université PARIS-SACLAY

FACULTÉ DE PHARMACIE

Electrophoretic separation methods

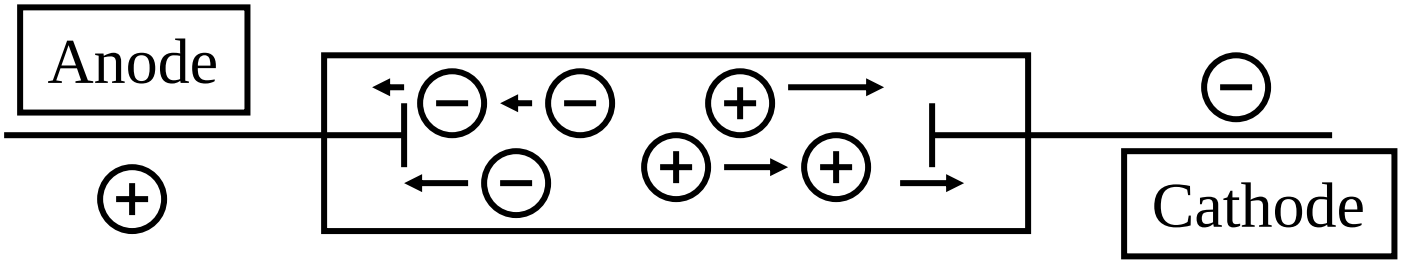
M1 – TU09
ANALYTICAL SCIENCE

Duc MAI
Université Paris-Saclay
PNAS - Institut Galien Paris-Saclay

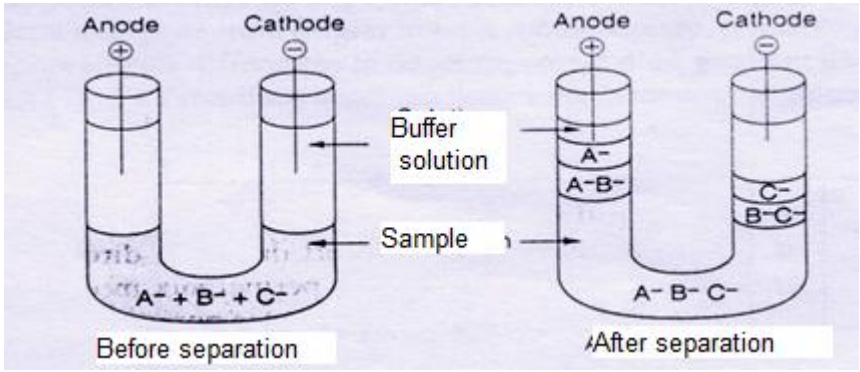
Definition

- **Electrophoresis**: Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field

  Tiselius 1948 : separation de proteins- Nobel price



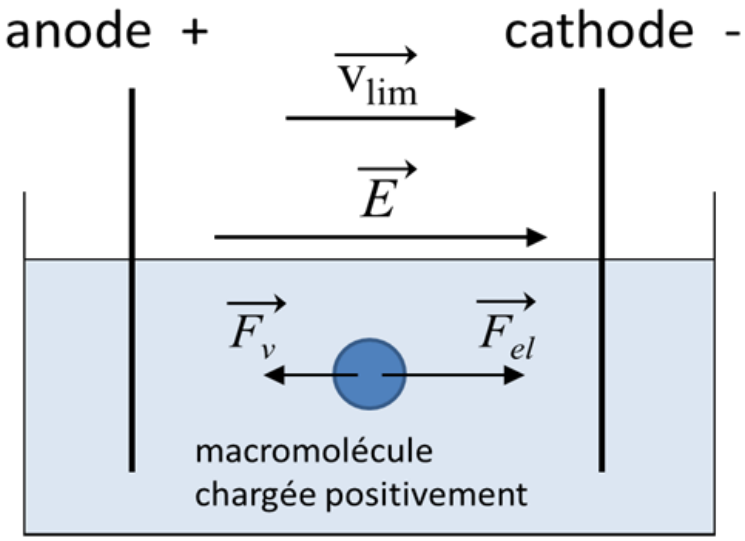
- Different categories of electrophoresis:
  - In tubing (very old version)
  - Zone electrophoresis on support
  - Isotachophoresis



Electrophoresis

1. **Theoretical**
2. **Agarose gel electrophoresis(ADN)**
3. **Polyacrylamide electrophoresis (proteins)**
4. **Capillary electrophoresis**

Theory of electrophoresis



Ions under an electrical field E:

- Electric force applied on the ion:

  F=qE q= ion charge

  E electrical field volt.cm^{-1}

- Friction force (Stokes' law)

  F'=6πηrv q=Ion charge

  η viscosity

  r: radius of the spheric ion

  V: velocity

$$qE = 6\pi\eta rv$$

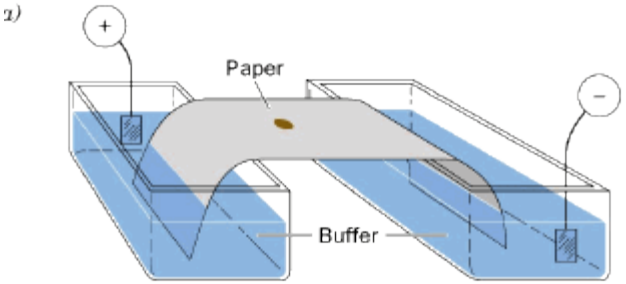
- At equilibrium (constant velocity), the two forces balance!

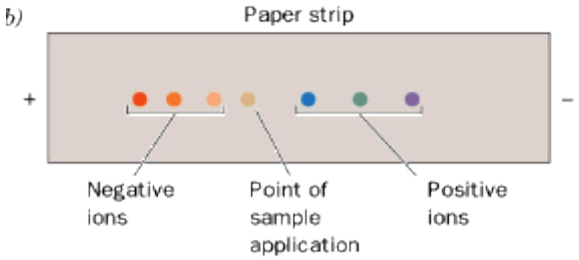
- The mobility of the target ion = velocity per unity of electrical field.

$$\mu = \frac{q}{6\pi r\eta}$$

Types of supports in electrophoresis

- **Paper** (adsorption, elevated electroendosmosis)

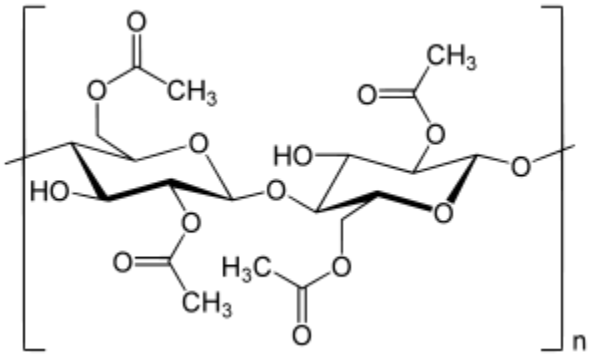




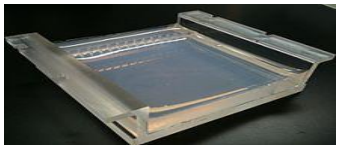
- **Cellulose Acetate**
  - Small molecules (amino acids, nucleotides)
  - Charged macromolecules (lipoproteins, hemoglobins, glycoproteins, isoenzymes)
- **Gels**
  - **Polyacrylamide Gel for proteins**
  - **Agarose Gel for nucleic acids**
  - Starch gel for the polymers of elevated masses

Starch: polysaccharide composd of several D-glucose molecules.

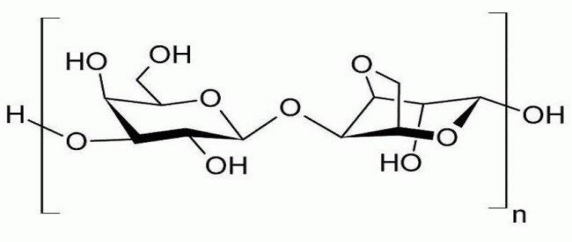
Electrophoresis on cellulose acetate



- 2 - 3 acetyl groups per unit of glucose.
- Less adsorption and sharper bands than paper
- No tailing of proteins or hydrophilic materials
- Wide range of sizes and layer thicknesses
- High resolution
- Possible application of a (relatively) high voltage

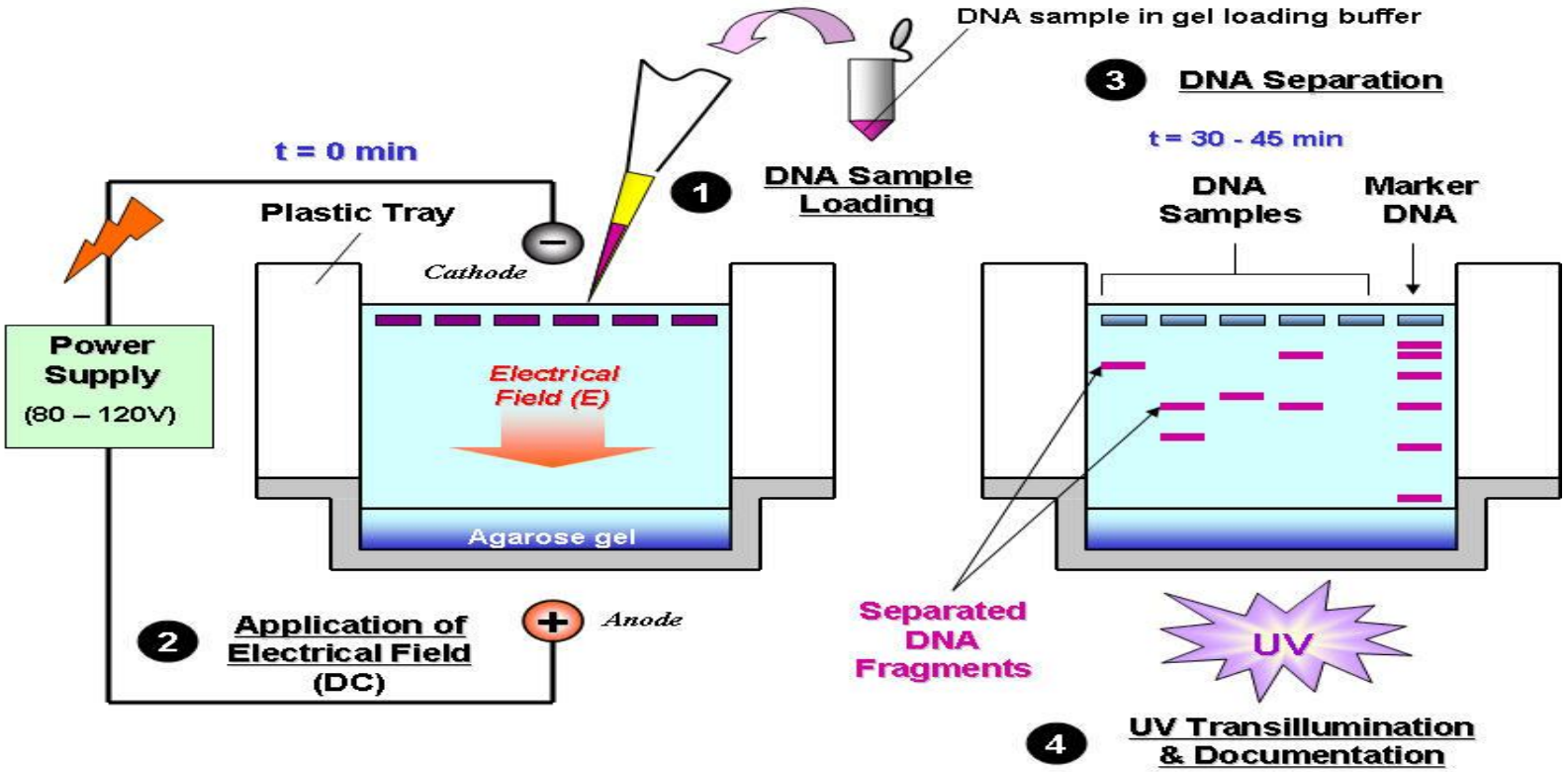


Agarose



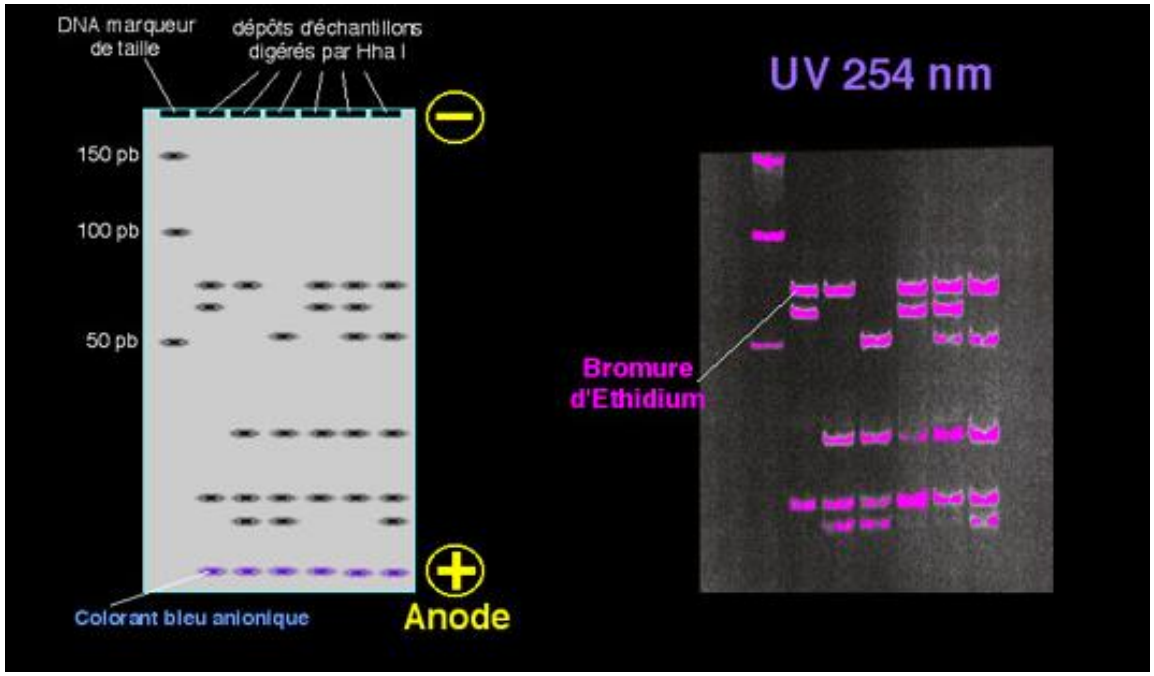
- **Neutral polysaccharide extracted from agar** (red seaweed)
  - **Non toxic**
- **Simple preparation:** concentrations : 0.5 to 2%.
  - Dissolution in hot H_2O bouillante then cooling down
- **Large pore sizes** (1% gel) : **100 nm - 500 nm**
  - Macromolecules of high molecular weight: **DNA (200 to 50,000 bp) and large protein molecules**
  - Larger molecules >> using a low concentration gel (0.15 - 0.9 %)
  - Smaller molecules >> high concentration gel. >> longer run times (sometimes days).
- **Low resolution.**
  - Lower resolving power than polyacrylamide gel

(Agarose gel) DNA electrophoresis



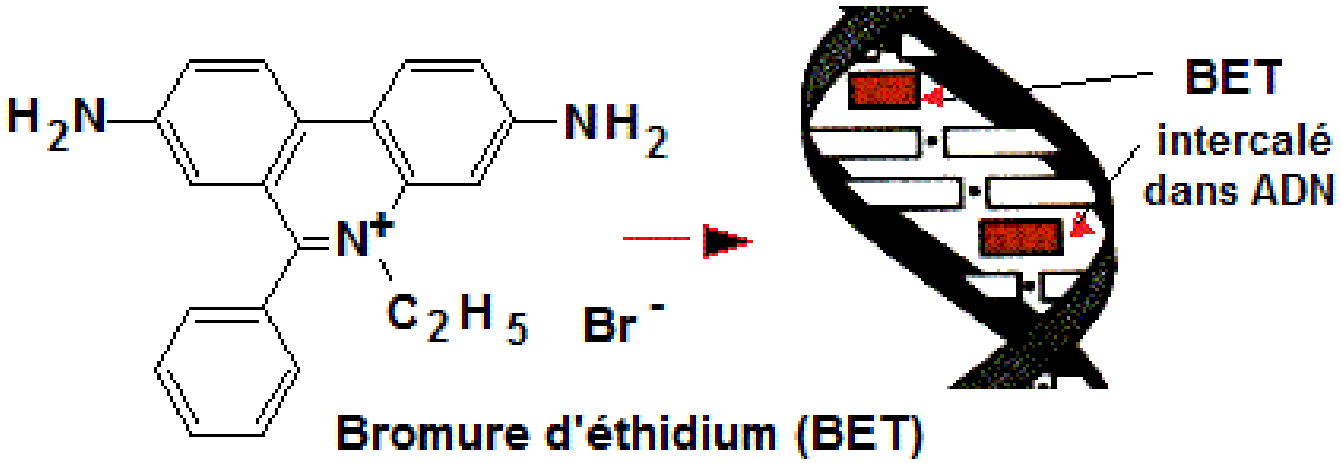
- **Linear DNA fragments migrate with mobility inversely proportional to Log(MW)**
- **DNA migrates towards anode (+)**
- **With identical weights, circular shapes migrate differently from linear shapes.**

DNA gel labeling



- DNA labeling after (in the gel) or before their separation
- More often used:
  - Intercalating agents: Ethidium (homidium) bromide, propidium iodide
  - The derivatives of Cyanine

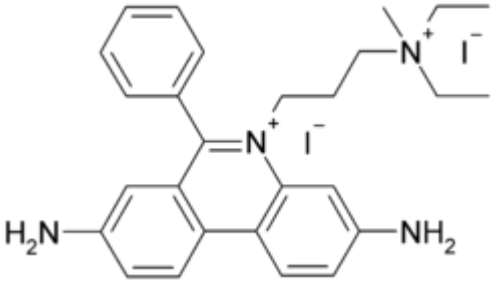
Ethidium bromide (BET, about 0,5 µg·mL^{-1})

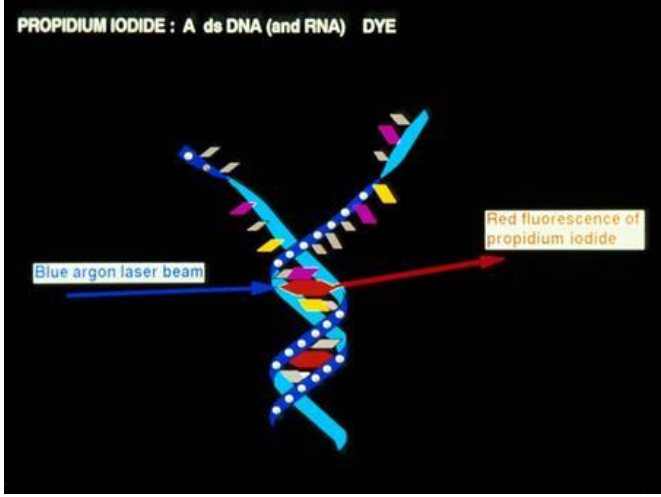


1. Exposed to UV >> fluorescent with a red-orange color, **20 times more intense when it is linked to ADN**.
2. RNAs are not double stranded >> at equal weights the RNA staining is markedly less intense than that of the DNA.
3. BET can be mixed with agarose when preparing the gel, or the gel can be immersed in a BET solution after migration is complete.

Propidium Iodide

(1IP / 4-5 base pairs)



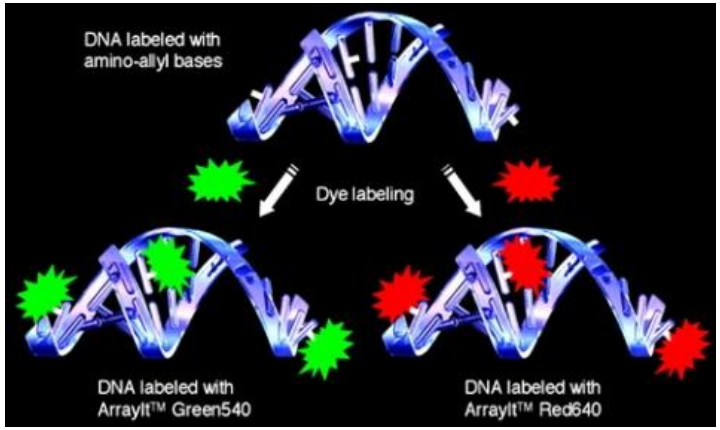


1. Fluorescent molecule (MW 668,403 Da).
2. Link to DNA bases with little or no sequence specificity
3. PI can also bind to RNA >> treatment with nucleases (RNases) in order to degrade the RNA and thus avoid any false positive.
4. A nucleic acid-binding PI molecule is 20 to 30 times more fluorescent than a free PI molecule in solution
5. **A powerful marker of cell viability**: DNA marker to mark the nucleus of cells that have lost their membrane integrity

The derivatives of Cyanine

The cyanines: a family of synthetic markers belonging to the polymethine group.

Fluorophore	Ex (nm)	Em (nm)	MW
Cy2	489	506	714
Cy3	(512);550	570;(615)	767
Cy3B	558	572;(620)	658
Cy3.5	581	594;(640)	1102
Cy5	(625);650	670	792
Cy5.5	675	694	1128
Cy7	743	767	818



Protocol

TD-P Revision 2.0

Creation Date: 1/10/2017
Revision Date: 1/24/2019

GelGreen™ Nucleic Acid Gel Stain, 10,000X

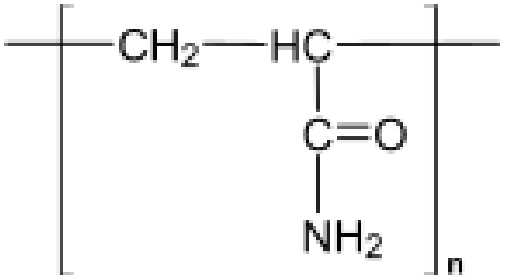
Procedure for staining dsDNA, ssDNA or RNA in gels

Introduction

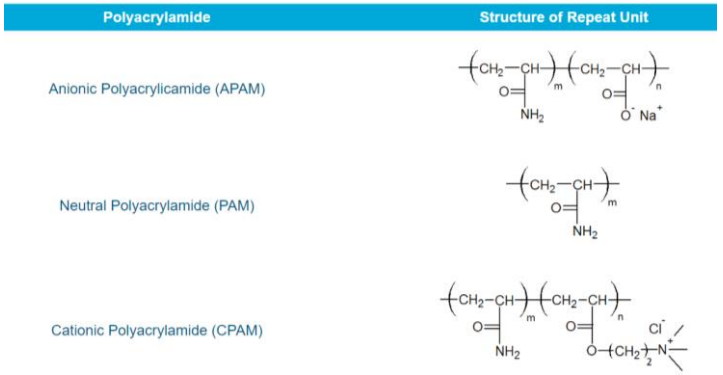
GelGreen™ is a sensitive, stable and environmentally safe green fluorescent nucleic acid dye designed to stain either dsDNA, ssDNA or RNA in agarose gels. GelGreen™ is far more sensitive than SYBR Safe. Unlike SYBR® dyes, which are known to be unstable, GelGreen™ is very stable, both hydrolytically and thermally. GelGreen™ is compatible with either a 254 nm UV transilluminator or a gel reader equipped with visible light excitation (such as blue LED light box, 488 nm laser-based gel scanner, or Dark Reader®).

- Safer than Ethidum Bromide
- Easy disposal
- Ultra-sensitive
- Extremely stable
- Simple to use
- Compatible with a standard UV transilluminator

Polyacrylamide gel

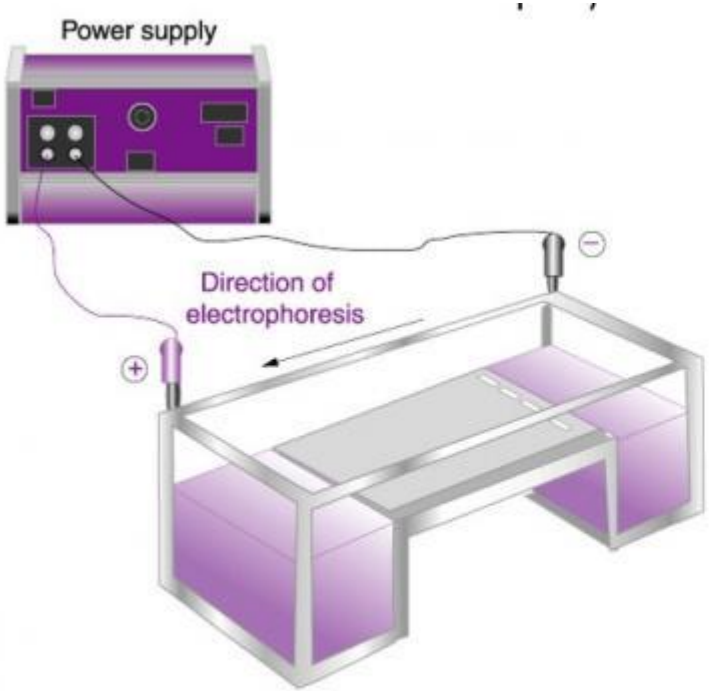


- Prefered method for **PROTEINS**
- Polymer [-CH2-CH(-CONH2)-]n, formed from acrylamide.
- The length of the polymer chain depends on the acrylamide concentration : 3.5 à 20%.

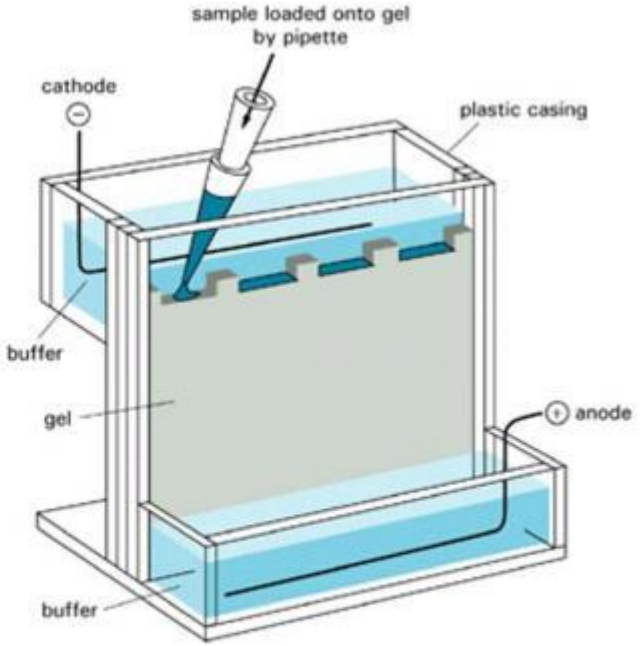


- **Polyacrylamide gel:**
  - **Restricted mass range**
  - **High resolving power**
  - Sometimes used as well for DNA

Protein Gel Electrophoresis



Électrophorèse horizontale
(gel d'agarose)



Électrophorèse verticale
(gel de polyacrylamide)

Gel Electrophoresis of proteins

1) **Native conditions (PAGE)**
   Separation based on the charge and size

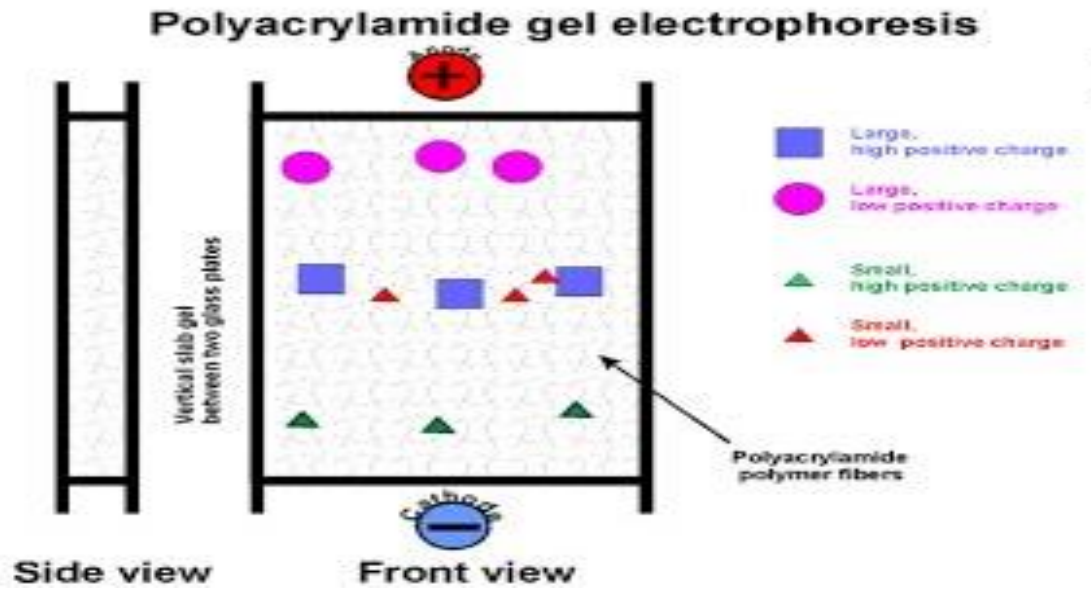
2) **Denaturing conditions (SDS-PAGE)**
   Separation based on sizes

3) **Isoelectric focusing (IEF)**
   Séparation based on isoelectric points

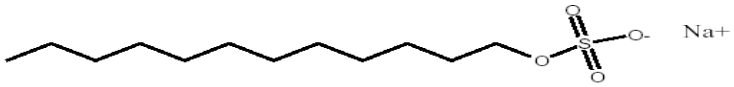
4) **Electrophoresis 2D**
   Separation based on charge and size

1) PAGE and native gel

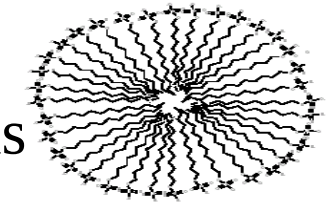
- Migration depends on shape + size + charge of the protein at working pH
- No denaturing, no dissociation



2) SDS PAGE



- **Sodium dodecyl sulfate**
  - Anionic detergent
  - Formation of micelles/complexes around proteins

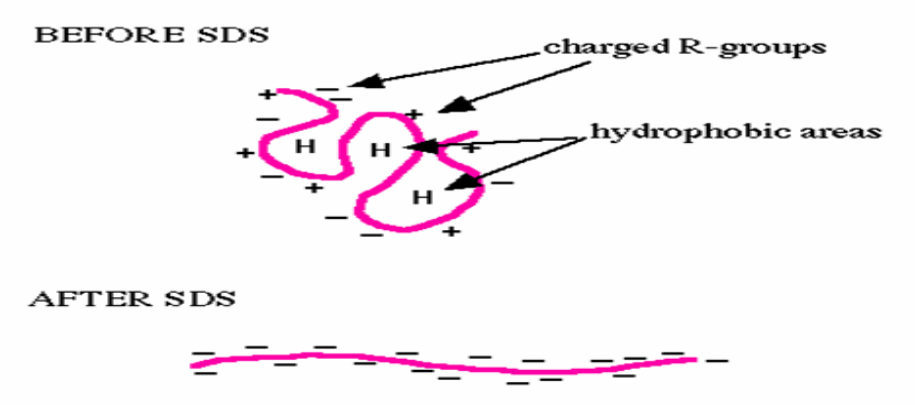


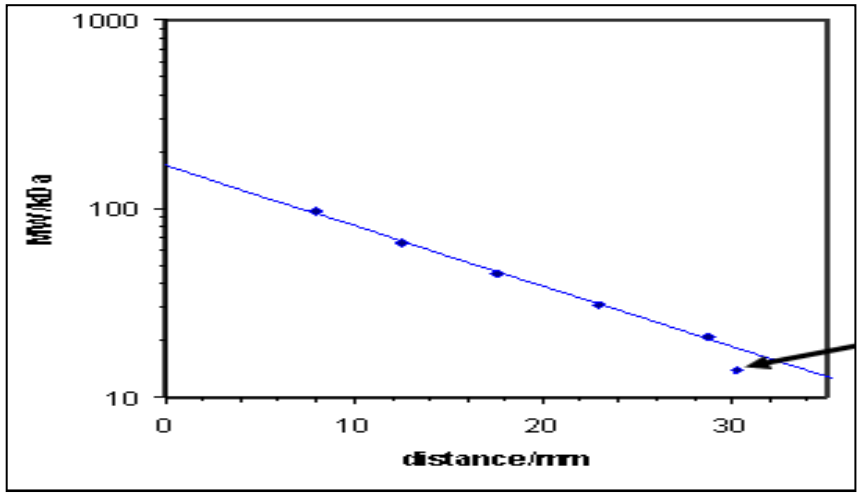
- All proteins become anionic
- Identical charge density
- Separation by size
-

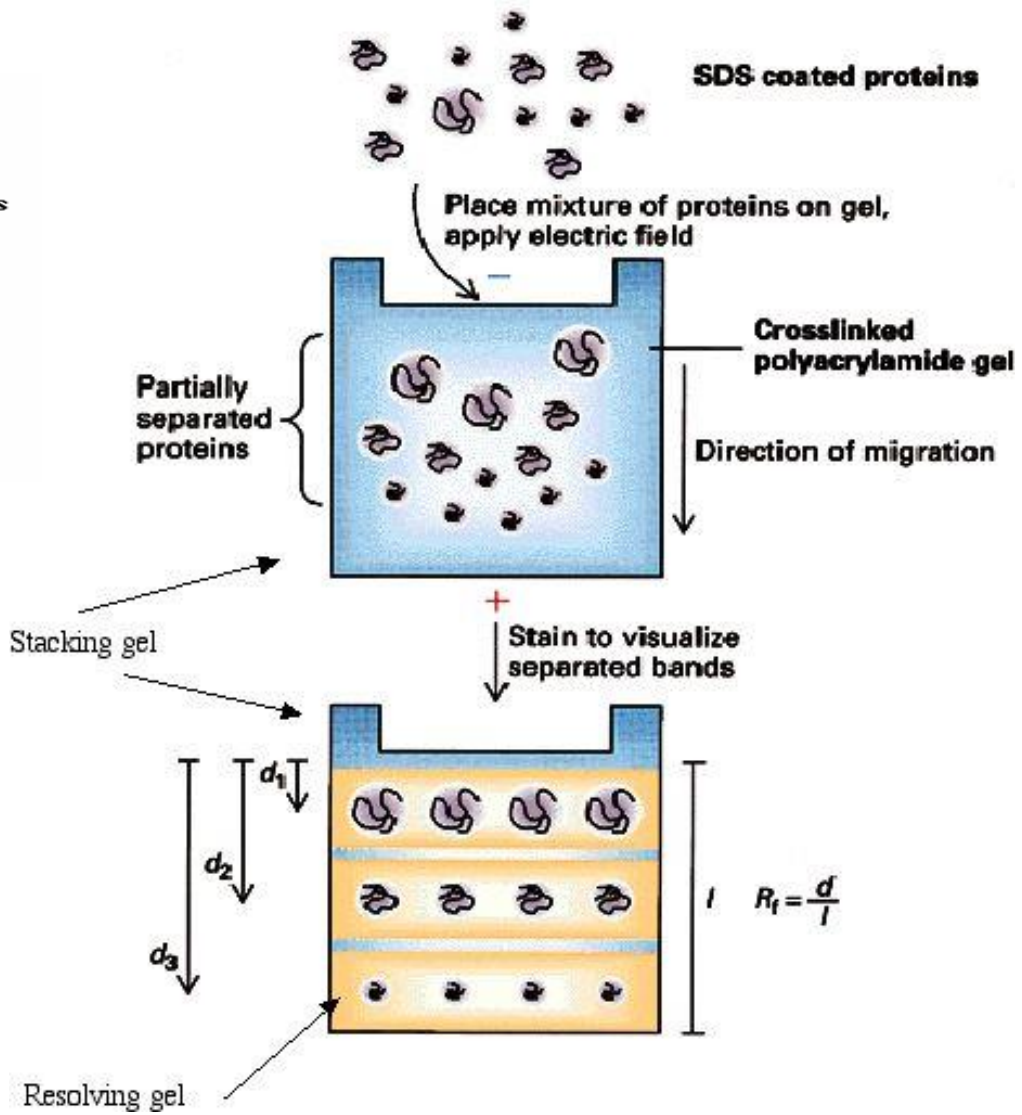
Tris-glycine SDS PAGE (Laemmli)
- 7.5% 40-400 kDa
- 12.5% 20-100 kDa
- 20% 3-25 kDa

Tris-Tricine SDS PAGE (Schaegger)
- 16.5% 2-25 kDa

2) SDS PAGE


BEFORE SDS
charged R-groups
hydrophobic areas
H
H
H
AFTER SDS


1000
100
10
MW/kDa
0
10
20
30
distance/mm


SDS-coated proteins
Place mixture of proteins on gel, apply electric field
Crosslinked polyacrylamide gel
Partially separated proteins
Direction of migration
Stain to visualize separated bands
Stacking gel
Resolving gel
d_1
d_2
d_3
l
$R_f = \frac{d}{l}$

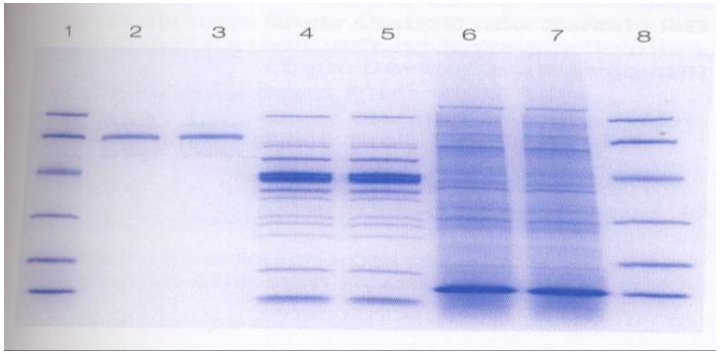
Revelation of PAGE gels

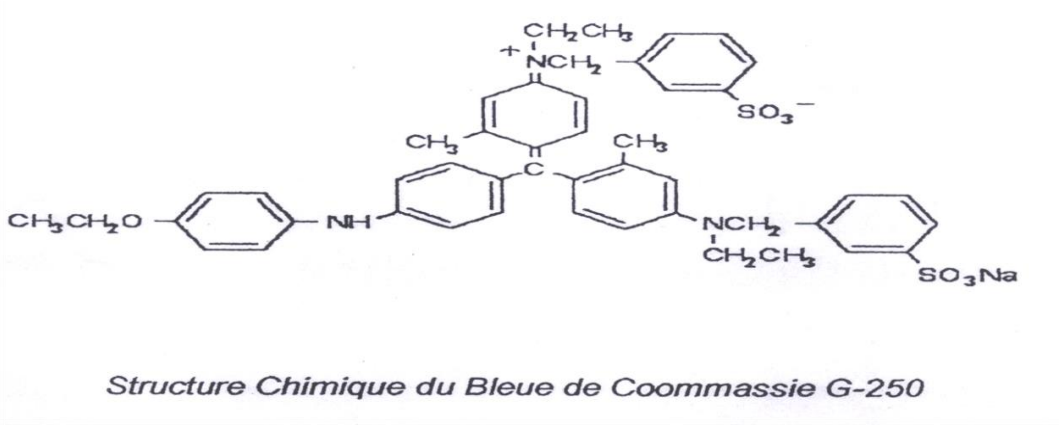
Direct methods

Coomassie blue

Non specific.

- Good sensitivity : 30-50ng
- Compatible with MS.





Structure Chimique du Bleue de Coommassie G-250

- Bound to protein: blue anionic form
- Linked to Arginine (8x) , Tyrosine, Trytophan, Phenylalanine, Histidine

Revelation of PAGE gels

Direct methods

- **Silver nitrate (0,5ng)**

- Longer protocol
- Very sensitive: 0.5-1.5ng de proteins
- Not specific.
- Narrow calibration range : less quantitative

Schägger 2006 (Nature protocols)

• Fixing solution: (deactivate contaminating ammonium aldehydes) :

methanol 50 %, ac. acétique 10 %,

•Ammonium acetate 100 mM

•Sodium thiosulfate : Na2S2O3 0,005%

• **AgNO3 0,1%**

•Developing agent : Na2CO3 2%, formaldehyde 0,036% (72 µl / 200 ml)

Revelation of PAGE gels

Direct methods

Zinc and Copper Dye

Negative labeling: proteins give light spots, Zn and Cu do not color SDS

Useful for protein analysis after separation

fast, easy and inexpensive

High sensitivity : 6-12 ng

Fluorescent markers

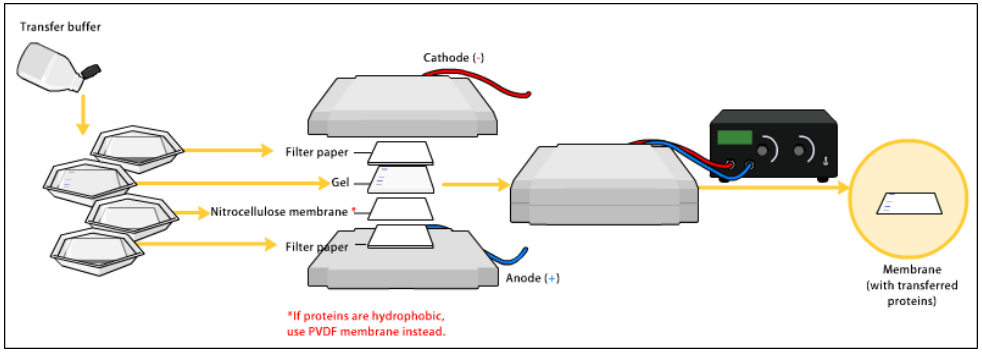
Easy to use but often expensive

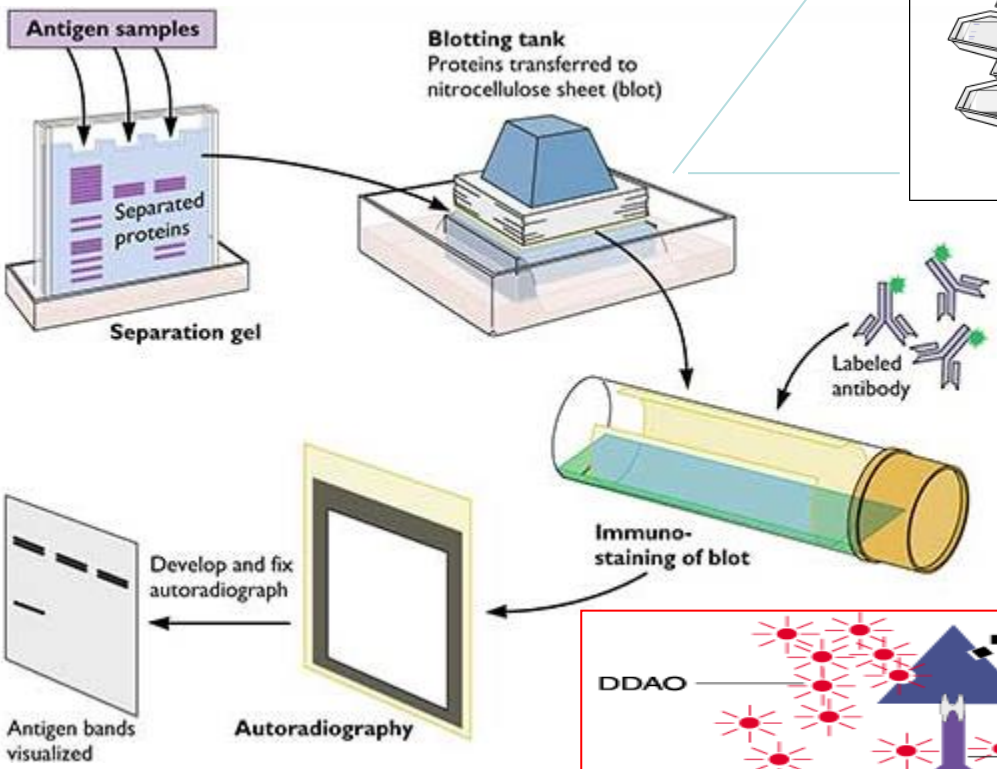
Selective

- Quantitative: Intensity of the spot directly correlated to the amount of protein
- Sensitivity : SYPRO ruby as sensitive as silver nitrate : **1-2ng**

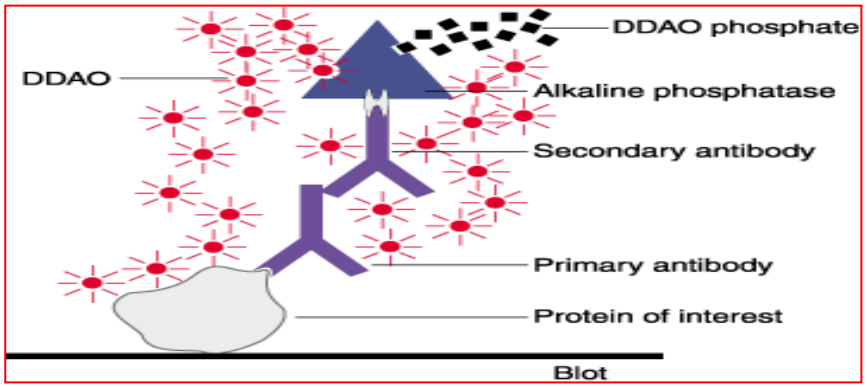
Revelation of PAGE gels

Immunodetection or Western





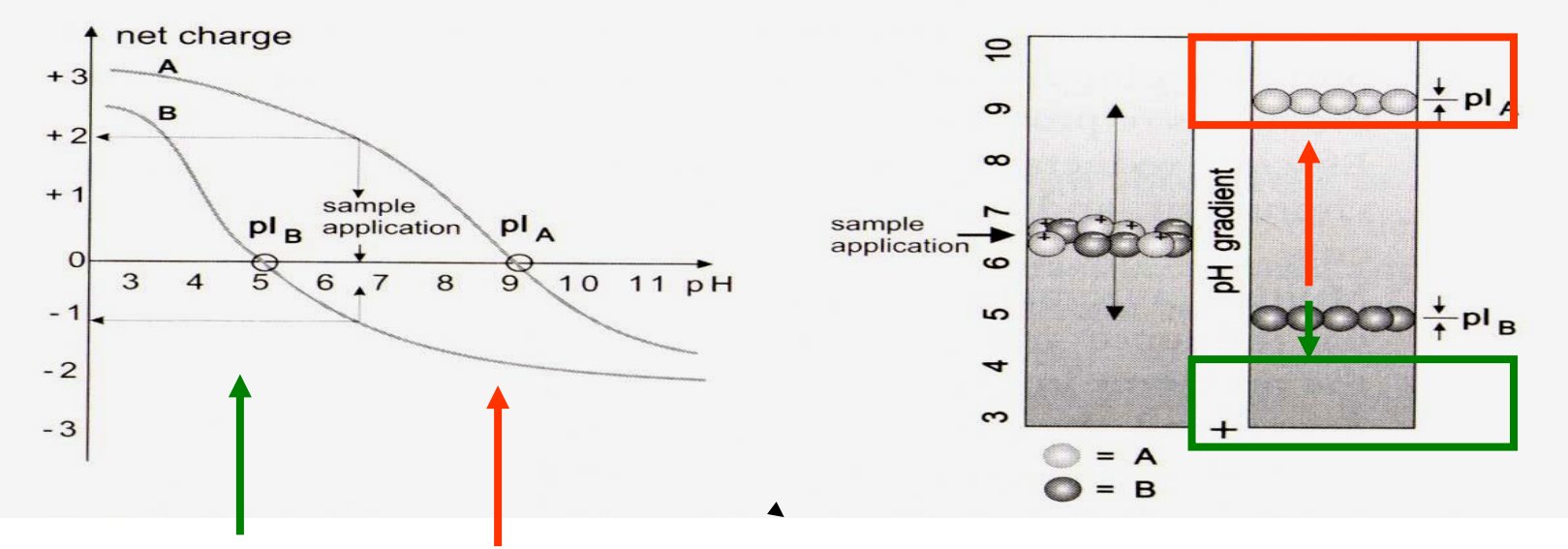
The phosphatase substrate **DDAO phosphate**
Hydrolysis product excited with the 633 nm
Excitation/emission maxima ~645/660).



Comparison of Southern, Northern, and Western blotting techniques

	Southern blotting	Northern blotting	Western blotting
Molecule detected	DNA (ds)	mRNA (ss)	Protein
Gel electrophoresis	Agarose gel	Formaldehyde agarose gel	Polyacrylamide gel
Gel pretreatment	Depurination, denaturation, and neutralization	-	-
Blotting method	Capillary transfer	Capillary transfer	Electric transfer
Probes	DNA Radioactive or nonradioactive	cDNA, cRNA Radioactive or nonradioactive	primary antibody
Detection system	Autoradiography Chemiluminescent Colorimetric	Autoradiography Chemiluminescent Colorimetric	Chemiluminescent Colorimetric

3) Principe of IEF- Separation by pI



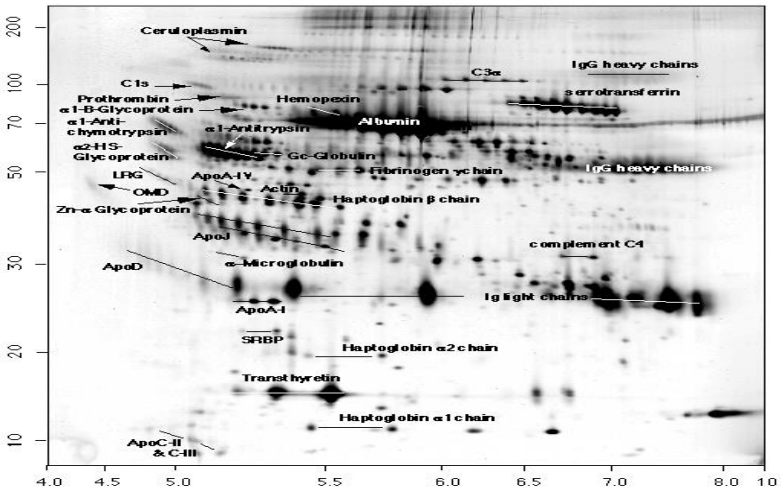
- Proteins are amphoteric molecules (acidic and basic groups with a buffering capacity)
  - Basic conditions >> negative charges
  - Acidic conditions >> positive charges
  - **Isoelectric point (pI): pH where the net charge =0**
- IEF is carried out in a pH gradient
- Highly resolutive

4) Electrophoresis 2D

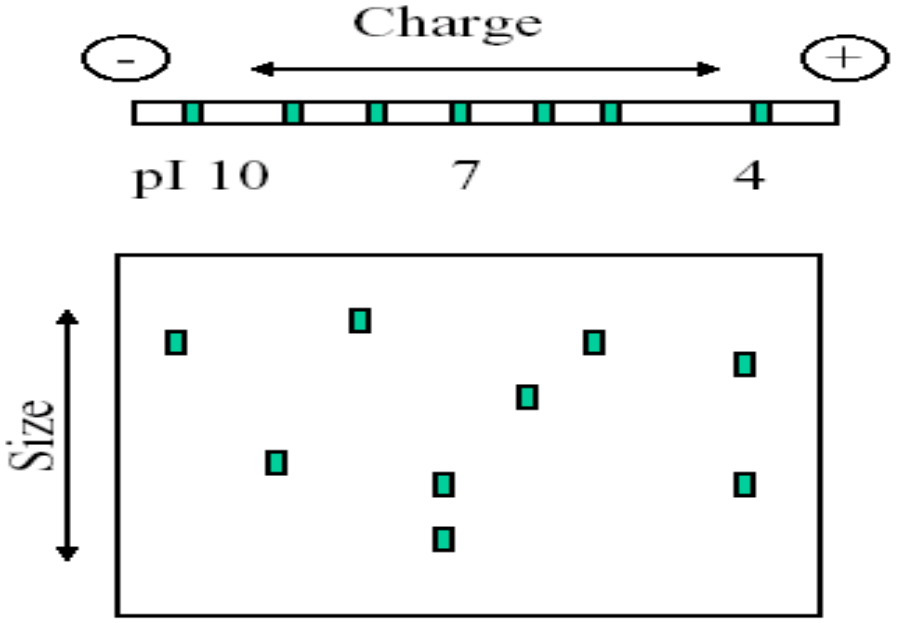
- Suitable for complex mixtures
- Proteomic analysis
- Two steps:

  IEF: first dimension

  SDS PAGE : second dimension

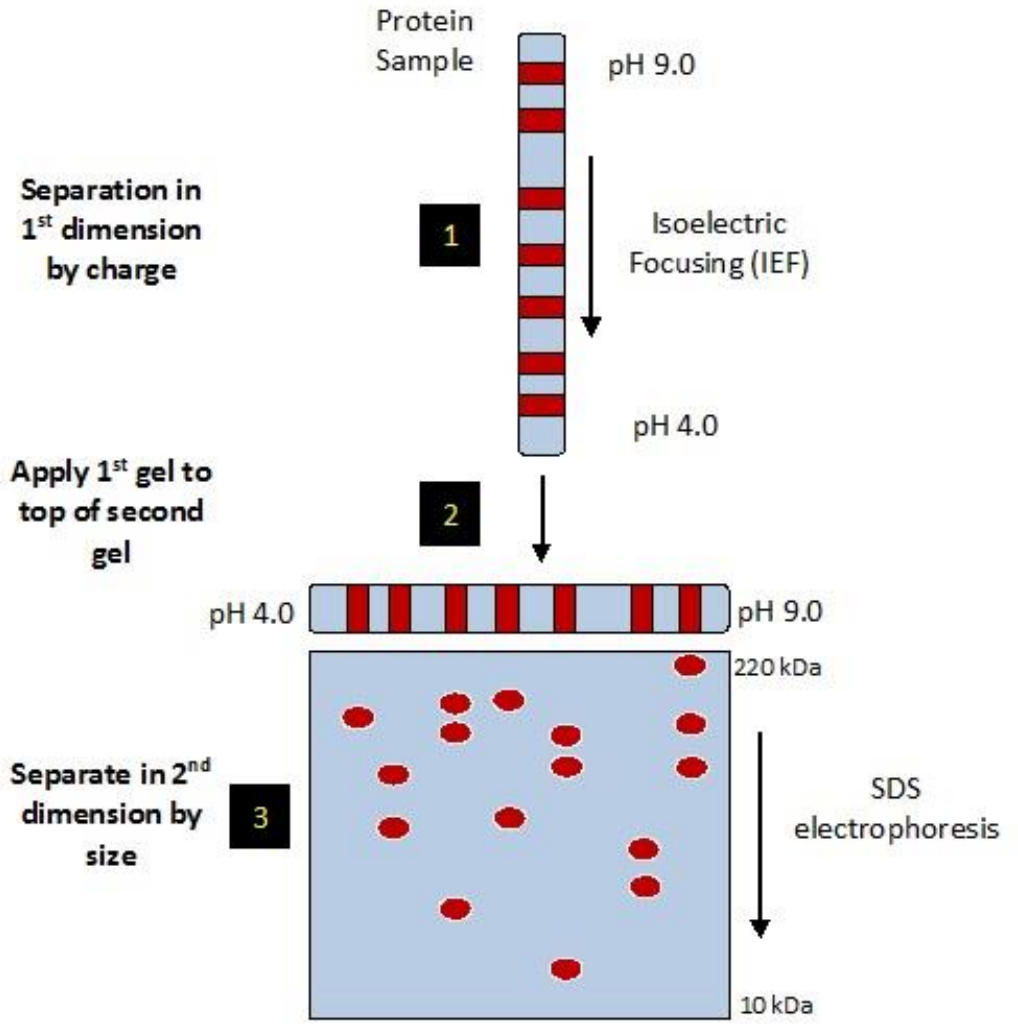


2DPAGE map



4) SDS-PAGE gel 2D

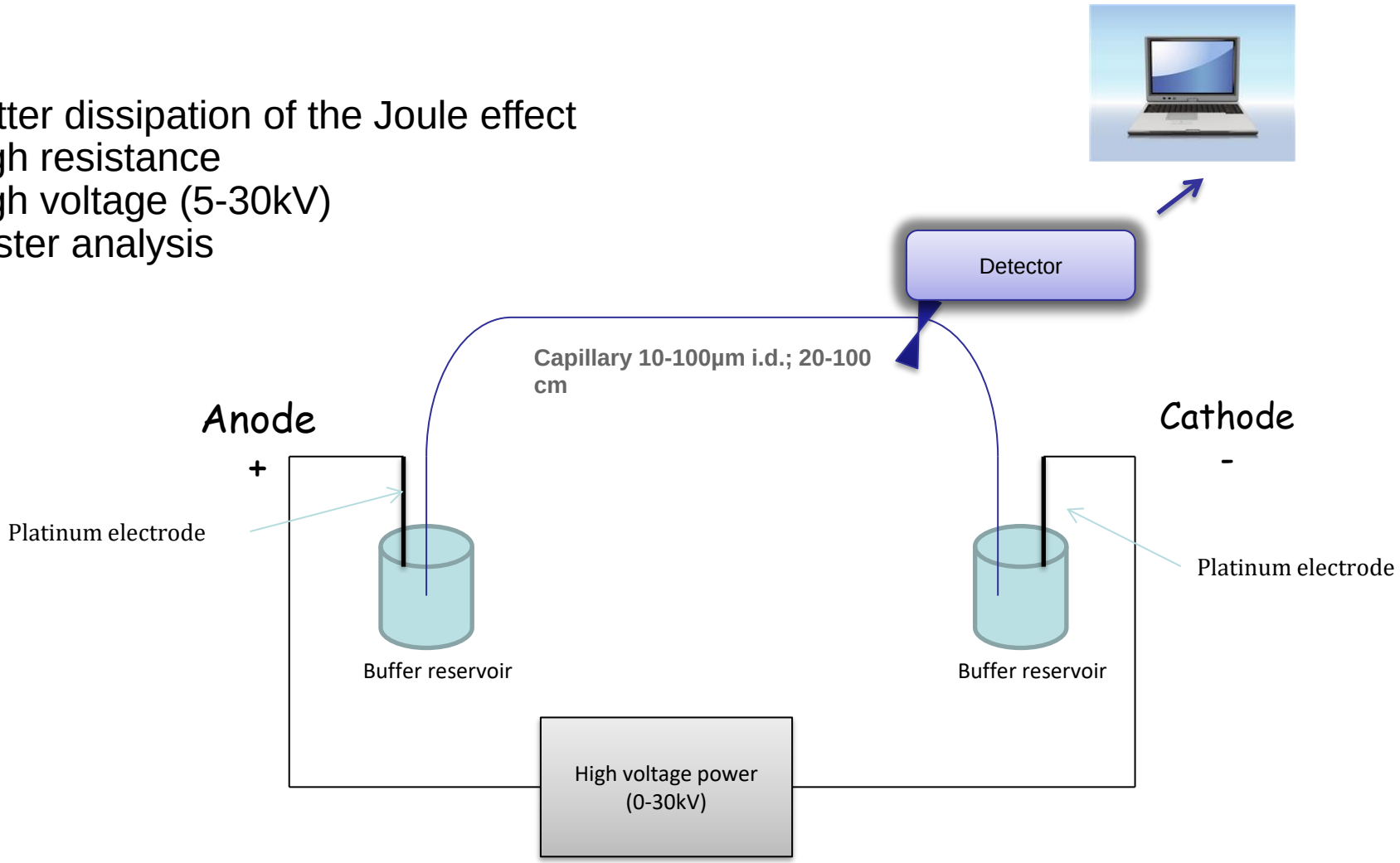
- Long protocol
- The most abundant proteins can mask the least abundant ones



Capillary electrophoresis: initiation

Schematic of capillary electrophoresis

- Better dissipation of the Joule effect
- High resistance
- High voltage (5-30kV)
- Faster analysis



Fused silica capillary
L=20-100 cm Di= 50-100µm

Migration mechanism

- **Electrophoretic migration**

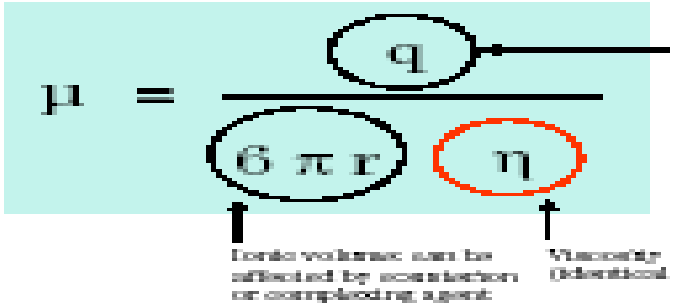
Charged species subjected to an electric field

Electrophoretic velocity $V_{ep} = \mu_{ep}.E$

Electrophoretic mobility:

$\mu_{ep} > 0$ cations

$\mu_{ep} < 0$ anions



Ion Velocity and Mobility

$$v = \frac{l_{eff}}{t_m}$$

$$\mu_a = \frac{v}{E} = \frac{l_{eff}}{t_m} \frac{L_{tot}}{V}$$

- * **Electroendosmosis :**

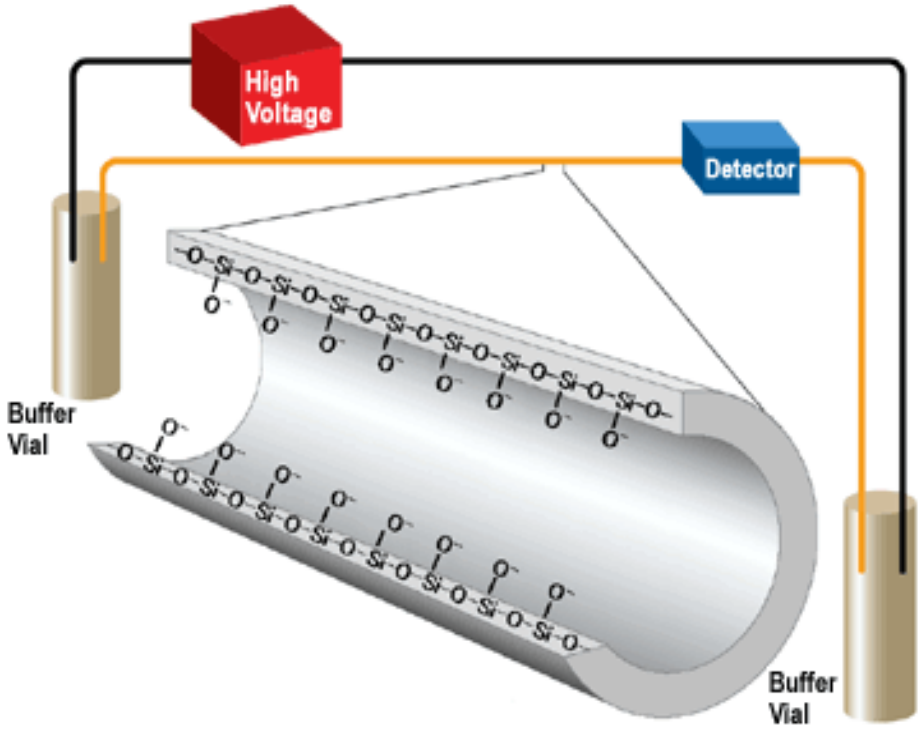
Presence of ionized silanol groups

Electrical double layer: zeta potential

Electroosmotic velocity

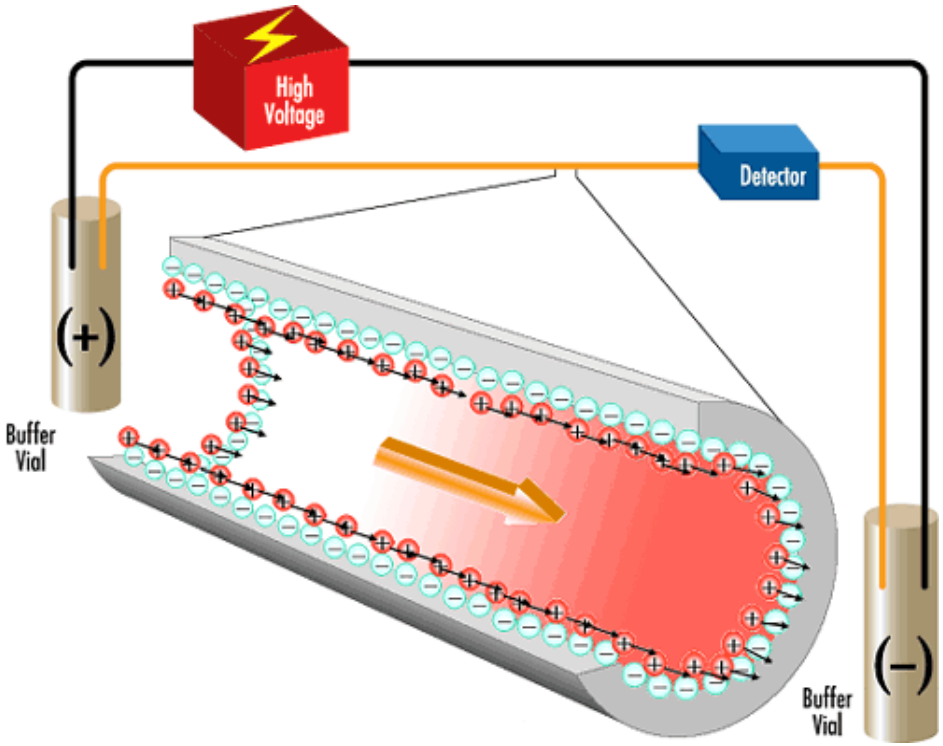
$V_{eo} = \mu_{eo}.E$

Electroosmotic flow



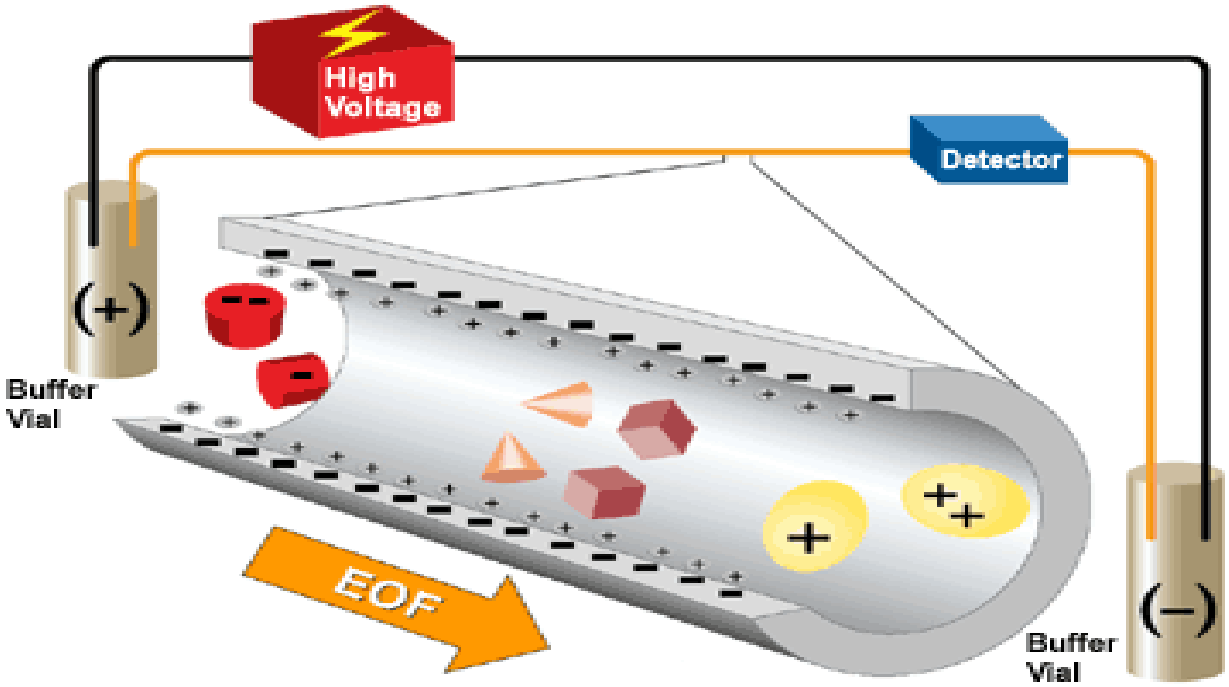
Ionization of the silanol group when in contact with an electrolyte solution

Electroosmotic flow

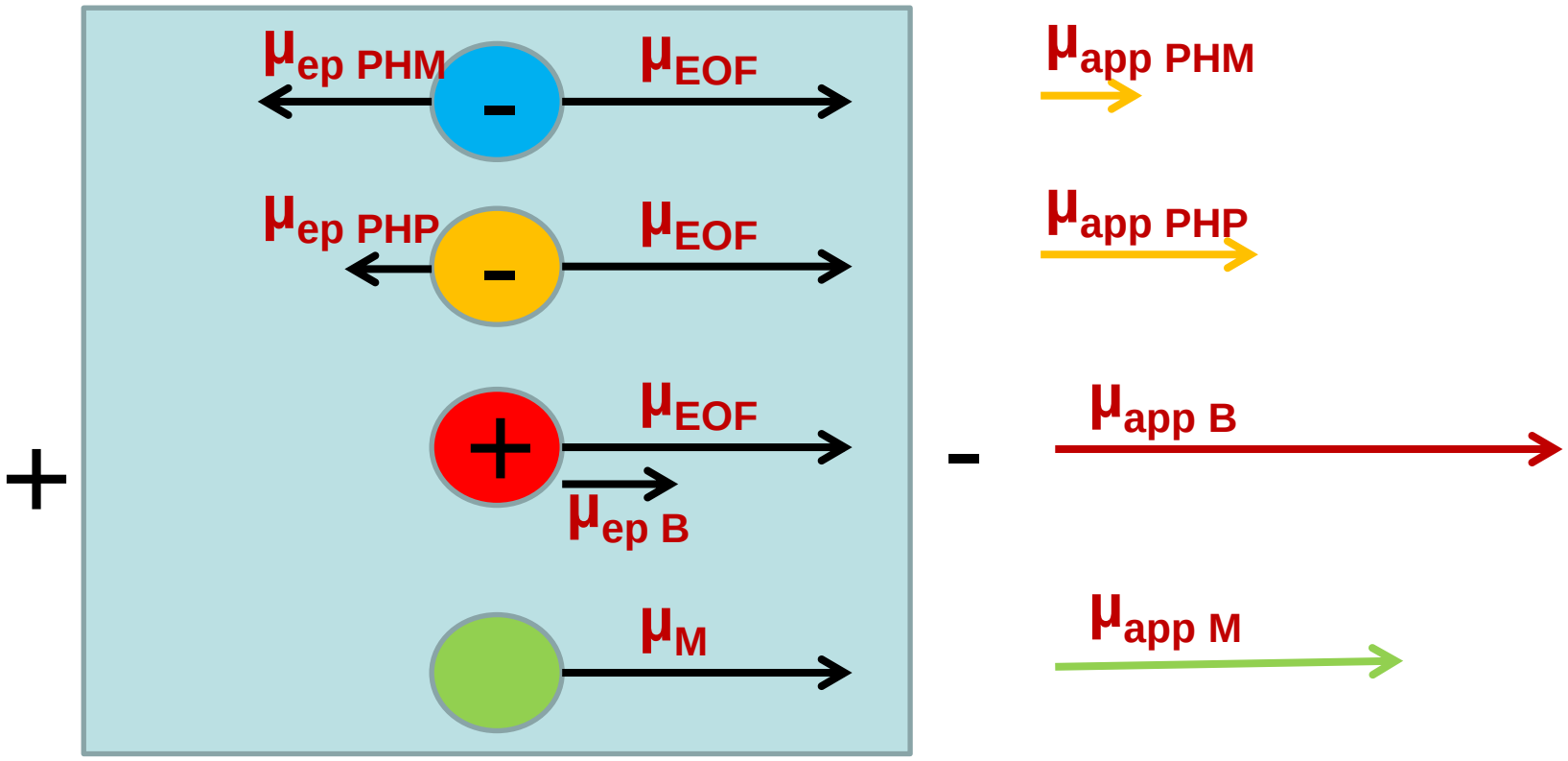


Movement of the positively charged ions when the high voltage is turned on

Electroosmotic flow

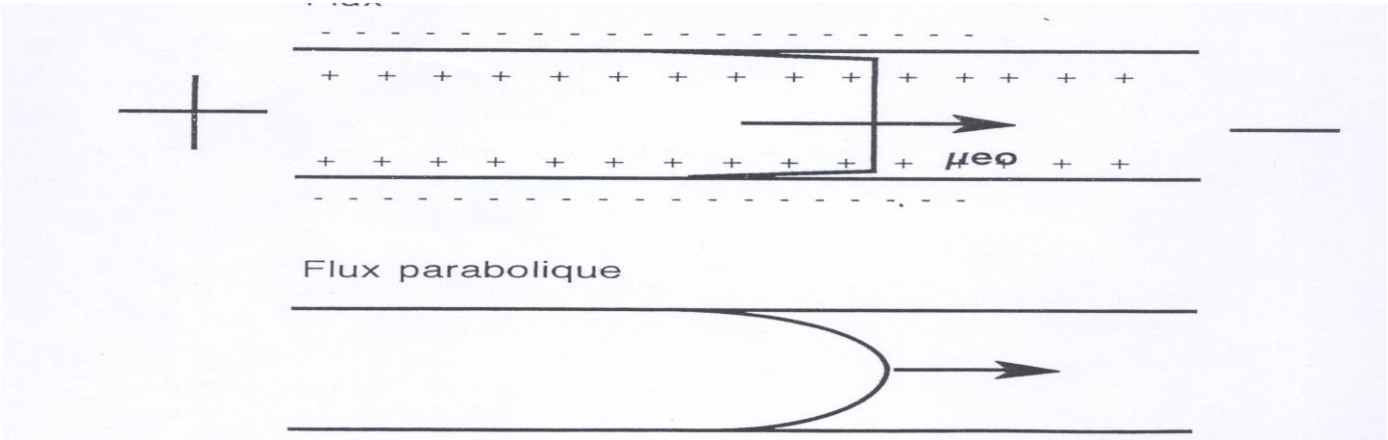


Migrations of ions under a high electric field


μep PHM
μEOF
μep PHP
μEOF
μEOF
μep B
μM
+
-
μapp PHM
μapp PHP
μapp B
μapp M

Electroosmotic flow

Flat front :
High N



<u>The EOF depends:</u>

- pH
- Nature et concentration of the electrolyte
- Surface and material of the capillary

Différent CE modes

- **Capillary zone electrophoresis**
- **Capillary gel electrophoresis**
- **Capillary electrochromatography**
- **Non aqueous capillary electrophoresis**
- **Chiral capillary electrophoresis**
- **Capillary affinity electrophoresis**
- **Capillary isofocusing**
- **…**

Injection modes

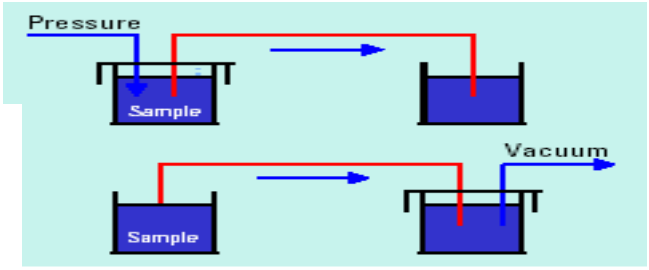
$$\text{Volume} = \frac{\Delta P\, d^4\, \pi\, t}{128\, \eta\, L_{tot}}$$

Capillary 50cm x 75µm: V_{tot}=5µl

$V_{injected}$= 1-50 nl

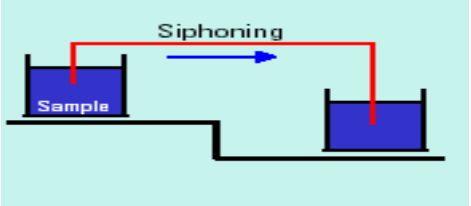
- **Hydrodynamic injection**

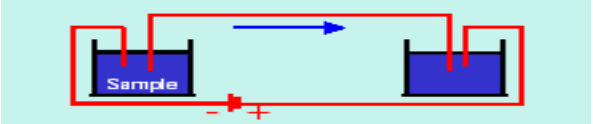
  △P=pressure difference [Pa]
  d et L_{tot}: diameter and total lenght of the capillary (m)
  t: time (s) ; η:viscosity [Pa.s]



- **Siphoning injection**

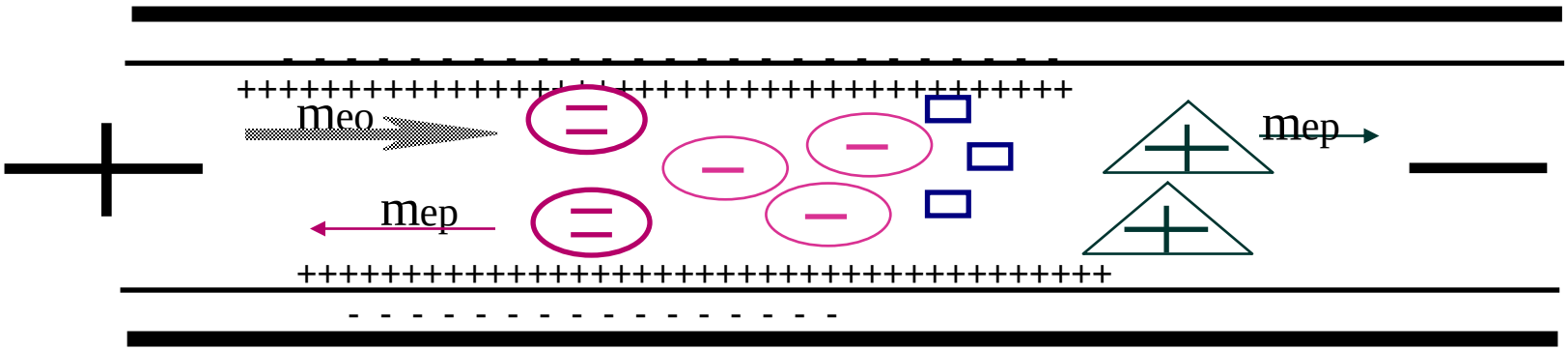
  Q=Quantity of the injected analyte
  µe et μ_{eof}: electrophoretic and electroosphotic mobilities
  E: electrical field ; r: capillary diameter
  C: sample concentration





- **Electrokinetic injection**

Capillary zone eletrophoresis

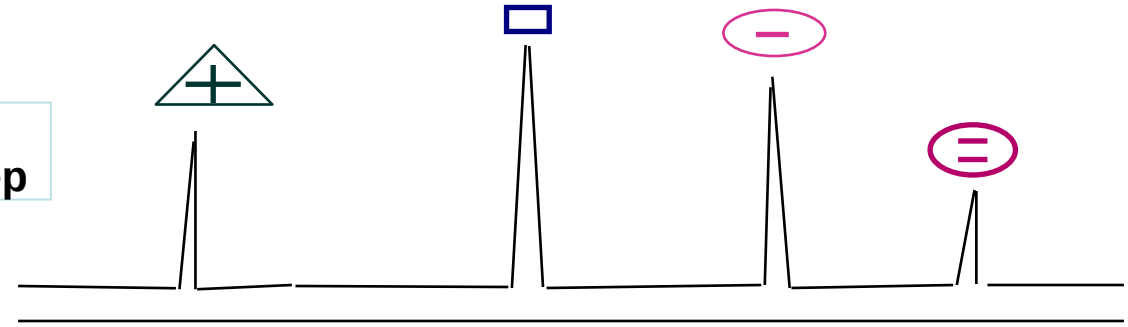


Positively charged molecules

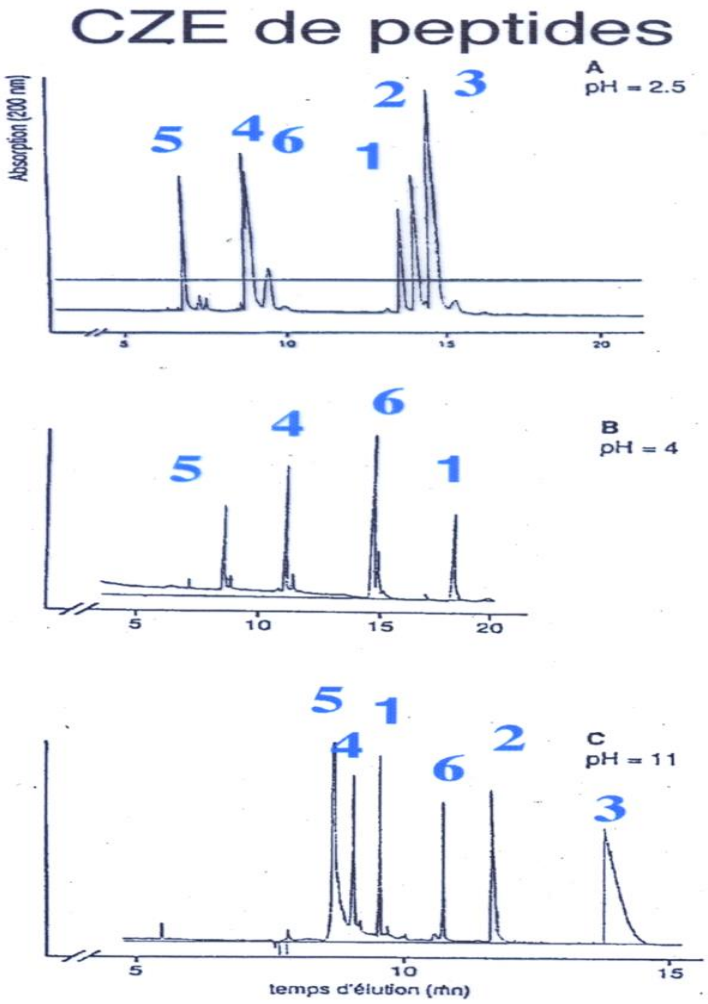
Negatively charged molecules

Neutral molecules

$\mu_{app}=\mu_{eo}+\mu_{ep}$



Influence of pH on CZE performance



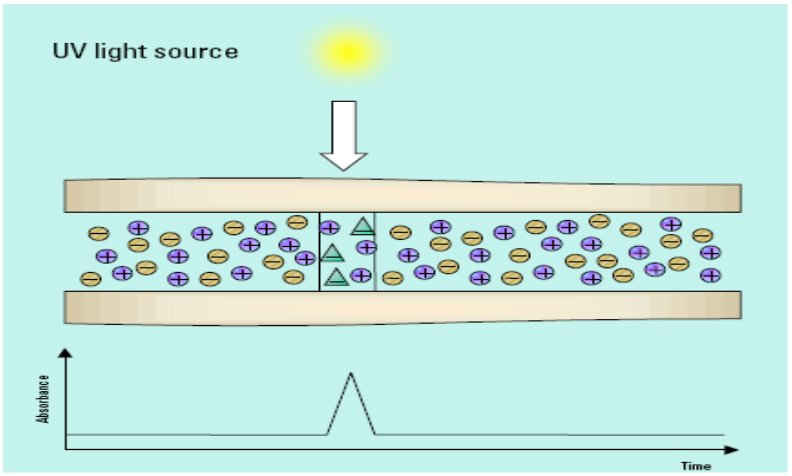
	séquence	Charge nette pH
1	AFAAING	0.41
2	AFDAING	0.37
3	AFDDING	0.33
4	AFKAING	1.41
5	AFKKING	2.41
6	AFKADNG	1.37

	pH=4	pH=11
1	0.02	-0.9
2	-0.54	-1.95
3	-1.09	-2.95
4	1.02	-0.71
5	2.02	-0.47
6	0.46	-1.71

Detection in CE

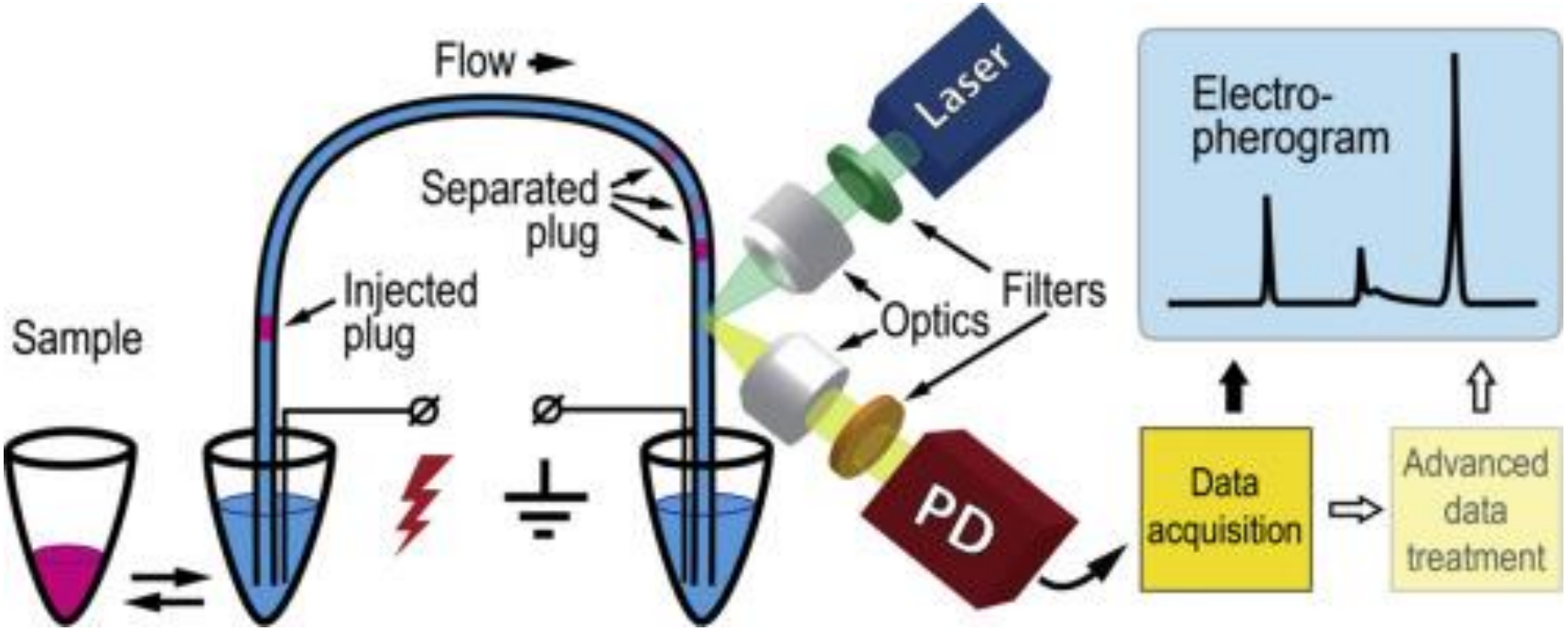
Principe de détection	***MDQ (mol)***	***MDC (M)***
Absorption UV-visible directe	10^{-13} -10^{-16}	10^{-5} -10^{-7}
Absorption UV indirecte	10^{-12} -10^{-15}	10^{-4} -10^{-6}
Fluorescence	10^{-14} -10^{-17}	
Fluorescence indirecte	10^{-13} -10^{-16}	
Fluorescence induite laser	10^{-18} -10^{-21}	10^{-9} -10^{-12}
Spectrométrie de masse		
interface: electrospray	10^{-17}	
interface: FAB	10^{-12} -10^{-14}	
Electrochimie		
conductimétrie	10^{-16}	
ampérométrie	10^{-17} -10^{-19}	
Réfractométrie		10^{-6} -10^{-7}
Résonance Magnétique Nucléaire		

UV-Vis detection

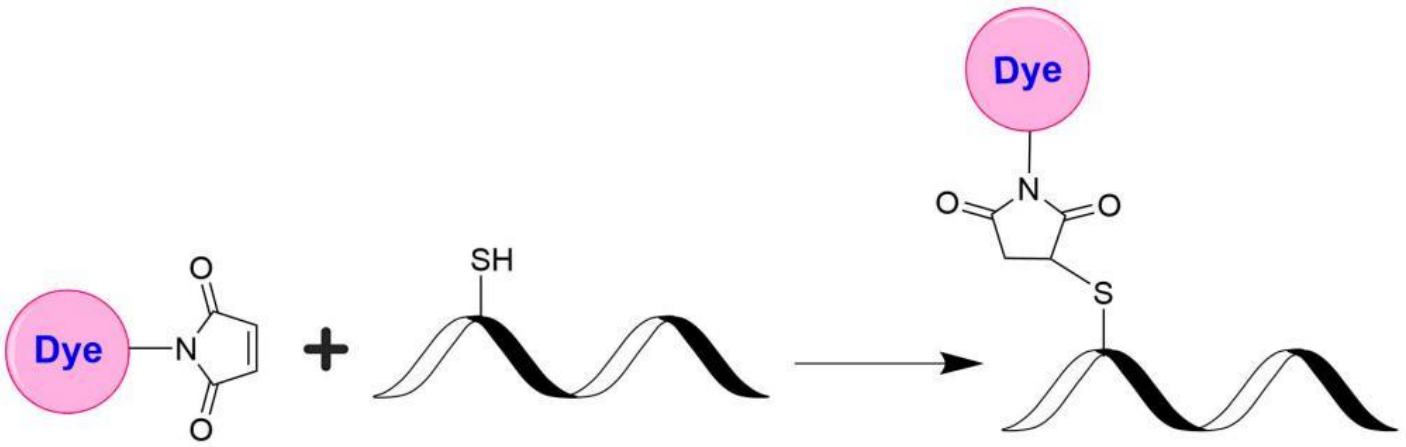


- Most used detection mode
- Large range185-800 nm
- 90% analytes absorb at 200-600 nm; 65% absorb at 254 nm
- Detection limit (M): 10^{-5} -10^{-7}
- Low pressure mercury or deuterium lamp (UV) / tungsten lamp (visible), selection l by filters and / or monochromator network
- Diode array detectors (DAD)

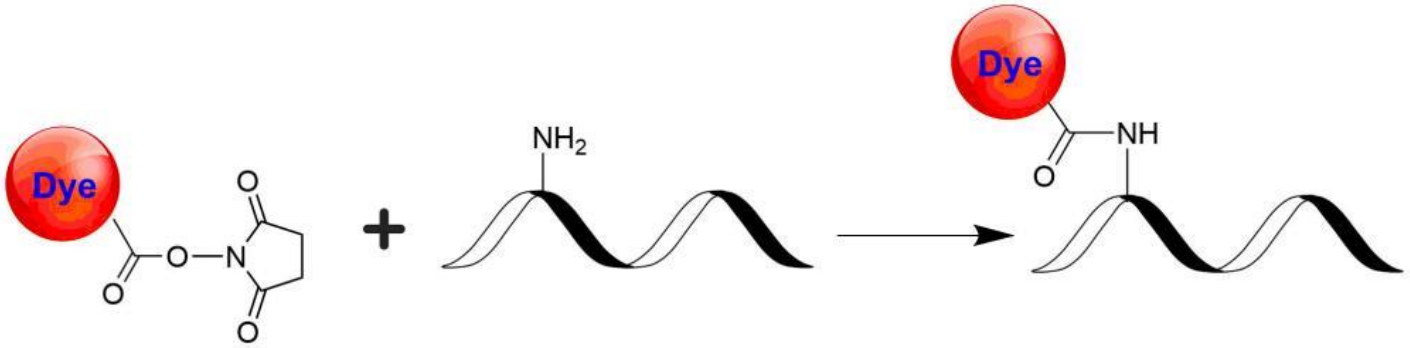
Laser induced fluorescent (LIF) detection



Fluorescent labeling of proteins and peptides

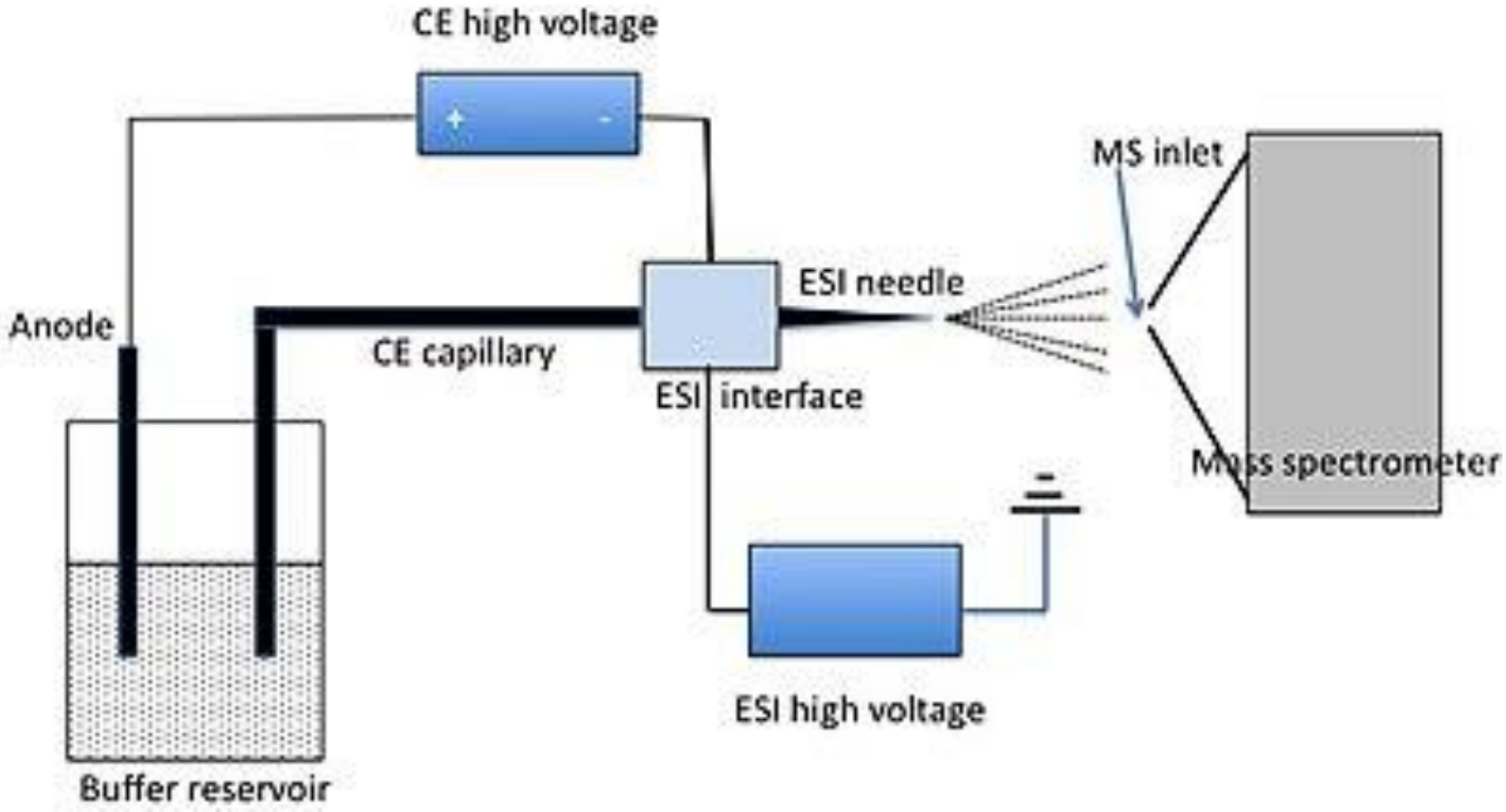


Fluorescent dye maleimides

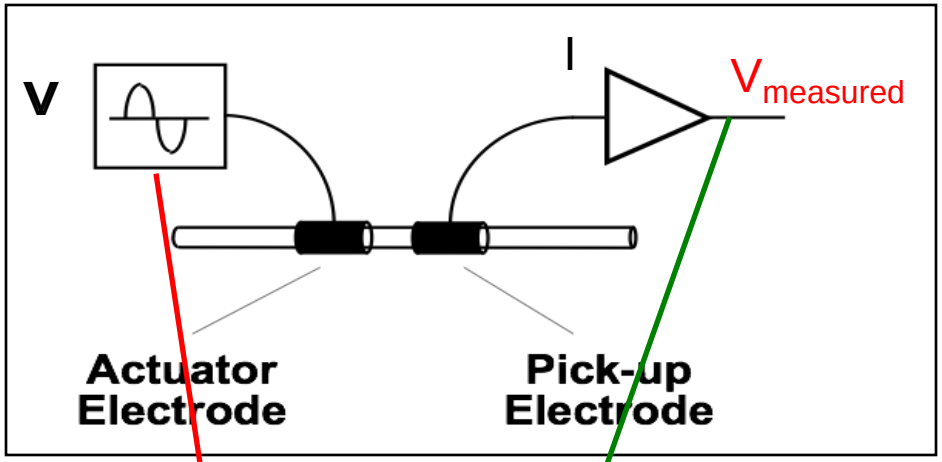


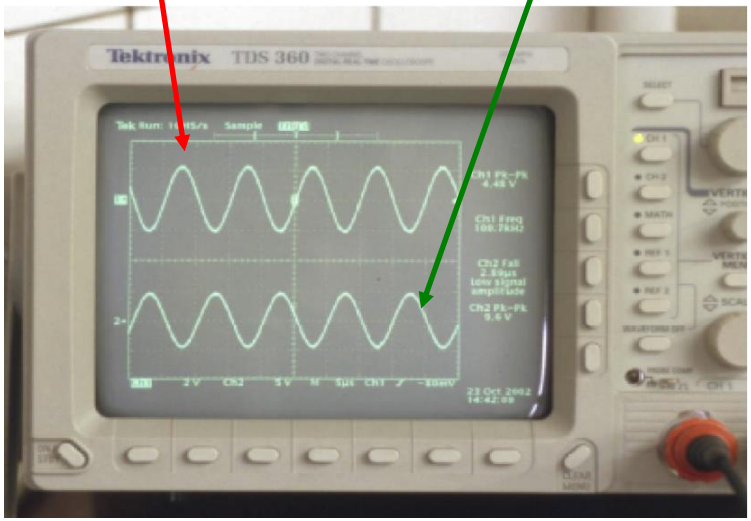
Fluorescent dye NHS esters (or succinimidyl esters)

CE-MS

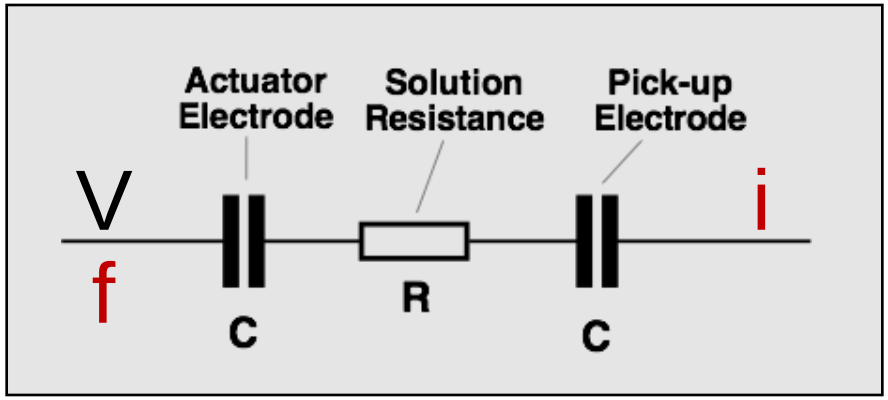

CE high voltage
+
-
MS inlet
ESI needle
Anode
CE capillary
ESI interface
Mass spectrometer
ESI high voltage
Buffer reservoir

Capacitively coupled contactless conductivity detection (C^4D)





Simplified equivalent circuit diagram



$$i = \frac{V}{\sqrt{R^2 + \left(\frac{1}{2\pi fC}\right)^2}}$$

$$V_{measured} = i \times R_{feedback}$$

Capillary Electrophoresis (CE) in the world

Bench-top CE

Portable CE

?

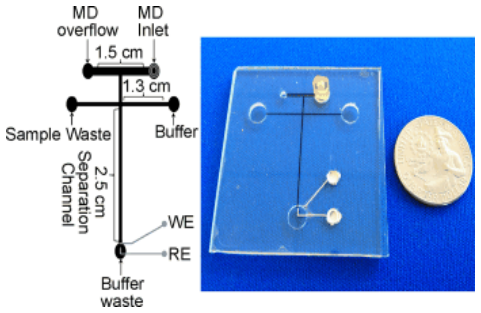
Microchip CE
(mostly for academic research)

Beckman Coulter

PrinCE:

Agilent:

Wyn CE:



Quelques applications

PHARMACEUTICAL QUALITY CONTROL

USE THE CAPILLARY ELECTROPHORESIS ADVANTAGES FOR THE CONTROL OF PHARMACEUTICAL PRODUCTS

ASCORBIC ACID IN VITAMIN C TABLETS

Determination and quantitation of ascorbic acid in Vitamin C effervescent tablets and orange juices with Wyn-CE capillary electrophoresis system and a contactless conductivity detection.

Read more

ASCORBIC ACID DANS IN ORANGE JUICES

Determination and quantitation of ascorbic acid in Vitamin C effervescent tablet and orange juice using Wyn-CE capillary electrophoresis system and an easy and sensitive contactless conductimetry detection (C4D).

Read more

CAPILLARY ELECTROPHORETIC METHOD FOR ANALYSIS OF CEFOTAXIME

Determination and quantitation of Cefotaxime by Capillary Electrophoresis with UV detection For Quality Control before injection to patients

Read more

DETERMINATION OF 5 AMINO ACIDS IN TOTAL PARENTERAL NUTRITION FORMULATION

This application describes the use of CE method with C4D detection for the determination of 5 amino acids (Valine, Leucine, Iso-Leucine, Tryptophan, and Tyrosine) in Total Parenteral Nutrition formulation.

Read more

DETERMINATION OF CATIONS IN TOTAL PARENTERAL NUTRITION FORMULATIONS

Determination of cations K+, Na+, Ca2+, Mg2+ in total parenteral nutrition formulation with the Wyn-CE Capillary Electrophoresis System and a contactless conductivity detection

GLUCOSE DETERMINATION IN TOTAL PARENTERAL NUTRITION FORMULATION

Determination and quantitation of Glucose by Capillary Electrophoresis with UV detection For Quality Control of

BIOCHEMISTRY

INITIATION TO MEDICAL DIAGNOSTIC WITH THE WYN-CE SYSTEM

PROTEINS SERUM ANALYSIS

The method presents the determination of relative amounts of albumin and globulins in human serum using the Wyn-CE system. Identification and quantitation of the analysed proteins is obtained by direct UV absorbance detection at 200 nmWyn-CE. L'identification et la quantification des protéines analysées ont été obtenues par détection UV direct à 200 nm.

Read more

SWEAT CHLORIDE QUANTITATION USING CE-C4D

This application compared the results obtained with the sweat test using the WynSep capillary electrophoresis (CE) method and coulometric measurement (Chlorochek chloridometer: CC) of sweat chloride in subjects with suspected CF.

Read more

SODIUM AND POTASSIUM CATIONS IN SERUM

The balance of electrolytes in the body is maintained by a function called homeostasis. Diagnosis of sodium and potassium levels are determined with a blood test. This Application Note describes K+ and Na+ cations analysis in serum with Wyn-CE system and a contactless conductimetry detection.

WATER MONITORING

SIMPLICITY AND ROBUSTNESS OF WYN-CE FOR WATER CONTROL

INORGANIC ANIONS IN DRINKING WATERS (ACCORDING US EPA6500)

Determination of chloride, nitrate, nitrite, bromide, sulfate, phosphate and fluoride anions in drinking water samples using Wyn-CE capillary electrophoresis system with indirect UV detection using a chromate-based electrolyte (according US EPA 6500 and ASTM D6508-00)

Read more

CATIONS IN WASTEWATERS AND SURFACE WATERS

Determination and quantitation of K+, NH4+, Na+, Ca2+, Mg2+ cations in wastewater and surface water using Wyn-CE capillary electrophoresis system and an easy to use and sensitive contactless conductimetry detection (C4D).

Read more

INORGANIC CATIONS IN DRINKING WATERS

Determination and quantitation of K+, NH4+, Na+, Ca2+, Mg2+ cations in drinking waters using Wyn-CE capillary electrophoresis system and an easy to use and sensitive contactless conductimetry detection (C4D).

Read more

MINOR INORGANIC CATIONS IN CALCIUM-MATRICES

The analysis of minor cations as potassium, sodium, magnesium and barium cations in samples with a high content of calcium has been achieved with the add of 2,6-pyridinedicarboxylic (PDC) acid in acetic acid/HIS buffer for C4D detection.

Read more

Source: WynSep