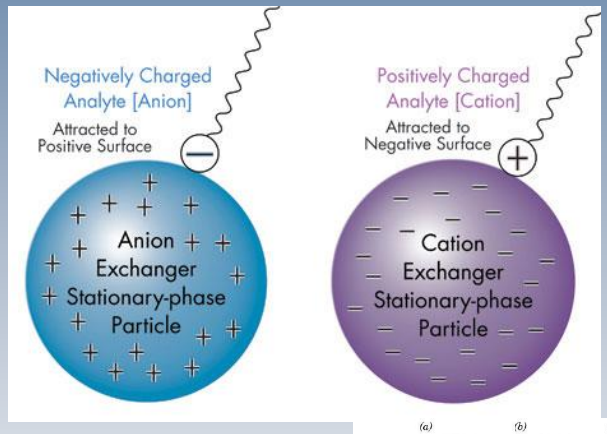
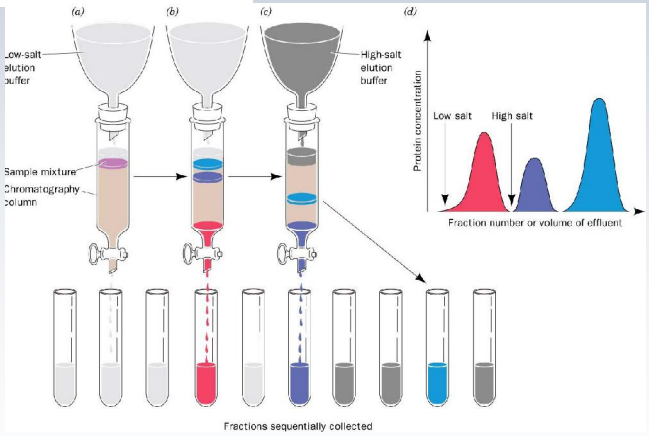
Ion exchange and affinity chromatography





Maciej M. Lalowski

Biomedicum Helsinki
Helsinki University

Introduction to Basic Protein Chemistry and Proteomics with Clinical Applications: 16.10.2012

Chromatography

- Separation of biomolecules which is based on their physicochemical characteristics:
  - Polarity (solubility, volatility, adsorption) HIC, RP
  - Size/mass (diffusion, sedimentation) Size exclusion Chr.
  - **Ionic characteristics** (charge) **Ion Exchange Chromatography**
  - **Shape** (ligand binding, affinity) **Affinity Chromatography**
- Based on these properties the molecules can be differentially separated between the stationary phase and mobile phases

Liquid chromatography (LC)

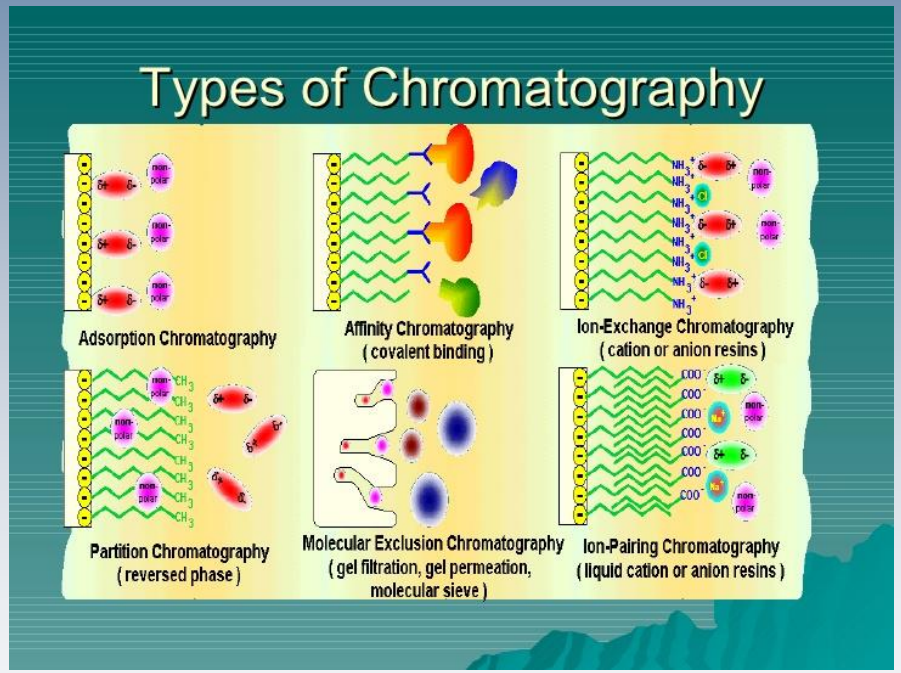
- IEX ➡ anion exchange
  ➡ cation exchange
  ➡ chromatofocusing
- Affinity chromatography

group separations vs. specific interactions

- **HIC** (Hydrophobic Interaction chromatography)
- **RP** (Reverse-Phase) **chromatography**

- Size exclusion/Gel filtration

  non-interacting & medium resolution



Adsorptive chromatography

- **Biomolecule adsorps to the matrix (stationary phase) reversibly**
- **Adsorption is controlled by the mobile phase - elution**

IEC: proteins in a low-salt mobile phase may be bound to the matrix, but when the composition of the mobile phase is changed to high-salt, the interaction is reversed and the proteins elute

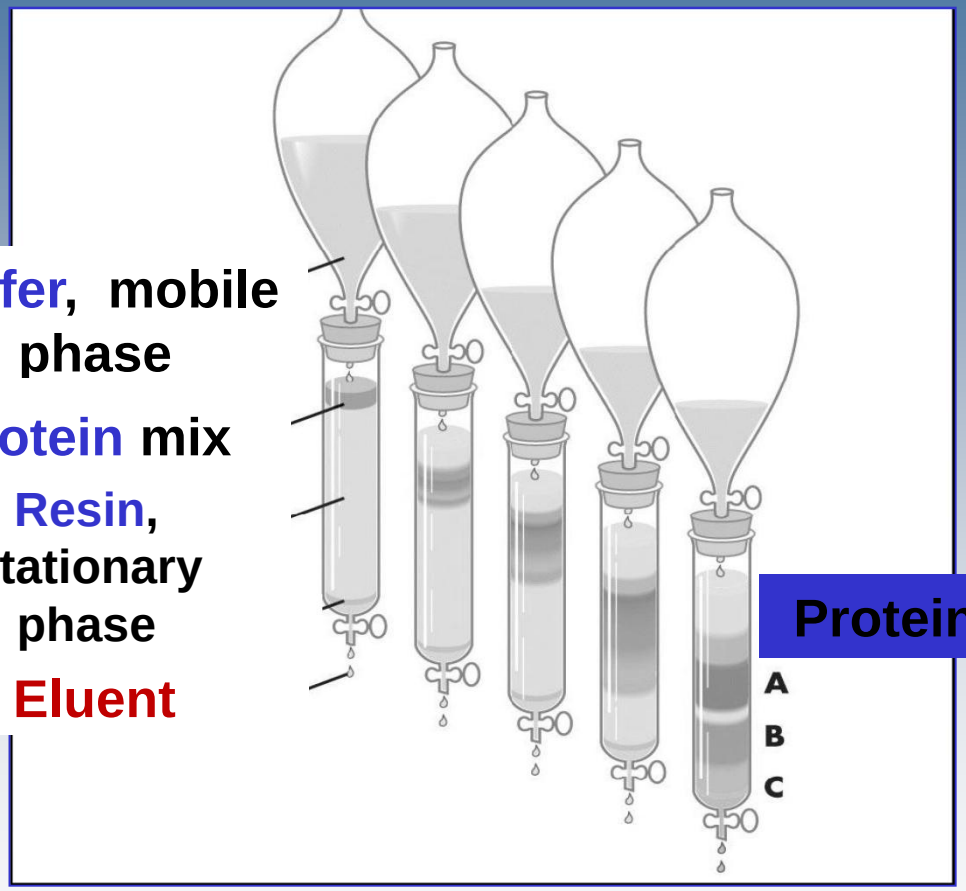
Liquid chromatography: basics

Buffer, mobile phase

Protein mix

Resin, stationary phase

Eluent

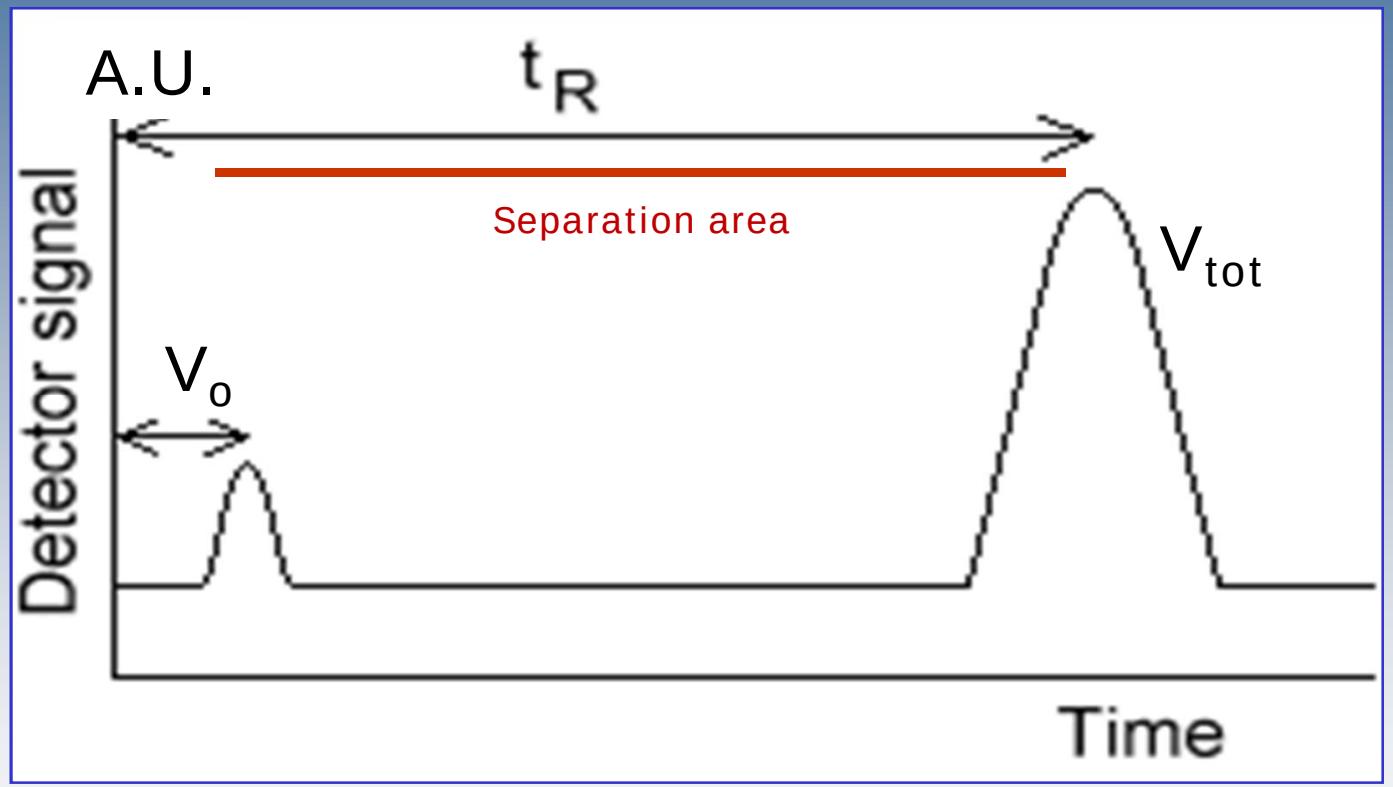


Chromatography steps:

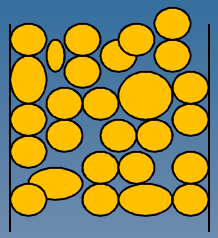
1. Equilibration
2. Injection
3. Elution
4. Washing

http://www.youtube.com/watch?v=Z54ec_G12QE

Chromatogram: basics



Matrix

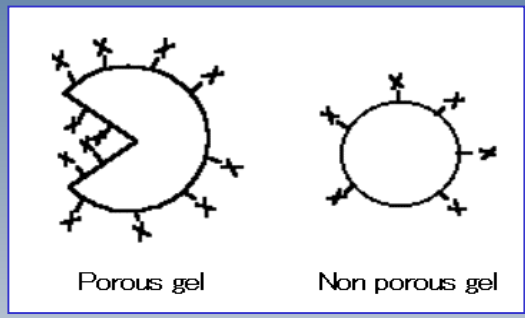


Porous

Cellulose
sugar polymers (agarose, dextran)
polymers (acrylamide, styrene)
silica coated with polymers

Nonporous

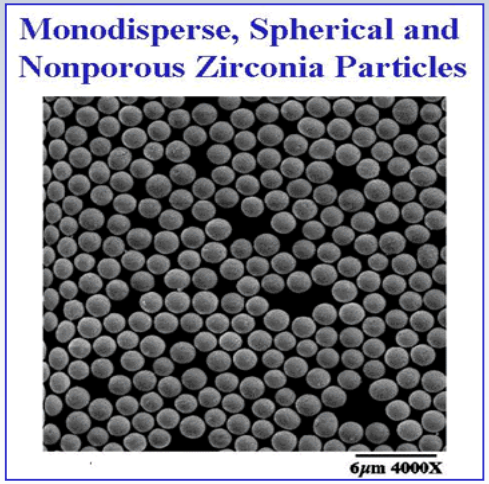
Styrene
Acrylates
Zirconia
Monolith
(*ProSwift*, *Dionex*)



- Particle size, ~5 μm ● 2 μm ●
- Pore size i.e. 100-200 Å

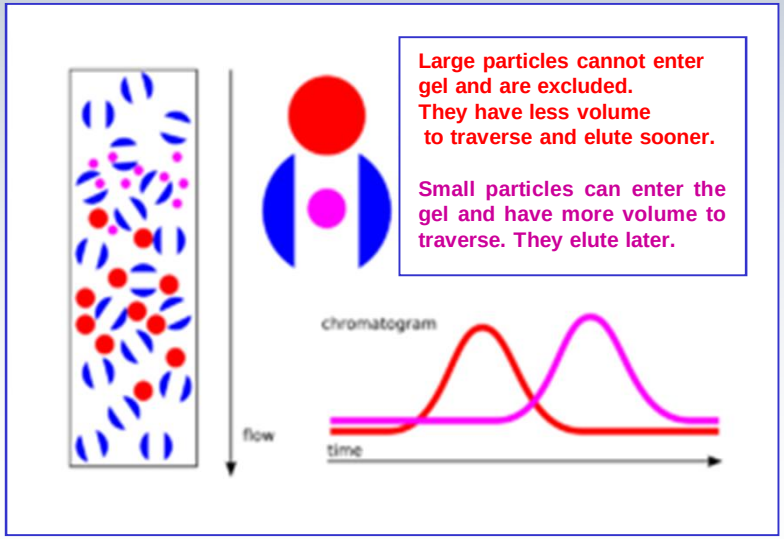
- Chemical and Physical stability
- Temperature <80°C vs >200°C)

•	Pressure	Porous < Nonporous
•	pH	Porous < Nonporous
•	Capacity	Porous > Nonporous



Diffusion and porous matrices

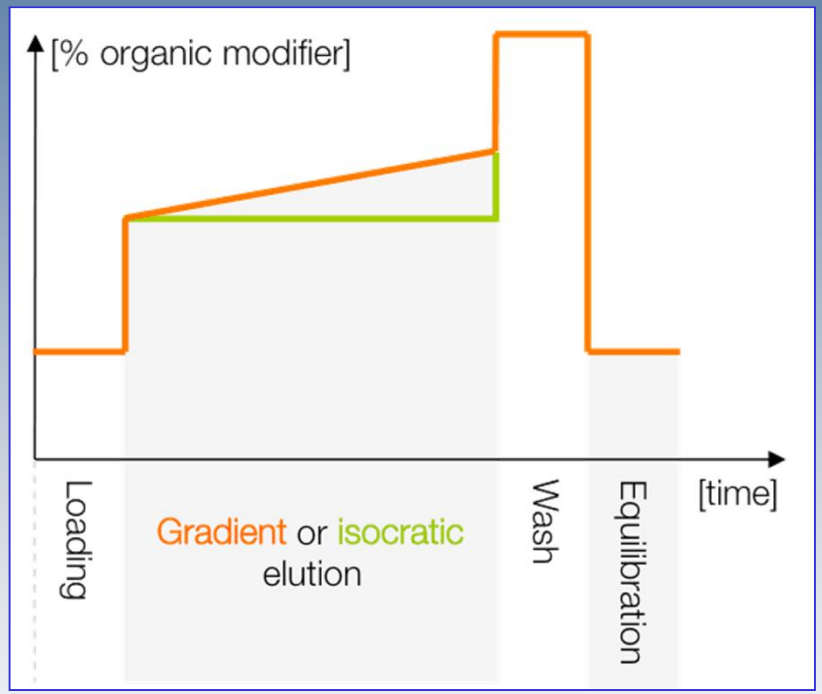
- **Size exclusion chromatography is based on *diffusion* and molecules in solution are separated by their size** (molecular weight)
- -Small molecules diffuse into smaller pores and travel slowly, therefore elute last, **close** to V_{total}
- Large molecules do not fit into pores and elute first, **close** to V_0
- **In *affinity* and *IE chromatography* the functional groups are attached to surface of particles inside the pores**



Mobile phase

- **Composition**
- **Type of elution**
  - **isocratic** vs **gradient elution (gradient shape)**
- **Flow rate**
  - **Depends on the type of matrix**

    ***Affects* resolution**
  - in **porous** matrixes should be **slow** enough to allow **diffusion** to pore cavities
  - in **nonporous** matrixes **higher** flow rates may be used

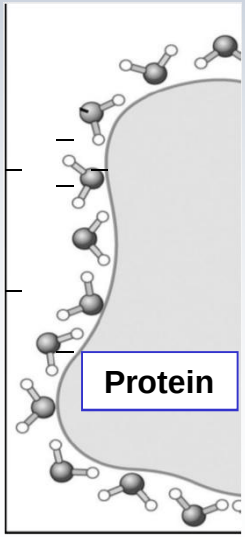


Ion exchange chromatography

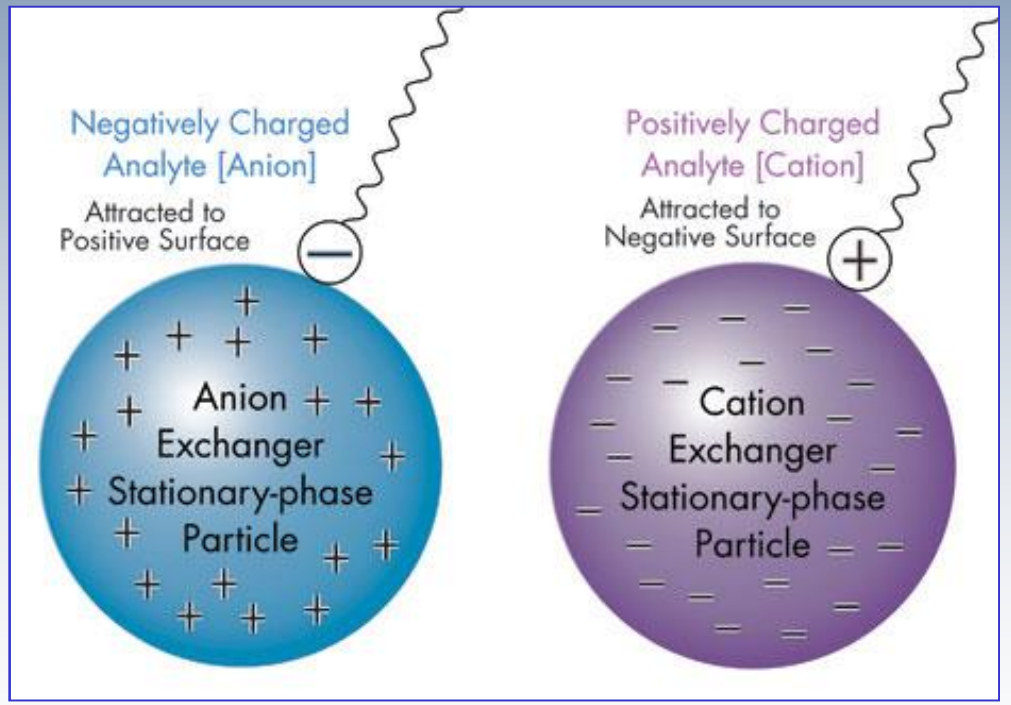
- **Based on ionic interactions**

Anion exchange +

Cation exchange –



local versus net charge



IEC in practise

1. Choose the **matrix type** according to your target protein
2. Equilibrate (**low salt**, 20 mM)
3. Inject protein sample (in **low salt**), balance
4. Apply gradient (**increasing salt**) to elute proteins

- Obey buffer instructions:
  AEC- cationic buffers, CEC- anionic buffers
- Non-ionic detergents (!)
- Elution:

1. *Increasing* salt gradient (0 ➡1M NaCl in 20 mM buffer)
2. *pH gradient* (ampholytes in chromatofocusing)
3. *Type of gradient*: linear gradient /step wise/shape of gradient

Titration curve of a protein

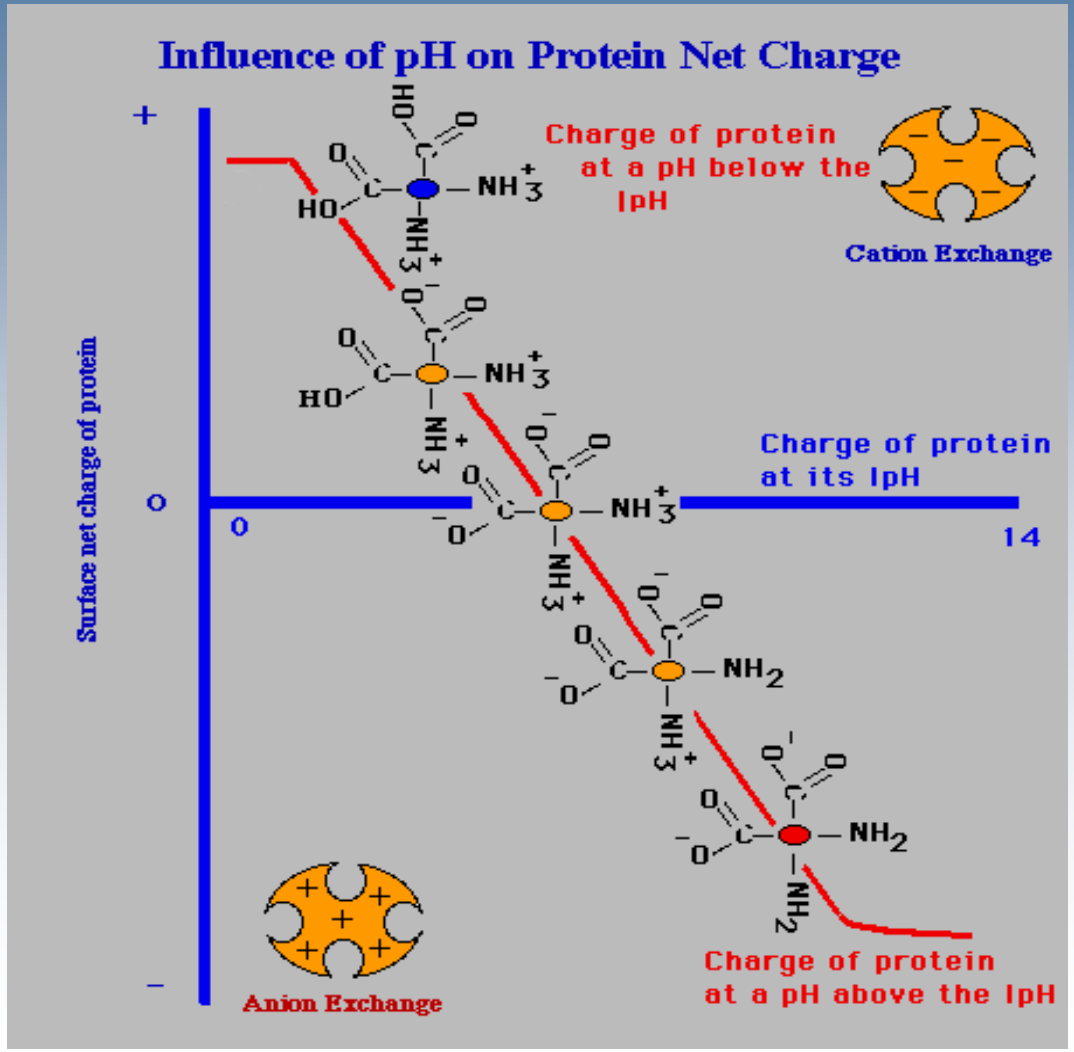
- **Charge of a protein is pH dependent !**

  Amino acids with **ionizable** side chains:
  Arg, His, Lys
  Asp, Glu
  Cys, Tyr

- pI =isoelectric point

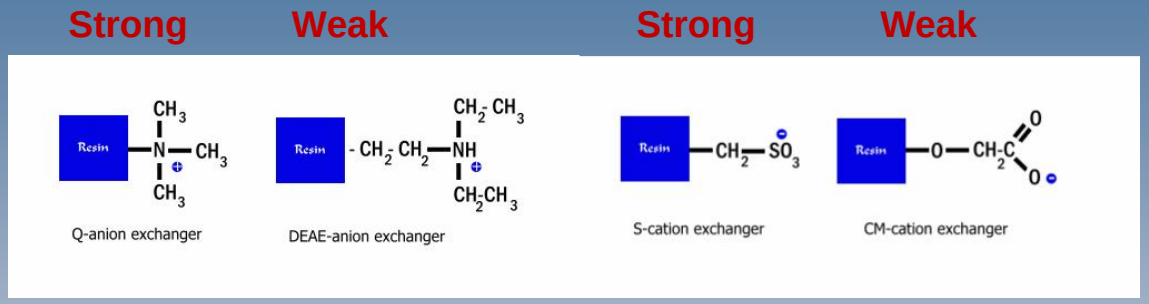
if pH > pI use anion EC

if pH < pI use cation EC

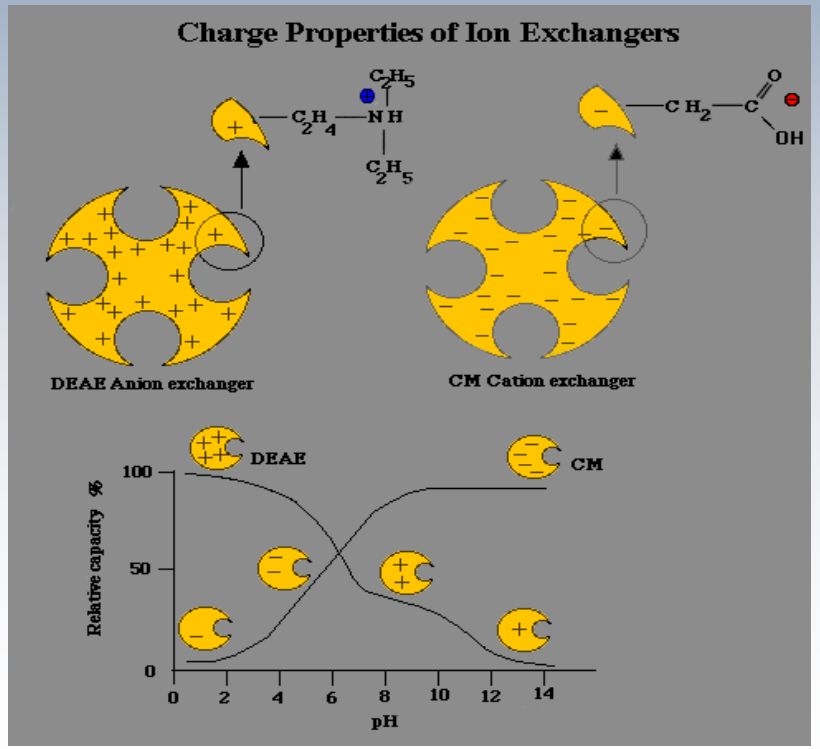


IEC matrices

- **Strong** versus **weak** ion exchangers



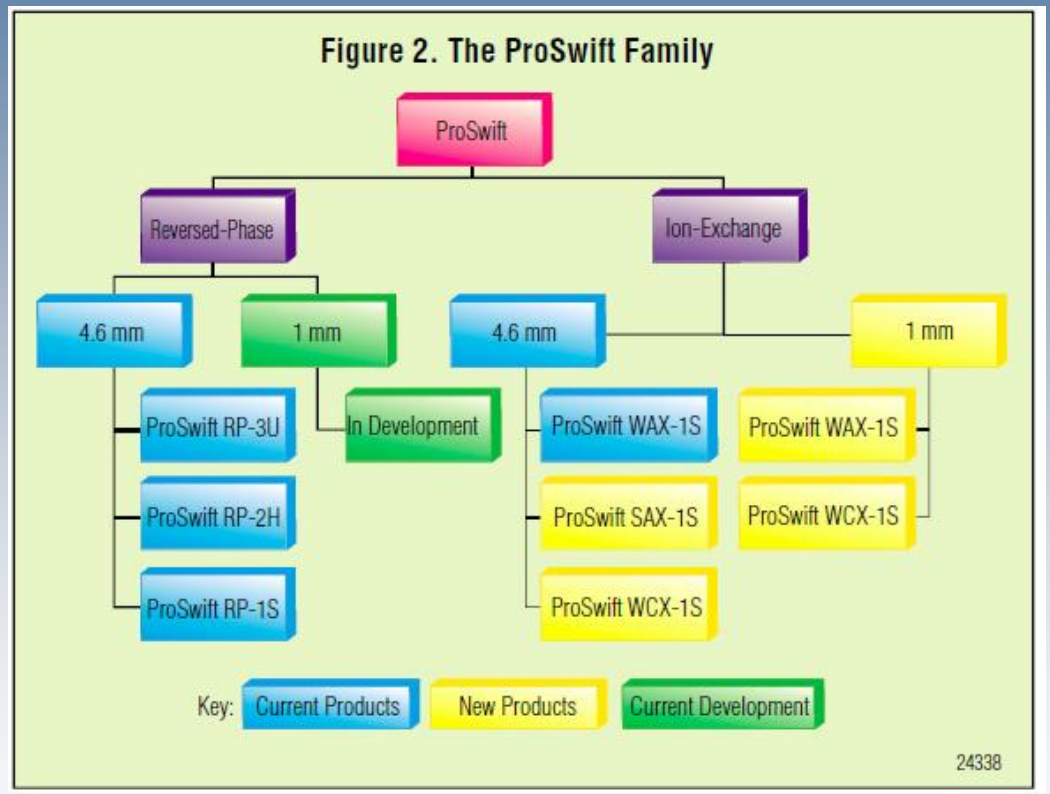
- **anion EC: positive matrix**
  - DEAE diethyl aminoethyl (W)
  - QAE quaternary aminoethyl
  - Q quaternary amine
- **cation EC: negative matrix**
  - CM carboxymethyl (W)
  - SP sulphopropyl
  - S sulphonate



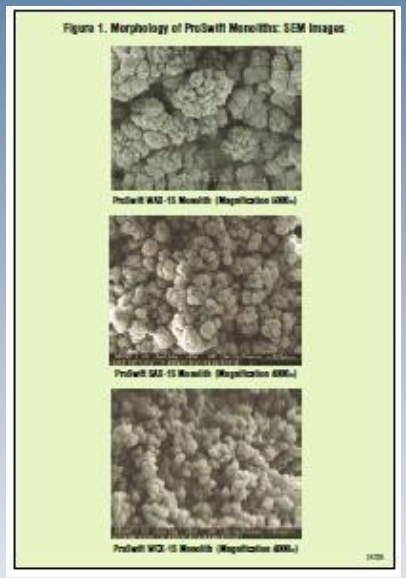
Buffers for exchange chromatography

Molecule (AEC)	pKa	dpKa/degree C.	Counter ion	Molecule (CAC)	pKa	dpKa/degree C.	Counter ion
N-methyl piperazine	4.75	-0.015	chloride	Maleic acid	2.00		sodium
piperazine	5.68	-0.015	chloride or formate	Malonic acid	2.88		sodium
L-histidine	5.96		chloride	citric acid	3.13	-0.0024	sodium
bis-Tris	6.46	-0.017	chloride	lactic acid	3.81		sodium
bis-Tris propane	6.80		chloride	formic acid	3.75	0.0002	sodium or lithium
triethanolamine	7.76	-0.020	chloride or acetate	butaneandioic acid	4.21	-0.0018	sodium
Tris	8.06	-0.028	chloride	acetic acid	4.76	0.0002	sodium or lithium
N-methyl-diethanolamine	8.52	-0.028	chloride	malonic acid	5.68		sodium or lithium
diethanolamine	8.88	-0.025	chloride	phosphate	7.20	-0.0028	sodium
1,3-diaminopropane	8.64	-0.031	chloride	HEPES	7.55	-0.0140	sodium or lithium
ethanolamine	9.50	-0.029	chloride	BICINE	8.35	-0.0180	sodium
piperazine	9.73	-0.026	chloride				
1,3-diaminopropane	10.47	-0.026	chloride				
piperidine	11.12	-0.031	chloride				
phosphate	12.33	-0.026	chloride				

Examples: ProSwift matrices (www.dionex.com)



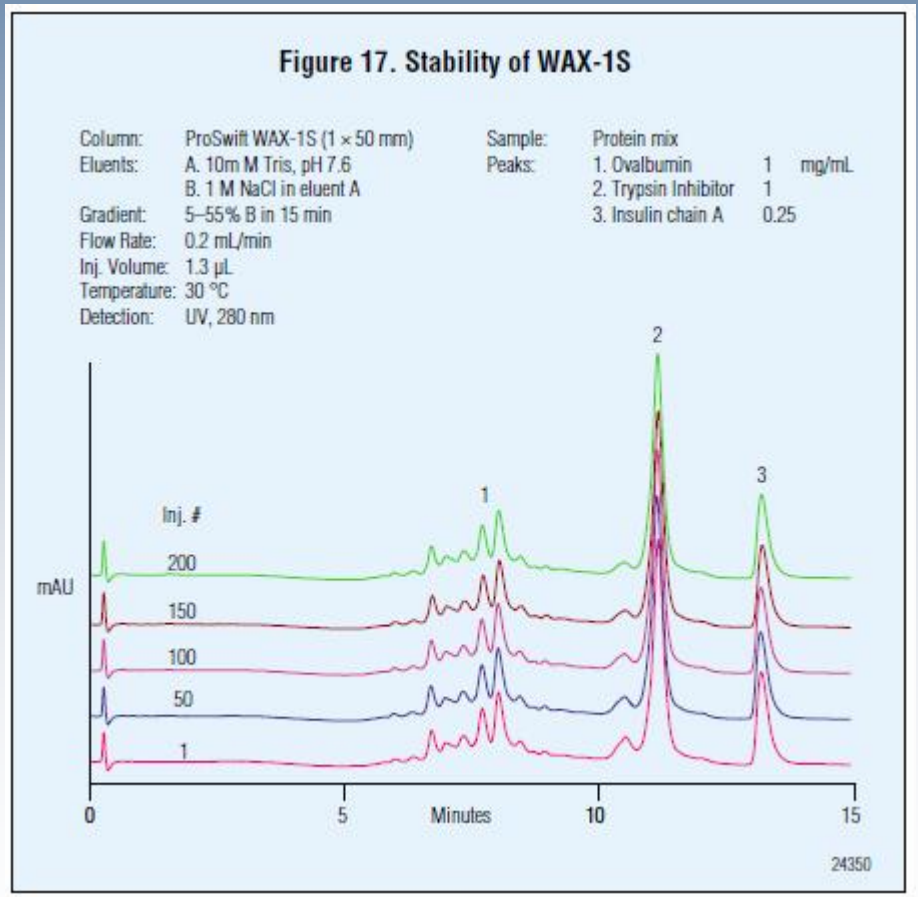
- **Monolith** matrix

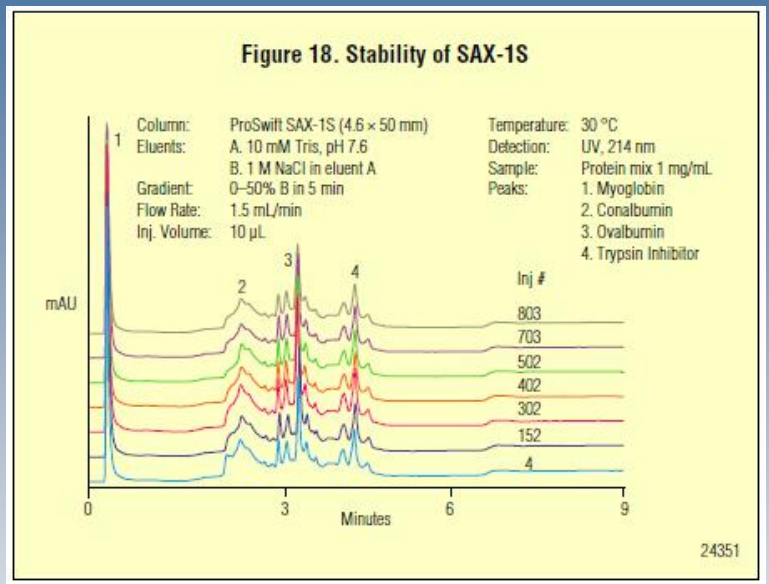


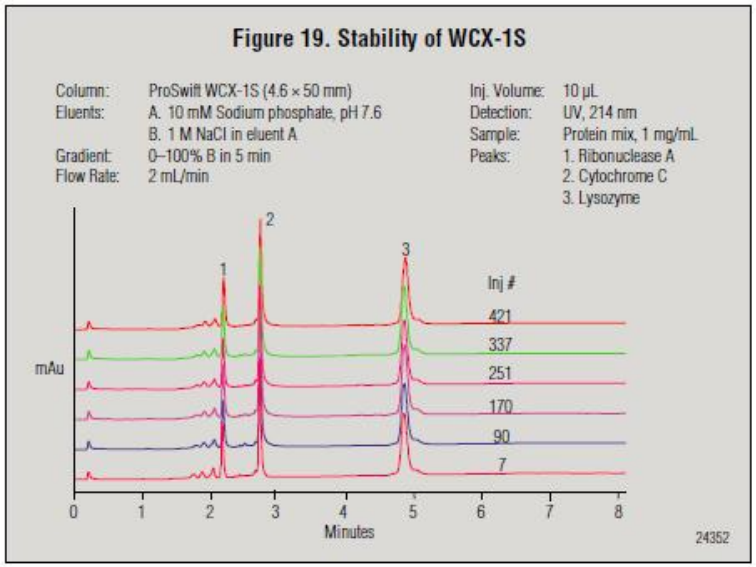
- Combines the **stability** of nonporous and **capacity** of porous matrices
- Optimal mass transfer ➡ minimal diffusion…
- High loading capacity and reproducibility!
- Can be used as1stdimension in multidimensional chromatography

ProSwift: IEC columns

Weak anion exchanger







Strong Anion exchanger

Strong cation exchanger

IEC: summary

PROS:

- **Concentrates** the sample
- Sample volume **is not** a restricting factor
- **Gentle**
- **High resolution**

  (strong vs weak)

CONS:

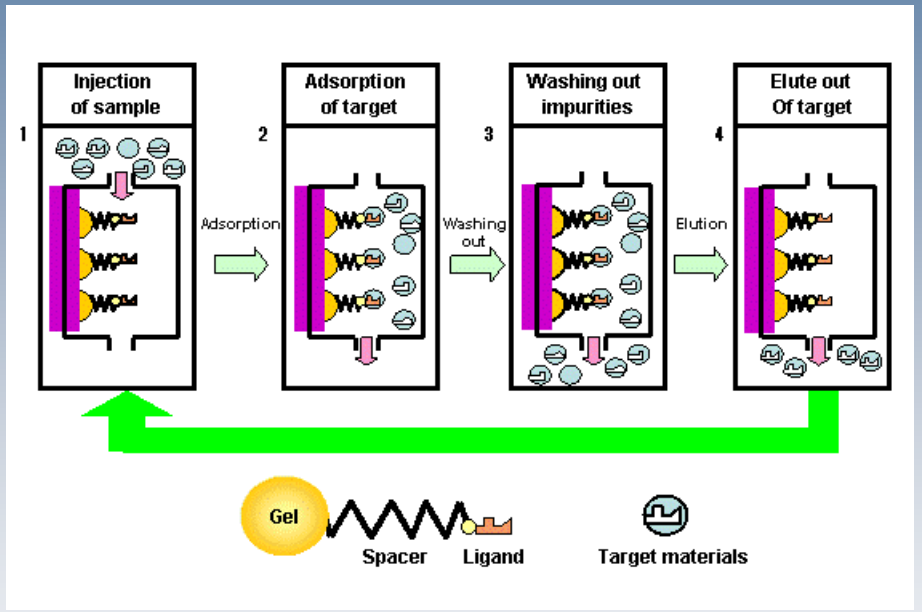
- **No salt** in binding phase
- Sample eluted in **high salt**

http://www.youtube.com/watch?v=q3fMqgT1do8

Affinity chromatography

- Discovered in 1968 by

  P. Cuatrecasas and M. Wilcheck
- Based on specific interactions
- Group affinity vs specific affinity



- Elution nonspecific vs specific
- Effective purification in one step
- Concentrating

- Various types of affinity chromatographies are used

Specificity of affinity chromatography

Specificity of Affinity Chromatography

- Specificity is based on three aspect of affinity

Matrix: for ligand attachment.

Spacer arm: used to bind ligand to matrix

Ligand: molecule that binds reversibly to a specific target molecule(site of interaction)

Matrices in affinity chromatography

- The matrix simply provides a structure to increase the surface area to which the molecule can bind
- The matrix must be activated for the ligand to bind to it but still able to retain it's own activation towards the target molecule

- Amino, hydroxyl, carbonyl and thiol groups located with the matrix serve as ligand binding sites
- Matrices are made up of agarose and other polysaccharides
- The matrix also must be able to withstand the decontamination process of rinsing with sodium hydroxide or urea

Examples of interactions in affinity chromatography

Ligand : Target

- **Antigen** : antibody
- **Enzyme** : substrate analogue
- **Binding protein**: Ligand
- **Receptor** : ligand
- **Lectin** : polysaccharide, glycoprotein
- **Nucleic acid** : complementary base sequence
- **Hormone, vitamin** : receptor, carrier protein.
- **Glutathione : glutathione-S-transferase** or GST fusion proteins.
- **Metal ions** : Poly (His) fusion proteins, native proteins with histidine or cysteine on their surfaces.

- **Other tags: tag affinity chromatography**

Ligands

- The ligand binds only to the desired molecule within the solution
- The ligand attaches to the matrix which is made up of an inert substance
- The ligand should only interact with the desired molecule and form a temporary bond
- The ligand/molecule complex remains in the column, while the contaminants are eluted
- The ligand/molecule complex dissociates by changing the pH

Applications

- **Purify** and concentrate a substance from a mixture into a buffering solution
- **Reduce** the amount of a substance in a mixture
- **Discern** what biological compounds bind to a particular substance, such as drugs
- **Purify** and concentrate an enzyme solution

- **Genetic Engineering** - nucleic acid purification
- **Production of Vaccines** - antibodies purification from blood serum
- **Basic Metabolic Research** - protein or enzyme purification from cell free extracts

Definitions

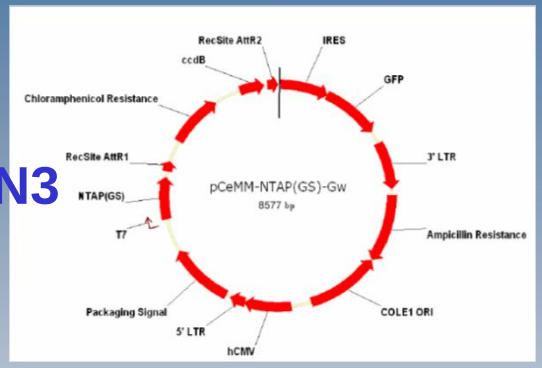
- Avidin (or Streptavidin) -biotin interaction is used to purify proteins
- Avidin: protein from egg white (birds, reptiles…)
- Streptavidin is a tetrameric protein purified from the bacterium *Streptomyces avidinii*
- Biotin: (vitamin H or B7) cofactor in the metabolism of fatty acids and leucine, and in gluconeogenesis

---

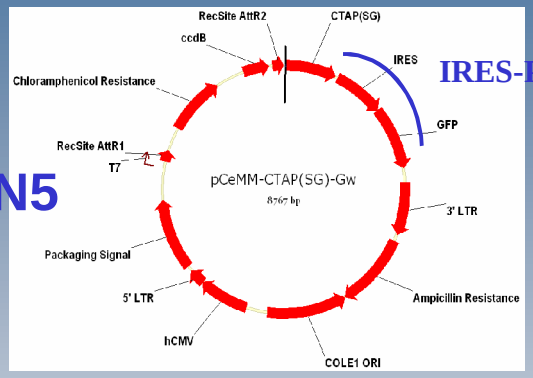
- The non-covalent bond formed between biotin and avidin or streptavidin has a binding affinity >most antigen and antibody bonds ~ strength of a covalent bond
- Affinity chromatography using immobilized avidin or streptavidin to separate the biotinylated protein from a mixture of other proteins and biochemicals

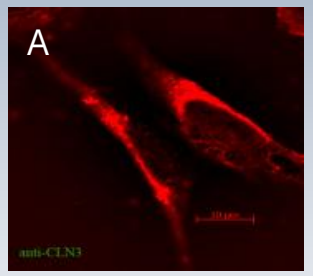
TAP purification of protein complexes from mammalian cells

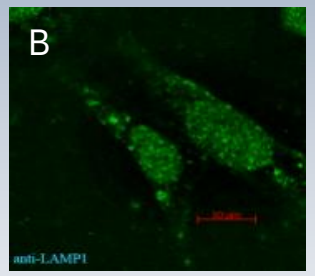
CLN3

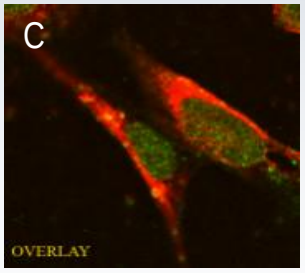


CLN5









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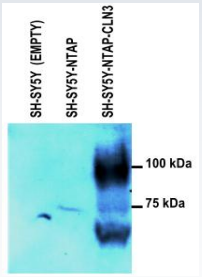
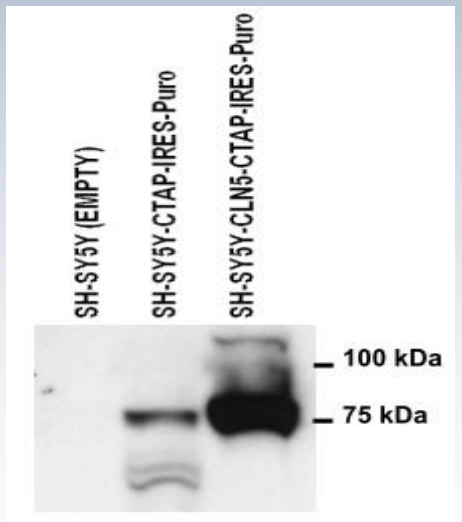




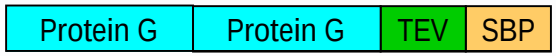
C-terminal vector CTAP:
- **folding and stability preference,**
- **different protein N-termini**

N-terminal vector NTAP:
- **allows handling of proteins requiring their C-terminus for cellular trafficking**

Protein complexes will be isolated by dual affinity chromatography including Protein G-Sepharose and Streptavidin affinity beads

PROS: GFP for tracking,
Retroviral packaging elements
CONS: NO mammalian marker

Design: **Bürckstummer et al. 2006**
Scifo et al. 2012



N-TAP (GS)

SG-

-C-TAP (SG)

TAP purification of protein complexes from mammalian cells

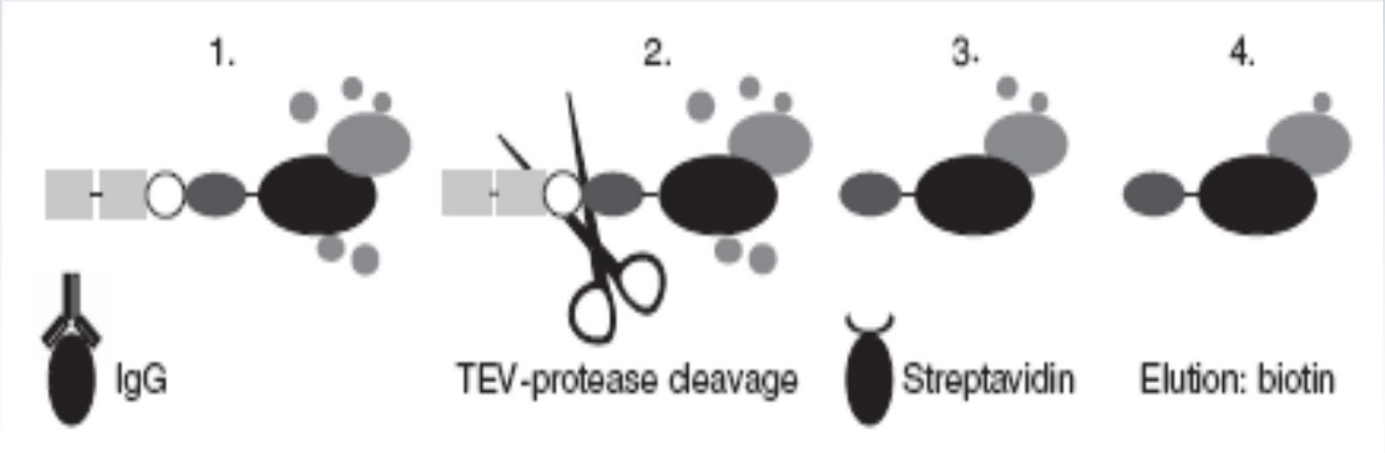
Benefits:

- Several members of a complex can be tagged, giving an internal check for consistency
- Detects real complexes in physiological settings

Drawbacks:

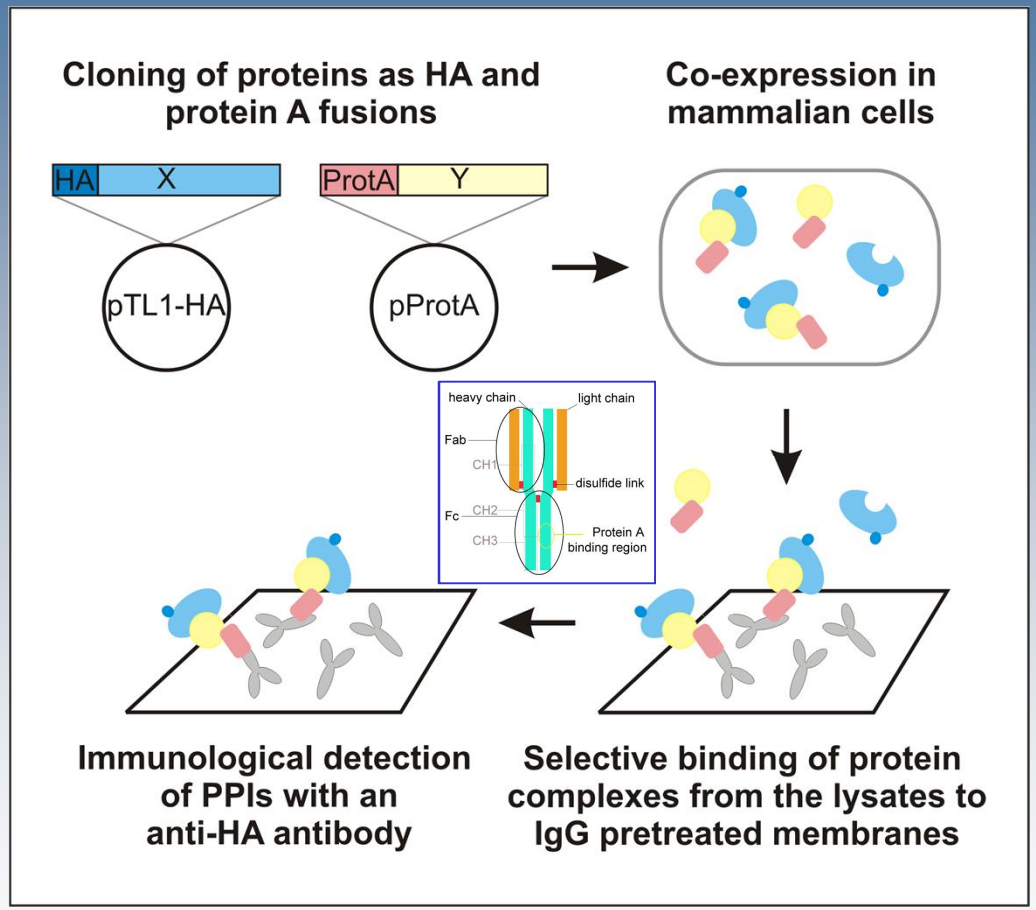
- Might miss some complexes not present under the given conditions
- Tagging may disturb complex formation
- Loosely associated components may be washed off during purification

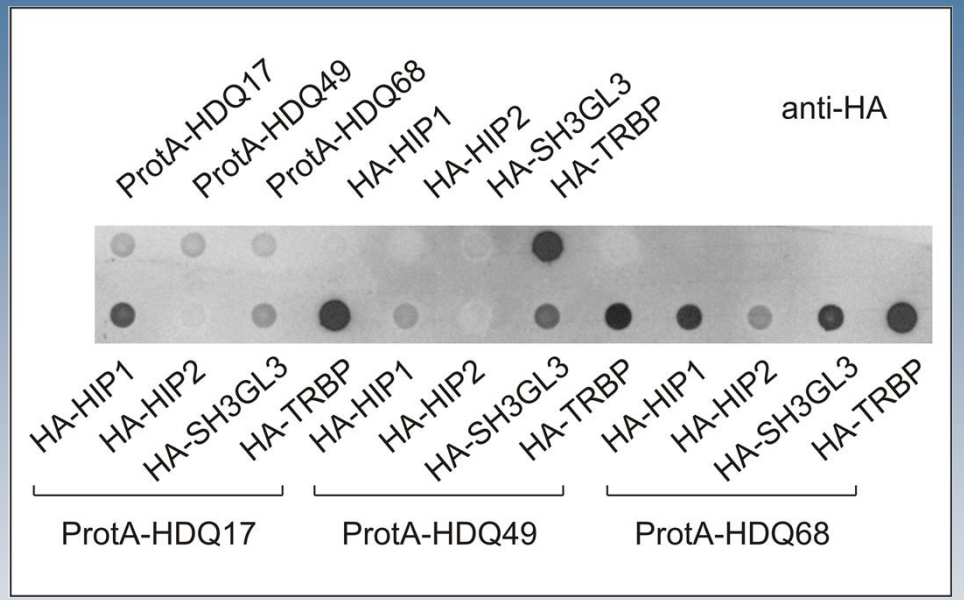
TAP Complexes purification



Buerckstummer et al. 2006

Validation of interactions using affinity-membrane technology

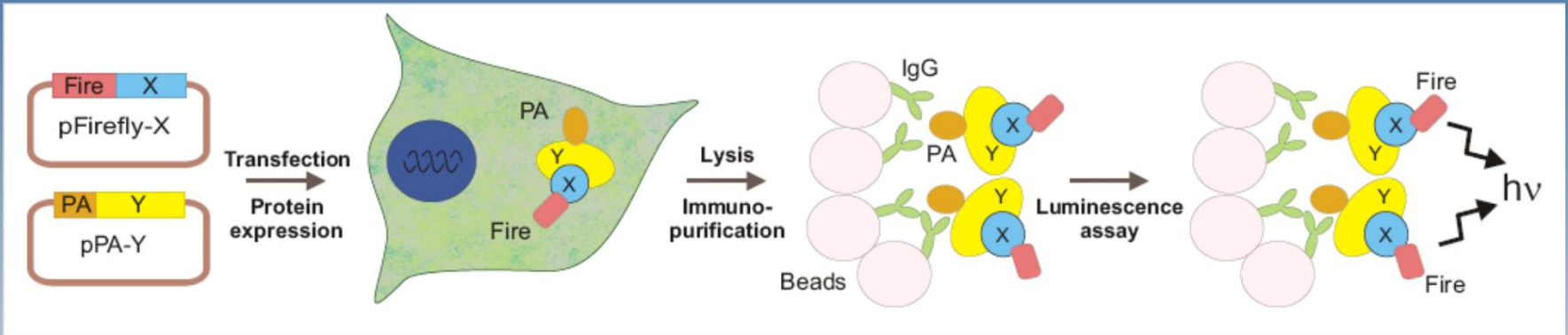




Interactions between huntingtin and known interaction partners

The principle

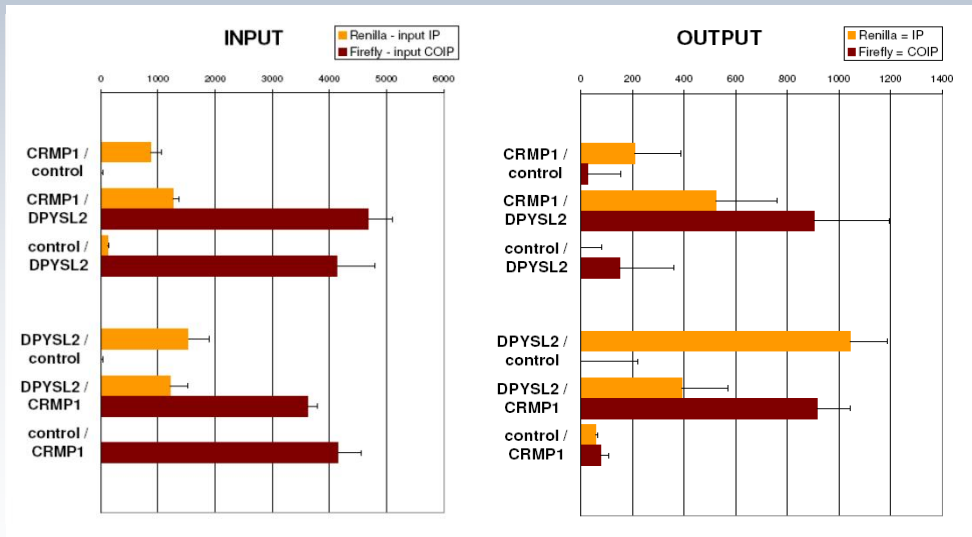
Validation of interactions using affinity-LUMIER technology



Adaptation of LUMIER (Barrios-Rodilles et al., 2005)

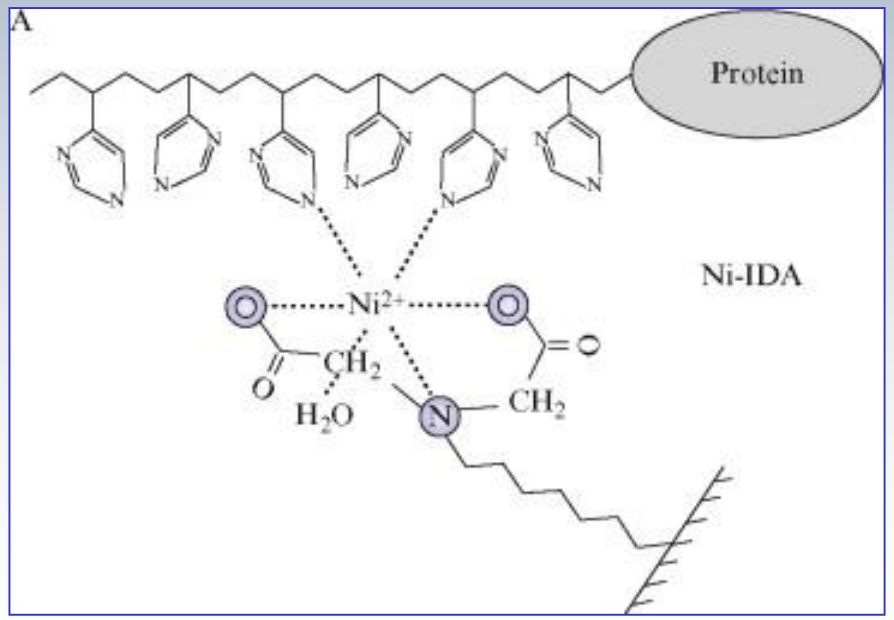
Reporter assay format:

- **Readouts for IP and Co-IP**
- **Determination of binding affinities**
- **Validating and quantification of binary interactions**



IMAC: Immobilized metal ion affinity chromatography

- Single step chromatography
- It is based on the known affinity of transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to histidine and cysteine in aqueous solutions – First described by Hearon, 1948 and later Porath et al., 1975
- For Zn-finger proteins and recombinant proteins with ≥6x His-tag



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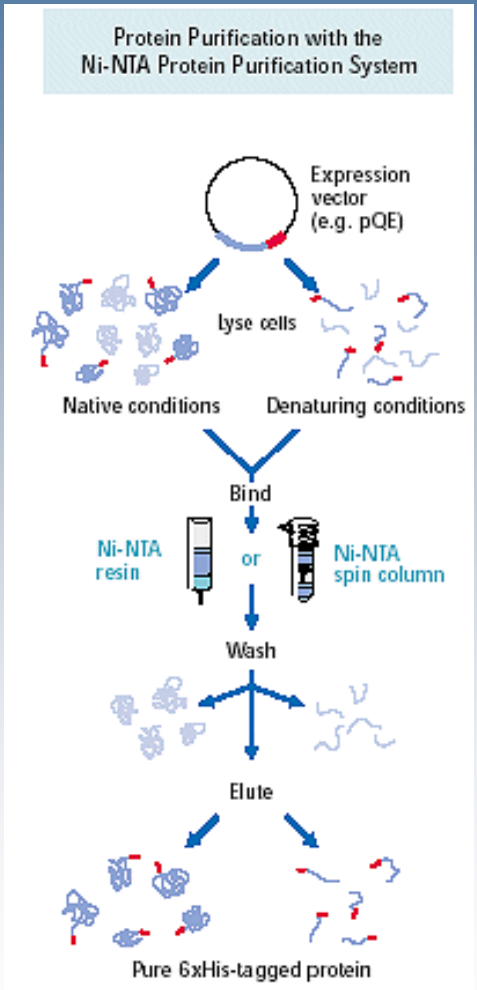
Model of the interaction between residues in the His tag and the metal ion in tri- (IDA), tetra- (NTA), and pentadentate IMAC ligands (TED)

Examples of purifications with IMAC



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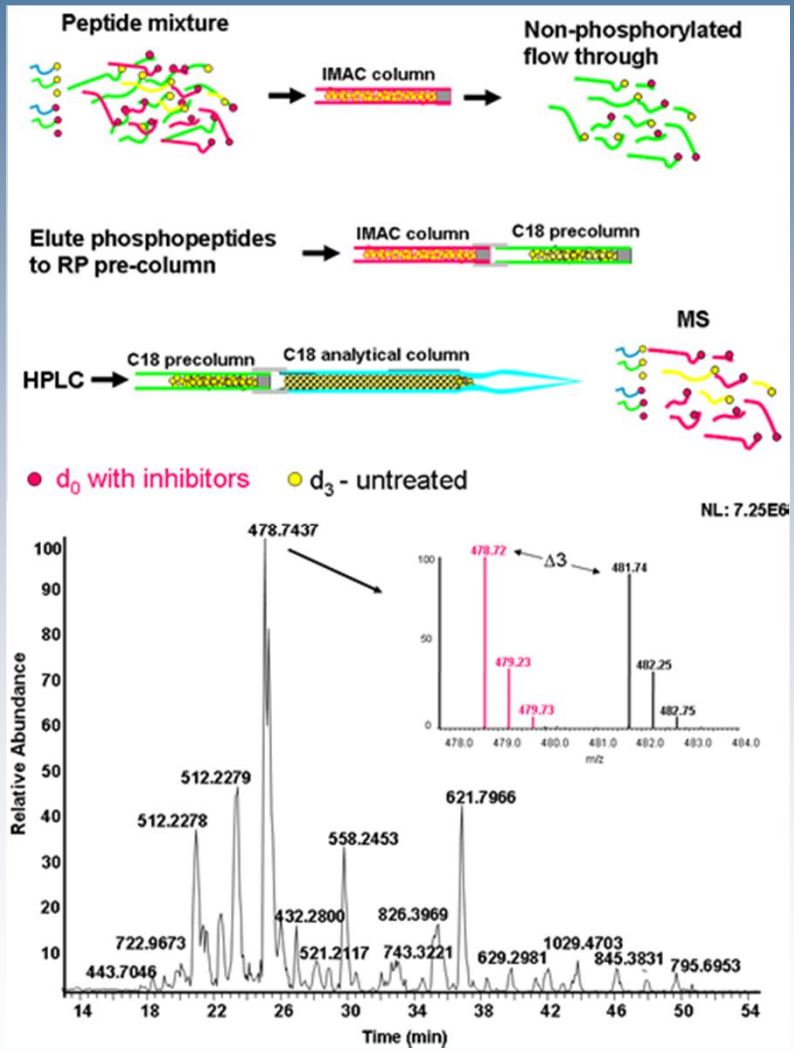


Chemical compatibility of purification of His-tagged protein using agarose-based IMAC (Ni-NTA) resins and its limitations

IMAC chemical compatibility

Component	Limitation (up to)	Component	Limitation (up to)
Buffers		**Salts**	
Na-phosphate	Recommended, limit not known	NaCl	4 *M*
Phosphate citrate	Limit not known	$MgCl_2$	4 *M*
Tris–HCl, HEPES, MOPS	100 m*M*	$CaCl_2$	5 m*M*[c]
Citrate	60 m*M*	$NaHCO_3$	Not recommended
Detergents (in 300 m*M* NaCl)		Ammonium salts	Not recommended
n-Hexadecyl-*β*-D-maltoside	0.0003% (w/v)	**Protease inhibitors**	
n-Tetradecyl-*β*-D-maltopyranoside	0.005% (w/v)	EDTA	1 m*M*[a]
n-Tridecyl-*β*-D-maltopyranoside	0.016% (w/v)	Commonly used protease inhibitors[d]	Compatible in effective concentrations
Brij 35	0.1% (v/v)	Complete cocktail (EDTA-free)	1× concentrated
Digitonin	0.6% (w/v)	**Denaturants**	
Cymal 6	1% (w/v)	Urea	8 *M*
n-Nonyl-*β*-D-glucopyranoside (NG)	1% (w/v)	Gu–HCl	6 *M*
n-Decyl-*β*-D-maltopyranoside (DM)	2% (w/v)	**Amino acids**	
n-Dodecyl-*β*-D-maltoside (DDM)	2% (w/v)	Histidine	1–2 m*M*[b]
C12–E9	1% (w/v)	Glycine	Not recommended
n-Octyl-*β*-D-glucopyranoside (OG)	1.5% (w/v)	Cysteine	Not recommended
Triton X-100, Tween, NP-40	2% (v/v)	Glutamate	Not recommended
Triton X-114	2% (v/v)	Aspartate	Not recommended
Fos-Cholines	0.05% (w/v)	Arginine	500 m*M*
Dodecyldimethyl-phosphineoxide	0.15% (w/v)	**Organic solvents**	
N,*N*-Dimethyldodecylamine-*N*-oxide (LDAO)	0.7% (w/v)	Isopropanol	60% (v/v)[e]
CHAPS	1% (w/v)	Ethanol	20% (v/v)
Laurosyl-sarcosine	1% (w/v)	**Reducing reagents**	
SDS	0.3% (w/v)[a]	*β*-ME	20 m*M*
Other		TCEP	20 m*M*
EGTA	1 m*M*[a]	DTT	10 m*M*
Imidazole	10–20 m*M*[b]	DTE	10 m*M*
Hemoglobin	Not recommended		
Glycerol	50% (v/v)		

Affinity chromatography: summary

PROS:

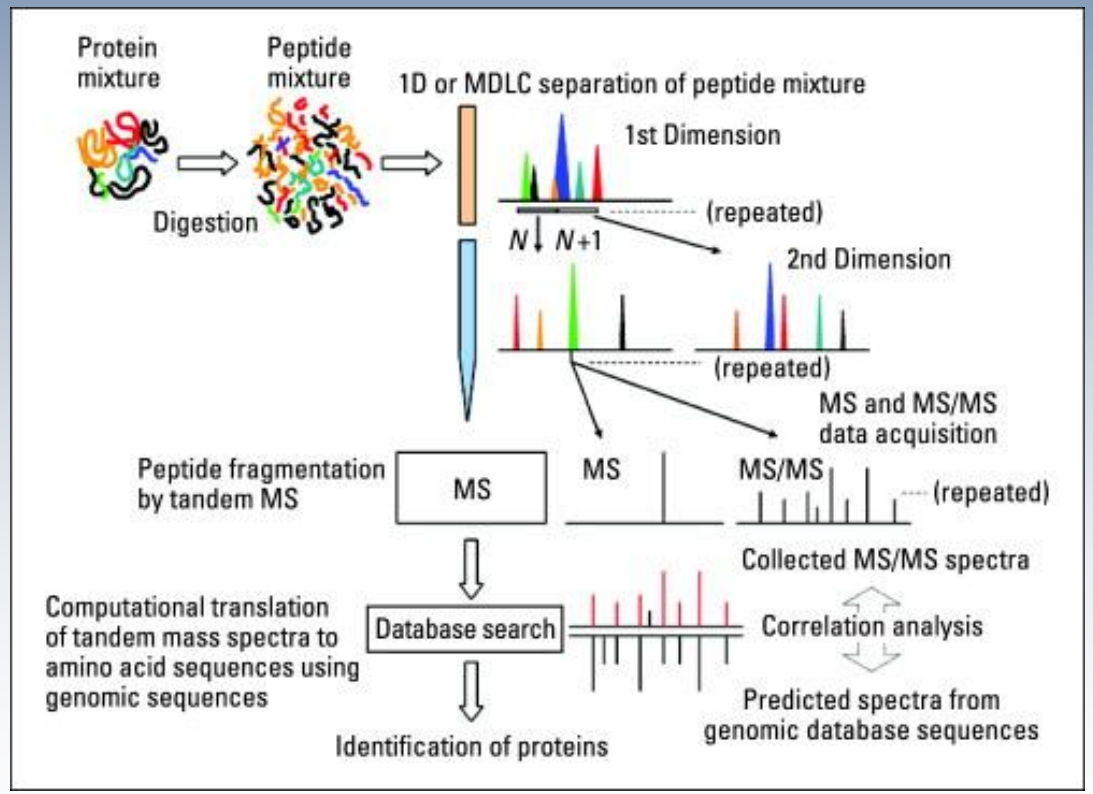
- Extremely high specificity
- High degrees of purity can be obtained
- The process is very reproducible
- The binding sites of biological molecules can be simply investigated

CONS:

- Expensive ligands
- Leakage of ligand
- Degradation of the solid support
- Limited lifetime
- Non-specific adsorption
- Relatively low productivity

MudPIT proteomics

- **MudPIT = Multidimensional Protein Identification Technology**
- Used for separation and identification of complex protein/peptide mixtures- alternative to 2D-electrophoresis



MudPIT

Mud Pit

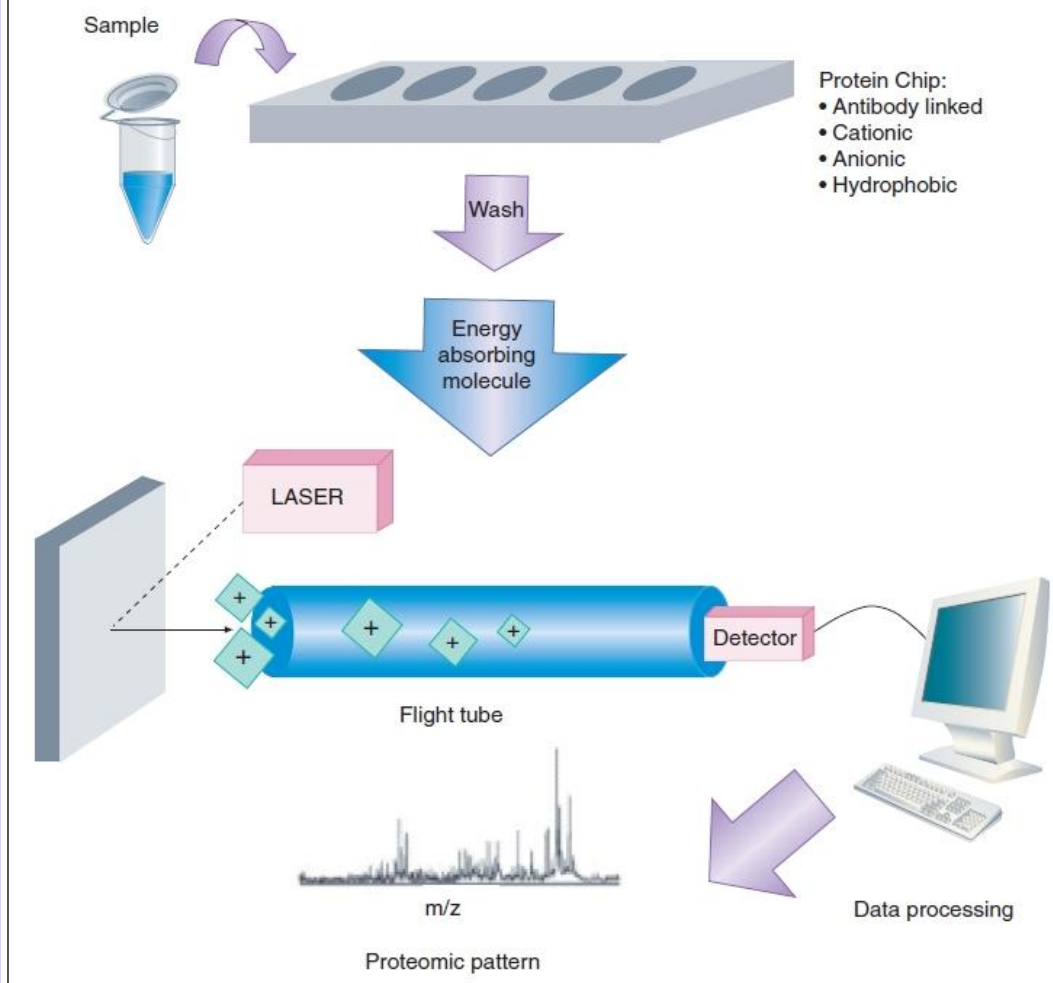
SELDI-TOF Mass Spectrometry

- **Surface-enhanced laser desorption/ionization-TOF Mass spectrometry (Ciphergen now Biorad Inc.).**

  - **Mass spectrometric technology on a chromatographic chip surface**
  - **Used to analyze complex biological mixtures such as serum, urine, milk, blood**
  - **Biomarker discovery**
  - **Differentially expressed proteins are determined by comparing protein peak intensities between mass spectra**

Proteomics using SELDI-TOF

Figure 1. SELDI-TOF MS schema.



1. A serum sample is loaded onto a chemically selective protein chip
2. Chip is then washed with buffer(s) to remove unbound proteins
3. Chip is then treated with energy absorbing molecules such as sinapic or cinnamic acid.
4. The bound proteins are then ionized with laser and acclerated through an electric field where they are separated based on their mass to charge to charge ratios (*m/z*).
5. The detector quantifies the proteins and a spectrum is generated with analytical software.

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Thank you for your attention!

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