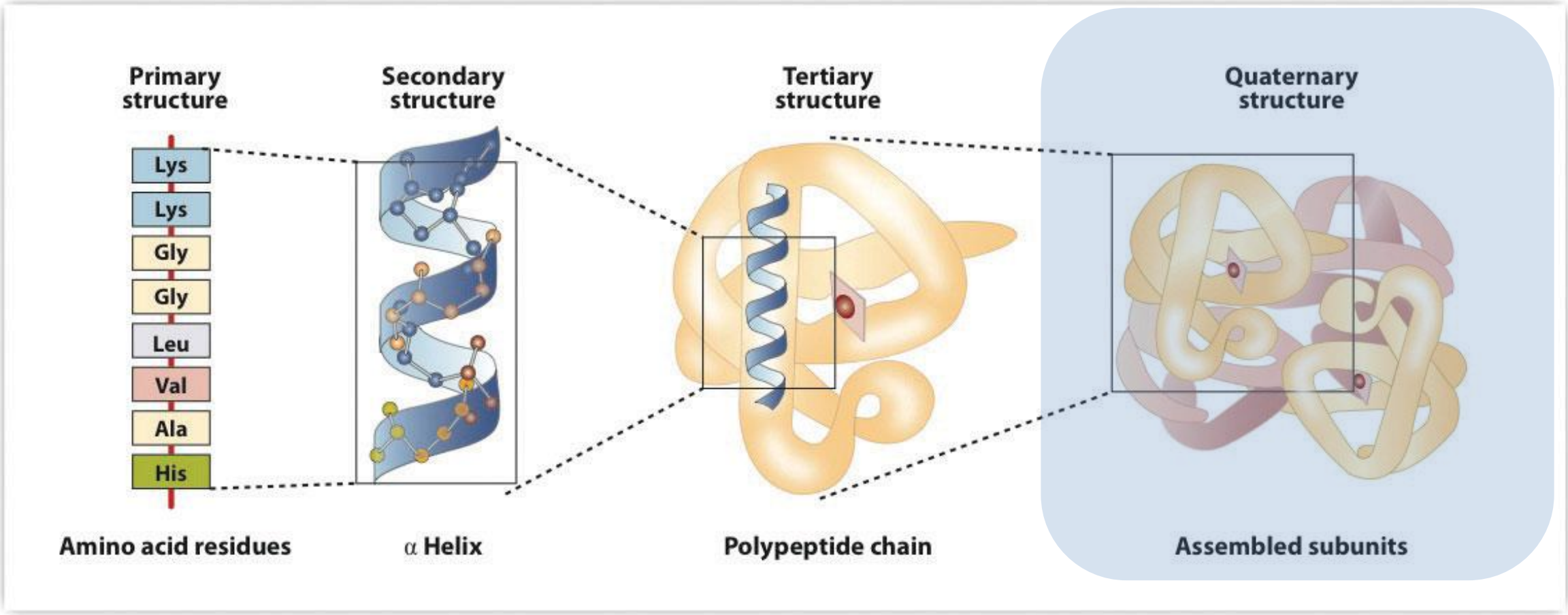
Tertiary Structure

- How the **substructures** of the polypeptide chain **fold in 3D space**
- The result of interactions:
  - Between amino acid R-groups
  - Between amino acid R-groups and the **fluid environment**
- These interactions include:
  - ***Spontaneous formation***
    - **Hydrogen** bonding
    - **Hydrophobic interactions** **Relatively weak**
    - **Ionic interactions**
  - ***Enzymatically mediated***
    - **Disulfide bridges** **Strong**



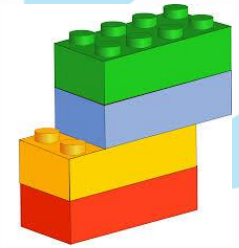


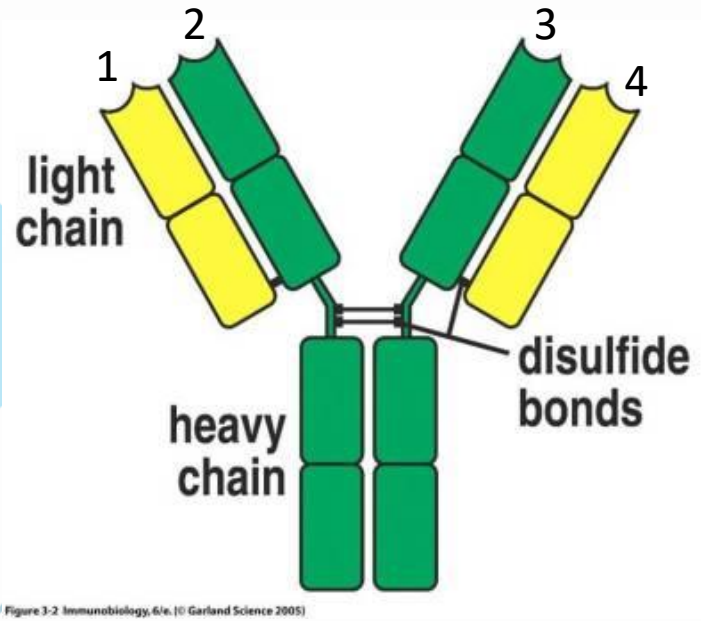
Protein Structure: Four Levels of Organisation

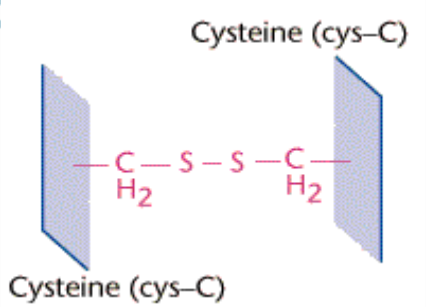


Quaternary Structure

- Proteins with **more than one polypeptide chain** have Quaternary Structure
- They can be joined together by
  - Disulfide bridges (mABs)
  - Charge interactions
  - Hydrophobic interactions
- mABs have 4 polypeptide chains in their quaternary structure their heavy chains are held together by a strong bond called a disulfide bond
- Disulfide bonds form between two cysteine amino acids

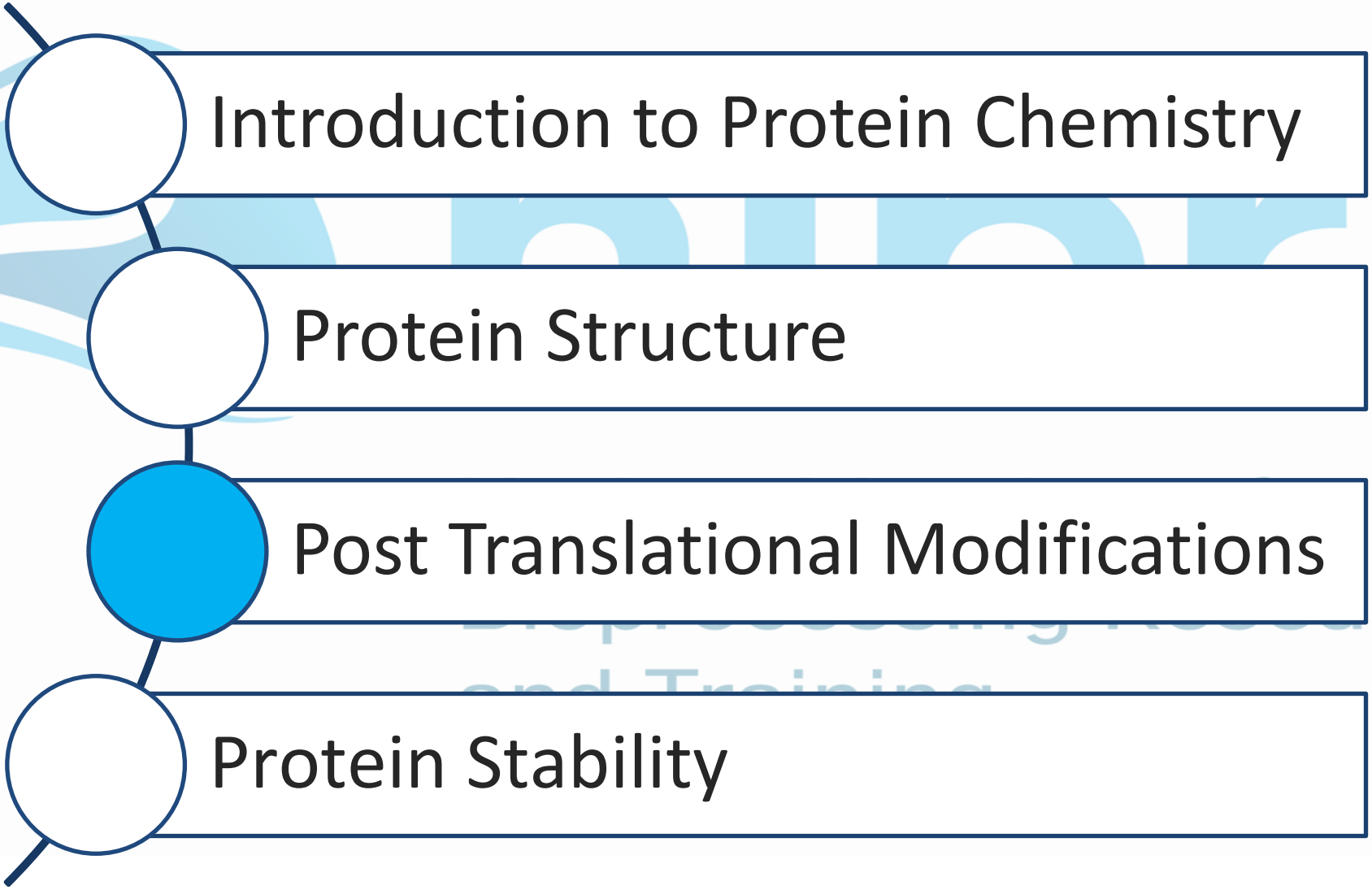






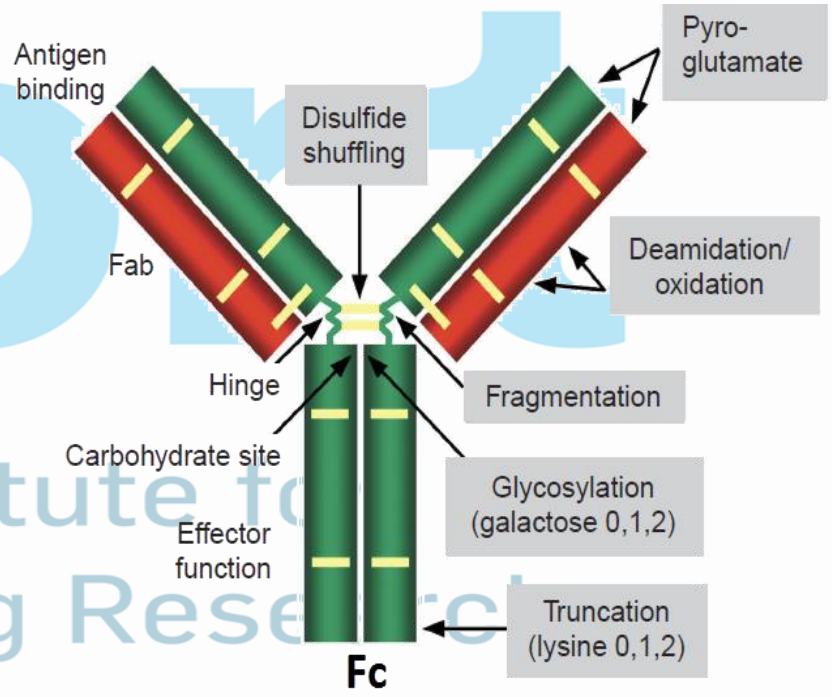
https://www.youtube.com/watch?v=OBJ95upPxuE&t=4s

Topics



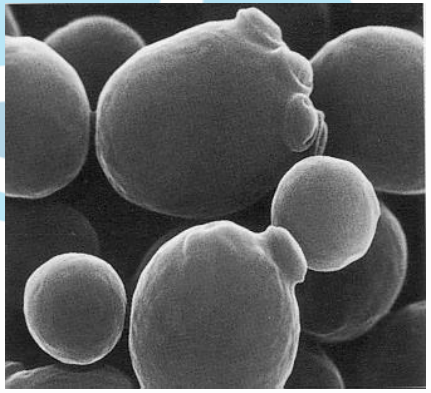
Post Translational Modification

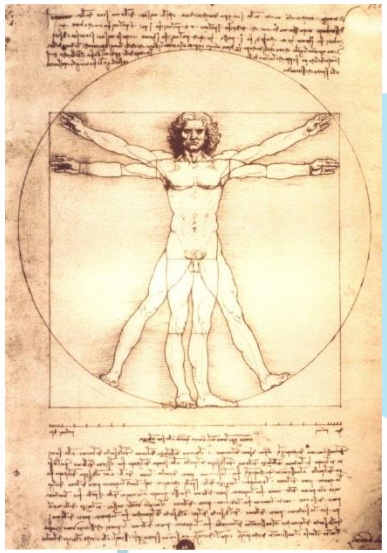
- After a cell creates a protein ('translation') **chemical modifications** (PTMs) may occur
  - Performed by enzymes in the cell
- Proteins can undergo 100s of PTMs
- PTMs influence the **structure** and thus the **function** of the protein
- PTMs are species specific
  - Bacteria versus mammalian cells



Chirino and Mire-Sluis, 2004

Why are post translational modifications important?





Yeast
~6000 genes that encode for proteins

Drosophila
~20,000 genes that encode for proteins

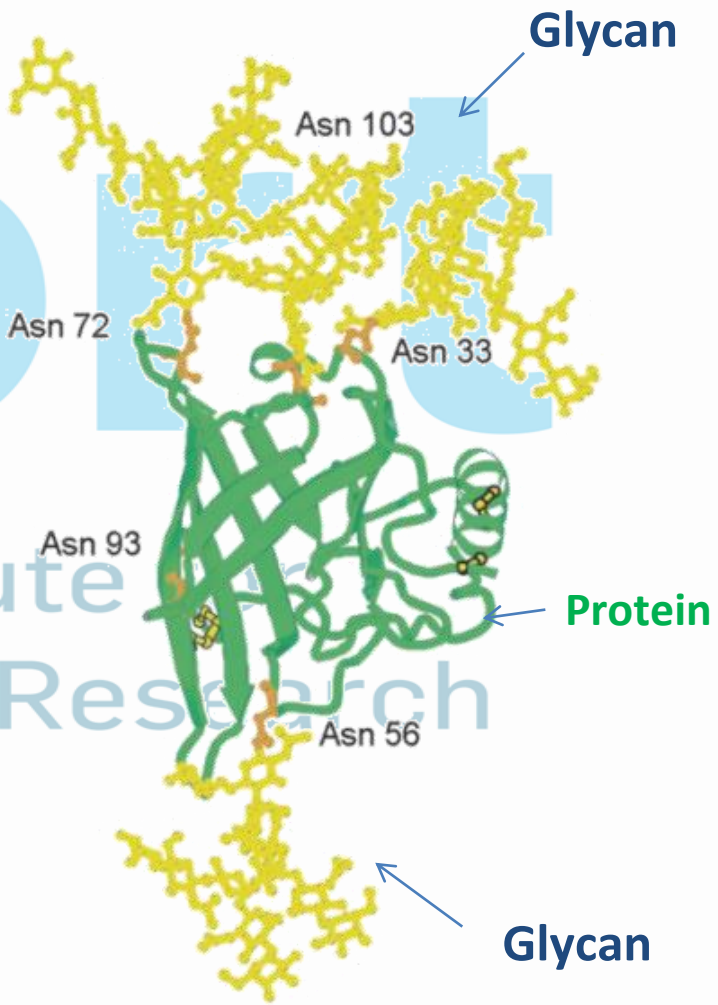
Human
~25,000 genes that encode for proteins

PTMs allow for the diversification of the protein pool as organisms become increasingly complex

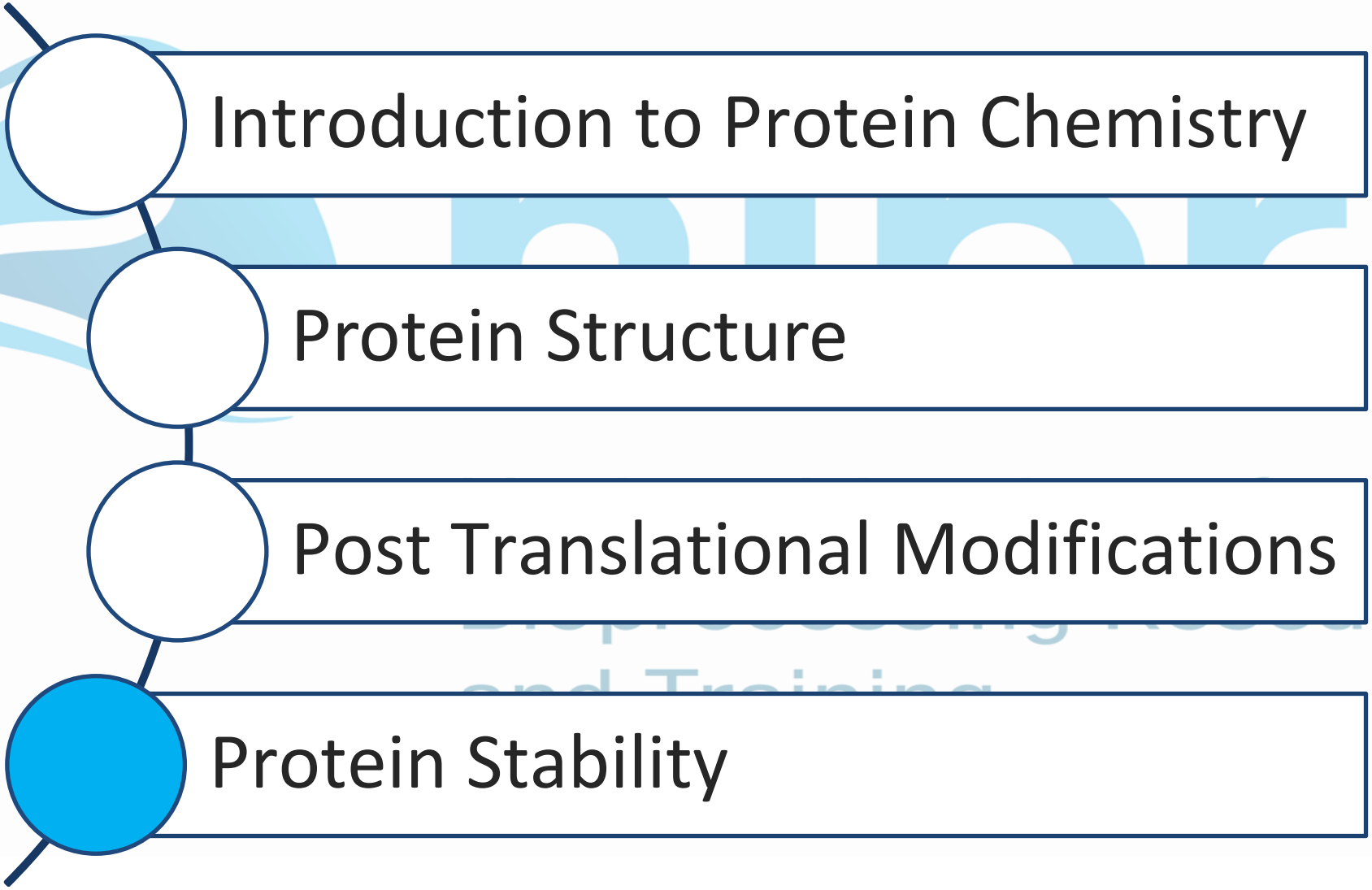
www.biochem.if.ua
www.iBioseminars.org
www.mycor.nancy.inra.fr
www.drawingsofleonardo.org

PTM Example: Glycosylation

- The addition of chains of carbohydrates ('glycans') to proteins
  - Performed by 'glycozymes'
- **66% of all biopharmaceuticals are glycosylated**
- Affects many aspects of protein **structure** and **function**
  - Folding, charge, solubility, stability, immune tolerance in patients
- Also affected by bioprocessing!



Topics

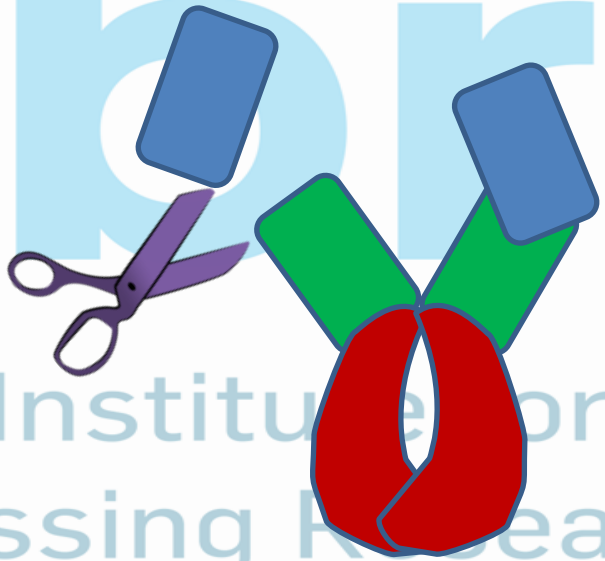

Introduction to Protein Chemistry
Protein Structure
Post Translational Modifications
Protein Stability

The structural features of biopharmaceuticals are not fixed!

- Many **physicochemical properties** are subject to change during all steps of manufacturing
- Rigorous **control** of the process and sensitive **testing** of drug **substance**, drug **product** and **raw materials** aim to minimise this
- Batches are still a **heterogeneous mix** of structurally related **isoforms,** but these must be defined and consistent from batch-to-batch

What happens if a protein becomes unstable?

1. Clipping
2. Denaturation
3. Aggregation
4. Precipitation
5. Chemical alterations



Clipping (Fragmentation/Truncation)

- Breaking apart of **subunits of a protein**
- Breaking **peptide bonds** between amino acids (hydrolysis) and degrading polypeptide chains
- Caused by:
  - **Contaminating proteases** (more on this later)
  - **Photooxidation:** reaction with light energy
  - Residual **caustic cleaning agents** (sodium hydroxide) in equipment or low pH

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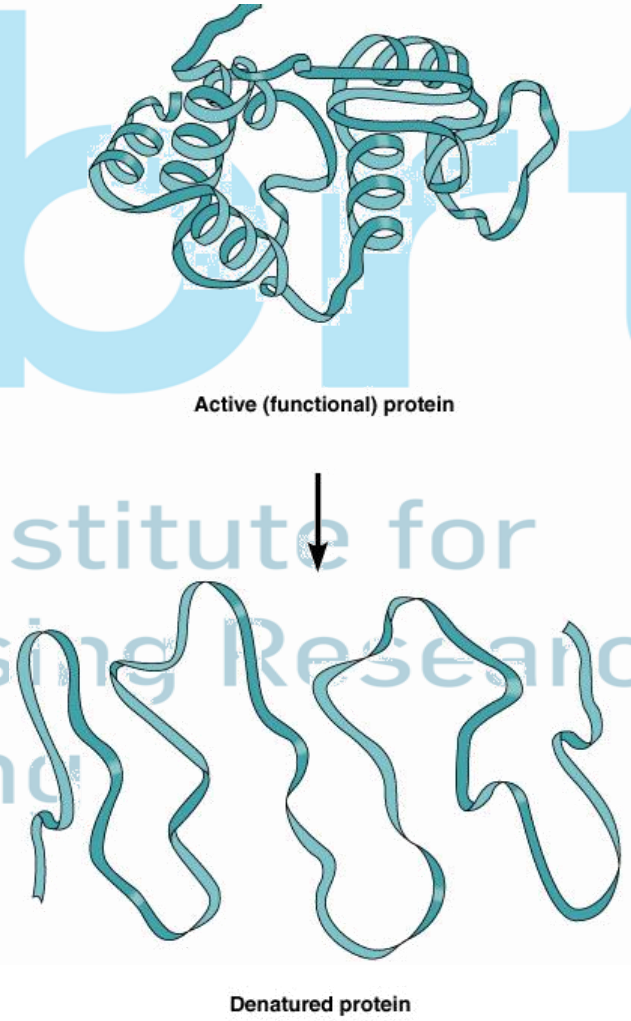
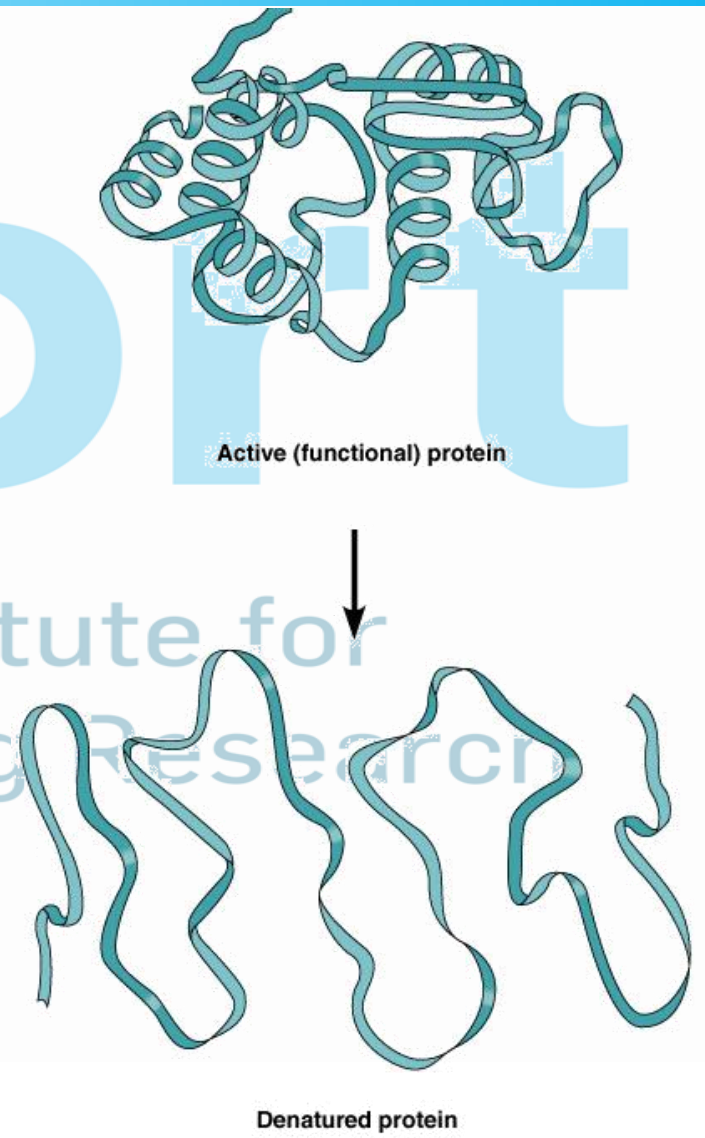
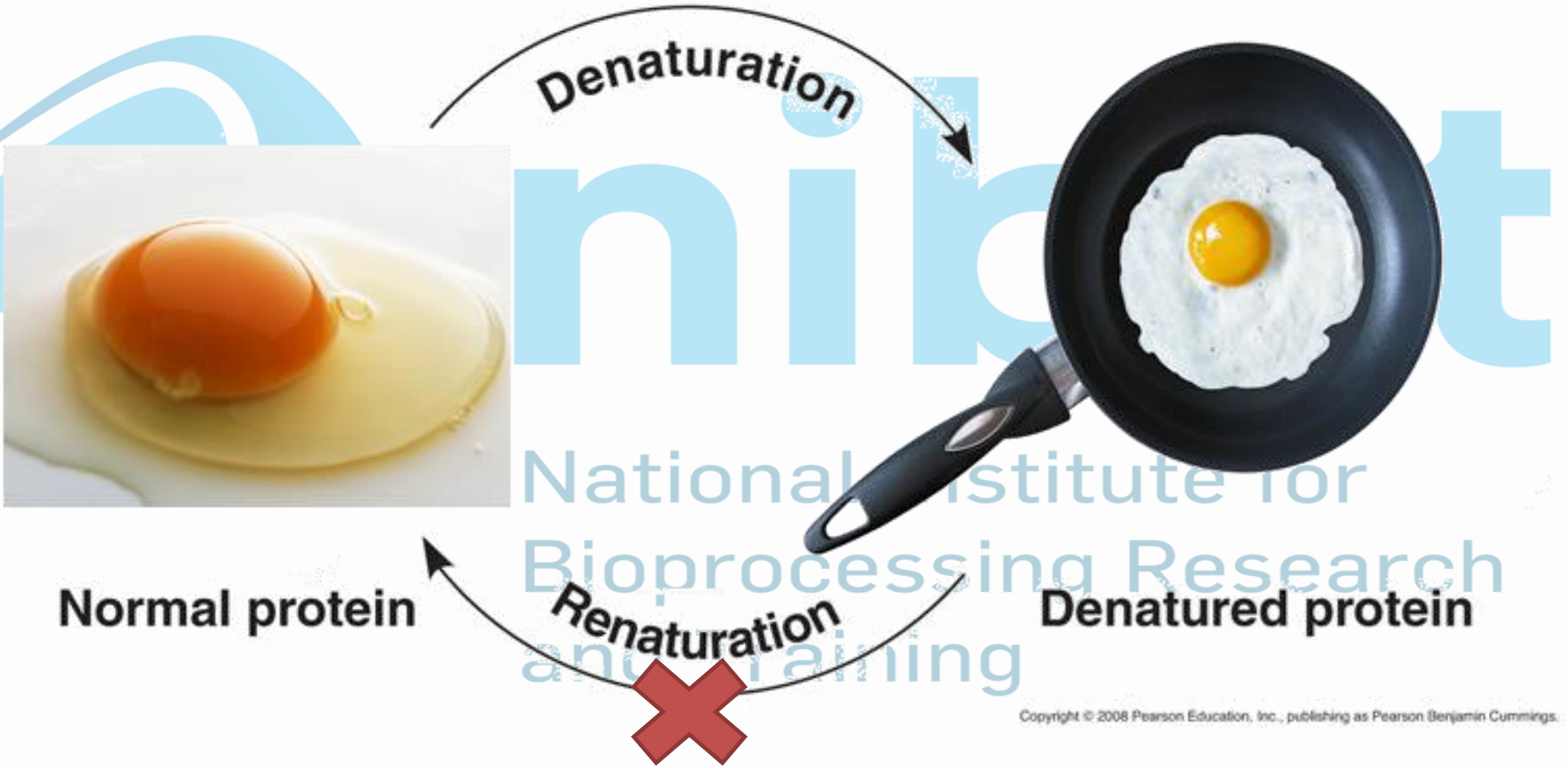


Denaturation

- Major change from the original native state without altering the primary structure
- Denatured proteins **lose their function** e.g. no therapeutic action in the patient
- Can be reversible or irreversible



Denaturation



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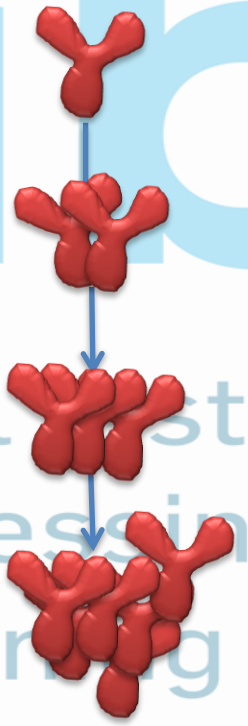
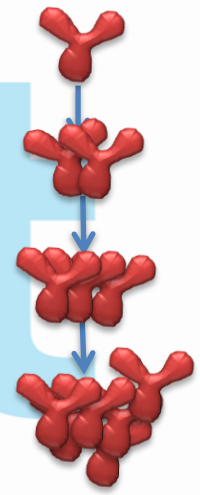
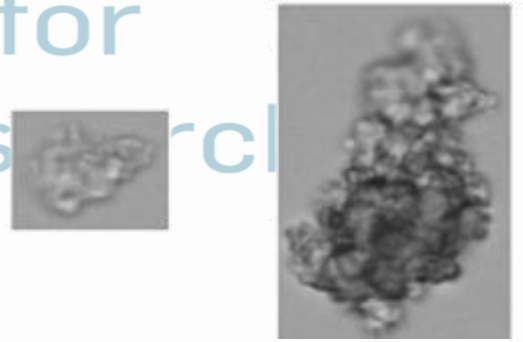


Aggregation

- Self-association of (usually mis-folded) proteins
- The monomeric (non-aggregated) form of the protein is typically the biologically active form
  - Dimers – 2 protein monomers aggregated
  - Trimers – 3 protein monomers aggregated
  - Multimers – multiple proteins aggregated
- Aggregation can be:
  - Reversible or non-reversible
  - Covalent or non-covalent
  - Visible or subvisible





IgG Protein Aggregates by FPIA Imaging

Wim Jiskoot (2010) at FIP Pharmaceutical Sciences World Congress

What happens if a protein becomes unstable?

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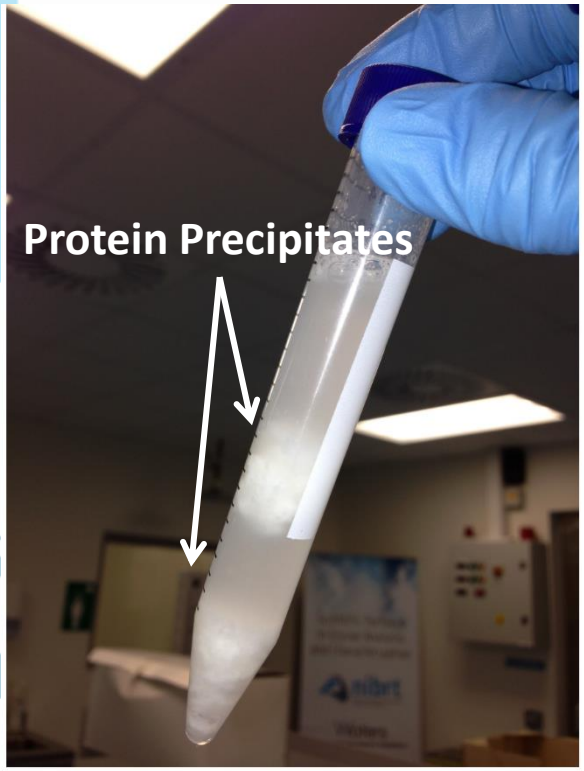
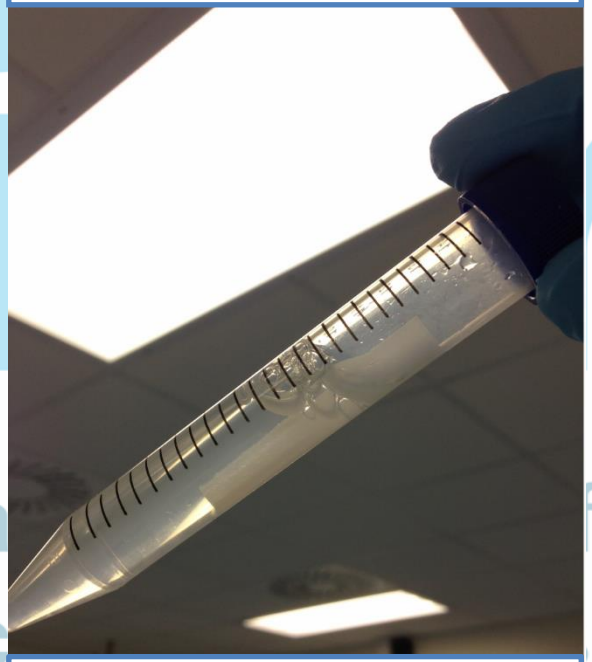


Protein Precipitation

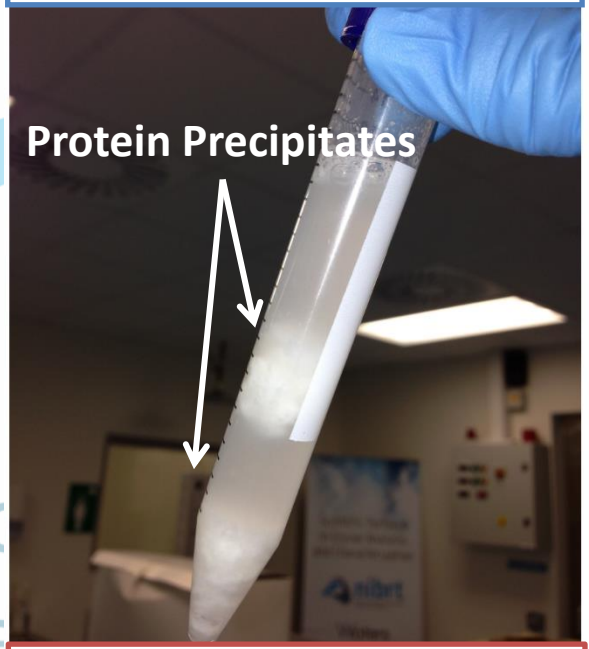
- Protein becomes insoluble
- Result of:
  - Denaturation (heat, pH effects)
  - Excessively high protein concentration
  - Excessively high salt concentration in the buffer
  - Exposure to organic solvents

Protein Solution



Protein is fully dissolved. Solution is clear.

+ 2ml HCl



Protein denatured by pH change and precipitates. Solution is cloudy.

What happens if a protein becomes unstable?

1. Clipping
2. Denaturation
3. Aggregation
4. Precipitation
5. Chemical alterations

Downstream processing can chemically alter proteins significantly

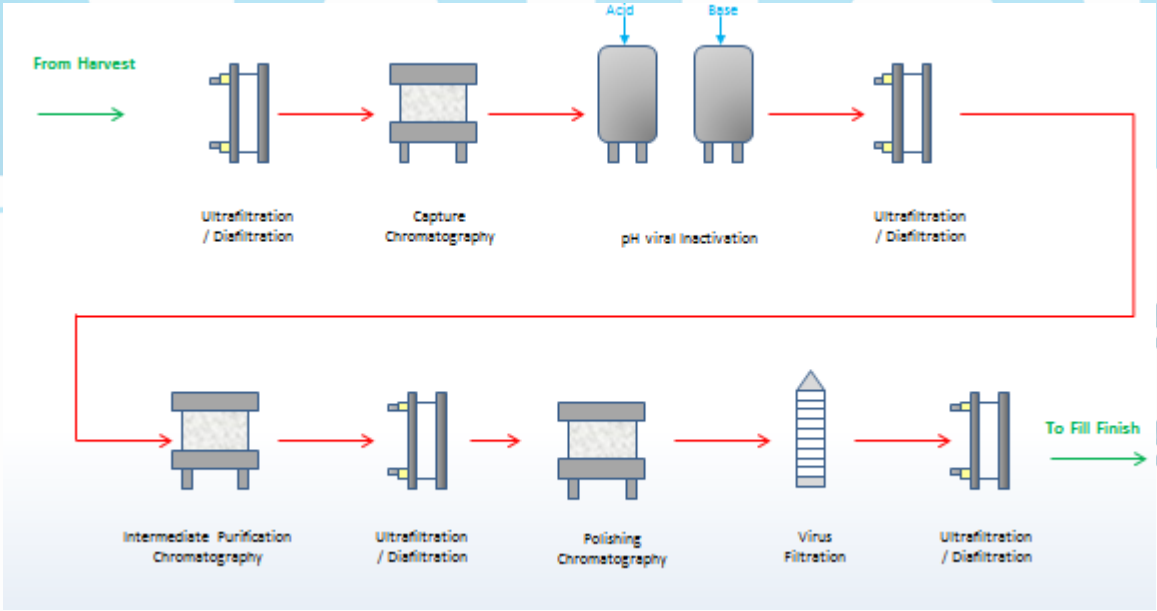
Hydrolysis

Denaturation

Aggregation

Desialylation

Precipitation



Glycation

Deamidation

Pyroglutamate

Disulfide scrambling

Oxidation

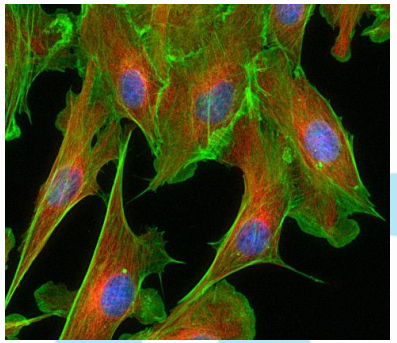
Walsh, Gary. *Pharmaceutical biotechnology: concepts and applications*. John Wiley & Sons, 2007.

What happens if a protein becomes unstable?

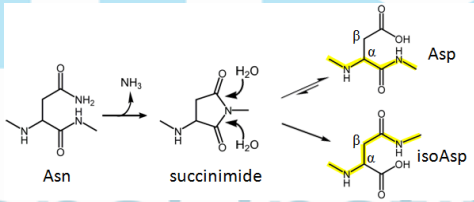
- Lose therapeutic efficacy
- Cause serious adverse effects in patients
  - Aggregation increases chance of immunogenic reactions
- Reduce production yield

Sources of protein instability

1. Biological origin



2. Chemical origin

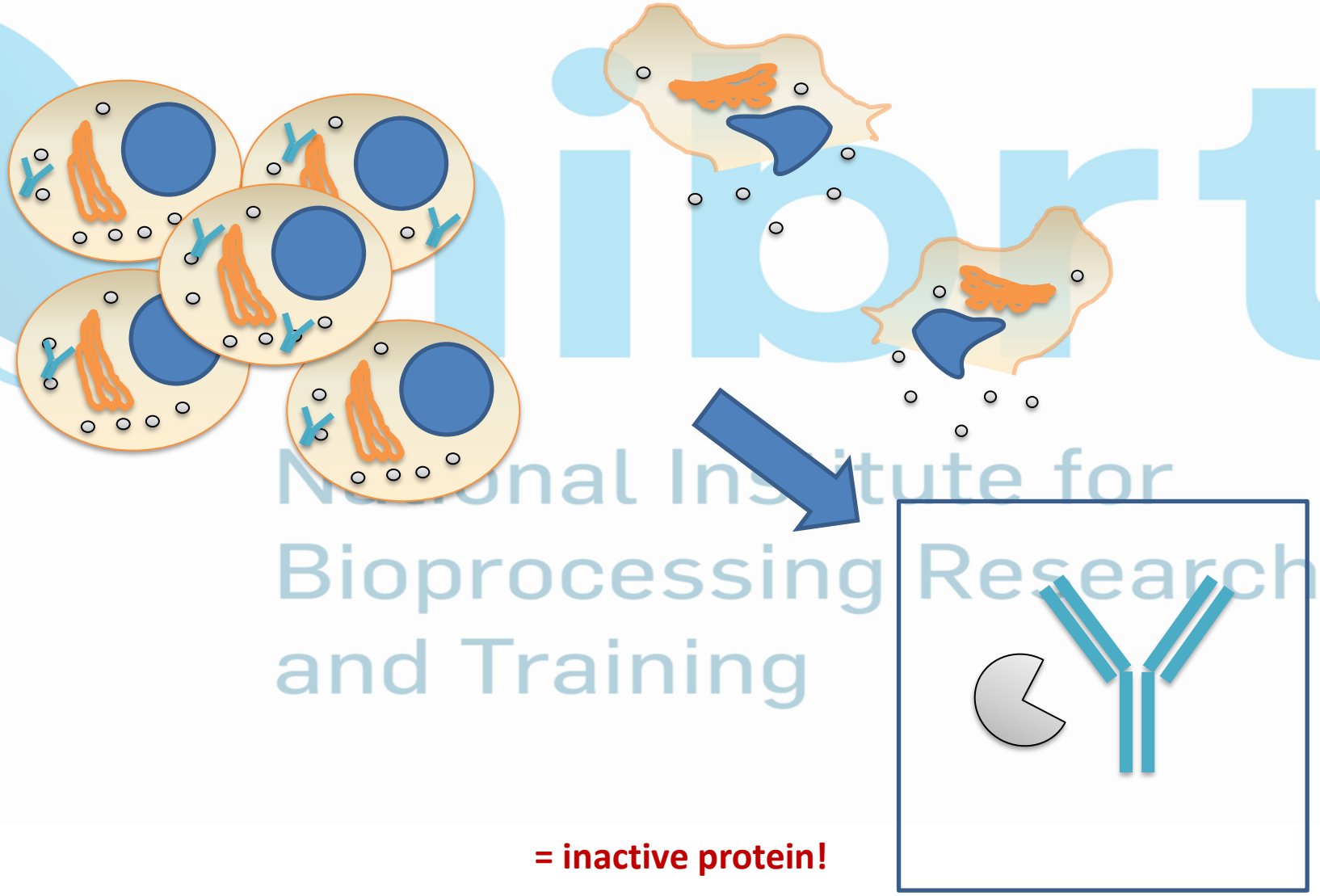


3. Physical origin

Biological Origin

- **Mutations** can lead to amino acid substitutions which lead to **structural changes**
- **Media composition** and culture conditions can affect PTMs, particularly **glycosylation**
- **Cell death** in the bioreactor can lead to degradation of the biologic due to release of **proteolytic enzymes**
- **Perfusion processing** can help to quickly separate fragile product from proteolytic enzymes in the bioreactor

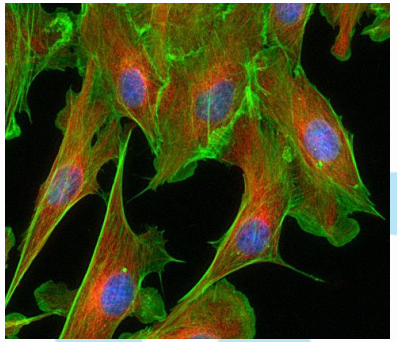
Proteolytic Enzymes Attack the Biopharmaceuticals in the Bioreactor



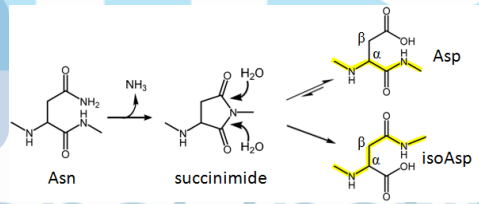
= inactive protein!

Sources of protein instability

1. **Biological origin**



2. **Chemical origin**



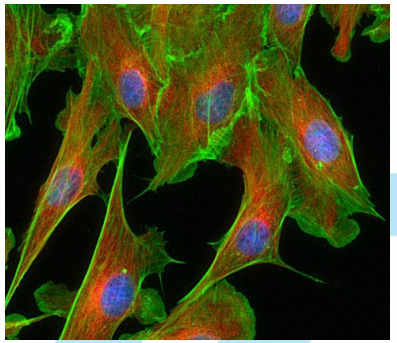
3. **Physical origin**

Chemical Origin

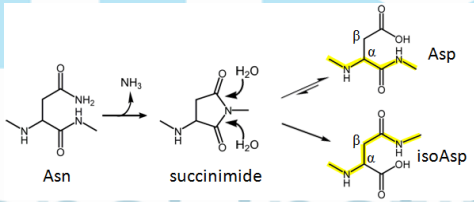
- Certain environmental factors during bioprocessing can change the chemical structure of the protein
  - **pH:** Proteins are very sensitive to pH changes, which can affect how amino acids interact and thus folding
  - **Ionic strength:** salt disrupts protein-protein interactions

Sources of protein instability

1. Biological origin



2. Chemical origin



3. Physical origin

Physical Origin

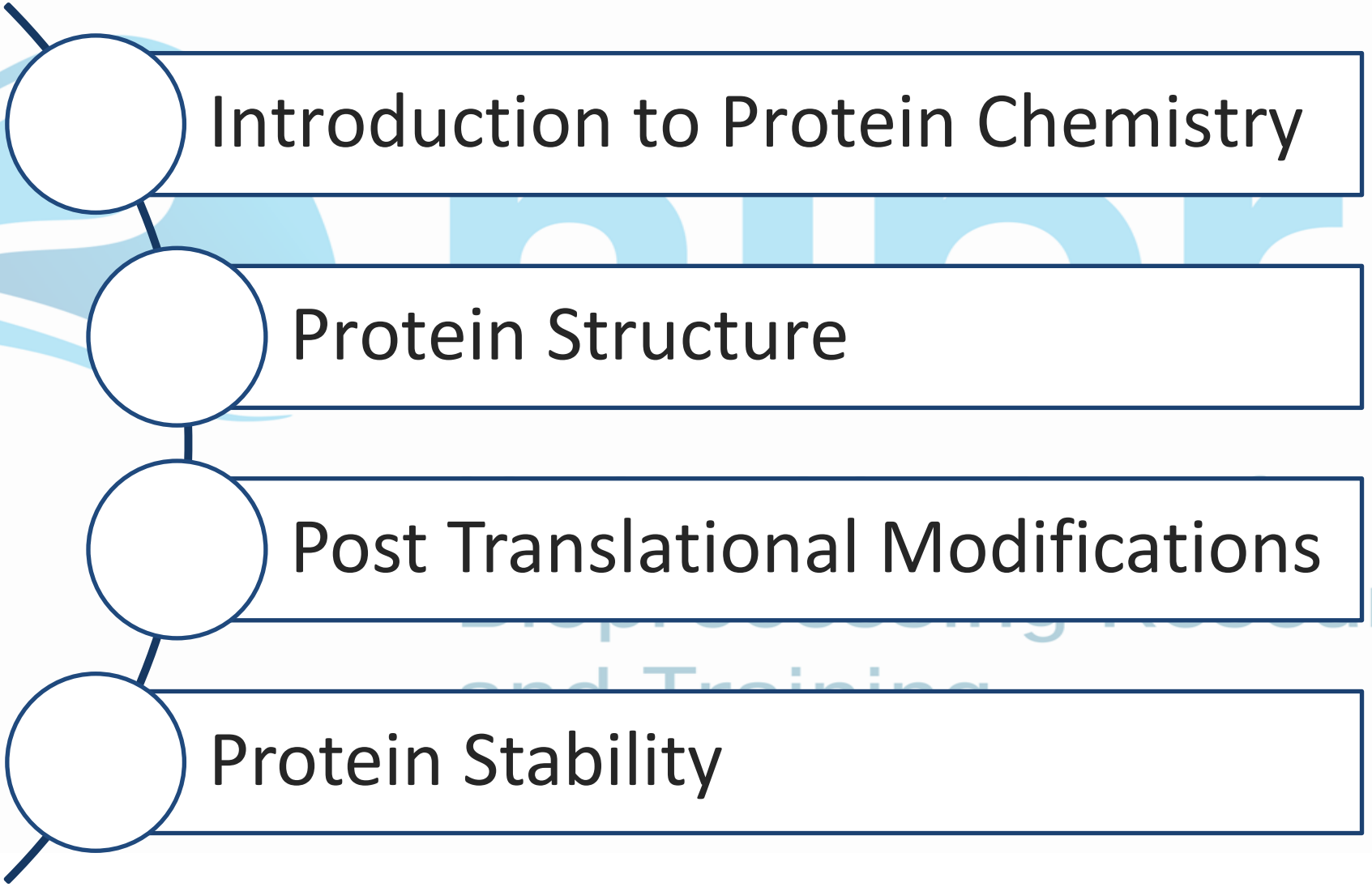
- **Mechanical shear**
  - Vigorous shaking (like whipping egg whites)
- **Temperature**
  - Proteins function at specific temperature
  - Freeze/thaw cycles
- **Protein concentration**
  - Too low: adsorption to container surface
  - Too high: precipitation
- **Light/ionising radiation**
  - Can trigger chemical chain reaction

Correct Protein Handling

- Prevent shear during transfers
- When purifying protein, maintain physiological conditions as much as possible
- Work at low temperatures if possible (4^{o}C)
- Limit processing timelines
- Avoid protein lingering at/near isoelectric point

Beware of introducing artefacts when handling QC samples!

Topics


Introduction to Protein Chemistry
Protein Structure
Post Translational Modifications
Protein Stability

Thank You