Biophysical and Biochemical Analysis of Recombinant Proteins

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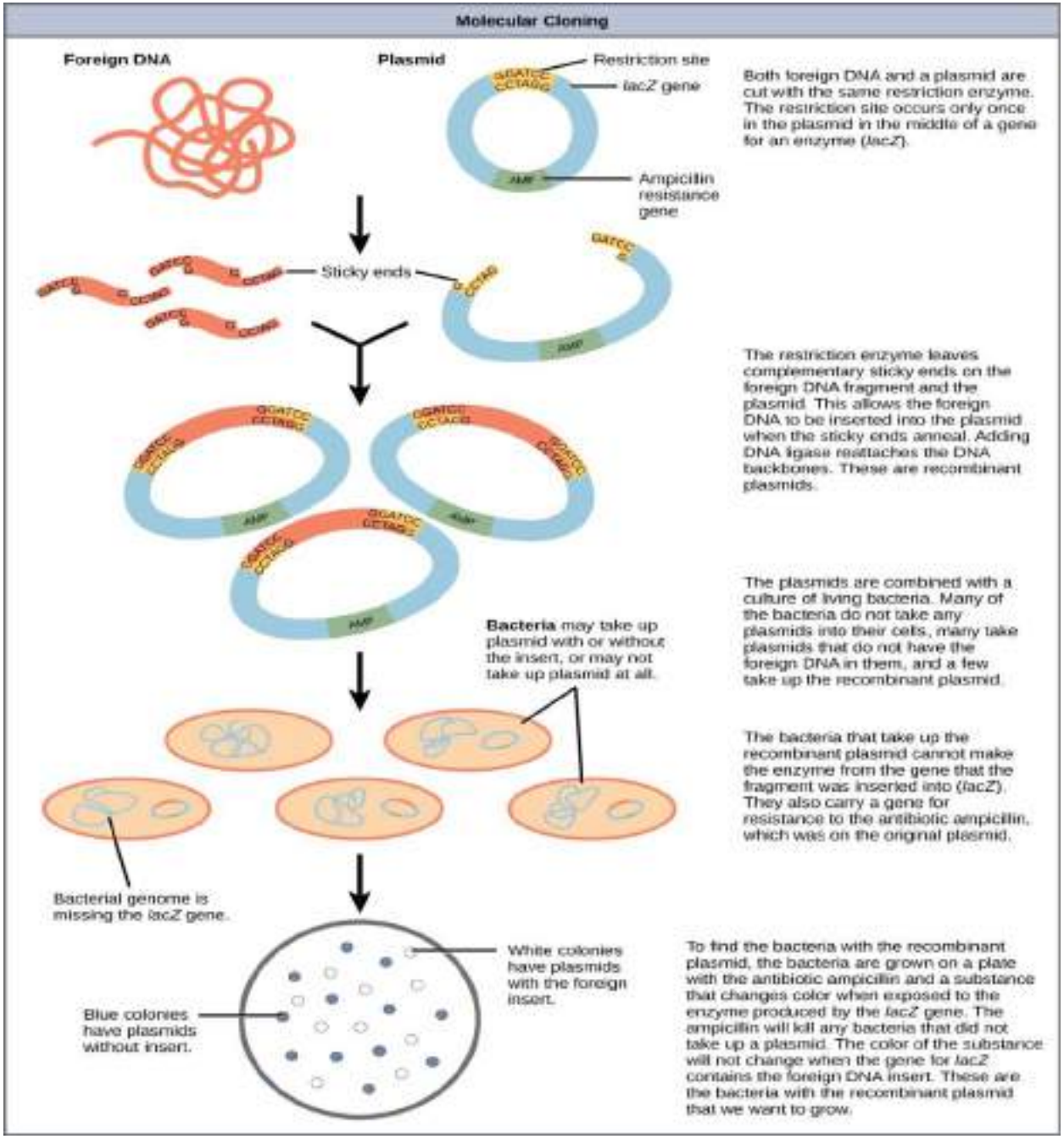
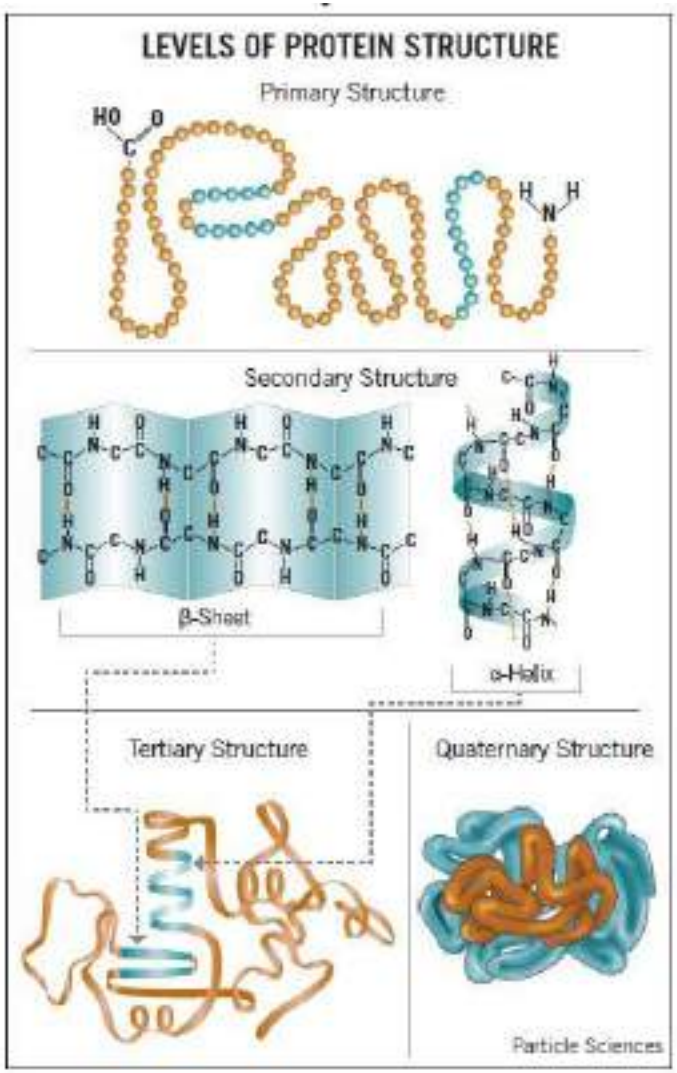
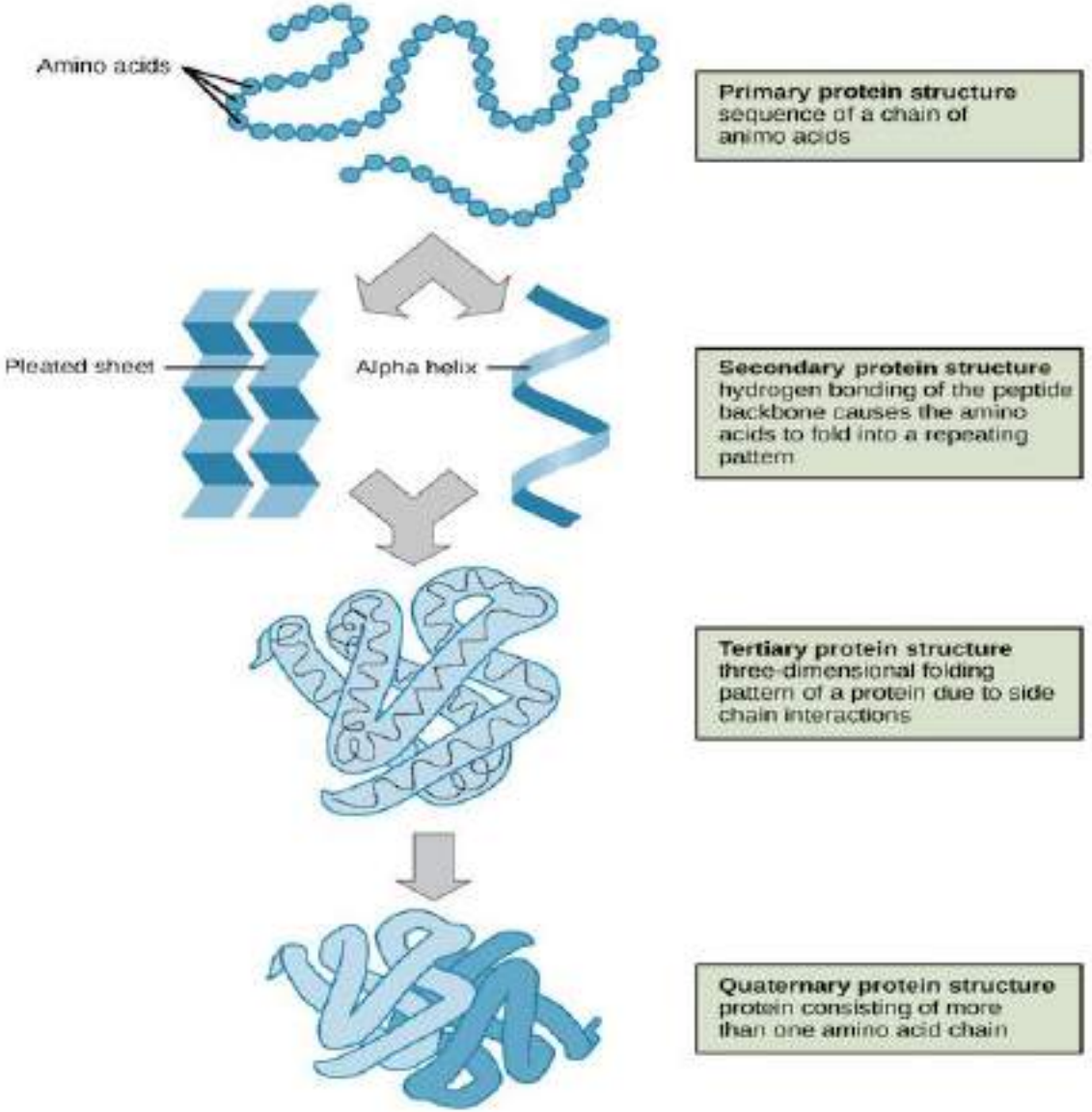
Overview (لمحة)

- For a recombinant protein to become a **human therapeutic**, its biophysical and biochemical characteristics must be well understood.
- يجب فهم الخصائص الكيميائية البيولوجية و الفيزيائية البيولوجية للبروتين المؤشب ليصبح بروتين علاجي جاهز للإستخدام البشري
- A number of techniques can be used to determine the biophysical properties of proteins and to examine their biochemical and biological integrity.



Protein structure (بنية البروتين)





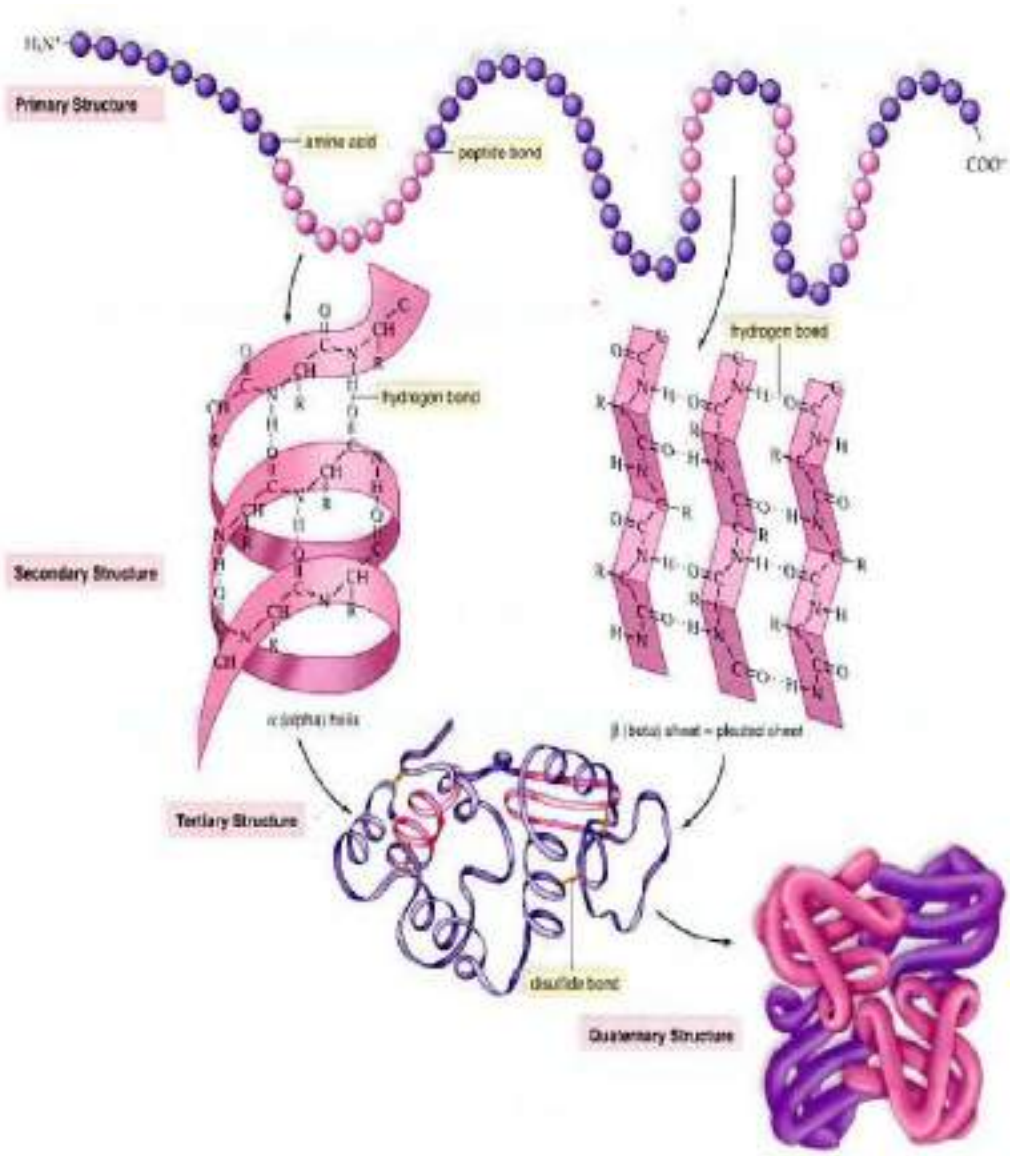
Protein folding (طى البروتين)

- Proteins become functional only when they assume a distinct tertiary structure and biological integrity.
- يصبح البروتنين وظيفي عندما يأخذ بنية ثالثية ثابتة و سليمة من الناحية البيولوجية

- When recombinant proteins are produced in Escherichia coli, they often form inclusion bodies (اجسام إشتمالية) into which they are deposited as insoluble proteins.(تترسب كبروتينات عير منحلة)

- Formation of such insoluble states does not naturally occur in cells where they are normally synthesized and transported.

- Unfolded (منتشر او ممتد) states of proteins are usually highly stable and soluble in the presence of denaturing agents (بوجود عامل محول للصفات الطبيعية.



Protein folding

- Therefore, an *in vitro* (خارج الجسم الحي)process is required to refold (اعادة طي)insoluble recombinant proteins into their native (الحالة الطبيعية) , physiologically active state (الحالة الطبيعية او الفعالة فيزيولوجيا).

- This is usually accomplished by solubilizing the insoluble proteins with detergents or denaturants, followed by the purification and removal of these reagents concurrent with refolding the proteins (متبوعة بتنقية وإزالة هذه العوامل مشكل متزامن مع إعادة طي البروتين) .

- **Refolding of the protein could be achieved by :**

  - **Dilution** of the denaturants with aqueous buffer (وقاء مائي) reduced its concentration.
  - **Dialysis** (الميز الغشائي) or the diafiltration of protein in the denaturant against an aqueous buffer.
  - First **binding** the protein in denaturants to a **solid phase** to a column matrix (الطور الصلب لمادة عمود التنقية), and then **equilibrating** it with an **aqueous buffer**

Protein stability (ثباتية البروتين)

- Proteins are neither chemically or physically stable because have surface that **chemically highly heterogeneous** (متغايرة المنشا) and contains reactive groups.
- The long term exposure to the environmental stress could make a various **chemical alteration** which could be (التغيرات الكيميائية):

- **Oxidation** (اكسدة)and disulfide exchange of the cysteine residues (ثمالات السيستئين)present in many proteins like growth factors and cytokines.
- **Hydrolysis** (حلمهة) can occur on peptide bonds and on amides of asparagine and glutamine residues.

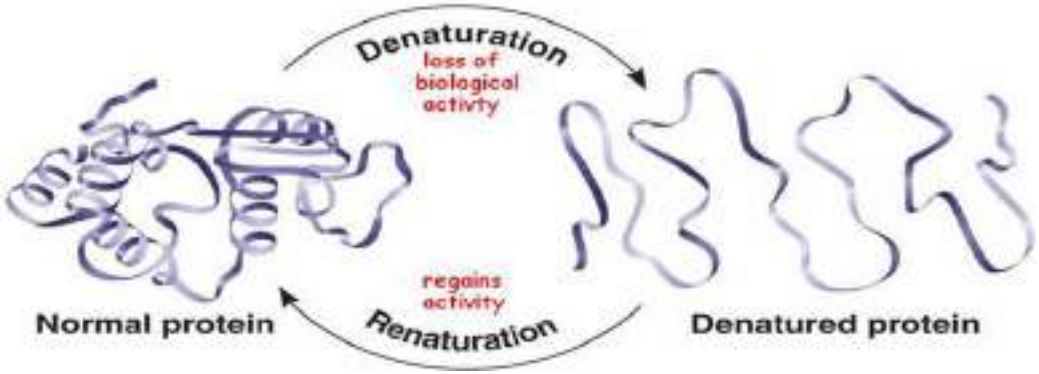
- **Physical stability** of the protein is the difference in **free energy** (الطاقة الحرة) , ΔGu, between the native and denatured states.

Common reactions affecting the stability of the proteins (تفاعلات شائعة تؤثر على ثباتية البروتين)

	Physical property affected	Method of analysis
Oxidation Cys Disulfide Intrachain Interchain Met, Trp, Tyr	Hydrophobicity Size Hydrophobicity	RP-HPLC, SDS-PAGE Size exclusion chromatography Mass spectrometry
Peptide bond hydrolysis	Size	Size exclusion chromatography SDS-PAGE
N to O migration Ser, Thr	Hydrophobicity Chemistry	RP-HPLC inactive in Edman reaction (used to determine amino acid sequence in peptide fragments of enzymatically degraded proteins) (Fig. 15)
α-Carboxy to β-carboxy migration Asp, Asn	Hydrophobicity Chemistry	RP-HPLC inactive in Edman reaction (used to determine amino acid sequence in peptide fragments of enzymatically degraded proteins) (Fig. 15)
Deamidation Asn, Gln	Charge	Ion exchange chromatography
Acylation α-amino group, ε-amino group	Charge	Ion exchange chromatography Mass spectrometry
Esterification/ carboxylation Glu, Asp, C-terminal	Charge	Ion exchange chromatography Mass spectrometry
Secondary structure changes	Hydrophobicity Size Sec/tert structure Sec/tert structure Aggregation Sec/tert structure, aggregation	RP-HPLC Size exclusion chromatography CD FTIR Light scattering Analytical ultracentrifugation

Protein stability

- In general the **unfolding** is reversible (عكوس) but in some cases like **heating** the denaturation is irreversible by forming subsequent aggregation (تكدس) .
- Therefore, any stress that decreases **ΔGu** and increases **k** will cause the accumulation of irreversibly inactivated forms of the protein.
- This **stress** could be a chemical like oxidation and hydrolysis.
- And could be **physical parameters** like **PH, temperature, ionic** strength and protein concentration.
- **The human use protein have to be in a forms provide prolonged shelf life(مدة طويلة من الصلاحية) .**
- Protein stabilizing agents (عوامل مثبتة للبروتين) **increase ΔGu** and could also **increase k.**
- **Lyophilisation** (التجفيد) is the process of choice for **therapeutic proteins**

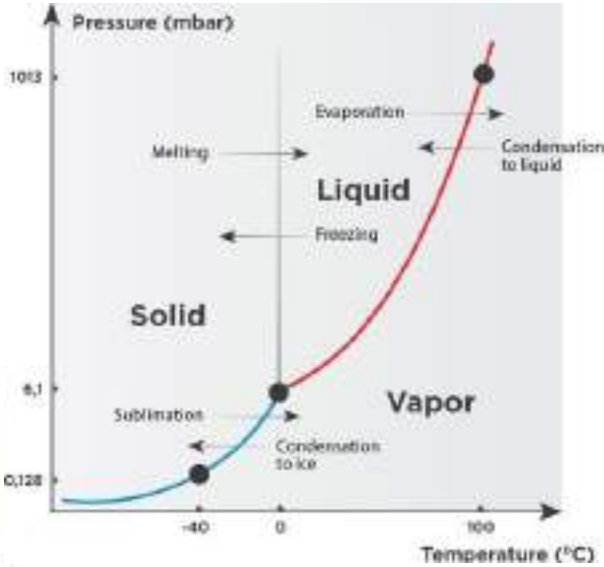


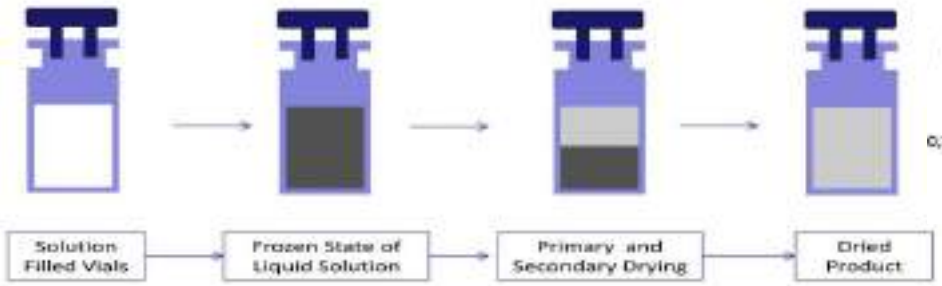
Stabilizing agents & mechanism of action (العوامل المثبتة للبروتينات وآلية عملها)

Stabilizing additive	Mechanism of action	Protein stabilized
Sugars-trehalose, sucrose Maltose, glucose	Increase Tg thereby enhancing thermal stability of proteins	Collagan, ribonuclease, ovalbomin
Salts- potassium phosphate, sodium citrate, amm.sulphate	Increase Tg of proteins and self association of proteins, reduce the solubility	Collagan, ribonuclease, ovalbomin
Cyclodextrins-hydroxypro-pylcyclo dextrins	Not clear; probably by changing the properties of solvent	Porcine growth hormone
Heparin	Increase the unfolding temperature by 15-30°	Acidic fibroblast growth factor
Metals - zinc	Complexation	hGH against urea induced denaturation Insulin
Chelating agent- EDTA	Complexation and decrease catalytic degradation by metal	Acidic fibroblast growth factor ribonuclease A
Surfactant - Non ionic-polysorbates Cationic-cetrimide	Preferential adsorption on hydrophobic interface of delivery matrix;	Nutropin^{R} (r-hGH) with polysorbates; hGH loaded PLG polymer matrix
Anionic - SLS	Membrane perturbation	

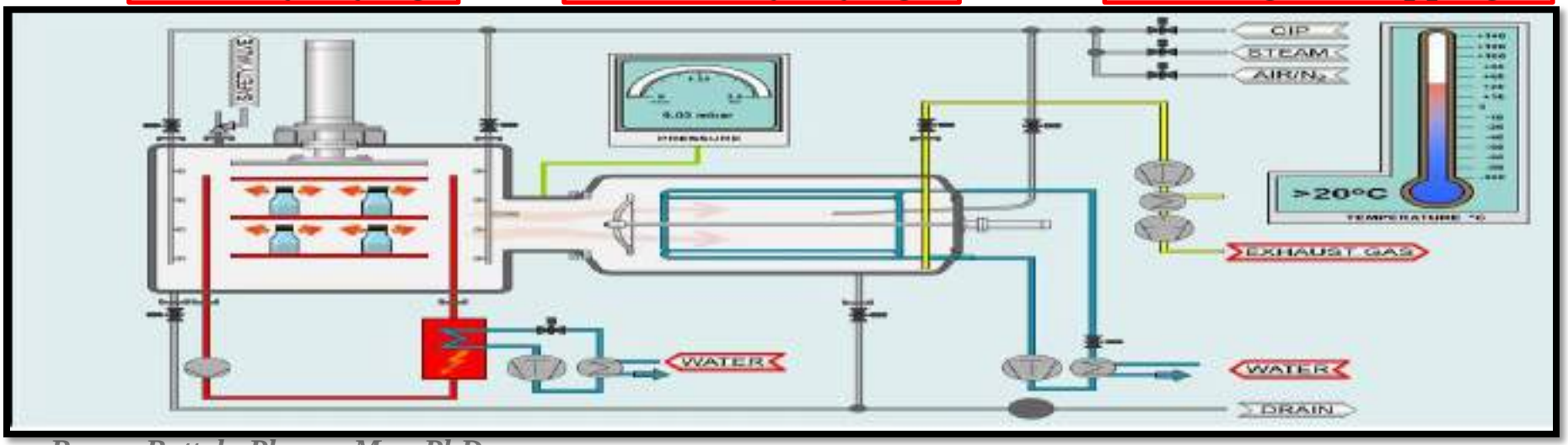
Summary of the lyophilisation steps

- **Freezing (تجميد)** of the product at (-60C°) with (1000mbar).
- **Primary drying** (تجفيف) at (+5 C°) (1-0.1mbar).
- **Secondary drying** (+20C°) (0.03mbar).
- **Sealing and capping (ختم و تعبئة)**.





Primary drying | **Secondary drying** | **Sealing and capping**

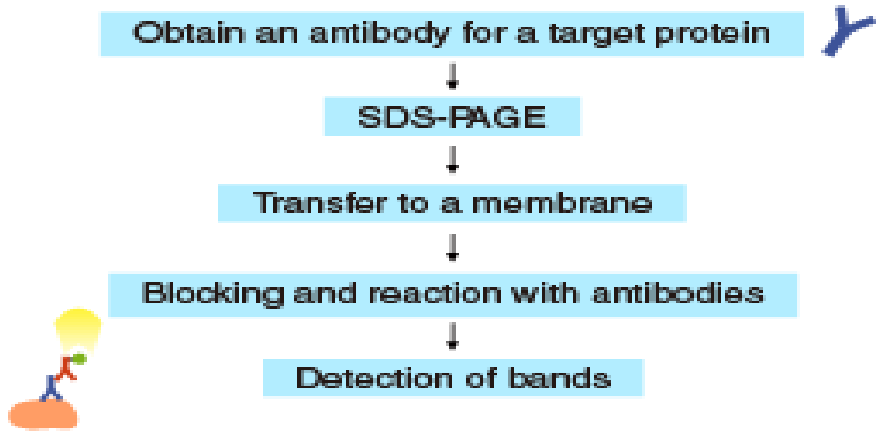


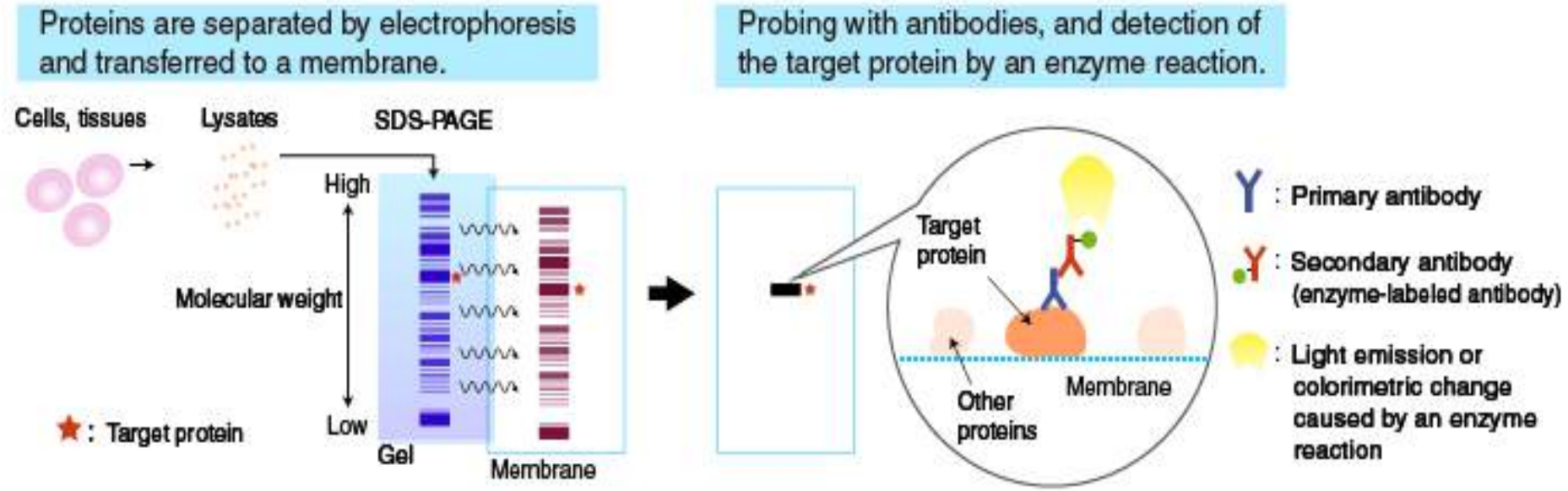
Analytical techniques for proteins (الطرائق التحليلة للبروتينات)

- **Blotting (التلطيخ)**

- **Blotting** is a technique used to detect a low level of molecules like **proteins** , **nucleic acid**.
- protein samples first subject to type of fractionation (تجزئة), such as **PAGE (polyacrilamide gel electrophoresis رحلان كهربائي)**, prior to the **blotting step** .
- **Western blots** involve the use of **labeled antibodies (أضداد واصمة)** to detect specific **proteins.**
- Blotting called **southern** for DNA and **northern** for RNA.
- Biomolecules are transferred to a membrane (blotting) by application of electric field .
- Then the membrane probed (يسبر الغشاء) with specific reagent or specific primary anti body (أضداد أولية) for a proteins.
- The membrane used are made from nitrocellulose or PVDF (polyvinylidine difluoride).
- Then the proteins being detected by **x ray film** or by **chemilumenecence ضيائة كيماوية) أو توهج) .**

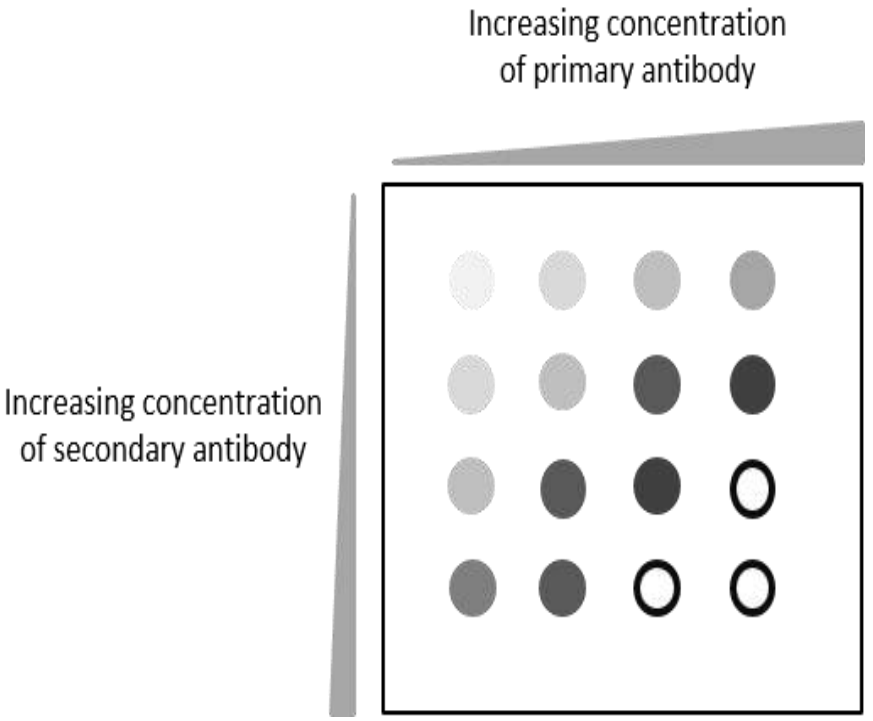
Blotting & SDS PAGE





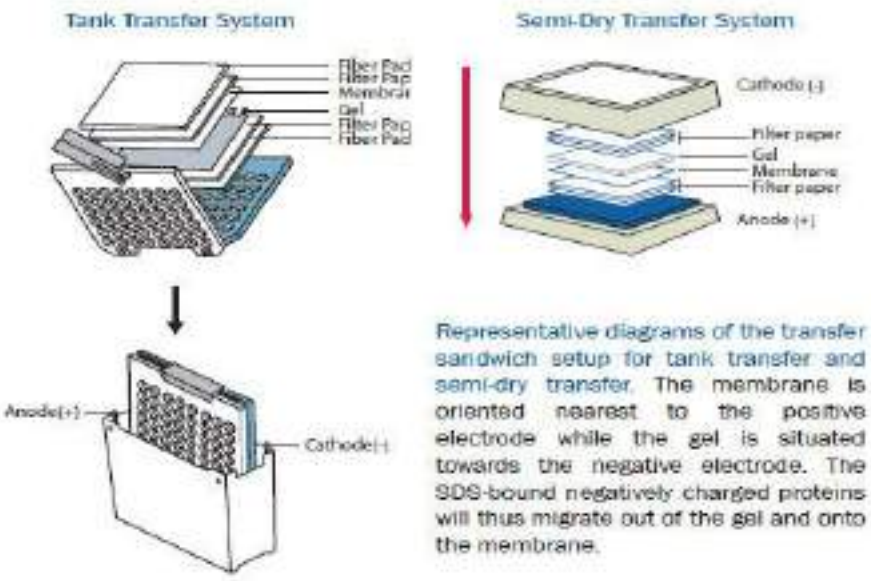
Dot blot (التلطيخ النقطى)

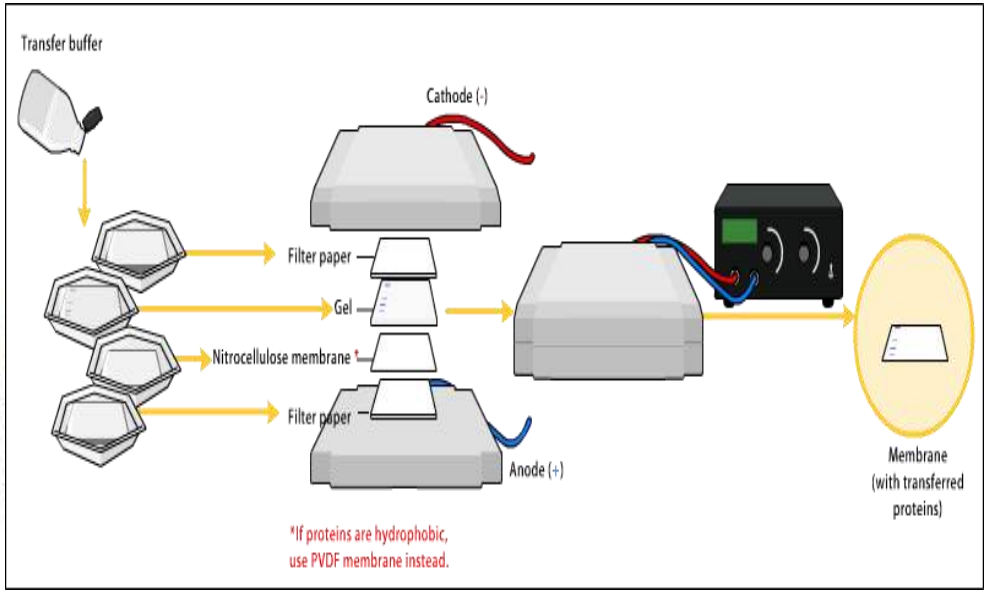
- A solution containing the biomolecules of interest is filtered through a membrane which captures the biomolecule.
- then the membrane will probe with specific primary antibody then with secondary marked antibody for detection.
- This technique **used for** detection of **known molecular weight of protein (بروتين معلوم الوزن الجزيئي)** .
- And could be used to determine the concentration of the primary and the secondary antibody used.



Transfer of protein (نقل البروتين)

- After **SDS PAGE,** the transfer of the protein from the gel to the membrane could be achieved by different ways:
  - **Capillary action** (الفعل الشعري) where the membrane is placed between the gel and absorbent paper.
  - **Electroblotting** (التلطيخ الكهربائي) where electric field is applied and the maximum distance the protein needs to migrate is only the thickness of the gel. Hence the transfer of proteins can occur very rapidly.



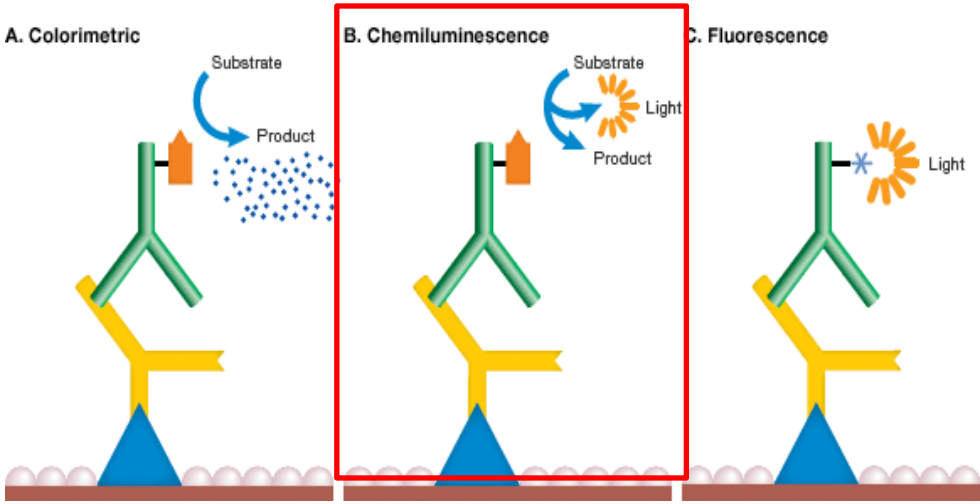


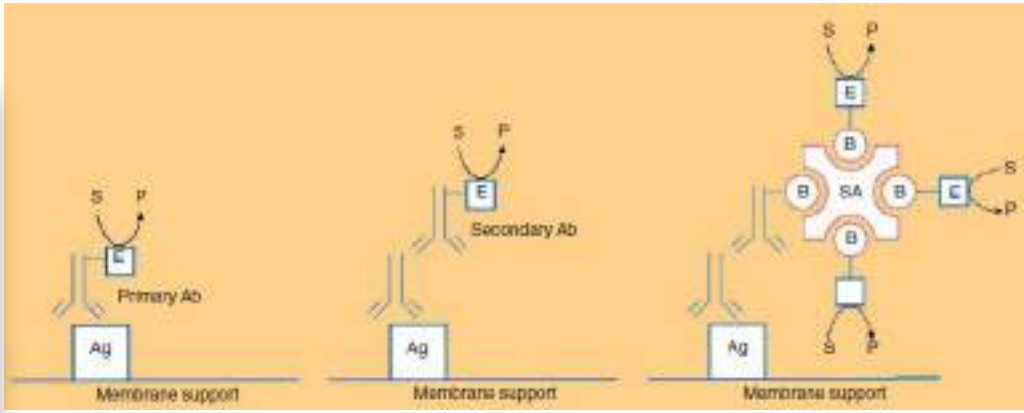
Major steps in protein blotting (الخطوات الأساسية فى تلطيخ البروتين)

1. Transfer protein to membrane, e.g., by electroblotting.
2. Block residual protein binding sites on membrane with extraneous proteins such as milk proteins.
3. Treat membrane with antibody which recognizes the protein of interest. If this antibody is labeled with a detecting group then go to step 5.
4. Incubate membrane with secondary antibody which recognizes primary antibody used in step 3. This antibody is labeled with a detecting group.
5. Treat the membrane with suitable reagents to locate the site of membrane attachment of the labeled antibody in step 4 or step 5.

Detection system (نظام التحري)

- Means identification of the desired protein by immunoblotting (التلطيخ المناعي) using **primary antibody (ضد أولي)** specific to the protein.
- The detection system is depending on the labelling of the primary or the secondary antibody using (**ChemiDoc™ XRS)**





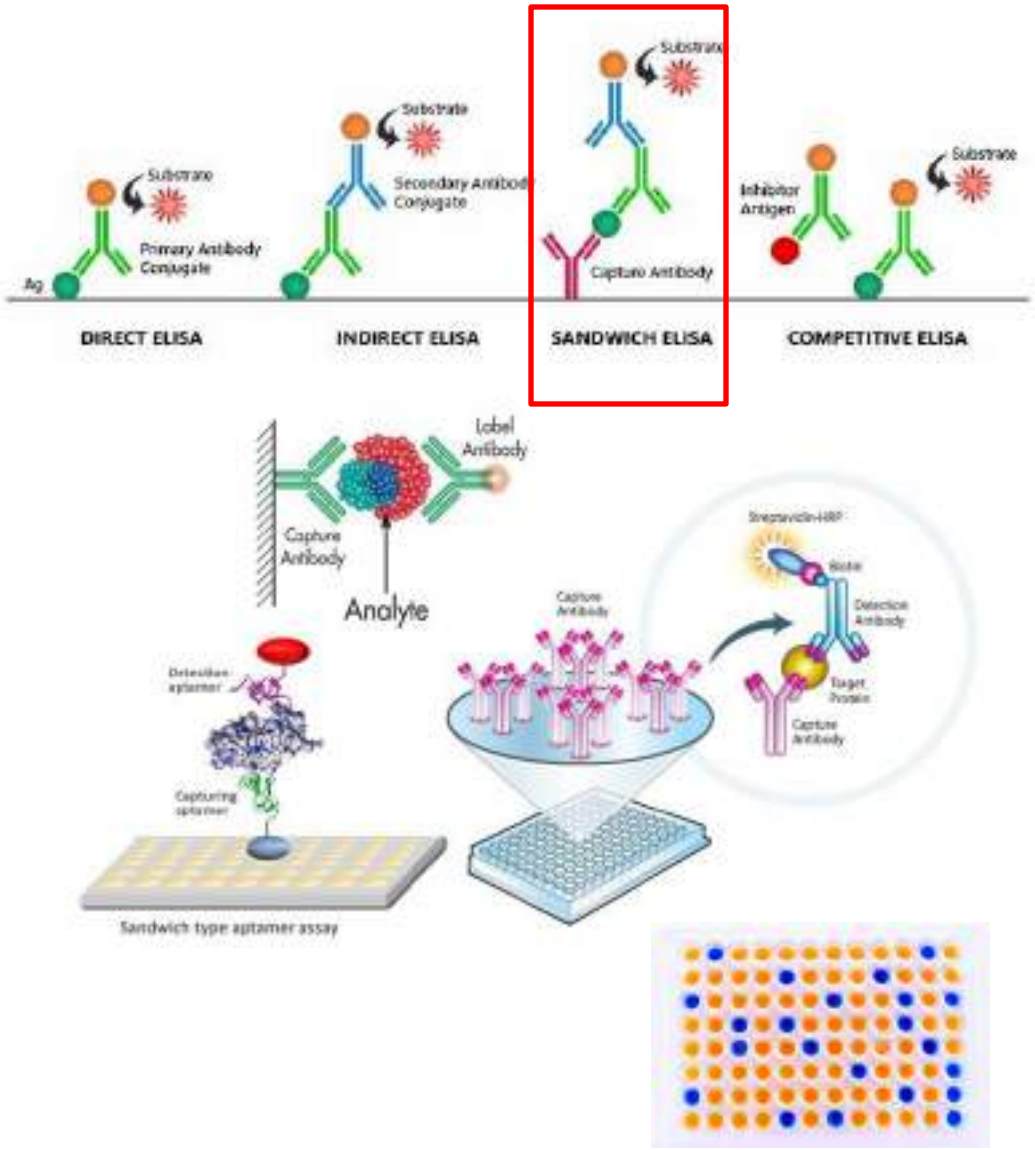
- Antibodies are labeled with radioactive markers such as ^{125}I.
- Antibodies are linked to an enzyme such as HRP or AP. On incubation with substrate an insoluble colored product is formed at the location of the antibody. Alternatively, the location of the antibody can be detected using a substrate which yields a chemiluminescent product, an image of which is made on photographic film.
- Antibody is labeled with biotin. Streptavidin or avidin is added to strongly bind to the biotin. Each streptavidin molecule has four binding sites. The remaining binding sites can combine with other biotin molecules which are covalently linked to HRP or to AP.

Immunoassays (ELISA)

- **Enzyme-linked immunosorbent assay (ELISA)** provides a means to quantitatively measure extremely **small amounts of proteins in biological fluids**.

- A number of **enzymes** can be used in ELISAs. However, the most common ones are HRP (horse radish peroxidase بيوكسيداز الفجل الحار) and AP(alkaline phosphatase).

- Plate readers, instruments which rapidly measure the absorbance of the colored product in all 96 wells of the microtitration plate



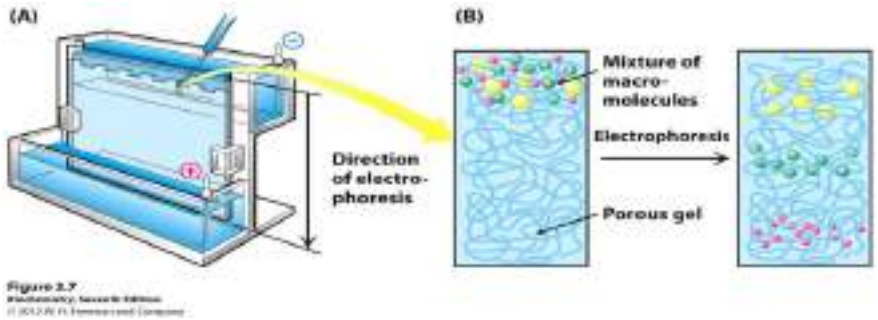
Electrophoresis (الرحلان الكهربائى)

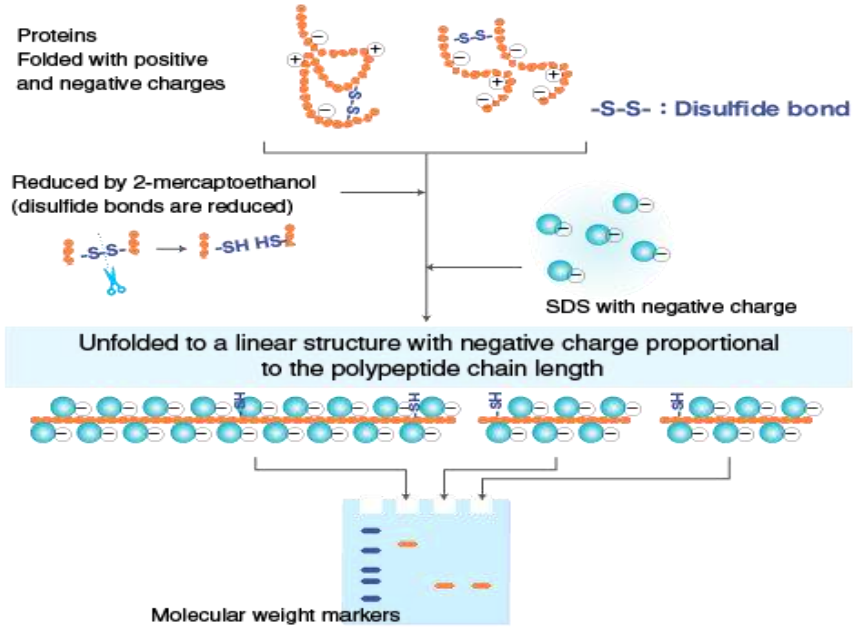
- **PAGE (polyacrylamide gel electrophoresis)** In this assay, proteins, being amphoteric (مذبذب) molecules with both positive and negative charge groups in their primary structure, are separated according to their net electrical charge.

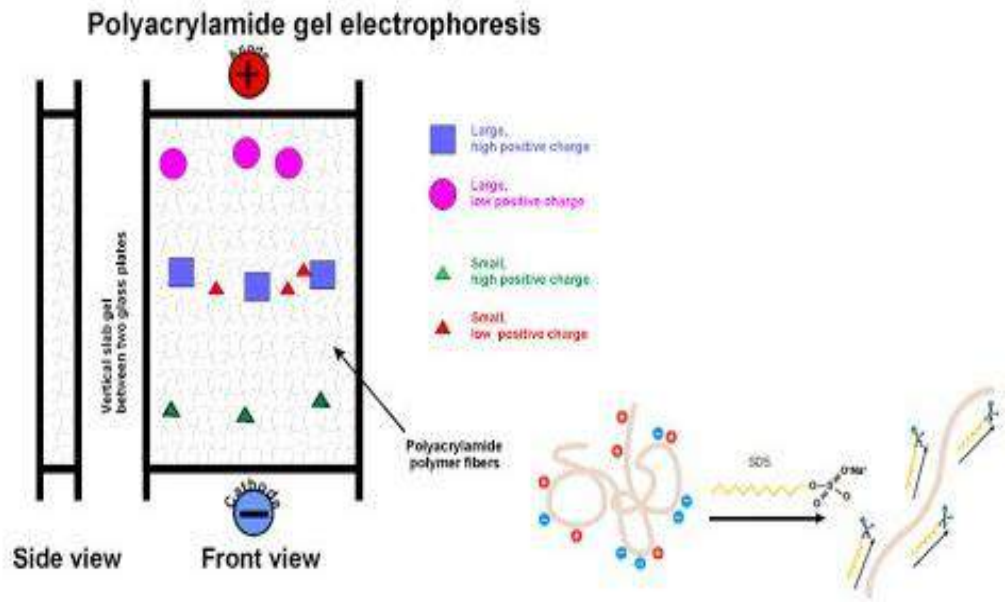
- The **mass (الكتلة)** of the protein is the **second factor responsible** for the protein separation.

- Thus, one can consider more precisely that the charge to mass ratio of proteins determines how they are separated in an electrical field

Electrophoresis







SDS PAGE electrophoresis

Native gel electrophoresis

Native PAGE

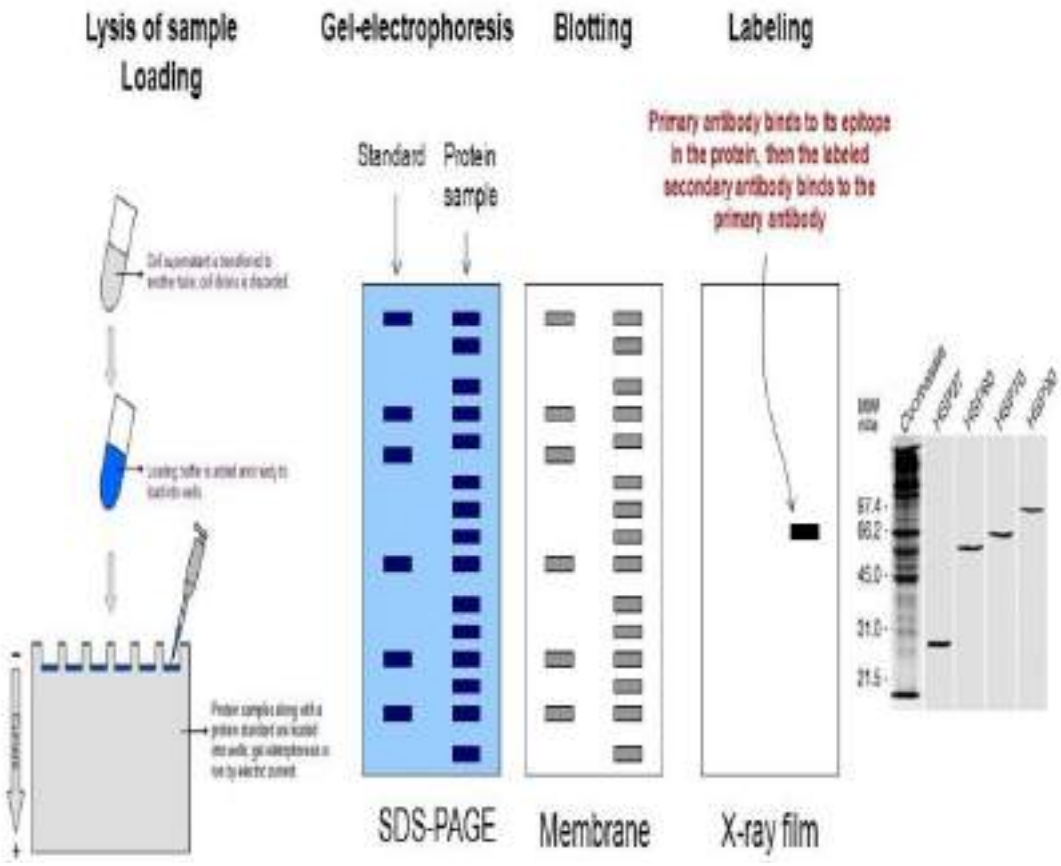
- In **this PAGE**, the charge of the protein can be controlled by the **pH** of the solution in which the protein is separated.
- The **direction** and **speed** of migration of the protein depend on the pH of the gel.
- If the pH of the gel is above its **pI (نقطة التعادل الكهربائي)** value, then the protein is negatively charged, thus migrates toward the anode.
- **The higher** the **PH** the gel the **faster** the migration.

SDS PAGE

- The addition of a detergent, sodium dodecyl sulfate **(SDS)**, to the electrophoretic separation system allows for the separation to take place primarily as a function of the **size of the protein.**

- Dodecyl sulfate ions form complexes with proteins, resulting in an **unfolding** of the proteins.

- Dodecyl sulfate is a negatively charged ion. When proteins are in a solution of SDS, the net effect is that the own charge of the protein is overwhelmed by that of the dodecyl sulfate complexed with it, so that the proteins take on a **net negative charge(شحنة سلبية إجمالية)** proportional to their mass (متناسبة مع كتلة البروتين) .



Native PAGE Vs SDS PAGE (الإختلافات مابين نوعين الرحلان)

Differences

Native PAGE	SDS PAGE
• Separation is based upon charge, size, and shape of macromolecules. • Useful for separation and/or purification of mixture of proteins • This was the original mode of electrophoresis.	• Separation is based upon the molecular weight of proteins. • The most common method for determining MW of proteins • Very useful for checking purity of protein samples

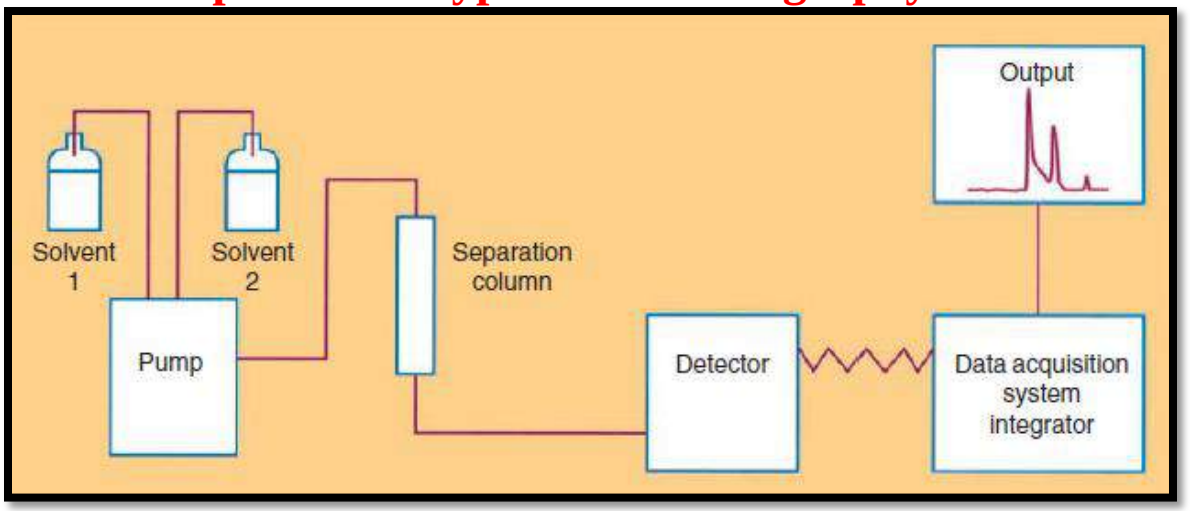
Chromatography (الإستشراب)

- **Chromatography** techniques are used extensively in biotechnology not only in protein **purification** procedures , but also in assessing the **integrity** of the product.

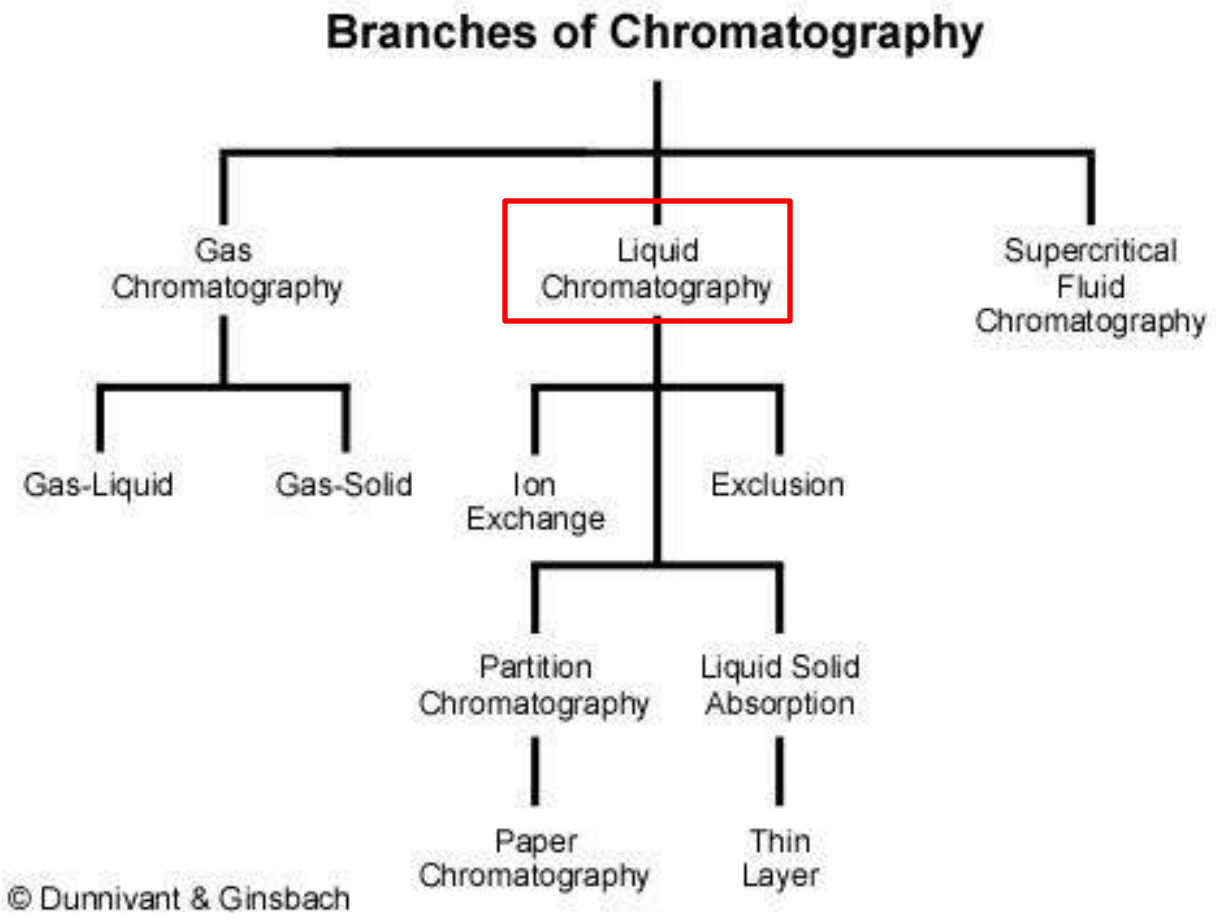
- Chromatographic systems offer a multitude of different strategies for successfully separating (فصل) protein mixtures and for **quantifying (تحديد كمية)** individual protein components.

Chromatography

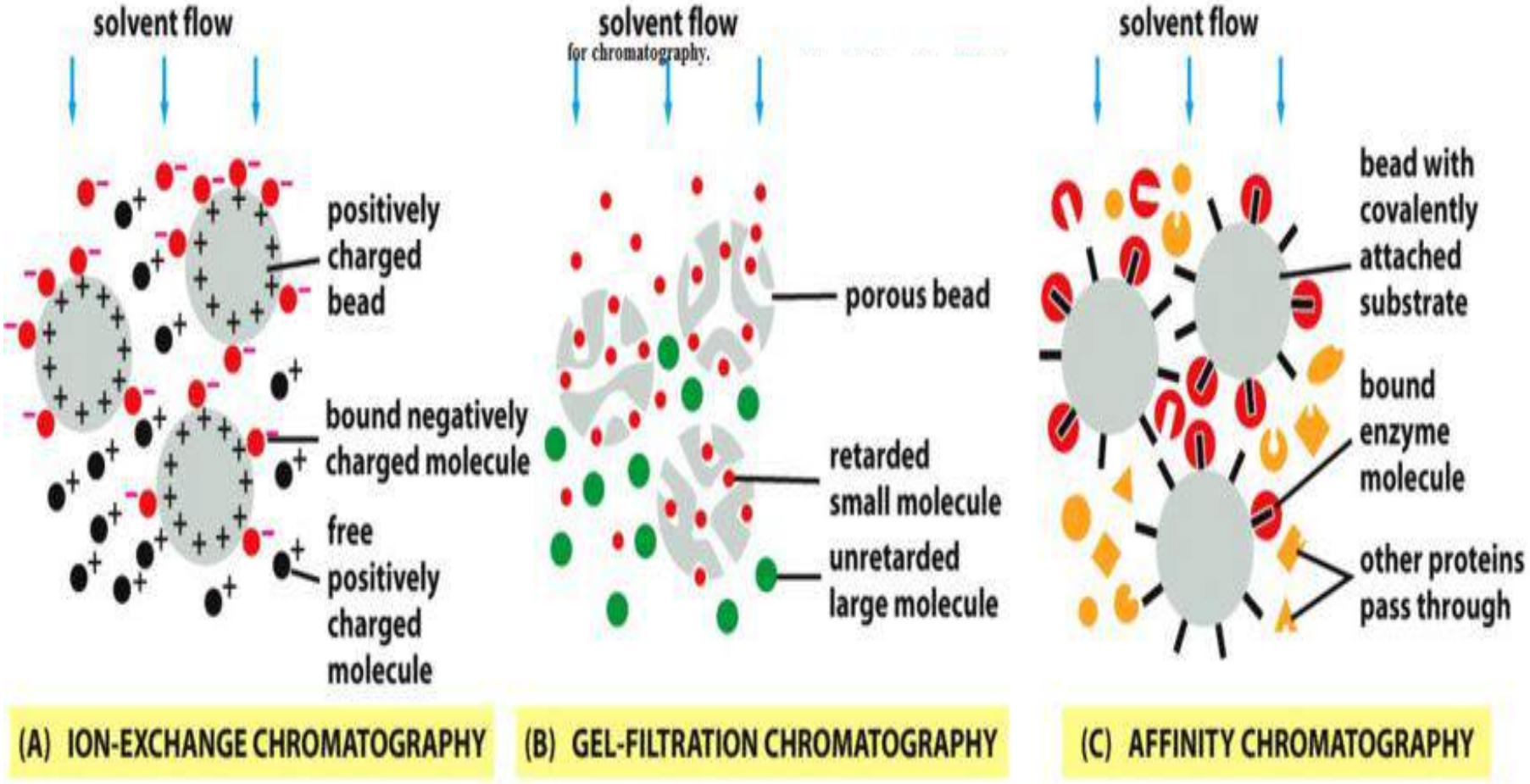
Components of typical chromatography station



Chromatography



Chromatography



Chromatography

- **Size exclusion chromatography (الإستشراب باستبعاد الحجم):**

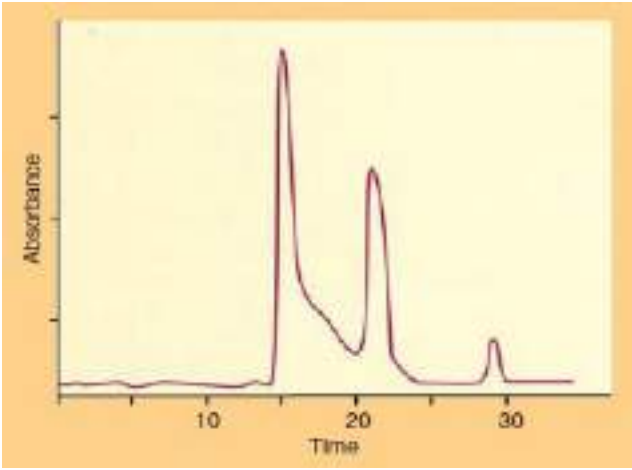
- this procedure separates proteins based on their size or molecular weight or shape.

- The matrix consists of very **fine beads** containing cavities and pores accessible to molecules of a certain size or smaller, but inaccessible to larger molecules.

- The **principle** of this technique is the **distribution of molecules** between the volume of solution within the beads versus the volume of solution surrounding the beads.

- **Smaller molecules** can reside within the pores for a finite period of time whereas **larger molecules**, **unable to enter** these spaces, continue along in the fluid stream.

- **Intermediate-sized** molecules spend an intermediate amount of time within the pores.



Size exclusion chromatography of a recombinant protein which, on storage, yields aggregates and smaller peptides. The peak at 22 minutes represents the native protein. The peak at 15 minutes is aggregated protein and that at 28 minutes depicts degraded protein yielding smaller polypeptide chains

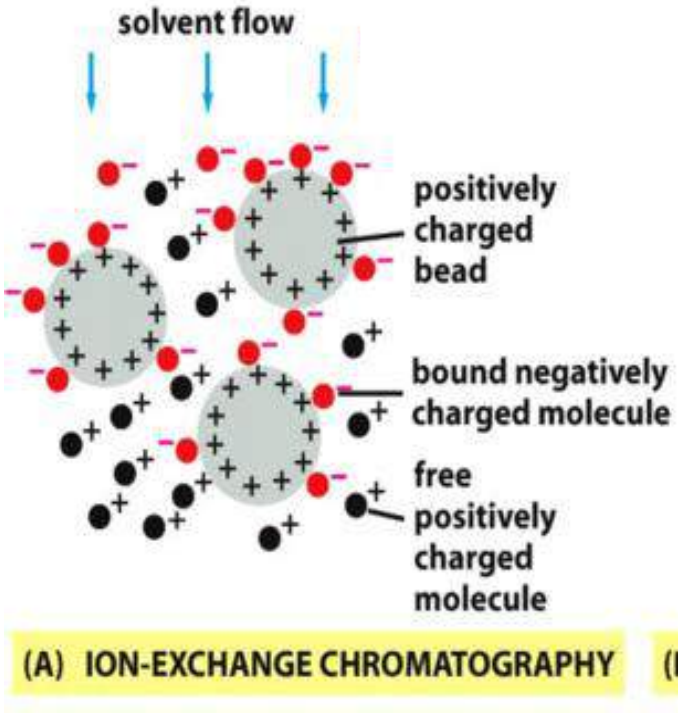
Chromatography

- **Ion exchange (الإستشراب بالتبادل الشاردي):**

- Some of the **amino acyl residues** are negatively charged and others are positively charged. The net charge of the protein can be modulated by the pH of its environment relative to the **pI value** of the protein.

- At a pH value **lower** than the **pI**, the protein has a net positive charge, whereas at a pH value greater than the pI, the protein has a net **negative charge**.

- The **resins** in this procedure can contain functional groups with **positive or negative charges** Proteins are displaced from the resin by **increasing salt**, e.g., sodium chloride, concentrations.

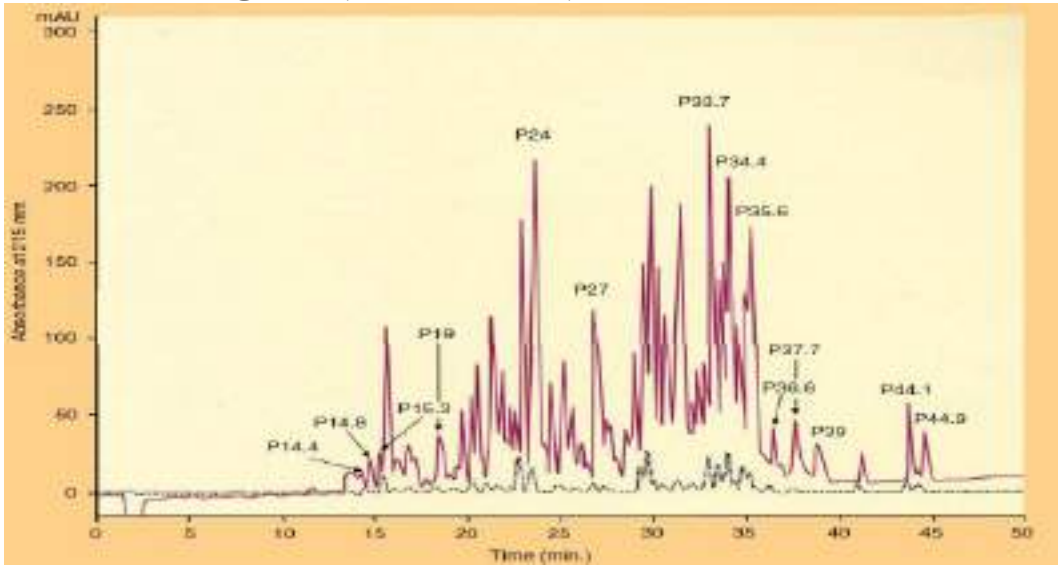


Chromatography

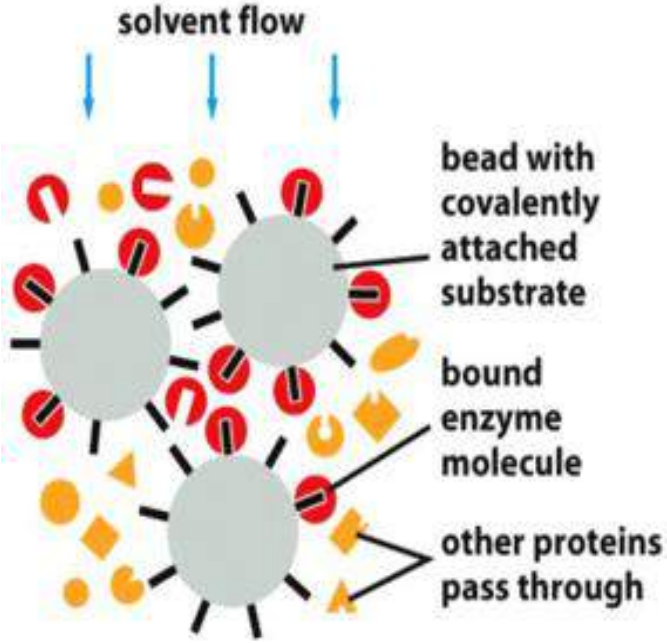
- **Affinity chromatography (الإستشراب بالإلفة) :**

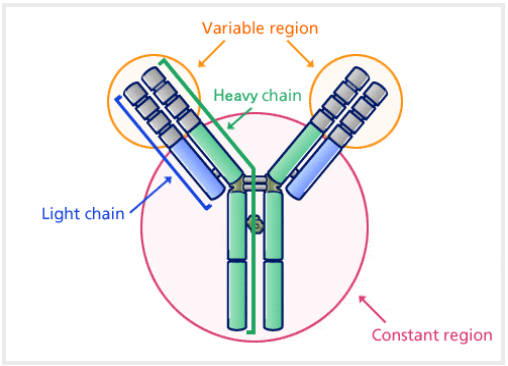
o These **affinity methodologies**, however, are more often used in the manufacturing process than in analytical techniques. For example, **conventional affinity** purification schemes of **antibodies** use Protein-A or -G columns.

o Protein-A or -G specifically binds antibodies in the **constant region (المنطقة الثابتة) .**



Peptide map of pepsin digest of recombinant human b-secretase. Each peptide is labeled by elution time in HPLC.



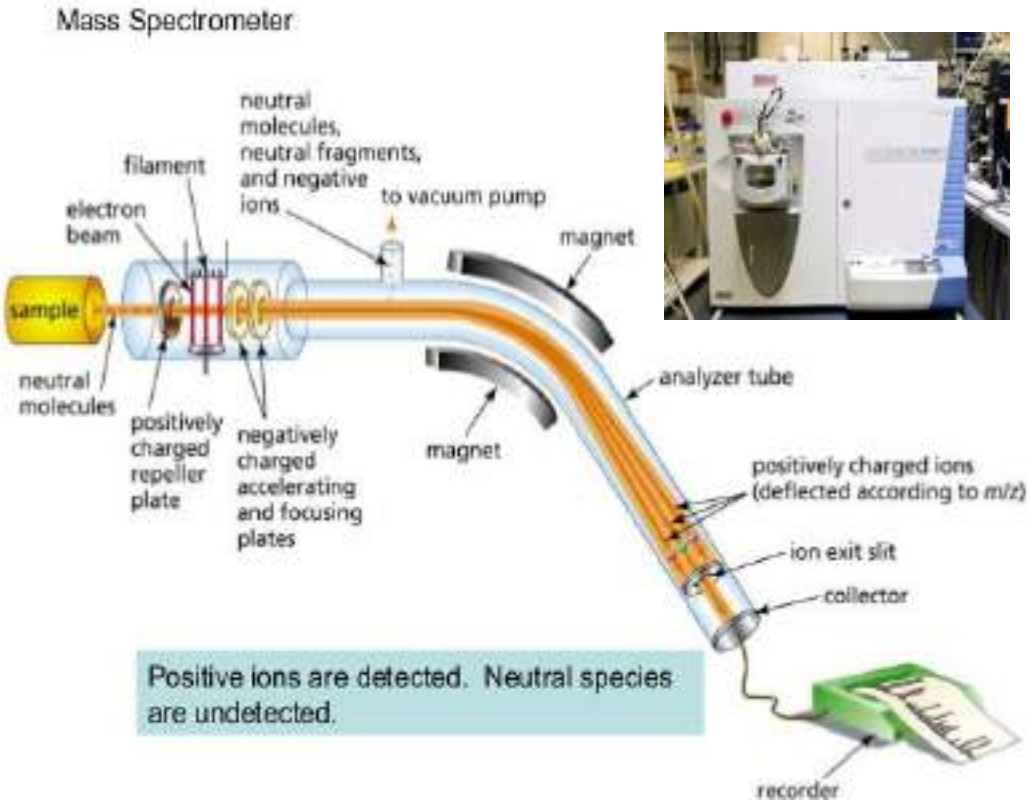


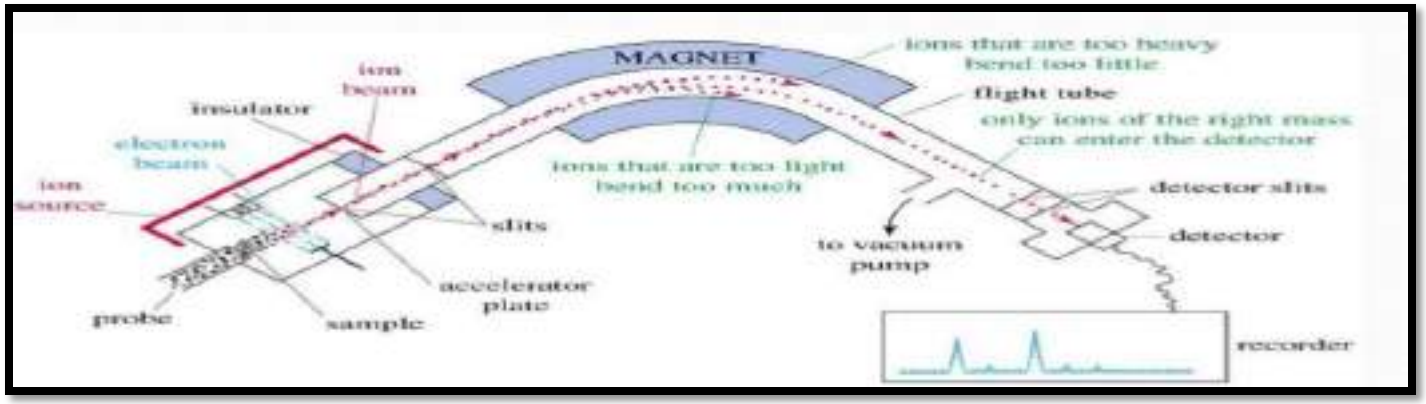
Mass spectrometry(مطياف الكتلة)

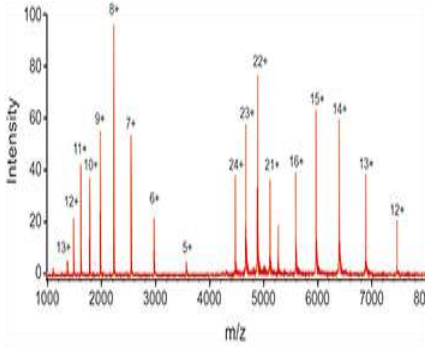
- Is an **analytical technique** that measures the **mass to charge** ration of ions. The results are typically presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio.

- could be used to analyze **small** and **high** molecular weight of charged protein **over 100KDa.**

- **Very precise (دقيقة جدا)** method even the **post translation** modification like acetylation or glycosylation can be detected.





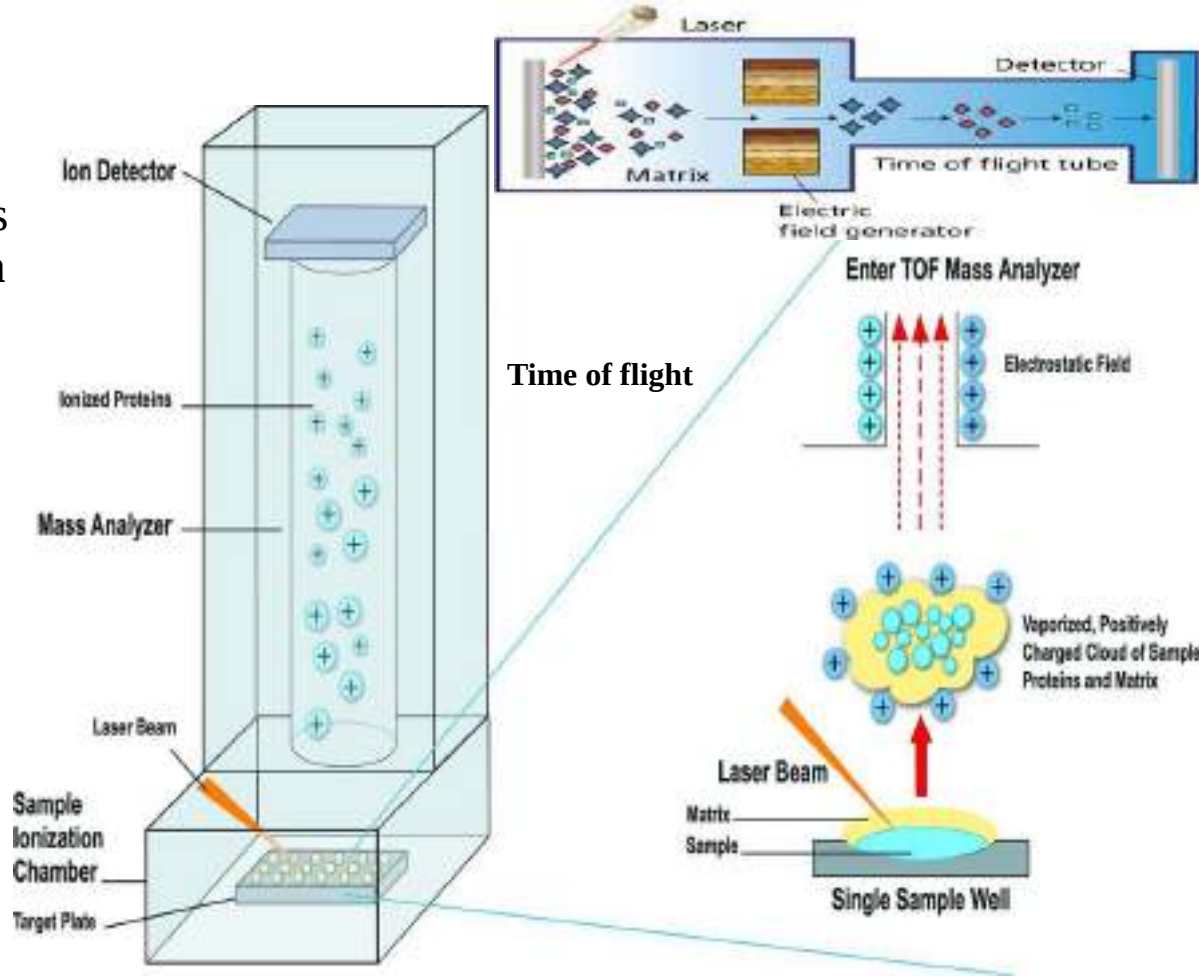


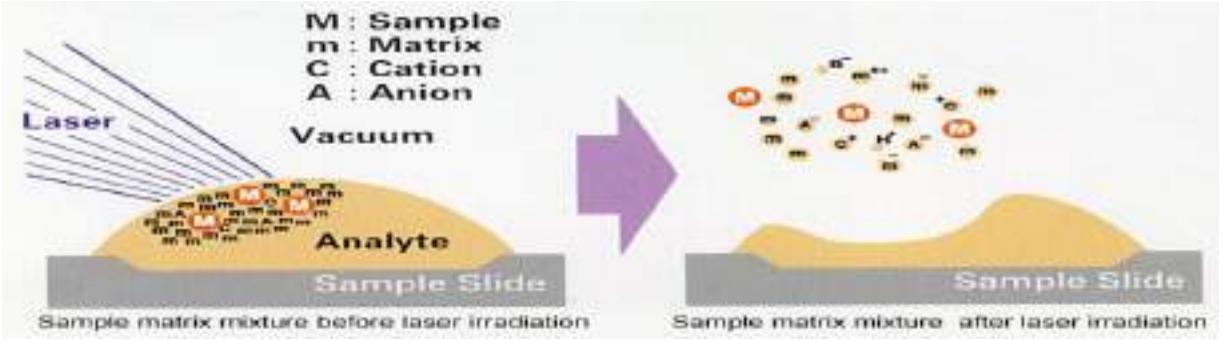
MALDI TOF

- changing three basic components of the mass spectrometer, the **ion source**, the **analyzer** and the **detector.** typical ion sources which volatilize the proteins are **electrospray ionization** تأين شاردي بطريقة البخ الكهربائي(EI) , fast atom bombardment and liquid secondary ion.

- **analyzers** include quadrupole, magnetic sector, and **time of flight instruments** مقياس زمن هجرة الجزيئات المتأينة, to separate the ionized biomolecules based on their mass to charge ratio.

- The **detector** measures a **current** whenever impinged upon by charged particles.

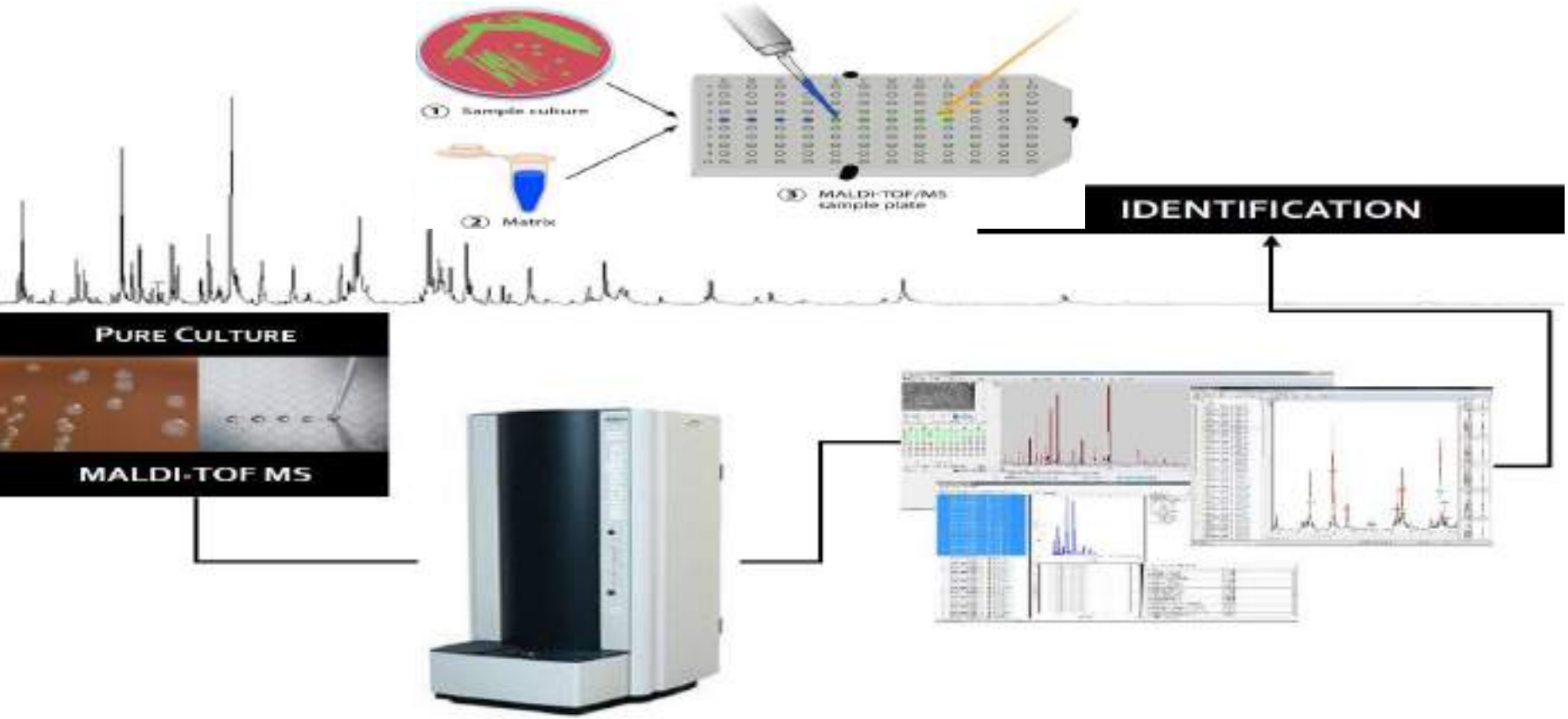




MALDI TOF

- **El** and **matrix-assisted laser desorption** **الإمتزاز الليزري المساعد بالماتريكس**(MALDI) are two sources that can generate high molecular weight volatile (متطايره)proteins.

- droplets are generated by **spraying or nebulizing** **تشكيل غيمة الكترونية** the protein solution into the source of the mass spectrometer. As the solvent evaporates, the protein remains behind in the gas phase and passes through the analyzer to the detector.



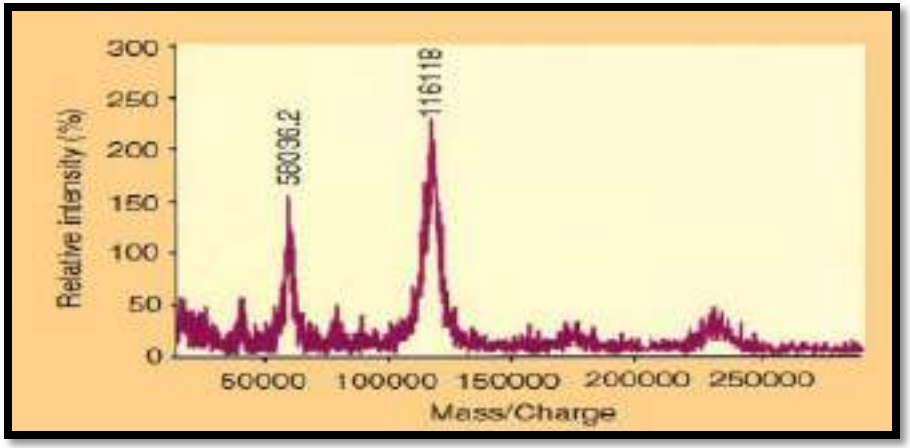
MALDI TOF

- In **MALDI**, proteins are mixed with a **matrix which vaporizes (تبخر)** when exposed to laser light, thus carrying the protein into the gas phase.

- For example: singly charged ion (116118 Dalton) and the doubly charged ion (58036.2) for a purified protein.

- proteins are multi-charge compounds, a number of components are observed representing mass to charge forms, each differing from the next by one charge.

- By **imputing** (إسناد) various charges to the mass to charge values, a **molecular mass** of the protein can be estimated.



MALDI mass analysis of a purified recombinant **human b-secretase**. Numbers correspond to the singly charged and doubly charged ions.

Thank you